A therapeutic perspective of the immunological function of the liver

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ABSTRACT

Therapeutic vaccination of chronic infectious diseases has been extensively explored because of its possible contribution to their eradication. In particular, therapeutic vaccination of hepatitis B virus chronic infections is especially interesting since this disease is characterized by a sustained necro-inflammatory process of the liver that may evolve into more severe conditions including cirrhosis and hepatocellular carcinoma. The basic role of the immune system in the healing process of this chronic infection suggests that it offers a favorable setting for immunotherapeutic treatments, either spontaneously or as a result of antiviral therapy. However, no vaccine has been able to cure this or any other chronic infection in spite of the large number of vaccine candidates tested. The knowledge of the liver as a lymphoid organ and the limited advances of therapeutic vaccination demand more thorough analyses within the rationale of current vaccine candidates. In the last ten years there has been an increased knowledge of innate immunity and intra- and extra-hepatic signaling mechanisms, to support a rational design of vaccine strategies. The high costs and low effectiveness of conventional treatments, and the large amount of chronic carrier patients for this virus, indicate a favorable setting for the development of immunotherapeutic products against chronic hepatitis B. It is possible to predict that adjuvant strategies that take into account the properties of the liver as a lymphoid organ would have an impact in the development of this new field of therapeutic vaccines.

Keywords: Liver, therapeutic vaccination, immune response, antigens, receptors, innate immunity

RESUMEN

Función inmunológica del hígado desde la perspectiva de la vacunación terapéutica. La vacunación terapéutica contra las enfermedades infecciosas crónicas se ha explorado ampliamente porque se estima que podría contribuir en mucho a combatirlas. Resulta interesante la vacunación contra la infección crónica por el virus de la hepatitis B, enfermedad que se caracteriza por un proceso necroinflamatorio sostenido del hígado, que puede evolucionar a formas severas de la enfermedad, entre ellas la cirrosis y el carcinoma hepatocelular. La función esencial del sistema inmune en la curación de la infección crónica por ese virus, bien de modo espontáneo o como resultado de tratamientos antivirales, sugiere que es un escenario propicio para el tratamiento inmunoterapéutico. Sin embargo, aún no hay una vacuna que cure esta u otra infección crónica; aunque sí se ha ensayado un grupo elevado de candidatos vacunales. El conocimiento acerca del funcionamiento del hígado como órgano linfoide, y los limitados avances en la vacunación terapéutica, obligan a revisar la racionalidad de los candidatos vacunales actuales. En los últimos diez años ha habido un desarrollo impetuoso del conocimiento de la inmunidad innata y los mecanismos de señalización intra y extrahepáticos, que permiten un diseño racional de las estrategias vacunales. Los precios elevados, la baja efectividad de los tratamientos convencionales y la gran cantidad de pacientes portadores crónicos de este virus, indican que hay un nicho oportuno para el desarrollo de un producto inmunoterapéutico contra la hepatitis B crónica. Es posible predecir que las estrategias de adyuvación que estén en línea con las características del hígado como órgano linfoide, tendrán un impacto en el desarrollo de esta rama de la vacunología.

Palabras clave: Hígado, vacunación terapéutica, respuesta inmune, antígenos, receptores, inmunidad innata

Introduction

From the anatomical viewpoint, the liver is a "strategic" organ with highly relevant functions. It has a critical function in the intermediary metabolism of carbohydrates, lipids and glycolipids, as well as in the synthesis and secretion of several plasma proteins, enzymes and bile salts among others. It is the main organ regulating the levels of most blood components.

Nearly 25% of the blood pumped by the heart passes through the liver, which is subjected to a double blood flow: the arterial blood carried by the hepatic artery intersects with venous blood returning from the intestines and the spleen through the portal vein. Therefore, toxic compounds ingested with the food are transported to the liver from the intestines by the portal vein for detoxification [1].

It was not until the year 2000 that the liver was widely recognized as having its own specialized defense mechanism against infectious agents, toxins and other bacterial products. It must also protect itself from undesirable responses against mild dietary proteins or against malignant cells transported by a massive blood flow. In fact the detoxification of potentially harmful dietary compounds determines a regular exposure to carcinogens that can generate mutations, which together with the high cell turnover rate of this organ, requires the action of a specialized tumor surveillance mechanism [2].

The particular immune system of the liver must be taken into account when designing therapeutic vaccine candidates against chronic hepatitis B (CHB). That

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system comprises tolerogenic and induction mechanisms of the immune response, with highly evolved signaling pathways. They allow the adaptation of the liver as a receptor organ of venous blood from the intestines and guarantee its complex and vital functions.

Therefore, a CHB therapeutic vaccination adjuvant strategy demands the thorough knowledge of the above signaling system, which under normal conditions may induce tolerance or an immune response of this organ. A first approach to these cellular components could provide a better understanding of the need of not only rescuing the antiviral effector cells from the state of tolerance to the chronic infection, but also of having them migrate into the liver parenchyma to exert their functions while escaping from this condition.

