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UNIVERSIDADE DO PORTO

Mechanisms of early immune activation in response to hepatitis B virus

Sílvia Margarida Teixeira Vilarinho

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Orientador – Dr. Jody L. Baron, Assistant Professor of Medicine, University of California San Francisco

Co-orientador – Professor Doutor Rui Appelberg, Professor Catedrático, Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

This dissertation is dedicated to the memory of my grandmother Emília Brandão,
who passed away victim of a liver-related disease
while I was performing these studies

Table of Contents

List of Figures

List of Tables

Abbreviations

Ab – Antibody
Ag - Antigen
α-GalCer – Alpha-Galactosylceramide
ALT - Alanine Aminotransferase
APAP - Acetominophen
APC – Antigen Presenting Cell
BCR – B Cell Receptor
cccDNA – covalently closed circular Deoxyribonucleic Acid
CMV – Cytomegalovirus
ConA – Concanavalin A
CTL – cytotoxic T lymphocyte
DAP10 - DNAX Activating Protein of 10 kDa
DAP12 - DNAX Activating Protein of 12 kDa
DC - Dendritic Cell
DHBV – Duck Hepatitis B Virus
DNA - Deoxyribonucleic Acid
ER- Endoplasmic Reticulum
FCH – Fibrosing Cholestatic Hepatitis
GI – Gastrointestinal
GSHV – Ground Squirrel Hepatitis Virus
HBcAg - Hepatitis B core antigen
HBeAg – Hepatitis B e antigen
HBsAg - Hepatitis B surface antigen
HBV - Hepatitis B Virus
HCMV – Human Cytomegalovirus
HCV - Hepatitis C Virus
HIV - Human Immunodeficiency Virus
HLA – Histocompatibility Leukocyte Antigen
HSV – Herpes Simplex Virus
IEL – intra-epithelial lymphocytes
IFN - Interferon
Ig - Immunoglobulin

IL – Interleukin

KSHV - Kaposi's Sarcoma-Associated Herpesvirus

LSECs – Liver Sinusoidal Endothelial Cells

mAb – monoclonal Antibody

MHC – Major Histocompatibility Complex

MHV - Mouse Hepatitis Virus

MIC(A or B) - MHC class I related-chain A or B

mRNA – messenger Ribonucleic Acid

MULT1 - Murine UL16-binding protein-like transcript-1

NASH – Non-Alcoholic Steatohepatitis

NK - Natural Killer

NKT - Natural Killer T

OVA - Ovalbumin

PBMC – Peripheral Blood Mononuclear Cell

PCR – Polymerase Chain Reaction

PI - Phosphatidylinositol

RAE-1 - Retinoic Acid Early inducible-1

RAG-1 - Recombinase Activating Gene-1

RNA - Ribonucleic Acid

RT-PCR – Reverse Transcription - Polymerase Chain Reaction

TCR – T Cell Receptor

Tg – Transgenic

Th – T helper

TNF - Tumor Necrosis Factor

ULBPs - UL-16-Binding Proteins

WHO – World Health Organization

WHV – Woodchuck Hepatitis Virus

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ABSTRACTS

ABSTRACT

Hepatitis B virus (HBV), a double-stranded DNA virus, is a major cause of acute and chronic hepatitis in humans. HBV infection itself is non-cytopathic, and it is the immune response to the viral antigens that causes hepatic pathology.

This work seeks to address the molecular and cellular mechanisms involved in the early immune recognition and subsequent acute immune response to HBV-infected cells, using a transgenic mouse model of primary HBV infection. It was previously established that the acute liver injury observed in these mice is mediated by non-classical, type II NKT cells, which are CD1d-restricted, but non-reactive to α-galactosylceramide (α-GalCer). We now have uncovered a molecular mechanism by which these non-classical NKT cells become activated in response to HBV-positive livers. Our studies demonstrated that surface expression of NKG2D and one of its ligands (Retinoic acid early inducible-1 or RAE-1) are modulated in a HBV-dependent manner. Furthermore, blockade of a NKG2D–ligand interaction completely prevented the HBV- and CD1d-dependent, non-classical NKT cell-mediated acute hepatitis and liver injury. A conclusion of these studies is that NKG2D, CD1d and HBV are required for non-classical, type II NKT cell activation and the initiation of acute hepatitis in our model. Thus, these studies have major implications for understanding activation of NKT cells and identifies a potential new therapeutic target in treating hepatitis B viral infection.

In mouse NK cells, NKG2D associates with DAP10 and DAP12 adapter molecules, but it is still unclear in NKT cells which adapter(s) are required for NKG2D signaling. Thus, we investigated which NKG2D adapter molecule(s) are involved in propagation of NKG2D signals at the time of acute hepatitis in our model of primary HBV infection. To address this question, we performed experiments using *Dap10*$^{-/-}$ and *Dap12*$^{-/-}$ mice. A conclusion of these studies is that ablation of the *Dap12* gene from the effector cells results in impaired acute hepatitis, suggesting that NKG2D requires DAP12 for signaling in NKT cells. However, in the absence of the *Dap10* gene a more severe acute hepatitis was observed.

Finally, we investigated whether other intra-hepatic innate immune cells might be involved in HBV recognition, potentially generating signals that could contribute to the hepatic immunological environment in which immune responses are primed. Our results demonstrated a mild hepatic necrosis in the HBV-Tg *Rag-1*$^{-/-}$ mice pre-adoptive transfer, and production of the cytokines IFNγ and IL-4. In addition, we demonstrated that the mild hepatic necrosis is IFNγ and NK cell-independent. Furthermore, our data indicate that the baseline

amount of IFNγ as well as the NK cell frequency within the livers of HBV-Tg *Rag-1*$^{-/-}$ recipient mice did not affect the non-classical type II NKT cell-mediated acute hepatitis. Understanding the mechanism responsible for the mild hepatic necrosis observed in HBV-transgenic mice that lack B and T lymphocytes should give insight into the role of innate immunity in HBV immunopathogenesis.

RESUMO

O vírus da hepatite B (VHB) é um vírus de ADN de cadeia dupla e uma importante causa de hepatite aguda e crónica no ser humano. O VHB, em si mesmo, não é citopático, sendo a resposta imunitária aos antigénios virais a causa da patologia hepática.

Este trabalho tem como objectivo o estudo dos mecanismos moleculares e celulares envolvidos no reconhecimento inicial do vírus pelo sistema imunitário e na subsequente resposta imunitária aguda contra as células infectadas pelo VHB, através da utilização de um modelo de ratinho transgénico que mimetiza a infecção primária humana pelo VHB. Estudos anteriores demonstraram que a patologia hepática observada nestes animais transgénicos é mediada por um grupo de células NKT, de tipo II, não clássicas, as quais são restrictas à molécula CD1d, mas não reactivas à α-galactosylceramide (α-GalCer). Este trabalho identifica o mecanismo molecular pelo qual estas células NKT, não clássicas, são activadas em resposta à infecção pelo VHB. Os nossos estudos demonstraram que a expressão à superfície do receptor NKG2D e de um dos seus ligandos (Retinoic acid early inducible-1 ou RAE-1) se encontram modulados de uma forma dependente da presença do VHB. Assim, o bloqueio da interacção entre NKG2D e o seu ligando previne totalmente a hepatite aguda neste modelo. Em conclusão, os nossos resultados mostram que é necessária a presença de NKG2D, CD1d e VHB para a activação das células NKT, não clássicas, e para a iniciação da hepatite aguda no nosso modelo. Estes resultados apresentam grandes implicações no estudo da activação das células NKT assim como identificam um novo potencial alvo terapêutico no tratamento da hepatite B.

