A Potentially Efficacious Approach for Treating HBV Viremia Using Combinatorial Cocktail Treatment

Yifei Hu[1], Junqing Yang[2] and Haotian Zhou[3,*]
[1]*University of California-San Diego, 9500 Gilman Dr, La Jolla, CA 92093, U.S.A.*
[2]*Lafayette College, Easton, PA 18042, U.S.A.*
[3]*Northwestern Polytechnical University, Xian, 710129, China*

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Abstract: Hepatitis B is one of the most prevalent diseases in the world and it covers a large population across the globe. The main cause–hepatitis B virus (HBV) has been studied for over 50 years. It targets hepatocytes and utilizes multiple mechanisms to replicate, survive and can cause acute or chronic infection. HBV viremia causes the impairment of T cell function which is a hallmark of chronic infection, making it an intractable virus to eliminate. Currently, common treatments for HBV viremia are using immunomodulatory agents such as nucleoside analogues. However, these drugs only suppress viral protein production and viral replication. HBsAg (hepatitis B surface antigen) remains on a certain level in serum and thereby the risk of hepatocellular carcinoma cannot be eradicated. In this study, we mainly review 3 pieces of researches, each of which introduces novel immunotherapy for chronic infection of HBV. And we hypothesize that the combinatorial treatment would potentially increase the efficacy for treating HBV viremia by increasing the number of HBV-specific CD8 T cells and would enhance the HBV-specific T cell functions, and further suppress the chronic infection.

1 INTRODUCTION

Hepatitis B is a prevalent viral infection that targets and injures the liver. As a potentially life-threatening infection, it continues to be a significant global health problem. Hepatitis B can cause both acute and chronic diseases. Moreover, persistent infection frequently results in decompensated cirrhosis and hepatocellular carcinoma. According to WHO data, more than 296 million people lived with chronic hepatitis B infection in 2019, and the diseased population increased by 1.5 million per year (World Health Organization, 2021).

Hepatitis B is caused by HBV, a type of double-stranded DNA virus in the family of Hepadnaviridae. Some markers used for serologic detection of HBV infection have been discovered, including HBsAg, anti-HBs (antibody to HBsAg), IgM anti-HBc (antibodies to hepatitis B core antigen), and IgG anti-HBc (immunoglobulin class G anti-HBc). Moreover, at least one of them is present during HBV infection, which assists the detection or quantification of HBV (Jennifer, 2015). Blood or infected bodily fluids are two common ways for HBV transmission, and HBV can survive (remains infectious) in the external environment for at least seven days.

After HBV enters the blood, an irreversible process of recognition and combination is followed. Na+-taurocholate co-transporting polypeptide (NTCP) plays an essential role in assisting HBV entering the hepatocyte as the functional cellular receptor. It binds to HBV, along with its protein shells (Yan, 2012). Then, the initiation of the cccDNA (covalently closed circular DNA) biogenesis is conducted by HBV with the participation of cellular DNA repair enzymes. After the cccDNA minichromosome is formed, it provides all essential viral RNAs and promotes viral replication and protein production. A high level of proteins in the serum maintains the chronic infection.

Although hepatitis B is vaccine-preventable, the vaccine is not effective in infected patients. Current treatments against hepatitis B include liver transplant, immunomodulatory agents such as conventional or pegylated type I interferons (interferon-α), and nucleoside analogues (direct-acting antivirals). Interferon-α produces several antiviral effects in infected cells, including the production of antiviral

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proteins and viral RNA suppression, but could only result in a cure among a small group of patients (Janssen, 2005; Lau, 2005). Nucleosides analogues (NAs), on the other hand, serve as direct-acting antivirals that can block the synthesis of viral DNA and thereby suppresses viral replication. Nowadays, NAs is considered as a well-tolerated drug that impactfully inhibits the activity of HBV polymerase with safety. However, nucleotide analogues could only suppress viral replication. After cessation of antiviral treatment, it has been found that the antiviral function is significantly constrained by the rebounding viremia and increasing resistance mutations of the drug (Locarnini, 2006; Zoulim, 2009). For these reasons, they can hardly eradicate HBV, so long-term treatment is required (Lannacone, 2021).

The functional exhaustion of virus-specific CD8 T cells is a significant trait of persistent HBV infection (Klenerman, 2005). According to previous researches on individual patients, impairment of HBV-specific T cells under viral load was revealed. Multiple essential cellular processes act simultaneously, forming the functional and quantitative defects of HBV-specific T cell response (Lopes, 2008; Bengsch, 2014). In the model of chronic infection to functional LCMV (lymphocytic choriomeningitis virus)-specific effector, the persistence of virus results in a hierarchical impairment and finally a depletion of T cell functions, with the sequential reduction of IL-2 production, cytotoxicity TNF-a, and IFN-c production (Wherry, 2007). According to the conceivable explanation of the dysfunction of HBV-specific T cells, the alternative pathway–therapeutic vaccination is regarded as a more efficient approach to induce immune responses against HBV viremia. Nevertheless, it failed to meet the expectations and does not always show efficiency, according to several studies (von, 2000; Dikici, 2003; Nevens, 2003). Furthermore, limited effectiveness is revealed under viral load conditions since the therapeutic vaccination cannot potently stimulate the dysfunctional T cells to produce antibodies (Wherry, 2005; Nisii, 2006).