Main immune response-related liver cell types

The complexity of the immune system of the liver is linked to the coexistence of conventional and non-conventional cell types, and their role during immune response activation or inhibition within the liver.

Hepatocytes comprise roughly two thirds of liver cells, the rest are cells that are not related to the liver parenchyma and have functions that are highly related to the defense of the organ. The latter third can be subdivided into endothelial cells (50%), Kuppfer cells (20%), lymphocytes (25%), bile cells (5%) and stellate cells (less than 1%) [3].

Lymphocytes are disseminated throughout the parenchyma and portal tracts. The normal human liver contains approximately 10^{10} lymphocytes, including both conventional and non-conventional lymphocyte populations of either the innate or the adaptive immune systems [3].

Conventional T cells comprise CD8+ and CD4+ T cells, both of them with diverse repertoire of α and β T cell receptors subunits that can recognize antigens within the context of the type I and II major histocompatibility complex molecules, respectively. CD8+ T cells outnumber CD4+ T cells and the effector/memory cell ratio is higher than in the blood.

Non-conventional T cells comprise several cell types that can be grouped according to the presence or absence of natural killer (NK) cell markers (known as NKT cells). At the same time, there are classical and non-classical NKT cells, the first one originating in the thymus and having a very restricted T cell repertoire, typically a Vα24/Vβ11 subunit T cell receptor recognizing antigens within the context of CD1 molecules. Classical NKT cells become activated by a-galactosyl-ceramide and can be either CD4+/CD8- or CD4-/CD8-.

The non-classical NKT cells are more frequent in the liver than in other organs, representing up to 30% of the intra-hepatic lymphocyte population [4]. Its migration into the liver and its expansion within this organ are both controlled by NK cells [5]; the latter being at an unusually high frequency among liver resident lymphocytes. *NK cells* represent a lymphoid population of cytolytic activity against tumor or virus infected cells, its function being regulated by activating and inhibitory receptors, with the inhibitory signal as the dominant one.

The *non-conventional T cells*, not expressing the NK cell markers, comprise the T cell subset bearing

$\gamma\delta$ subunits ($\gamma\delta$ T cells) that accounts for 15 to 25% of intrahepatic T cells. This makes the liver one of the main sources of $\gamma\delta$ T cells in the body. These cells bear invariant or oligoclonal T cell receptors that recognize a limited range of antigens, such as stress proteins and non-protein antigens.

The complexity of the hepatic immune system is also evidenced at the level of antigen presenting cells (APCs). The liver has several types of APCs, which are able to link antigens passing through sinusoids or those released when the pathogen-infected hepatocytes die. Resident APCs included *Kupffer cells*, *liver sinusoidal endothelial cells* (LSEC) which represent an unusual vascular endothelial cell type, and *dendritic cells* (DCs). It is considered that these three APCs types are essential in inducing tolerance under non-inflammatory conditions [6].

Kupffer cells are the main subset of resident macrophages in the body, originating from circulating monocytes developed from bone marrow parental cells [7]. These cells are located throughout the sinusoidal vascular space, prevailing in the periportal space, where they are advantageously located for the elimination of blood endotoxins that pass through the sinusoids, for the phagocytosis of cellular debris and microorganisms. Their slow migration through the sinusoids frequently disrupts the flow intermittently and facilitates the contact of the circulating lymphocytes with the different cell types present. Kuppfer cells can pass through the Disse space and get into direct contact with hepatocytes, phagocyting them when they become apoptotic.

LSEC are lined up with the sinusoids in a similar way than the arterial, portal and central vein vascular endothelia. Nevertheless, their morphology differ considerably forming a filter-shaped fenestrated endothelium whose cells express molecules that promote antigen assimilation. The mannose and *scavenger* receptors are among them; this cell layer also express molecules promoting antigen presentation, such as: the co-estimulatory CD40, CD80 and CD86 molecules. The receptor-mediated endocytosis and phagocytosis and the antigen processing and antigen presentation of sinusoidal endothelial cells are similar to those of DCs [8].

Due to the small diameter of the sinusoids, slightly wider than lymphocytes, a minimal increase of the systemic venous pressure and the disruption of the sinusoidal flow, slow down the flow enough to promote the contact between lymphocytes and APCs, leading to lymphocyte extravasations. This process is facilitated by the fenestrations of the sinusoidal endothelial cells, enabling the access of lymphocytes into the Disse space and their contact with the extracellular matrix, stellate cells, resident hepatic DCs, Kuppfer and endothelial cells, and hepatocytes. This liver-specific tissue morphology facilitates the direct and indirect sensitization of lymphocytes, modulates the immune response against hepatotropic pathogens and contributes to the immune response induced by this organ [2].

Resident dendritic cells originate in the bone marrow [9] and surround the central veins and portal tracts. In the normal liver, DCs are predominantly at an immature state [10] able to capture and process antigens. Sinusoidal endothelial cells, together with

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Kuppfer cells, produce IL10 and TGFβ; these cytokines are inducible in stellate cells, contributing to the unique cytokine environment of the normal liver that becomes resident DCs tolerogenic [10, 11]. The inactivated DCS can inhibit proliferation and cytokine production of infiltrating lymphocytes through the CTLA-4 and PD-1 receptors [12]. In contrast, when they become activated, DCS down regulate these receptors and increase their capacity to migrate through the Disse space into lymphatic vessels within the portal tracts, finally reaching the extrahepatic lymph nodes [12].