O receptor NKG2D, nas células NK do ratinho, associasse com ambas as moléculas adaptadoras DAP10 e DAP12. Porém, ainda não se conhece quais a(s) molécula(s) que se associam com o NKG2D nas células NKT. Assim, propusemo-nos investigar quais são as moléculas necessárias à transdução do sinal via NKG2D aquando da hepatite aguda no nosso modelo. De forma a responder a esta questão realizámos experiências usando ratinhos "knock-out" para o gene *Dap10* ou para o gene *Dap12*. Em conclusão, estes estudos mostram que a hepatite aguda é prevenida quando as células efectoras não possuem o gene *Dap12*, o que sugere que o receptor NKG2D necessita da molécula DAP12 para a transdução do sinal nas células NKT. No entanto, na ausência do gene *Dap10* observasse uma hepatite aguda mais grave.

Por último, investigámos se outras células intra-hepáticas do sistema imunitário inato poderão estar envolvidas no reconhecimento do VHB, gerando sinais que poderão

contribuir para o ambiente imunológico hepático basal onde as respostas imunitárias são induzidas. Os nossos resultados indicam que os ratinhos transgénicos para o VHB, mesmo antes de receberem a transferência de células, apresentam uma necrose hepática moderada bem como secreção das citoquinas IFNγ e IL-4. Porém, a necrose hepática observada nestes animais é independente da presença de IFNγ e de células NK. Os nossos resultados também mostram que a quantidade de IFNγ assim como a frequência de células NK nos fígados dos ratinhos transgénicos para o VHB *Rag-1*$^{-/-}$ não afecta a hepatite aguda resultante da activação das células NKT de tipo II, não clássicas. A compreensão do mecanismo responsável pela necrose hepática moderada presente nos ratinhos transgénicos para o VHB, na ausência de linfócitos B e T, deverá ajudar a decifrar a função do sistema imunitário inato na patologia causada pelo VHB.

RÉSUMÉ

Le virus de l'hépatite B (VHB), un virus à ADN double brin, est une cause majeure d'hépatite aigue et chronique chez l'humain. L'infection par VHB n'est pas cytopathique en soi, et c'est la réponse immunitaire aux antigènes viraux qui cause la pathologie hépatique.

Ce travail cherche à adresser les mécanismes moléculaires et cellulaires de la reconnaissance immunitaire primaire puis de la réponse immunitaire aigue aux cellules infectées par VHB, en utilisant un modèle de souris transgénique qui mime une infection VHB (VHB-Tg). Il a été préalablement démontré que la lésion hépatique aigue observée dans ces souris dépend des cellules NKT de type II non classiques, qui sont restreintes à CD1d, mais non réactives à α-galectosylceramide (α-GalCer). Nous révélons un mécanisme moléculaire par lequel ces cellules NKT non classiques sont activées en réponse à une infection hépatique par VHB. Nos études démontrent que l'expression en surface de NKG2D et d'un de ses ligands (Retinoic acid early inducible-1 ou RAE-1) est modulée d'une manière qui dépend de VHB. Aussi, le blocage de l'interaction entre NKG2D et son ligand prévient entièrement l'hépatite aigue et la lésion hépatique dépendante de VHB et CD1d. En conclusion, nous démontrons que NKG2D, CD1d et VHB sont nécessaires pour l'activation des cellules NKT classiques de type II et pour l'initiation de l'hépatite aigue dans notre modèle. Ces résultats ont donc des implications majeures dans l'étude de l'activation des cellules NKT, et identifient une nouvelle cible potentielle dans le traitement de l'infection virale de l'hépatite B.

Dans les cellules NK de souris, NKG2D s'associe aux molécules adaptatrices DAP10 et DAP12, mais la molécule adaptatrice s'associant a NKG2D dans les cellules NKT reste incertaine. Nous avons donc examiné quelles molécules adaptatrices sont importantes pour propager les signaux reçus par NKG2D lors de l'hépatite aigue dans notre modèle d'infection primaire par VHB. Pour adresser ce sujet, nous avons utilisé un souris knock-out pour Dap10 et Dap12. En conclusion, l'absence de Dap12 dans les cellules effectrices résulte en une hépatite moins aigue, suggérant que NKG2D nécessite la molécule adaptatrice DAP12 pour sa signalisation dans les cellules NKT. Cependant, l'absence de Dap10 résulte en une hépatite plus aigue.

Enfin, nous avons étudié la possibilité que d'autres cellules immunitaires innées pourraient être impliquées dans la reconnaissance de VHB, potentiellement en créant des signaux qui pourraient contribuer a l'environnement hépatique dans lequel les réponses immunitaires sont induites. Nos résultats démontrent une nécrose hépatique bénigne chez

les souris VHB-Tg *Rag-1*$^{-/-}$ préalablement au transfert adoptif et une production des cytokines IFNγ et IL-4. Nous avons également démontré que la nécrose hépatique bénigne est indépendante des cellules NK et de INFγ. Aussi, nos résultats démontrent que la quantité basale de IFNγ ainsi que la fréquence des cellules NK dans les foies des souris VHB-Tg *Rag-1*$^{-/-}$ récipientes n'affectent pas l'hépatite aigue résultant des cellules NKT non classiques de type II. Comprendre le mécanisme responsable de la nécrose hépatique bénigne présente dans les souris VHB-Tg en l'absence des lymphocytes B et T serait important pour déchiffrer le rôle de la réponse immunitaire innée dans la pathologie de VHB.

CHAPTER I

INTRODUCTION

Hepatitis B virus (HBV), discovered in 1965, is a major cause of human liver disease worldwide. More than 2 billion people have been infected with HBV, a virus one hundred times more contagious than human immunodeficiency virus (HIV) and ten times more contagious than hepatitis C virus (HCV). Although a highly effective vaccine against HBV infection has been available for more than 20 years (since 1982), universal vaccination still remains a goal rather than an accomplishment. According to the World Health Organization (WHO) 400 million individuals are chronically infected worldwide, and 10 to 30 million people will become infected each year. An estimated 1 million people die annually from HBV-associated diseases, such as active chronic hepatitis, cirrhosis and hepatocellular carcinoma.

Although there has been significant progress in understanding HBV pathogenesis, several important questions remain to be answered. These include the receptor(s) required for HBV entry in human hepatocytes and the role of the innate immune system in controlling HBV infection. A large body of evidence indicates that the outcome of HBV infection and the pathogenesis of the attendant liver diseases are determined by immune-mediated host-virus interactions. While a great deal is known about the effector limb of the $CD8^+$ cytotoxic T cell responses, the factors that determine whether a given individual will mount an effective immune response to HBV are poorly understood, representing a great challenge in the HBV field.

The work presented in this thesis is aimed at understanding the molecular and cellular mechanism(s) of early immune recognition of HBV-expressing hepatocytes in a mouse model of primary human HBV infection.

Hepatitis B virus

Evidence for a second form of hepatitis, transmitted from blood and body fluids, began appearing in the 19^{th} and early 20^{th} centuries. Widespread acceptance did not occur until the 1940s, based on investigations of outbreaks of hepatitis following vaccination for measles, mumps, and yellow fever. However, HBV was only discovered in 1965 when Baruch Blumberg described the Australian antigen (later known to be Hepatitis B surface antigen, HBsAg) in the blood of Australian aboriginal people (1), a discovery for which Dr. Blumberg received the Nobel Prize in Physiology and Medicine in 1976 (2). In parallel with

work from Blumberg's laboratory, Prince *et al* were using a direct approach to search for a viral cause of serum hepatitis. Their idea was to use antiserum from patients with acute hepatitis to detect virus in the liver via immunofluorescence microscopy of liver tissue sections. Using this technical approach, Prince successfully identified a serum protein (designated as SH) in patients with post-transfusion hepatitis (3), which later on was shown to be identical to the Australian antigen. The discovery of this antigen had an important practical benefit because it allowed the detection of contaminated blood, resulting in an approximately two-fold decline in the incidence of post-transfusion hepatitis. Unlike the feco-oral transmission of the first form of hepatitis (hepatitis A), HBV is spread by sexual contact with an infected host or from parenteral exposure to virus-containing blood or blood products. In 1970, D.S. Dane and others discovered the virus particle by electron microscopy (4). In the early 1980s the genome of the virus was sequenced (5), and in 1982, an effective recombinant HBV vaccine was developed and made available.