To better understanding the mechanism and treatment method of HBV Viremia, this paper will introduce three key primary researches, each of which puts forward a novel and potentially effective therapy either for blocking HBV entry or reinforcing the antiviral effects of HBV-specific T cells. A combinatorial treatment is discussed, and the effect is predicted based on reviewed primary researches.

2 PRIMARY RESEARCH

2.1 Enhancing Virus-Specific Immunity in Vivo by Combining Therapeutic Vaccination and PD-L1 Blockade in Chronic Hepadnaviral Infection (Jia, 2014)

It is suggested that one of the main causes of the T-cell exhaustion is the interaction between the inhibitory receptor programmed death-1 (PD-1) and its ligands (PD-L1) (Barber, 2006). Figure 1 shows the interaction structure of PD-1 and PD-L1.

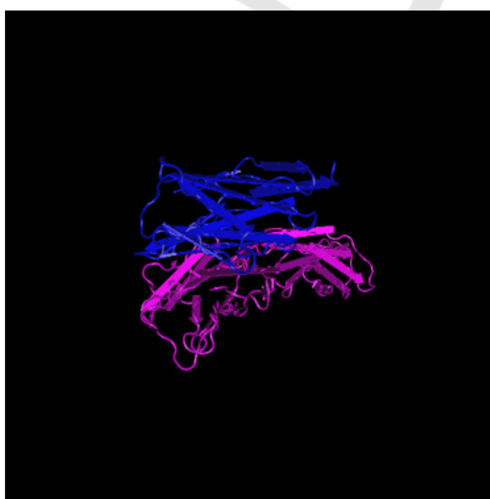

Figure 1. Crystal Structure of the PD-1/PD-L1 Complex. Molecular that colored in blue is the PD-1; molecular that colored in purple is the PD-L1. They form a dimeric complex (Barber, 2006).

The expression of PD-1 was found in different human chronic infections, including HBV. In this study, the T cell function was improved through inhibiting the PD-1/PD-L1 in vivo in the WHV model, and the study designed a combinatorial therapy that included therapeutic vaccination, traditional antiviral treatment of nucleosides analogous ETV, and the blockade of PD-1 receptor on T-cell, which was shown to be able to continuously suppress WHV replication comparing with NAs treatment or with therapeutic vaccination. This study further examined the effect of this combinatorial therapy in WHV transgenic mice, which disrupts the immune tolerance to WHV antigens and decreases viral load. The result suggested that this is a potentially new approach for producing efficacious therapeutic vaccination against chronic infection of HBV.

2.2 A Practical Approach to Immunotherapy of Hepatocellular Carcinoma using T Cells Redirected Against Hepatitis B Virus (Koh, 2013)

It is shown that the current treatment for HCC cells has excellent limitations, which induces exogenous HBV-specific TCR by viral vectors. The process is dangerous due to the non-exclusiveness of HBV antigen. Adoptive T-cell therapy could cause severe damage to the liver because normal hepatocytes may also express HBV antigens. Moreover, it could increase the risk of oncogenes activation, not to mention the costs and complexity of the process. These limitations could be overcome using mRNA electroporation, which could rapidly produce a significant number of HBV-specific T cells.

2.3 Blocking Entry of Hepatitis B and D Viruses to Hepatocytes as a Novel Immunotherapy for Treating Chronic Infections (Maravelia, 2021)

Nucleoside analogues (NAs) are a commonly used HBV therapy. By suppressing the reverse transcriptase (of HBV polymerase), it can inhibit the production and secretion of viral protein and the biogenesis of cccDNA, which is the primary cause for HBV chronicity. However, NAs only limit the replication of the virus and requires long-term treatment. It cannot eradicate the HBsAg in serum and thus cannot avoid the risk of hepatocellular carcinoma (HCC) (Papatheodoridis, 2011; Zoulim, 2012). During the chronic infection, an important characteristic is the dysfunction of HBV-specific T cell response (Chen, 2000; Chen, 2005; Chen, 2004; Milich, 2016). With the overproduction of small HBsAg, antibodies to small HBsAg are blocked, which enhance the middle and large HBsAg, containing PreS1, PreS2, etc (Short, 2009; Rydell, 2017). PreS1 domain is essential for transporting HBV to hepatocytes (Ni, 2010; Ni, 2014). Therefore, in this study, a novel therapy against HBV and potentially HDV was designed, using a PreS1 sequence linked to the HDV antigen, circumventing the dependence and participation on HBV-specific T cell response, which is exhausted in chronically infected hosts, in the purpose of inducing the production of endogenous antibodies specific to the PreS1 antigen. In this therapy, HDV serves as a heterologous T-cell epitope carrier. It supports the production of PreS1 antibodies and finally aims to block the entry of HBV.