*B*iology of the liver immune response

Many liver cells have been described as having a potential capacity for antigen presentation, including LSEC, hepatocytes, DCs, Kupffer cells, and more recently stellate cells. All of them present antigens to naïve T cells [13]; however, Kupffer cells and LSEC are specifically well located to interact with naïve T cells coming from the blood and circulating within the sinusoids. In this section, we will first analyze what happens with liver DCs in this organ and with other cell types later on.

The role of DCs

All the APCs compete with lymphoid tissue DCs for the activation of naïve T cells, irrespective of the type of APCs involved in presentation. There are two opposite ways for that activation. While activated T cells within lymph nodes acquire a complete effector function and take part in immunity, T cells activated within the liver become no responsive or are eliminated, in a process causing an antigen-specific tolerance [14]. This model explains why there could exist an effective immune response in the liver against some pathogens while maintaining this organ its intrinsic capacity to induce tolerance.

Freshly isolated DCs from the liver are relatively immature and less immunogenic than spleen DCs. It is considered that intra-hepatic DCs are relevant to the tolerogenic function of the liver [15], since all liver DCs secrete high levels of IL10 and TGFβ and negatively regulates the immune response, also inducing a regulatory T cell response [16].

Most of the hepatic DCs are sequestered within the portal tracts rather than in the sinusoids [17]. It is improbable within this context for immature DCs to get into contact with circulating naïve lymphocytes, in spite of the evidence of DCs translocation through the sinusoids [18]. Therefore, it is probable that immature DCs could activate circulating naïve CD8+ T cells. The finding that LSEC interfere the presentation capacity of DCs, particularly affecting its co-stimulatory function (required to activate CD8+ T cells), could be an adaptative strategy of the liver. On the contrary, in the absence of interaction with LSEC, DCs fully recover their cellular proliferation capacity. This is an LSEC property which is absent in hepatocytes and B cells. It is known that LSEC reduces the expression levels of CD80, CD86 and IL12 in DCs upon the interaction with LSEC. In other words, LSEC are not only able to tolerogenize T cells directly (see below), but also to suppress the capacity to induce T cell immunity in the neighboring DCs [19].

Regardless of the above finding on the regulatory capacity of LSEC on DCs, it is relevant to notice that DCs, which are mostly located at the portal tracts, are poorly stimulatory because of their immature condition rather than because of being tolerogenized during their passage through the hepatic sinusoids.

This network of regulatory mechanisms acting on DCs shows the control of the liver on a potentially harmful cytolytic immune response, which is advantageously used by the pathogens to develop a persistent infection. Therefore, for a therapeutic vaccine candidate to eliminate a persistent pathogen it must overcome these mechanisms controlling liver immune response.

Immunological functions of LSEC

Several studies have focused LSEC since this cell type bears scavenger receptors and efficiently captures and presents circulating antigens. It has been demonstrated in mice that these cells express low levels of MHC II and CD80/CD86 co-stimulatory molecules, also evidencing their ability for antigen presentation and cross-presentation to CD4+ and CD8+ T cells, which as a whole favors the induction of tolerance [20, 21].

Naïve CD4+ T cells start producing IL4 and IL10, instead of IL2 and IFNγ, in response to antigens presented by LSEC. Dominance of IL10 in the liver is not only determined by CD4+ T cells, but also by Kupffer cells, as recently demonstrated by intrahepatic CD8+ T cells [22]. This environment modifies the expression of chemokine receptors in DCs, reducing their migration toward the drainage lymph nodes [23]. T cells sensitization in the presence of IL10 reduce their capacity to produce cytokines and also their effector functions [24].

Antigen presentation by LSEC to CD8+ T cells also results in a tolerogenic rather than an effector function. CD8+ T cells co-cultured with LSEC show a reduced capacity to produce IL-2 and IFNγ, low cytotoxicity, low proliferative response and are also prone to apoptosis. These properties can be re-established by exogenously adding IL12 to the co-cultures of CD8+ T cells and LSEC [21].

Under non-inflammatory conditions lacking IL2, LSEC antigen presentation contributes to tolerance. On the contrary, within a pro-inflammatory context, LSEC down-regulates MHC expression, also reducing its tolerogenic effect.

Functions of Kupffer cells

Kupffer cells are activated by several bacterial stimuli, including lipopolysaccharides (LPS) and superantigens. Cytokines released by Kupffer cells are relevant to modulate proliferation and differentiation of other cell types. These cells produce TNFα and IL10 in response to physiological concentrations of LPS [25], downregulating receptor-mediated antigen uptake and MHC II expression by the LSEC and DCs, further decreasing the activation of T cells [24].

Kupffer cells also produce prostanoids, nitric oxide and reactive oxygen intermediaries which suppress T cell activation [26]. In fact, the systemic tolerance to antigens within the portal vein depends on these cells, decreasing considerably by eliminating them [27].