HBV is the prototype member of the family *Hepadnaviridae* and consists of enveloped, 3.2 Kb partially double stranded deoxyribonucleic acid (DNA) virus that specifically targets hepatocytes for viral replication. This family contains two genera, the orthohepadnaviruses, which infect mammals, and the avihepdnaviruses, which infect birds (6).

Chronic active hepatitis and hepatoma were frequently observed in captive woodchucks during necropsy. These findings led veterinarians to search for HBV-like particles in serum from these animals. This investigation resulted in the identification of the first non-human hepadnaviruses, namely the woodchuck hepatitis virus (WHV). This virus genome shares about 60% nucleotide sequence similarity with its human counterpart and is morphologically indistinguishable from HBV (6). Other similar viruses include the ground squirrel hepatitis virus (GSHV) and duck hepatitis B virus (DHBV), which have been recovered from ground squirrels and ducks, respectively. Despite important distinctions, the similarities between all of these viruses outnumber their differences, since they share several common properties, such as (a) enveloped virions of 3 to 3.3 kb partially double stranded DNA; (b) virion-associated polymerases with ability to repair the gap in the virion DNA template; (c) a narrow host range; and (d) persistent infections exhibiting marked hepatotropism (6). Less well-characterized viruses have been isolated from arctic ground squirrels, wild herons, varieties of wild and domestic geese, marsupials, and orangutans (6).

It is widely accepted that hepatocytes are the primary site of HBV infection. However, the mechanisms by which HBV enter hepatocytes or other susceptible cells are still largely

unknown, mostly because of the lack of infectible cell culture systems. Nevertheless, several studies suggested that the initial step of HBV infection may involve interaction of the envelope polypeptides of HBV with a variety of molecules (6). These include endonexin II (7), IL-6 (8), annexin V (9) and apolipoprotein H (10).

Following virion entry into hepatocytes, the HBV nucleocapsid is released into the cytoplasm and the HBV DNA is transported into the nucleus (Fig. 1.1). There, the relaxed circular viral DNA genome is repaired by cellular polymerases into the covalently closed circular (ccc) DNA, which represents the viral transcriptional template (11) of four viral ribonucleic acids (RNAs). These are then exported to the cytoplasm and used as messenger RNAs (mRNAs) for translation of the HBV proteins (Fig. 1.1). The longest, pre-genomic, RNA is translated to produce the viral core and polymerase proteins, which occurs in nucleocapsids in the cytoplasm of the hepatocyte. Specifically, viral replication occurs within these capsids by reverse transcription of the pre-genomic RNA to produce a single-strand DNA copy that serves as the template for second-strand DNA synthesis, producing a circular double-stranded DNA genome (Fig. 1.1). Viral capsids containing double-stranded DNA traffic either back to the nucleus to amplify the viral cccDNA genome or to the endoplasmic reticulum (ER), where they engage the viral envelope proteins (small - S, medium - M and large - L) and exit the cell as virions that can infect other cells (12), after passing through the Golgi complex (Fig. 1.1). In addition to the 42 to 47nm virions, HBV-infected patients contain in circulation 20nm spheres that consist of HBsAg and host-derived lipids. Notably, these spheres outnumber the virions by a factor of 10^4 to 10^6 (13).

With the availability of polymerase chain reaction (PCR)-based assays, eight genotypes, A to H, have been identified. Different genotypes tend to have distinct geographical distributions and possibly distinct clinical manifestations and outcomes (14).

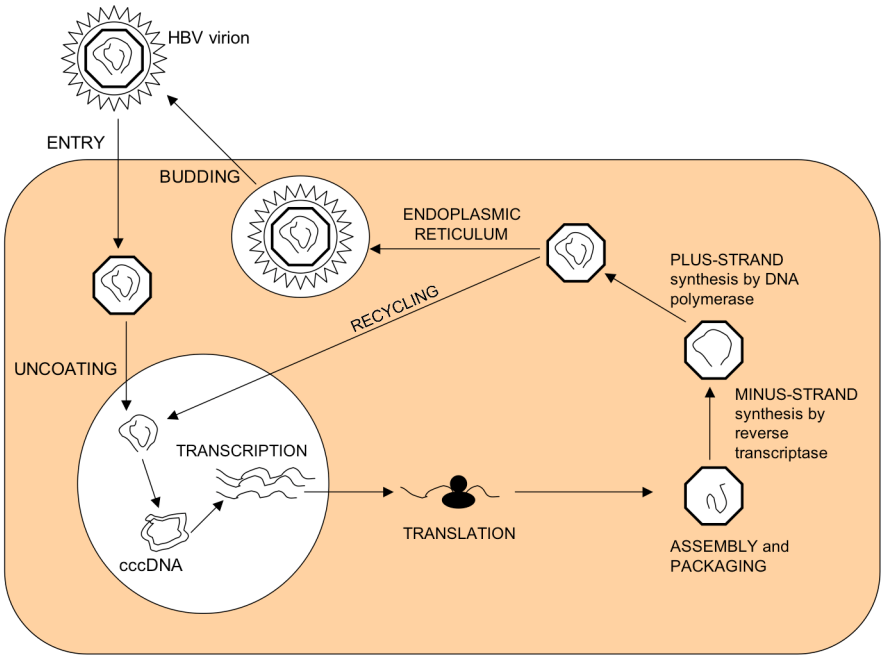

Figure 1.1. Schematic representation of the replication cycle of hepatitis B virus. Adapted from Fung, SK and Lok, ASF, Nature Clinical Practice Gastroenterology& Hepatology (2004) 1, 90-97.

The liver as an immunological organ

The liver is the largest solid organ, both in rodents and humans, and its structural organization has profound implications for its immune function (15). It has a dual blood supply in which venous blood from the intestinal tract enters via the portal (presinusoidal) venules, and arterial blood enters via the hepatic arterioles. These two blood supplies converge at the sinusoids, which drain the blood into the central (postsinusoidal) venules (16) (Fig. 1.2). Interestingly, this unique sinusoidal structure of the liver seems to allow a more efficient access of immune cell populations to HBV-infected hepatocytes. By contrast, the microvascular anatomical barriers present in other tissues (e.g. brain, kidney, testis, pancreas, gastrointestinal (GI) tract) seem to make the HBV extra-hepatic reservoirs of more difficult access to the immune system, and thereby contribute to viral persistence.

For many years, hepatic research was performed using mainly histological analysis. This methodology identified liver-specific cell populations such as Kuppfer cells (resident macrophages within the liver) and stellate cells (Fig. 1.2), but did not suggest the presence of many other intra-hepatic immune cells (17). However, when the liver is digested, a very distinct scenario appears (18). The nonparenchymal cells comprise one-third of the total number of cells within the liver whereas the hepatocyte population comprises the two other thirds and approximately four fifths of the total organ volume (19). The intra-hepatic nonparenchymal cell populations are diverse, being around 50% liver sinusoidal endothelial cells (LSECs), 20% Kuppfer cells, 5% biliary cells, 25% intra-hepatic immune cells, and stellate cells accounting for less than 1% (Fig. 1.2).