Nevertheless, the third approach will not be put to use in the following research since we intend to enhance the HBV-specific T cell response instead of bypassing it. Nevertheless, blocking the entry of HBV provides us with an innovative way to induce stable loss of HBsAg that may be applied in the future.

Here, based on the researches above, we aim to examine whether in vivo blockade of the PD-1 pathway and therapeutic vaccination in combination with NAs treatment and mRNA electroporation anti-HBV TCR on T cells could improve the outcome of chronic HBV treatment and finally educe a solution of chronic infection of HBV in the mice model. HBV transgenic mice would first receive entecavir (ETV) to suppress the viral replication. They would be treated with therapeutic vaccination, followed by introducing HBV-specific TCR on T cells (using mRNA electroporation) and in vivo blockade of the PD-1 receptor. We hypothesize that this combinatorial treatment would increase HBV-specific CD8 T cell number and enhance the immune response of T cells.

3 MATERIAL AND METHODS

3.1 HBV Transgenic Mice

In this study, 1.3x HBV transgenic mice will be used as the experiment model. Hepatitis B virus could perform viral replication and produce HBV-infected protein in the mice. With this advantage, the measurement of HBV concentration will be easy to

perform by examining the amount of HBV genome, HBsAg, and HBeAg in the serum. The amount of functional T cell concentration in the serum is also able to be examined, providing a pathway to determine the efficiency of anti PD-1/PD-L1 and mRNA electroporation therapies.

3.2 Entecavir and Therapeutic Vaccine

Common and traditional treatments will be set for multiple control or combinatorial groups to compare the results and highlight the differences.

3.3 In vivo PD-L1 Blockade

Rat anti-mouse PD-L1 antibody (10F:9G2) will be received by intraperitoneal injection. (5 times every 3 days.)

3.4 Electroporation of TCR

During electroporation, the host cell from peripheral blood mononuclear cells will be suspended. TCR mRNA is then added into the solution as the desired material to be electroporated into the cell. The mixture is placed in a cuvette. An electrical circuit is placed closed around the mixture for the electroporation process. An electrical pulse at a specific voltage that only lasts a few microseconds is discharged through the mixture. This process disturbs the phospholipid bilayer of the cell membrane that forms temporary pores around it. The increased electric potential created during electroporation across the membrane will allow mRNA to be driven across the membrane through the pores into the nucleus of the cell. After the electroporation, the cells will be resuspended and cultured until analysis. Large scale electroporation is done similarly, but with larger amount of TCR mRNA ("Electroporation." Thermo Fisher Scientific – US; Potter, 2018; Koh, 2013).

3.5 HBV DNA Quantification

Platinum SYBR Green Kit will be used for real-time PCR, to quantify the HBV DNA.

3.6 Analysis of cccDNA and HBV Replication

The QIAamp Tissue Kit will be used to extract entire DNA from transgenic mice. HBV replication will be analyzed by Southern blot hybridization, and PCR is once more to be conducted for determining HBV cccDNA.

3.7 Evaluation of Glutamic Oxaloacetic Transaminase Levels

According to standard diagnostic procedures in the Central Laboratory of the University Hospital Essen, the level of glutamic oxaloacetic transaminase is to be measured. And it is thought to be promoted if the values are greater than 50 international units per ml.

4 EXPERIMENTAL SEQUENCE

The experiment contains 9 groups of transgenic HBV mice, each group has 5 mice in total with different combinations of therapies. The experiment will last 25 weeks, and blood will be drawn from the tail of the mice weekly to test the amount of HBV DNA and TCR of the mice. All 9 groups of experiments will be administered at the same time, and detailed data and observations will be recorded after each treatment is given to the mice and at the end of each week.

Group A is the mock trial that contains 5 transgenic HBV mice with no treatment, given.

Group B mice will be given ETV (oral gavage) treatment. The appropriate dosage of ETV is given to each mouse daily. For the previous 12 weeks, 0.2mg of ETV will be given to each mice per day. For the remaining weeks, the amount of ETV will be increased to 1.5mg per week per mouse.

Group C mice will be given a total of 13 doses of therapeutic vaccination, in which they will receive a pre-treatment of cardiotoxin by intramuscular injection a week before the start of the first dose of vaccination. After that, they will receive one dose of vaccination via intramuscular injection every two weeks for 12 doses.