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It is recognized that Kupffer cells produce IL12 and IL18, these cytokines that regulate NK cell differentiation and promote their local expansion, lead to NK cell secretion of large amounts of the antiviral IFNγ. Other cytokines secreted by Kupffer cells promote neutrophil infiltration and antimicrobial activity.

NK cells, activated through their activating and inhibitory receptors, modulate the liver damage by establishing a balance between the local production of proinflammatory (Th1) and anti-inflammatory (Th2) cytokines.

Considering the effect of LPS on the functions of Kupffer cells and its relevance to liver immunity, it would be logical to question if monophosphoryl lipid A (MPL) –an LPS-derived compound– can be considered a therapeutic vaccine candidate able to break hepatitis B virus (HBV) tolerance in this organ. It is assumed that the association between MPL and the vaccine antigen within an oily vaccine formulation can reach the blood flow and be assimilated in the liver to a certain extent, generating a signal not different from that normally triggered by LPS. LPS stimulates the secretion of IL10 and TNFα, creating subsequently a tolerogenic environment that opposes the proper activation of liver T cells. On the other hand, a signal favoring the migration of specific CD8+ T cells into the liver has been described related to the Toll-like receptor activation via TLR-3 (as further explained below).

Functions of NK cells

Liver NK cells modulate liver damage by equilibrating the local production of pro- and anti-inflammatory cytokines through their respective activating or inhibitory receptors. The NK cell receptors involved in NK cell activation and lysis of target cells are activated in the absence of inhibitory signals and in the presence of type I IFNs and IFN-induced CCL3 ligands [28]. Activation also implies IFNγ boosting that stimulates hepatocytes and LSEC to secrete the CXCL9 chemokine, the latter responsible for recruiting T cells into the liver.

Functions of NKT cells

Most NKT cells recognize non-peptide antigens, such as lipids and glycolipids from the cell walls of microorganisms. Recognition is restricted to the CD1 molecule that can be expressed by hepatocytes and APCs (including DCs, macrophages and B cells). Most of the classic NKT cells are activated by the IL12 produced by DCs and NK, mostly resulting in a Fas-mediated lysis [29, 30].

Given the NKT cell capacity to produce high levels of IFNγ and IL4, it has been considered that these cells are related to the polarization of both the local and systemic adaptive immune responses, either pro- or anti-inflammatory. These cells carry out a relevant function for liver infections, since NKT cell or CD1d deficient mice are more susceptible to certain viral [31] and bacterial [32] infections. Besides, it was demonstrated in a transgenic mouse model that activation of NKT cells with the synthetic CD1d ligand α-galactosyl ceramide (α-GalCer) increases the production of IFNγ by downregulating HBV replication [33].

This property of NKT cells and specifically the result obtained by Kakimi *et al*. give an opportunity to the use of NKT cell activation as a therapeutic tool. More recently, in 2008, it was reported that the coadministration of αGalCer together with the surface antigen of the HBV (HBsAg) enhanced the induction and proliferation of anti-HBsAg cytotoxic T cells [34]. This result can represent a new adjuvanting method for therapeutic vaccination against CHB, which is more suitable for this hepatotropic pathogen, when applying the most recent knowledge on liver immunity.

Liver T cell response and NASVAC potential

The T cell-mediated protection against hepatotropic viruses, such as HBV or the hepatitis C virus (HCV), depends on a CD8+ T cell immune response that is able to control the emergence, distribution and expansion of the pathogen. In spite of a series of studies to characterize the phenotype and function of pathogen-specific T cells, both in the blood and the liver, only a few have addressed the mechanisms that make it possible for intrahepatic antigens to induce a T cell-mediated response as well as the sites where they are presented to CD8+ T cells *in vivo*.

Unprimed CD8+ T cells are localized within secondary lymphoid compartments and require two independent signals to become fully activated. The first signal is provided by the interaction with the peptide-MHC class I complex through the specific T cell receptor. The second signal, the co-stimulatory one, is antigen receptor-independent and critical for the full activation of CD8+ T cells [35]. In this way, while primed CD8+ T cells can be activated by any target cell presenting the antigen within the MHC I context, only "professional" and properly licensed bone marrow-derived APCs can initiate CD8+ T cell responses by expressing co-stimulatory molecules. These cells take the antigen from the infection site to the lymphoid organs.

It is currently discussed whether the antigen can be efficiently presented within the liver, or if this presentation is confined to the drainage lymph nodes. It should not be excluded that the antigens of pathogenic agents expressed within the liver could be passively up taken by LSEC and Kuffer cells and later presented to unprimed T cells which re-circulate through hepatic sinusoids or those localized at lymphoid aggregates within the portal tracts. Nevertheless, under non-inflammatory conditions, the induction of tolerance surpasses the enhancement of T cell immune responses [21].

It has been traditionally established that cells should migrate and that CD4+ and CD8+ lymphocytes activation must occur within the regional lymph nodes, where mostly unprimed CD8+ T cells can be found. Once activated, antigen-specific lymphocytes enter the blood stream and get access to the liver, where they exert their effector functions.