Every minute, around 30% of the total blood passes through the liver (20). Thus, in 24 hours, approximately 100 million peripheral blood lymphocytes recirculated through this organ (21). The liver sinusoids are composed of a fenestrated monolayer of LSECs, and each lymphocyte (~7-12μm) that passes through the sinusoids (~6-15μm) is in direct contact with these endothelial cells (Figure 1.2) (15). Therefore, any increase in systemic venous blood pressure results in stasis, prolongs the interaction between lymphocytes and antigen presenting cells (APCs) and contributes to lymphocyte extravasation (15). The space of Disse, or the perisinusoidal space, contains stellate cells that are star-shaped cells in the liver and which mediate intra-hepatic non-immunological as well as immunological functions (22). In addition to its pivotal role in the metabolism of vitamin A and in the storage of 80% of total body retinol, upon activation, stellate cells differentiate to myofibroblasts for production of extracellular matrix and can contribute to liver fibrosis (22). The microvilli of hepatocytes can extend into the space of Disse, allowing components from the sinusoids to be taken up by hepatocytes (15).

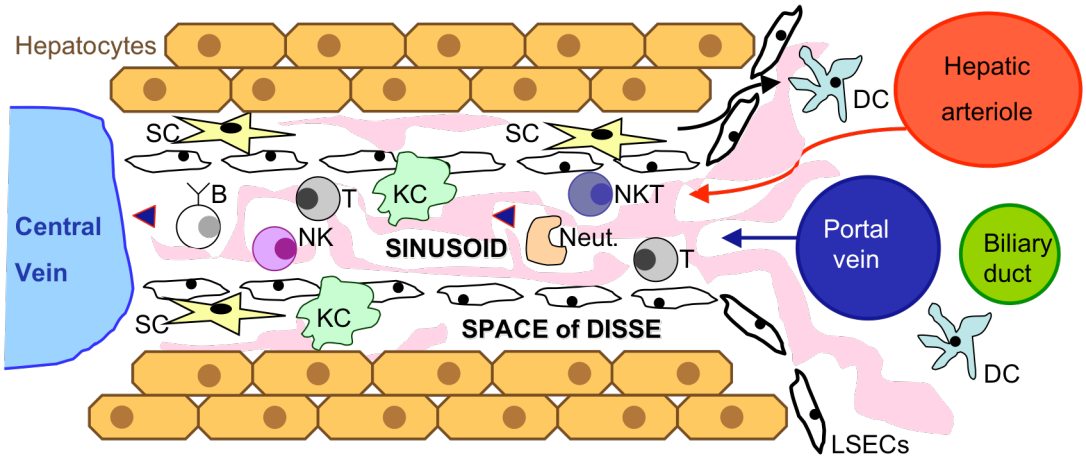

Figure 1.2. Anatomy of a healthy liver and its immune cells. SC – stellate cell; KC – Kuppfer cell; DC – dendritic cell. Adapted from Racanelli V. and Rehermann B. in Hepatology (2006) 43:S54-S62.

Approximately 25% of the liver nonparenchymal cells are immune cells. The absolute number of lymphocytes in the liver is 10 to 20 million cells per gram of tissue (23), which is remarkable for a non-lymphoid organ (18). Natural Killer (NK) and Natural Killer T (NKT) cell populations are abundantly present in a healthy liver, accounting for approximately half of the resident intra-hepatic immune cells. Thus, they are likely to be important in immune responses to hepatotropic pathogens, such as HBV. Moreover, other immune players are found within the liver such as granulocytes, macrophages, dendritic cells (DCs), αβ T lymphocytes, γδ T lymphocytes and B cells (Table 1.1). Notably, $CD8^+$ T lymphocytes predominate in the liver when compared with $CD4^+$ T lymphocytes, being the hepatic CD4/CD8 ratio the opposite of that in the lymph nodes (18).

Cell type	Function
Granulocytes (neutrophils, eosinophils and basophils)	Quickly recruited to the site of certain viral infections. Produce significant amount of the antiviral molecule TNFα.
NK cells	Key cells of the immune system in antiviral defense and tumor immunosurveillance. These cells can produce large amounts of IFNγ upon activation.
Dendritic cells	Specialized APCs. Upon encounter with potentially pathogenic antigen, DCs traffic to draining lymph nodes to prime and activate αβ T cells.
NKT cells	Rapidly activated. Produce large amounts of IFNγ, IL-4 and TNFα.
αβ T lymphocytes	Highly polyclonal αβ TCR and can be $CD4^+$ or $CD8^+$. $CD8^+$ T cells differentiate into cytotoxic cells; $CD4^+$ T cells can differentiate into Th1 (IFNγ) or Th2 (IL-4, IL-5, IL-10, IL-13).
γδ T lymphocytes	γδ TCR of limited diversity. Function not well defined within the liver.
B lymphocytes	Not much is known about their function within the liver. Antiviral antibodies contribute to viral clearance by blocking virus entry into susceptible cells and by removing infectious virions from the circulation.

Table 1.1. Summary of the intra-hepatic immune cell types and its function. Adapted from Mehal, WZ *et al*, Gastroenterology (2001) 120: 250-260.

The liver also performs crucial metabolic functions. It receives oxygenated blood from the hepatic artery and blood enriched in nutrients from the gut via the portal vein. In addition, the blood that enters the liver from the intestines is rich in commensal gut bacterial products and food-derived antigens. Thus, the constitutive presence of non-self and microbial components within the liver provides a unique environment in this organ which is thought to favor tolerogenic immune responses (24). This hepatic tolerogenic predisposition is believed to protect the organ from constant inflammation and consequent damage. Early in the history of experimental transplantation, it was found that allogeneic liver transplants are accepted and maintained by the recipient without immunosupression (25). In humans, liver transplants require less immunosuppressive therapy and experience less severe T cell-mediated rejection episodes than other vascular organ grafts (26), despite histocompatibility leukocyte antigen (HLA) disparities between donor and recipient cells. Direct injection of antigen or allogeneic cells into the portal vein results in tolerance (27, 28). And, oral tolerance (when antigen is administered via the GI tract) does not develop if the blood flow

from the intestine bypasses the liver (29). Thus, a key feature of the liver as an immunological organ is its ability to remain tolerant to certain antigens while retaining the capacity to repel pathogens effectively. However, acute inflammatory reactions can occur in response to certain harmful stimuli, leading to hepatic necrosis (hepatitis) and subsequent regeneration, or to hepatic fibrosis if the stimuli are sustained. Both in rodents and humans, liver injury is generally measured by the level of serum alanine-aminotransferase (ALT), which is an enzyme that is released into the bloodstream during hepatocyte necrosis. Actually, there are situations in which hepatic infections result in a robust immune response, clearance of the pathogen and functional memory. This is observed in almost all hepatitis A infections and to a variable extent in patients infected with HBV and HCV. It has been suggested that activated $CD8^+$ T cells within the liver are predisposed to undergo apoptosis, and that $CD4^+$ T lymphocytes, upon interaction with LSECs and many intra-hepatic DCs, are skewed into cells with regulatory functions (24). However, how this default state of hepatic tolerance might be interrupted to allow T cell activation and effector responses to occur remains to be understood. Interestingly, chronic hepatitis B infection illustrates both tolerance and immunity within the liver. In general, chronic HBV infection is characterized by a period of immune tolerance (elevated HBV-DNA and low ALT values), followed by immune clearance (elevated ALTs and decreased of HBV-DNA), which usually leads to immune control (low HBV-DNA and low ALTs). However, for reasons that are not yet known, the intra-hepatic immune system against HBV-infected cells is reactivated in one third of these patients. Understanding the dynamic interplay between immune control and immune activation against HBV-infected cells may provide insight into tolerance versus immunity within the liver.