Group D mice will receive anti-PD1 mAB treatment. 200 ug of anti-mouse PD-L1 antibody (10F:9G2) will be received 5 times every 3 days.

Group E mice will receive electroporation treatment. Peripheral blood will be drawn out from each mouse to undergo electroporation of mRNA TCR, then reinjected into the mouse. Electroporation treatment will be administered at the beginning of each week, and blood will be drawn at the end of each week for testing.

Starting from Group F, mice will be administered with mixtures of treatment. For Group F, mice will be receiving ETV and therapeutic vaccination. ETV will be given in the same dosage as Group B mice, with 0.2mg per mouse given daily for the previous 12 weeks and 1.5 mg throughout the week per mouse given for the remaining weeks. Therapeutic vaccination is also given to each mice similar to those

of Group C, in which 13 doses of intramuscular injection treatments will be given. The two kinds of treatments will be administered simultaneously.

Group G contains three treatments. The mice will be receiving ETV and therapeutic vaccination in a similar fashion as Group F, as well as anti-PD1 treatment similar to that of the Group D mice, in which each mouse will be given 200ug of anti-mouse PD-L1 antibody 5 times every 3 days. The three treatments will be administered simultaneously.

Group H contains three treatments. In addition to ETV and therapeutic vaccination treatment as the mice from Group F, the Group H mice will also be receiving electroporation of mRNA TCR treatment similar to that of group E, in which the mice will be administered one electroporation treatment per week, simultaneously with the other two treatment.

The last group, group I, of mice will receive the combination of all 4 kinds of treatments: the ETV, therapeutic vaccination, anti-PD1, as well as electroporation of mRNA TCR. The treatments will be given independently and simultaneously of each other, each in the same way as previously described for the whole duration of 25 weeks.

5 PREDICTED RESULTS

For the mock group, normal viral replication and damage to hepatocytes are expected.

For ETV treated group, we expect the viral replication will be reduced as the treatment begin, and the viral replication will rise again after stopping the treatment.

For therapeutic vaccination, as mentioned in the introduction, due to the large viral load in the model, the effectiveness of the vaccination will be relatively low, which should be shown as the HBV genome is not decreased very much.

With known information on HBV, the expression of PD-1/PD-L1 from HBV could exhaust the T-cells, so for anti-PD-1 treatment, this inhibition of PD-1/PD-L1 receptor could help reactivate the dysfunctional T-cells and help to control the HBV genome concentration in the blood sample. However, we expect the unregulated viral replication will decrease the efficiency of this inhibition process.

From the former study, HBV-specific TCR mRNA electroporation treatment is expected to increase the amount of HBV-specific T-cells in a short period and help suppress the viral replication. In this group, we also expect the number of active T cells will increase as the treatment start and decrease significantly after 72 hours20, and this reduction of active T cells is assumed as the result of the PD-1 expression of HBV.

In the combinatorial treatment of ETV and therapeutic vaccination, an increase of effectiveness of therapeutic vaccination is expected which should be indicated by the further decrease on HBV genome compared to ETV group, but as the treatment stops a bounce of the concentration of HBV DNA are expected.

As we added anti-PD-1 treatment in the former group, a prolonged suppression is expected as indicated before. 18

However, when the mRNA electroporation of HBV-specific TCR is added to the therapy, a further decrease in HBV genome concentration in serum is not very likely, because the new HBV-specific T cells are still affected by PD-1/PD-L1 which leads to the dysfunction of T cells.

For the final group, we expect to see the number of HBV-specific T cells increase drastically, and they will constantly exist and perform antiviral functions to consistently decrease the HBV genome. Also, because of the robust immune response caused by the excess amount of T cells, the side-effect is possible to be observed.

6 CONCLUSION

From the prediction of the result of the nine groups, we conclude that control on the PD-1 receptor is seemed to be the precursor of a prolonged and consistent suppression on HBV because groups without the regulation on PD-1 receptor are not able to provide the model a constant decrease on HBV. The combinatorial therapies work and possibly cure only when the immune system works effectively. A better understanding and strategy on regulating PD-1 receptors could help treat HBV and other related viruses with similar mechanisms in later studies. Also, there is a concern that if the number of T-cells is too large and does not have a system to regulate their quantity due to the TCR electroporation and anti-PD-1 treatment, the immune system could not activate or inactivate the T cells properly. Some data suggested that PD-1 is an essential cause for the exhaustion of T cells. Because immunotolerance is highly preserved and autoimmunity is impeded as well. In addition, among patients with cancer, the PD-1 stoppage with monoclonal antibodies usually leads to immune-related severe adverse events in various tissues such as the liver, skin, and lung.

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Yifei Hu, Junqing Yang, and Haotian Zhou contributed equally to this work and should be considered co-first authors.

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