An alternative view considers that antigens are presented *in situ* by bone marrow-derived DCs, further activating the unprimed infiltrating lymphocytes. Interestingly, a recent study suggests that the priming of CD8+ T cells within lymph nodes and in the liver results in qualitatively different effector functions. In that study, the authors used a transgenic mouse model simultaneously expressing the antigen, both within

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the liver and the lymph nodes. They were able to demonstrate that CD8+ T cells induce hepatitis when T cells are primed within the lymph nodes. In contrast, a defective cytotoxic immune response with a decreased half-life of CD8+ T cells was observed when the priming occurred within the liver [14].

When the APCs are not infected, they are unable to properly process the antigen exogenously, with a deficient direct priming of CD8+ T cells. In this scenario, the only way to initiate a CD8+ T cell response is through cross-priming. In this process, an antigen within a cell is endocyted by another cell and "cross-presented" by the latter within the MHC I context to CD8+ T lymphocytes for priming.

At this point, it is relevant to notice that particulated antigens are especially efficient for cross-priming. HBsAg and HBcAg, which are present in the NASVAC formulation, are antigens that can be cross-primed by different presenting cell types. The HBcAg induces cross-priming after being assimilated by B cells, activating them very efficiently, and making them professional APCs to activate naïve T cells, even without T cell help [36, 37].

In the cross-priming models available, the cells infected by a virus are not a simple source of antigens, but they can also develop an active role for the quality and specificity of T cell priming, by delivering pre-processed antigens. Additionally, a certain adjuvanting effect can be attributed to the dying or already dead cells [38]. In this way, the balance between immune tolerance and the induction of the immune response can be modulated.

The efficacy of this mechanism depends on the number of infected cells, their viability, the amount of endogenously-expressed antigens and the inflammatory environment. While an exuberant inflammatory immune response could obviate some of the co-stimulatory requirements for CD8+ T priming, a limited response could be too weak to effectively present some antigens to CD8+ T cells [38].

In the case of the NASVAC therapeutic vaccine candidate, the coexistence of two particulated antigens with capacity to be cross-presented favors a pro-inflammatory scenario. This is not only due to the RNA associated to the HBcAg particle [39], but also to the presence of 180-230 monomers forming each particle, that increases the efficiency of the APC by the high number of antigens assimilated in a single endocytic event, compared to the assimilation of soluble proteins, that favors the efficiency of the presentation/cross-priming process.

*T*LRs and innate immunity activation within the liver

The reciprocal stimulation between Kupffer and NKs cells is triggered by their activation through TLRs by pathogen-derived antigens [40, 41], initiating the innate immune response.

The liver is constantly exposed to non-pathogenic dietary antigens and LPS from the intestinal flora. LPS as a TLR-4 ligand is a potent innate immunity stimulus, activating the professional APCs. Therefore, the liver must develop a mechanism to avoid developing a harmful immune response against dietary antigens directly reaching the intestine together with the LPS through the portal venous blood [42, 43].

The understanding of how the liver innate immunity works is very relevant for the study of hepatotropic viral infections. It is known that secretion of IL10 by Kupffer cells is a mechanism to modulate the host immune response against the pro-inflammatory cytokines secreted by the same Kupffer cells [25, 44, 45]. What favors the balance to one or the other side?

The binding of ligands coming from microorganisms to their respective TLRs on the cells of the innate immune system activates two different signaling pathways: one dependent on the myeloid differentiation factor 88 (MyD88), which finally results in the activation of the NF-κB transcription factor and the secretion of pro-inflammatory cytokines and the other, a MyD88- independent pathway, whose signal is transduced through the TRIF-IRF-3 complex, responsible for the synthesis of type I IFNs and pro-inflammatory cytokines associated to the activation of NF-κB [41, 46, 47]. Both MyD88 and TRIF can stimulate pro-inflammatory cytokine production through NF-κB, but IFN production is limited to the signaling through TRIF-IRF-3.

Gram-positive bacterial cell wall components such as lipoteichoic acid (LTA) are detected through TLR-2, and those from gram-negative bacteria through TLR-4. The TLR-2 pathway is strictly MyD88-dependent, while the TLR-4 pathway can use both MyoD88 and TRIF-IRF-3 pathways. Viral products are detected through the TLR3, which is strictly MyD88 independent and uses TRIF-IRF-3 [48, 49].

The effect of the LTA, poly-IC and LPS on TLRs 2, 3 and 4, respectively, were studied in cell cultures containing freshly isolated liver sinusoidal leukocytes from live donors of hepatic transplants. Results evidenced that IL10 produced by Kupffer cells through the MyD88 dependent pathway attenuated the levels of IFNγ secreted by NK cells. The double stranded RNA analogue poly-IC, which is detected by the TLR-3 via TRIF-IRF-3, did not induce a substantial synthesis of IL10, causing a strong secretion of IFNγ by the liver NK cells [50].