Despite the tremendous progress towards understanding how extra-hepatic immune responses are initiated and regulated, much of this knowledge has not been yet effectively translated into a better understanding of human liver diseases. General mechanisms of immune regulation should be considered in the context of very unique populations of lymphocytes and APCs that are enriched in the liver. Advances in unraveling the functioning of the intra-hepatic immune system in tolerance and in immune activation in the mouse may be very relevant for the development of treatments of liver diseases in humans.

Hepatitis B as an immunological disease

HBV infection and replication are not cytopathic to hepatocytes; rather it is the immune response to the viral antigens that causes the hepatic necrosis and liver damage. Very early events in immune activation and reactivation are essential in determining whether this disease process is set in motion, since continued immune tolerance to HBV antigens avoids hepatitis. One mechanism whereby infected cells can limit the initial viral spread is the induction of apoptosis. The evidence that HBV can induce apoptosis is contradictory, as both pro- and anti-apoptotic effects have been detected in cultured cells and transgenic mouse models expressing different viral products (30). Importantly, during the early phase of HBV infection in chimpanzees (before virus-specific T cells enter the liver), there is an absence of histological or biochemical evidence for hepatocyte damage (31, 32). Furthermore, when cellular immune responses are deficient or pharmacologically suppressed, HBV can replicate at high levels in the livers of either patients or HBV-transgenic mice, without inducing any detectable pathological consequences (11, 33). Collectively, these results indicate that HBV replicate non-cytopathically within the primary hepatocyte in vivo and suggest that hepatocyte damage during viral hepatitis is an immune-mediated event.

Primary HBV infection is asymptomatic in most adult individuals, but may result in varying degrees of acute liver injury (acute hepatitis). Approximately 95% of acutely HBV-infected adult population clear the virus intra-hepatically and from the bloodstream and recover completely from the infection without long-term sequelae. Therefore, only up to 5% of the HBV-infected adults develop chronic hepatitis B of varying severity. In contrast, over 90% of the newborn population vertically exposed to HBV will develop chronic hepatitis B. For example, ten percent of the entire Chinese population and its diaspora, which comprises approximately 130 million people, are chronically infected with HBV. Although approximately two thirds of this latter population can remain immunotolerant (especially in terms of cellular immunity) and never develop chronic hepatitis, around one third of the chronically infected patients will break HBV-specific immune tolerance and develop active, and often progressive hepatitis with life-threatening consequences, such as cirrhosis and hepatocellular carcinoma. Symptoms of chronic liver disease are insidious and often overlooked because the liver has a remarkable regeneration capacity, which masks progressive scarring despite decades of injury. Notably, the asymptomatic carriers are the

major global epidemiologic reservoir of HBV, and it is mainly they who spread HBV to susceptible hosts.

Virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T-helper (Th) cells play key effector and regulatory roles, respectively, in antiviral immunity. These T cells participate in viral pathogenesis either directly, by killing infected cells, or indirectly, by producing soluble factors such as cytokines and chemokines. In this way, they contribute to the inflammatory process and/or inhibit viral replication. Although APCs that have internalized viral antigens secreted by other cells can efficiently prime Th cells, activation of CTLs usually requires the processing of viral proteins that are either endogenously produced or phagocytosed by professional APCs (34). Acute HBV infection is characterized by a polyclonal HBV-specific $CD8^+$ T cell response. There are CTLs responding to most HBV proteins, leading to increased recognition of the target epitope and reduced viral "escape" via mutation (35). Depletion of $CD8^+$ T cells following acute HBV infection of chimpanzees led to persistence of HBV infection and showed the importance of both cytolytic and non-cytolytic activity of this population of lymphocytes. In human HBV infection, not all individuals who recover from acute HBV infection show hepatocyte necrosis or clinical symptoms, suggesting that non-cytolytic mechanisms, such as those induced by cytokines like interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) are used to clear acute HBV infection (36). The importance of these cytokines was confirmed in HBV-transgenic mouse models, where administration of anti-IFNγ and anti-TNFα antibodies blocked the ability of $CD8^+$ T cells to clear HBV RNA intermediates and nucleocapsid protein (Hepatitis B core antigen - HBcAg) (37). Although IFNγ is mainly produced by HBV-specific $CD8^+$ T cells, it can also be produced by NK, NKT cells and HBV-specific Th1 $CD4^+$ cells (38). Thus, despite mature $CD8^+$ T lymphocytes play a major role in HBV clearance, coordinated effect of cytokines and activation of different immune cell players appears to be necessary to achieve viral control and clearance. This is consistent with the kinetics of viral replication and lymphocyte recruitment and proliferation observed in chimpanzees following acute HBV infection (39).

It is very well established that Th cells can be divided in two subsets, Th1 and Th2 cells, based on the profile of cytokine production. Th1 cells secrete interleukin-2 (IL-2), TNFα and IFNγ and are involved in antiviral functions and in the regulation of cellular immune responses. Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which are known to promote humoral immune responses (40). A direct, cytokine-dependent antiviral role of Th1 cells has been shown in HBV- transgenic mice, when transfer of HBV-specific Th1 cells into

these immunologically tolerant mice induced cytokine release and suppression of viral replication in the liver. During an acute infection, HBV-specific $CD4^+$ T cells can be detected at the time of elevated HBV DNA concentration (before the peak of liver damage) and persist long after recovery from HBV infection (41). $CD4^+$ T cell proliferation or/and antiviral cytokine production are more commonly detected to the core than to any other HBV protein (41-43), such as HBV-envelope or HBV-polymerase (43, 44).

In a typical acute HBV infection, HBV DNA is detectable in circulation within a month of infection, but remains at a relatively low level of 10^2 to 10^4 genome equivalents per mL for a period of four to six weeks before the HBV DNA and the secreted HBV e antigen (HBeAg) and HBsAg peaks (6). Approximately ten to fifteen weeks after infection, serum ALT levels start to rise, which is indicative of T-cell mediated liver injury. Interestingly, HBV DNA in the serum and in the liver can be cleared before the ALT peak, as shown in experimentally infected chimpanzees (31). As mentioned previously, approximately 90% of acutely infected adults resolve all clinical symptoms, develop HBeAg- and HBsAg-specific antibodies, clear free HBeAg and HBsAg from the circulation and maintain lifelong protective immunity. Despite complete clinical recovery, trace amounts of HBV DNA persist and are controlled by humoral and cellular immune responses (13). In patients who have recovered from acute HBV infection, activated Th2 cells induce B-cell production of anti-HBs, anti-HBc and anti-HBe antibodies (HBsAb, HBcAb, HBeAb). HBcAg-specific immunoglobulin (Ig) M is an early marker of infection, whereas antibodies specific for HBeAg and HBsAg are detected later and indicate a favorable outcome of infection (13). Anti-HBsAb are synthesized early in infection but are undetectable because they are complexed with an excess of envelope antigens produced during viral replication (45). HBsAb is then detected later in HBV-infected patients as well as in vaccinated individuals. HBsAb is used in the latter group as a marker to evaluate immunity to the virus. In general, HBcAg-specific IgG and HBsAg-specific antibodies persist for life after clinical recovery.

Regulatory T cells compose a distinct T lymphocyte population. These cells are described phenotypically as $CD4^+CD25^{hi}FoxP3^+$, and functionally as immunological suppressors against self (46) and foreign antigens (47) through suppressive cytokines or direct cell-cell contact. Not much is known about the role of regulatory T cells in the liver or during HBV infection, but recent data from three independent groups suggested that $CD4^+CD25^+$ T regulatory cells were linked to the chronicity of the disease in patients with chronic hepatitis B (48-50). In chronic severe hepatitis B patients, the frequencies of $CD4^+CD25^+$ T regulatory cells in both peripheral blood mononuclear cells (PBMC) and intra-

hepatic infiltrating lymphocytes were significantly increased and there was a dramatic increase of $FoxP3^+$-cells in the liver compared with healthy controls. In acute hepatitis B patients, the frequency of circulating $CD4^+CD25^+$ T regulatory cells was initially low, increased in number during the convalescence phase and returned to normal levels upon resolution (50). Nevertheless, a detailed analysis of the intra-hepatic numbers and function of these cells in healthy and HBV-infected livers awaits to be experimentally addressed.