The ability of the liver to modulate inflammation through IL10 is an adaptation of the liver to the constant exposure of bacterial products coming from the intestine. It also points out to the TLR-3 as the receptor activating an inflammatory response mediated by NK cell stimulation, with fast production of IFNγ that stimulates hepatocytes and LSEC to secrete the CXCL9 cytokine to recruit T cells into the liver, modulating the hepatic damage [50].

Results of that study highlighted the existence of local immunological states within the liver that modulate towards tolerance by secreting IL10 in response to stimulation through TLR-2 and 4. This mechanism has a preserved antiviral activity mediated by the stimulation of TLR-3 through a MyD88-independent signaling pathway [50].

The release of IL10 by DCs through a MyD88-dependent signaling pathway was demonstrated through the activation via TLR-2 [52]. The secretion of IL10 by Kupffer cells following the stimulation with LPS has been well documented [25, 52].

The liver is an organ where activated CD8+ T cells are entrapped and undergo apoptosis during the development of a systemic immune response. The constant exposure to endotoxins coming from comensal bacte-

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ria in the intestine acts through TLR-4 and promotes the adhesion of activated T cells. It has been demonstrated that the liver loses its capacity to sequester activated CD8+ T cells in the absence of TLR-4, with an inverse correlation between the frequency of CD8+ T cells entrapped within the liver and the frequency of these cells in the circulation. In the absence of inflammation, TLR-4 ligands are relevant for the capacity of the liver to sequester activated CD8+ T cells. Therefore, the immune response is regulated under basal conditions [53].

The role of the liver in the systemic immune response is currently under scrutiny, since this organ is the second largest reservoir of CD8+ T cells in the body after the spleen. It is considered that recently activated lymphoblasts are localized at the hepatic sinusoids, depending on the adhesion molecules expressed by the sinusoidal epithelium. These cells are eliminated by Kupffer cells, decreasing the excess of activated T cells. This process, however, does not occur in memory cells, which can repopulate the memory of the systemic immunity. In this sense, it is considered that the liver regulates the peripheral immune homeostasis [54].

The association of therapeutic vaccine antigens to TLR-3 ligands allows the induction of a type of proinflammatory response in the case that those antigens reach the liver. That signal opposes the response produced by TLR-4 activation.

In summary, the immune response is reshaped within the liver by elements of the local environment, by detecting pathogen-associated molecular patterns (PAMPs). This mechanism is optimized to preserve the balance between self tolerance and the host defense. Given that the liver is continuously exposed to bacterial products, including the TLRs-2 and 4 ligands, it is inappropriate for these signals to promote inflammation or the innate immunity in the liver. In contrast, TLR-3 stimulation occurs in response to signals coming from viral infections. Therefore, the immune response, mediated by signaling through TRL-3 is appropriate due to its capacity to induce proinflammatory cytokines.

TLR-3 activation modulate the immunoprivileged condition of the liver

In the field of transplantology, the capacity of the liver to induce tolerance has been known for a long time. Liver transplantation induces the acceptance of other solid organs transplants from the same donor, which are rejected under other circumstances [55]. Autoimmune hepatitis due to the attack by B and T cells is a rare manifestation of autoimmune disease [56, 57]. Interestingly, diagnostic autoimmune hepatitis markers –such as anti-mitochondrial antibodies– are also found in healthy people [58]. Taken together, all these findings suggest the existence of mechanisms to protect this solid organ from the attack of the immune system. This is the reason why the liver is considered an immunoprivileged organ.

Some immunoreactivity studies against components of solid peripheral organs, such as pancreatic islet cells, salivary glands or thyroid antigens indicate that B or T cells could not be enough by their own to induce the disease. Additional inflammatory signals are required for an efficient induction of the disease

[59]. In line with these clinical observations, results coming from studies in animal models suggest that naïve T cells reactive against antigens expressed within the liver ignore the antigen [60] or are tolerogenized there [61, 62].

Inflammation resulting from naturally occurring systemic infections can positively regulate co-stimulatory molecules within the liver and this lead to the break of tolerance [63, 64]. Viruses, in addition to priming an adaptive immune response, can promote inflammatory responses due to their capacity to activate the innate immune system through TLRs [65].

It has been recently determined that activation of TLR3 and 7, which recognize double- and single-stranded RNA, respectively, promote autoimmunity in mice exhibiting a high frequency of functional autoreactive CD8+ T cells. The appearance and progression of the disease correlates with the production of IFNα [66, 67]. This suggests that production of proinflammatory cytokines such as IFNα and TNF-α can influence the development of autoimmunity.

The immune-mediated protection or destruction of the liver depends on two mechanisms: a) sensitization of T cells for antigens liver-expressed antigens, and b) migration of T cells to the target organ where they will exert their lytic function. Sensitization is controlled by first line factors comprising co-stimulatory factors, signals of the innate immune system and regulatory T cells [68, 69]. However, this is insufficient since the liver does not attract antigen specific cells due to a low level expression of chemokines. Thus, even at high levels of primed T cells in blood, very few of them migrate into the liver.

A recent study addressed the requirements for immune destruction of the liver in a mouse model expressing a protein of the lymphocytic choriomeningitis virus. A second line of the liver immunoprivileged condition is based on the TLR-3 signaling evidenced in that work. The proinflammatory signals triggered by the TLR-3 signaling can redirect CD8+ T cells towards the liver causing its destruction [70]. The mechanism mediating this process involved the upregulation of IFNα- and TNFα-dependent genes that resulted in CD8+ T cell relocation and migration [70].

In summary, the activation of the innate immunity by the stimuli provided through TLRs by microorganisms affects the balance between tolerance and immune response in the liver. The knowledge of mechanisms controlling this balance directly influences the selection of the most attractive adjuvant strategy for therapeutic vaccine candidate antigens against CHB and other chronic infection of the liver.

NASVAC : a vaccine formulation containing soluble and particulated antigens associated to TLRs 3 and 7 ligands

Innate immunity activation defects of an immature immune system during HBV infection can lead to a deficient response and chronicity. A given vaccine candidate must fulfill the antigenic and the immune system requirements of the liver innate immune system and, at the same time, fill the gaps on the innate response, in order to establish an immune response able to control HBV. The latter can be achieved by including ligands able to trigger signals similar to the

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pro-inflammatory ones that follow the stimulation through TLR-3, which finally redirect CD8+ T cells towards the liver [70].

Nevertheless, only a few currently available adjuvants able to activate TLR-3 are being assayed for this purpose. Poly-IC derivatives, the most studiedTLR-3 ligands, have caused relevant adverse reactions limiting their use. High fever in most of the volunteers, lymphopenia and hypotension episodes are among the most frequent adverse events reported [71].

The HBV nucleocapsid (HBcAg) antigen produced at the Center for Genetic Engineering and Biotechnology (CIGB), is a particulated nucleoprotein (Figure 1A and B) with an electron-dense core (Figure 1A). It contains an RNA-like nuclear component (Figure 1C) that has been identified in the literature as double-[72] and single-stranded [39] RNA. The simultaneous deploy in time and space of both the antigen and the adjuvant in this 28 nm HBcAg particle favors that antigens and their TLR-3 and -7 ligands could co-localized within the same endocytic vacuole together where TLR-3 and -7 are present and able to be stimulated. Therefore, this is an effective way to optimize the specific activation.

The physical association between HBsAg and HBcAg also favors the simultaneous deploy with its TLR-3 and -7. This aggregation confers a marked enhancement and modulation of the immune response against both antigens [73, 74].

Another advantage of using nuclear material nucleocapsids is that it is cost-saving from the production viewpoint. But its main impact is at the clinical investigation phase, related to its regulatory suitability, because of the advantage of minimizing contaminant RNA, that become an adjuvant, to the level required for safety concerns. Additionally, a recent study [75] showed that after enzymatic removal of RNA from the HBcAg particle, the modulatory effect of the nucleic acid that it contains can be reproduced by adding an amount of free RNA 1000 times higher than the amount contained in the particle. This fact confirmed the immunomodulatory effect of the trace RNA present in the HBcAg preparation.

Another aspect is related to the protection of the nucleic acid by the protein itself. RNA frailty is well known and in this particular study, the RNAse treatment required as high as 2 mg/mL concentrations of the enzyme incubated for 6 h at 37 ºC, much higher than the concentrations naturally found for this enzyme. This protection favors the "contaminant" RNA become adjuvant to reach unaltered the endocytic vacuoles (Figure 1C). The absence of any other compound at trace levels in addition to this, confers safety properties to the HBcAg nucleocapsid formulation, as proven in healthy volunteers and chronic patients [76, 77].

A number of immunological properties of HBcAg are related to its particulated nature, favoring its use for therapeutic vaccination, such as: a) its capacity to simultaneously perform both as a T-dependent and –independent antigen [78]; b) the immunogenicity of the particulated variant primes over that of the soluble antigenic form, estimated as 1000-times [79]; c) its capacity to enhance mainly Th1 responses vs. the HBcAg, preferentially priming those cells that particularly promote a Th2-like response [80]; d) the capacity of HBcAg-specific Th cells to help not only HBcAg-specific B cells but also anti-HBsAg responses [81]; and e) the property of being an excellent carrier protein [82]. All these properties are associated to the particulated structure of the HBcAg and its physical-chemical nature as a nucleoprotein [73].

HBV infection in neonates, which has been characterized as immune tolerant due to their immature immune system, becomes persistent. This is, infection extends over the time and can "peacefully" coexist without liver damage, or activate the immune system against the organ after the second or third decade of the patient's lifetime. The concentrations of the viral antigens during this initial phase of the infection are very high.