A complex interaction exists between HBV and the host in the initial clearance of HBV, the long-term persistence of HBV and the pathogenesis of HBV liver disease. Thus, understanding the pathogenesis of HBV infection mandates uncovering the immune responses underlying these processes. A great deal is known about the adaptive immune response to HBV-infected cells, both cellular and humoral components, but the role of innate immunity against HBV requires further investigation. However, the study of HBV immunopathogenesis has been problematic because natural hepadnaviral infections occur only in outbreed species whose immune systems are difficult to experimentally manipulate. In addition, conventional experimental systems for the study of HBV immunopathogenesis are not available because HBV is not infectious, even for human cells, in vitro.

Overview of the models used to study HBV immunopathogenesis

Cell culture systems

Numerous strategies have been used to develop cell culture systems for HBV propagation. The majority of these advances were driven by the goal to develop and evaluate potential antiviral agents for activity against HBV. Some examples are the stably transfected line of HepG2 cells, 2.2.15 cells (51, 52), the HepAD38 cell line (in which replication of HBV can be regulated with tetracycline) (53, 54), and the HepAD79 cell line (which was developed to determine the relative susceptibility of viruses with mutations in the YMDD motif in cell culture) (53).

However, despite many attempts to develop an HBV-cell culture system, a successful in vitro system to address immunological questions has not been generated.

HBV natural hosts as animal models

As mentioned previously, natural hosts for the duck (DHBV), woodchuck (WHV), and ground squirrel (GSHV) are considered hepadnaviral homologues of HBV. Nevertheless, DHBV, WHV and GSHV are genetically divergent from HBV, with WHV being the most closely related (55).

These three animals are outbred species whose immune systems are difficult to experimentally manipulate due to the lack of reagents able to characterize their immune responses against these viral antigens. Therefore, although these animals have contributed to our understanding of the natural history and pathogenic potential of these viruses, they do not allow definitive analysis of the role played by the immune system in viral clearance, disease pathogenesis and hepatocarcinogenesis (45, 56, 57). In fact, these animal models of HBV natural infection have been predominantly used by virologists rather than immunologists.

Mouse models of HBV infection

HBV-transgenic mouse models

A reproducible tissue culture model of HBV infection does not exist, nor is HBV infectious for immunologically well-defined laboratory animals, such as mice. Therefore, in the late 80's with the arrival of embryo microinjection technology, several laboratories, using DNA constructs encoding HBV-derived regulatory sequences, generated transgenic mice that preferentially express all of the viral gene products, and even replicate the virus, within the hepatocyte (58-65). Experiments using these mice showed that HBV can replicate efficiently within mouse hepatocytes. This finding suggested that once the viral transgene is integrated into the host genome there are probably no species-specific constraints to viral replication. One of the earliest studies with HBV transgenic mice involved strains that produced the three viral envelope proteins, S being the most abundant and L the least, which reflects in part the relatively weak PreS promoter in comparison to the S promoter (66). However, a strong promoter (albumin promoter) was used to direct synthesis of the PreS mRNA in a few strains of transgenic mice generated by Chisari and colleagues, one of which will be discussed in this dissertation. As a result of the use of such artificial promoter HBsAg is accumulated in the ER. In addition, the hepatocytes derived from this strain of mice are more susceptible to the effects of IFNγ. This is reflected in higher ALT levels seen after disease induction when compared to mice that express the entire viral genome under

the control of the viral promoters. These mice recapitulate, to some extent, a rare clinical event designated as fibrosing cholestatic hepatitis (FCH), which is characterized by an exaggerated accumulation of HBV large envelope protein in human hepatocytes that leads to fulminant hepatitis due to the exacerbated immune response to HBV-infected hepatocytes.

Based on observations of infected patients, it was generally assumed that a major histocompatibility complex (MHC)-restricted cytolytic immune response to virally encoded antigens expressed at the surface of the hepatocytes plays an important role in viral clearance and in the pathogenesis of HBV-induced liver disease. Using HBV-transgenic mice, Chisari and colleagues sought to examine this assumption. For this purpose, non-transgenic mice were immunized with a vaccinia virus expressing HBV envelope proteins (Vacc-HBs). Splenocytes isolated from these immunized mice were cultured to produce CTL lines that were cloned and characterized in vitro. MHC-class I-restricted $CD8^+$ CTL that recognized HBsAg-positive target cells and secreted IFNγ and TNFα were injected intravenously into syngeneic HBV-transgenic recipient mice whose hepatocytes express HBsAg. The pathogenic and anti-viral consequences of CTL activation in the liver were monitored. Interestingly, HBV-transgenic mice that express the complete viral genome reveled relatively little liver injury after CTL adoptive transfer. These data suggested that hepatocellular HBV gene expression was greatly suppressed by non-cytolytic signals, most likely cytokines, delivered by the HBsAg-specific CTLs (67).

Collectively, Chisari and colleagues have contributed significantly over the past two decades in characterizing the role of strong and polyclonal CTL responses to HBV-expressing cells in HBV clearance and/or in the context of chronic hepatitis B infection, using HBV-transgenic mice as well as HBV-infected chimpanzees.

HBV hydrodynamic tail vein injection model

HBV-transgenic mice are immunologically tolerant to the virus and it is necessary to adoptively transfer T lymphocytes previously primed to a maximum response to HBV antigens for hepatitis to occur, thereby compromising the greatest potential strength of a mouse model of HBV infection. Therefore, a new mouse model was developed in an attempt to alleviate these experimental constraints. Hydrodynamic tail vein injection of a head-to-tail dimer of *adw* HBV genome (pHBV*adw*HTD) into immunocompetent mice generated HBsAg and HBeAg expression in both serum and hepatocytes followed by seroconversion. This way, a transient liver-targeted transgenic mouse was generated (66, 68). This technique

requires tail vein injection into 6 to 9 weeks old wild type mice of a volume of saline equivalent to approximately 8% of the body mass of the mouse within 5-7 seconds. Thus, this method causes an acute circulatory volume overload, resulting in a mortality rate of 20-30%. One day after injection a sharp rise in serum ALTs is detected and return to baseline levels by day 7 after transfection. In addition to the previous limitations, this method only allows HBV transfection of 5 to 10% hepatocytes in vivo (68).

Other HBV mouse models

Other mouse models have also been developed in an attempt to study HBV. It was demonstrated that long-term engraftment of primary human hepatocytes transplanted in a matrix under the kidney capsule of mice was achieved with administration of an agonistic antibody against c-Met. These mice were susceptible to HBV infection and able to support complete viral life cycles. In addition, super-infection of the HBV-infected mice with HDV was shown. These findings described a new xenotransplant model that seemed to allow the study of multiple aspects of human hepatitis viral infections. Despite this model (69) being extremely appealing, its dependence on human hepatocytes (which are not easily available) limits follow-up studies.

Primate model of HBV infection

Higher primates are susceptible to HBV, and the chimpanzee, in particular, has been used to study virus transmission, the host response and vaccination. Chimpanzees can be infected at a specific time with defined inocula and studied in the early phase as well as throughout the course of infection, by performing sequential liver biopsies and blood analysis. The chimpanzee model contributed to the understanding of viral hepatitis B as a transmissible disease, to the assessment of the neutralization capacity of different HBV-specific antibodies, and to the characterization of antibody production and T-cell mediated immune responses (31).