The immune response against a highly replicating hepatotropic pathogen such as HBV that infects a high number of cells could be devastating and affect the vital functions of the liver. A mechanism has been described in the liver that eliminates the infection without affecting the organ. It is called cytokine-media-

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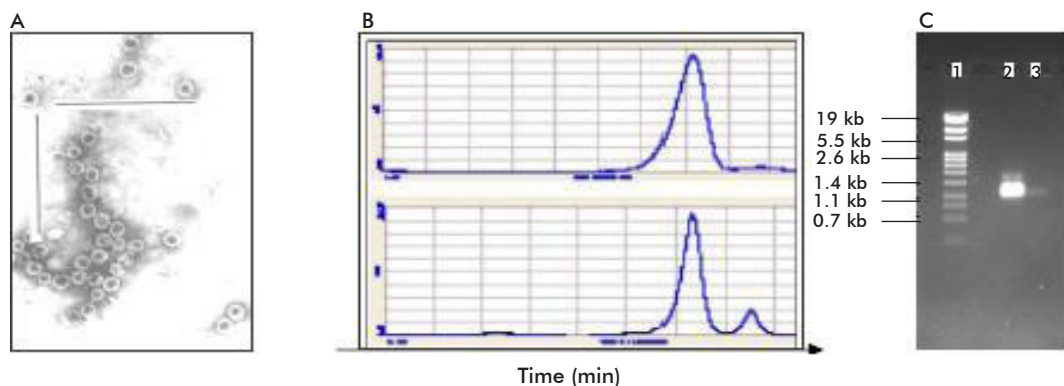
Figure 1. Study of the physical aspect, chromatographic profile and presence of nucleic acids associated to the HBcAg antigen. A) transmission electron microscopy. Bars represent 200 nm. B) Study of the size and homogeneity of the HBcAg particle by HPLC. The uppert and lower charts show the HBsAg and HBcAg chromatographic profiles. Runs were made on TSK-6000 columns at a flow rate of 0.25 mL/min in PBS, applying 100 μL of each protein. Detection was carried out at 280 nm. Both particles had a retention time of 73 min. The second peak in the HBcAg chart belongs to a nonproteic component in the solution where the HBcAg was diluted (EDTA). C) Detection of the presence of nucleic acids associated to the HBcAg by agarose gel electrophoresis. Lane 1-DNA molecular weight marker; lane 2- HBcAg; lane 3-HBcAg treated with RNAse (2 mg/mL) at 37 ºC for 6 h. The HBcAg subjected to treatment was at 0.447 mg/mL.

ted viral control, and is a way to preserve the physical integrity of liver cells and control viral replication, simultaneously [83].

Regarding the safety of a vaccine candidate, it is also important to eliminate the virus without damaging the liver. This is associated to the reinforcement of a Th1 response pattern as that generated by NAS-VAC and the cytokine-mediated virus elimination process. Preliminary findings from chronic patients demonstrate the elimination of the virus with a slight increase of the transaminase levels, suggesting that the control of the virus is established by mechanisms similar in nature to that mentioned above but without ruling out cytolytic processes [77].

In summary, even in the presence of CD8+ T cells induced by therapeutic vaccination, signaling through TLR-3 must be considered to subvert the immunoprivileged condition of the liver. This allows immune cells to migrate into the liver. The presence of TLR-3 ligands within the recombinant HBcAg particle is a significant step for that subversion and, up to now, the clinical evidence demonstrates that NASVAC is a vaccine candidate that can be regarded as safe.

Conclusions

Given the strategic anatomical situation of the liver, this organ is constantly exposed to dietary antigens and also to the degradation debris of comensal and pathogenic bacteria. In this antigenic environment and because of the requirement of preserving the multiple and essential functions of the liver, a particular immunological system has evolved within this organ.

Both conventional and unconventional innate immune cells are unusually abundant in the liver compared to the systemic immune system. In addition to DCs and Kupffer cells, a subset of liver non-hematopoietic cells (which include LSEC), stellated and parenchymal cells, all function as APCs. These cells present antigens in a context of immunosuppressive cytokines

and inhibitory cell surface ligands, determining that the immune response against the antigens in the liver frequently leads to tolerance.

A group of relevant human pathogenic agents, including the HBV and HCV viruses, exploit this tolerogenic environment of the liver and subvert immunity, establishing persistent infections.

The detection of the PAMPs by cells with antigen presenting functions in the liver constantly reshapes the resulting immune response. This mechanism is optimized for maintaining the balance between tolerance and host defense. Bacterial products able to stimulate TLR-2 and -4 promote anti-inflammatory signals in the liver as an adaptation, due to the high influence of this type of signals (LPS and LTA) coming from the intestine in the blood stream. On the other hand, the stimulation of TLR-3 in response to signals of viral infections promotes inflammatory responses. Unraveling this complex signaling is very useful to optimize therapeutic vaccine candidates as NASVAC, which associates very small and effective amounts of TLR-3 and -7 to its antigens.

The immune response or tolerance can be manipulated by administering therapeutic vaccines, based on the analysis of mechanisms able to induce immune responses similar to those naturally shown as effective to control chronic infections. In addition to the nature of the immune response in the liver, the gaps or functional problems in the subsets of presenting, effector and regulatory cells and the immunopathogenic mechanisms of the different viruses must be studied.

Similarly, the requirements for immune response activation under non-physiological conditions must be determined, to face stimuli of different nature within the context of abnormal liver conditions. By these means, the hepatic immune system would be manipulated in such a way to use innate immune activators that resemble the effect of those normal mediators of a real activation of liver immunity.

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