Despite being the only animal that is naturally infected by HBV other than humans, chimpanzees seem to have a milder clinical course of hepatitis and a weaker and more restricted humoral immune response as compared to humans. It is possible to argue, however, that the human studies have a selection bias, since asymptomatic patients do not seek medical attention while all of the experimentally infected chimpanzees are narrowly studied. Nevertheless, vertical transmission, which is the main route of HBV transmission in humans, is not common in chimpanzees (13).

On the other hand, experiments in primates are limited owing to high costs and limited availability of chimpanzees for research, as many studies are restricted to two to three primates (13). In addition, ethical considerations are also an issue which limit biomedical research in primates.

Thus, understanding the immune response to HBV is still incomplete, largely due to the narrow host restriction of this pathogen and the limitations of existing experimental models.

Disease model: transgenic mouse model of primary hepatitis B virus infection

Being aware of the limitations in the study of potential innate immune responses to human HBV and its implications, our laboratory recently established a new transgenic mouse model of primary HBV infection. This in vivo system allows, for the first time, the study of mechanisms underlying both major arms of immune responses to HBV, namely the innate and the adaptive immune systems. To generate this new mouse model of primary HBV infection (Fig. 1.3A), we took advantage of HBV-transgenic mice originally generated by Chisari and colleagues. Two strains of mice were used; animals that express the small, middle, and large envelope proteins of HBV as transgenes in the liver under the constitutive transcriptional control of the mouse albumin promoter (hereafter designated HBV-Env$^+$) (70), and mice that express a terminally redundant HBV DNA construct as a transgene, resulting in intra-hepatic HBV replication and release of infectious progeny virions (hereafter designated HBV-Replication$^+$). These latter mice have high level of viral replication in their hepatocytes. The replication level is comparable to that observed in the infected livers of patients with chronic persistent HBV hepatitis, but with no evidence of cytopathology (64). The system developed in our laboratory introduced two major modifications to Chisari's adoptive transfer model. First, the resident adaptive immune system of both HBV-transgenic mice was ablated by crossing to mice deficient in recombinase activating gene-1 (*Rag-1*). Second, the immune system was reconstituted by the adoptive transfer of naïve, un-immunized splenocytes isolated from syngeneic, wild-type mice. This method allowed a liver full of HBV-expressing hepatocytes to be exposed for the first time to a healthy, un-tolerized, naïve immune system – mimicking an acute HBV infection (Fig. 1.3). Therefore, bias introduced by immunization of the wild-type donor mice or by selection for particular

immune effector subpopulations is completely avoided in this model. This system results in a biphasic illness, with a rapid acute hepatitis, which will be the focus of this dissertation, followed by a smoldering chronic hepatitis (Fig. 1.3B) (71).

To ensure that acute pathology observed in this model is HBV-dependent, we used immunodeficient *Rag-1*$^{-/-}$ mice that express an OVA-transgene under the control of the same albumin promoter. As expected, these mice lacked a rise in ALT and an intra-hepatic cytokine production (Vilarinho & Baron, unpublished data).

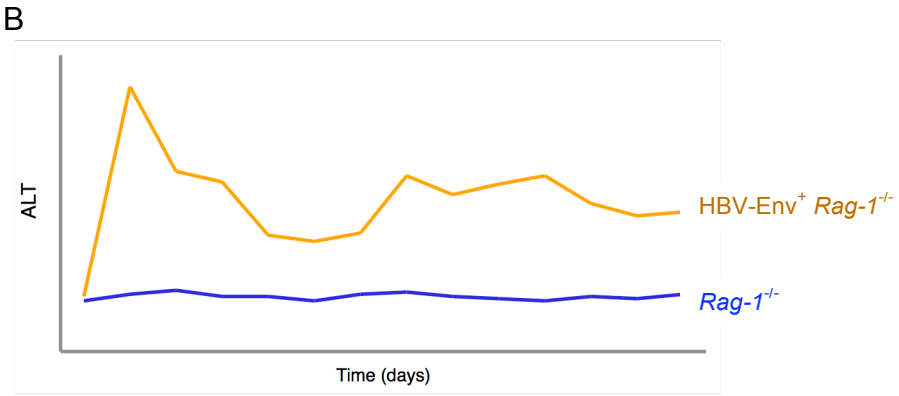

Figure 1.3. (A) Transgenic mouse model of primary hepatitis B virus infection. (B) Hepatic injury as measured by serum ALTs from HBV-Env^{+} *Rag-1*$^{-/-}$ (orange line) and *Rag-1*$^{-/-}$ (blue line) after adoptive transfer of syngeneic naïve wild type splenocytes, adapted from Baron *et al.*, Immunity (2002) vol.16, 583-594.

Since this experimental system resembles primary human HBV infection, it is possible to explore aspects of pathogenesis not directly addressed by the previously discussed models. In particular, the innate immune response(s) to HBV and its potential implication in chronic hepatitis can be studied. In addition, it would be possible to dissect apart the possible contributions of the individual components of the innate immune system in response to HBV.

Using this mouse model of primary HBV infection, Baron *et al* have demonstrated a role for the innate immune system (71). The cells causing hepatitis in this model are prevalent in the liver, as 100-fold fewer intra-hepatic lymphocytes are still sufficient to induce disease. A combination of sorting and depletion experiments demonstrated that a population of CD1d-restricted NKT cells specifically mediate the acute liver injury observed post-transfer (71). They further showed that the NKT cell population does not express the invariant Vα14 T cell receptor (TCR). Adoptive transfer of naïve splenocytes from Vα14 TCR transgenic mice were unable to cause acute hepatitis, and adoptive transfer of naïve wild type splenocytes into HBV-transgenic *Rag-1*$^{-/-}$ *CD1d*$^{-/-}$ mice also did not induce acute hepatitis. Data using this model suggest that the presence of HBV leads to alterations in the MHC class I-like molecule, CD1d, and subsequently affects activation of NKT cells and hepatitis (71). The NKT cell-mediated, CD1d-dependent, acute hepatitis described in this model of primary HBV infection was confirmed in both HBV-transgenic mouse strains mentioned above, namely HBV-Env^{+} and HBV-Replication^{+} (71).

Although the severity of hepatitis observed in the two lines of HBV-transgenic mice is different (due to an increase in hepatocyte sensitivity to cytotoxic effects of IFNγ in the HBV-Env^{+} mice), histological analyses confirm that both lines of transgenic mice develop significant and reproducible hepatitis with a similar disease pattern (71). Specifically, a biphasic ALT rise that is seen in the HBV-Env^{+} *Rag-1*$^{-/-}$ mice is also observed in the HBV-Replication^{+} mice, but as expected the ALT rise is significantly more modest than the one seen in the HBV-Env^{+} mice — typically, serum transaminases were elevated no more than 2-fold above background. Like most cases of acute viral hepatitis in humans, the disease in the HBV-Replication^{+} mice is transient, relatively mild and non-fatal. On the other hand, the severe hepatitis seen in the HBV-Env^{+} mice resemble features of human HBV-induced fulminant hepatitis, such as FCH (72, 73), described previously.

This model allowed the discovery of a role for NKT cells and a “bridge” between the innate and the adaptive immune system in response to infection by a human viral pathogen. This system contributes not only to the study of pathogenesis of HBV infection but also

enhances our understanding of NKT cell biology. The focus of this thesis will be to use this transgenic mouse model of primary human HBV infection to address the molecular and cellular mechanisms involved in the early immune recognition and subsequent immune response to HBV-expressing cells.

Natural Killer T cells and their function

NKT cells are unusually abundant in the liver, where they constitute one third of all resident lymphocytes under baseline conditions. Thus, they may have a "special" role in the control of hepatic infections since they do not need to be recruited into that organ. In contrast, NKT cells represent 0.5% or 0.1% of total cells, in the spleen or lymph nodes, respectively (74).

Research carried out in many laboratories over the past 20 years led to the discovery and definition of NKT cells as a distinct cell population (75-85). The term "NKT cells" was first published in 1995 by the Taniguchi group (86) and defined as a subset of mouse T cells that express NK1.1 (Nkrp1c or CD161c), a marker of the NK cell lineage, in addition to markers of the T cell lineage. However, a number of cellular subpopulations, with different properties and functions, is comprised within this definition (87). In fact, this simplistic classification of NKT cells has been complicated by the fact that most commonly used mouse strains, with the exception of C57BL/6, do not express the NK1.1 marker, and expression of NK1.1 and its human homologue (CD161) is not limited to the so-called NKT cells (88).

Further evidence for the unique characteristics of NKT cells came from data showing that their development is independent of MHC class II expression but requires β2-microglobulin (although mouse NKT cells do not express CD8) (89-91). More importantly, Bendelac *et al* showed that NKT cells are reactive to CD1d, which is a MHC class I-like molecule (92). At the same time, it was established that a large majority of the NKT cell population expresses an invariant TCRα chain: Vα14- Jα281 (currently known as Vα14-Jα18) in mice and Vα24- JαQ (currently known as Vα24- Jα18) in humans. Therefore, the best characterized and the most predominant NKT cell subset is designated as type I, classical or invariant NKT cells. This subset is generally defined by exhibiting the following characteristics: expression of a canonical Vα14 receptor which is conserved in mice and humans and pairs with a limited number of Vβ chains (Vβ8, 7, 2) (74), and by the recognition

of the glycosphingolipid α-galactosylceramide (α-GalCer) in the context of CD1d (93). Mouse type I NKT cells are the group of lymphocytes that can be detected by flow cytometry using tetramers of CD1d loaded with α-GalCer. These cells do not express CD8 and are either $CD4^+$ or double negative (Table 1.2). In humans, however, type I NKT cells can be $CD8^+$. This invariant subpopulation accounts for approximately 70% of all NKT cells in the body, but they are less abundant in humans than in mice (94). The correlation between invariant expression of Vα14 and reactivity with α–GalCer-CD1d is very strong (93, 95), although there are some exceptions (96, 97). More recently, a particular subset of invariant type I NKT cells was identified in the lung that do not express the NK1.1 marker on their surface and secrete high amounts of IL-17 and low levels of IFNγ and IL-4 upon synthetic (α–GalCer) as well as natural (lipopolysaccharides or glycolipids derived from *Sphingomonas wittichii* and *Borrelia burgdorferi*) ligand stimulation (98).

Two other subsets of NKT cells, type II and type III NKT cells, have been identified and designated as non-classical NKT cells because they fail to recognize CD1d tetramers loaded with α-GalCer (99), since they do not express the semi-invariant Vα14- Jα18 in mice and the Vα24- Jα18 in humans. Whereas type II NKT cells do not recognize α-GalCer in the context of CD1d, but require CD1d for activation, type III NKT cells do not recognize CD1d in any circumstance (Table 1.2).

Since the NKT cell field is relatively new and in permanent expansion, it may be possible that other subsets remain to be identified. For instance, it seems that type II NKT cells can be divided into two populations: CD1d-sulfatide tetramer positive and CD1d-sulfatide tetramer negative. In addition, some of the type II NKT cells are auto-reactive, as they recognize the endogenous myelin-derived glycolipid sulfatide and help protect mice against the development of experimental autoimmune encephalitis (100).

	Type I NKT (classical or invariant NKT cells)	Type II NKT (non-classical or non-invariant NKT cells)	Type III NKT (CD1d-independent NK1.1$^+$T cells or NKT-like cells)
CD1d-restricted	+	+	-
Self-reactive	+ or -	+ or -	-
α-GalCer reactive	+	-	-
Sulfatide reactive	-	+ or -	-
TCR α-chain	Invariant: Vα14- Jα18 (mice) Vα24- Jα18 (humans)	Diverse (some Vα3 in mice)	Diverse
TCR β-chain	Vβ8.2, 7, 2 (mice) Vβ11 (humans)	Diverse (but some Vβ8.2 in mice)	Diverse
NK1.1 (CD161)	+ (resting mature) low (immature or activated) - (IL-17-producing NKT)	+ (resting mature) low (immature or activated)	+
Subsets	CD4$^+$ and DN (mice) CD4$^+$, CD8$^+$ and DN (humans)	CD4$^+$ and DN (mice)	CD4$^+$, CD8$^+$ and DN
IFNγ secretion	+	+	+
IL-4 secretion	+	+	-
IL-17 secretion	- (majority) + (NK1.1$^-$)	-	-

Table 1.2. Classification of different subsets of NKT cells. Adapted from Godfrey, DI *et al*, Nat. Rev. Immunol. (2004) 4: 231-237.

NKT cells recognize lipids and glycolipids, rather than peptides, in the context of the non-polymorphic MHC-like class I molecule, CD1d (101). Interestingly, human and mouse NKT cells have functional and phenotypic homologies to the extent that mouse CD1d-restricted NKT cells recognize human CD1d and vice-versa (102). NKT cells have a surface phenotype reminiscent of memory cells, and have been shown to rapidly produce large amounts of cytokines, such as IFNγ and IL-4, upon activation. Because of their rapid response to activation, NKT cells have been suggested to play a role in several different immunological situations, such as clearance of pathogens, progression or suppression of autoimmune diseases, clearance of tumors and maintenance of immune tolerance (103). The action of NKT cells in many disease models depends on the production of either IL-4 or

IFNγ alone. This is quite intriguing given that activation of these cells usually leads to the production of both cytokines together. Studies of NKT cells and their effects on disease usually examine the consequences in the absence of NKT cells, the effects of activating NKT cells on the progression of the disease, or both. Thus, the broad spectrum of their activities is a striking factor that suggests that functionally different subsets of NKT cells might exist. Most of the studies conducted to date on NKT cells have focused on investigating the biological functions of type I rather than type II NKT cells. Only recently, it has been demonstrated that type II NKT cells have critical functions in regulating tumor surveillance, diabetes, and intestinal inflammation (104-106).

Furthermore, NKT cells despite being potent producers of IFNγ, IL-4 and TNFα, do not seem to exert natural cytotoxicity as key effector mechanism. However, these cells can express perforin and FASL, as well as other receptors, such as NKG2D, that mediate cytotoxicity.

NKT cells have attracted a great deal of attention due to their potential to link the innate and adaptive immune systems. Characteristically, they respond very quickly to stimuli and are then able to activate a number of effector cells. Thus, NKT cells are likely important players in the early control of viral infections if they become activated in the infected tissue.

Natural Killer cells and their function

NK cells, recognized for their ability to provide first line of defense against viral pathogens, account for approximately 5 to 10% of the total lymphocyte population, being predominantly found in the blood, spleen and liver. NK cells were first defined as a “null cell” since they do not express a TCR or a B cell receptor (BCR) on their surface. However, today they are considered highly sophisticated lymphoid effector cells whose activity is extremely well controlled by a balance between activating and inhibitory receptors, many of which recognize the amount of MHC class I or MHC class I-like molecules (107). NK cells are a heterogeneous population in terms of its receptor repertoire. Different NK cell subsets express different combination of receptors (activating and inhibitory) (108).

According to the “missing-self” hypothesis (109), NK cells were proposed to provide immune surveillance for cells that had down-regulated MHC class I, which frequently occurs in transformed or viral-infected cells. Until recently, it was thought that NK cells attack any cell lacking MHC molecules, with the exception of erythrocytes, because the potential target

cell cannot engage an inhibitory NK cell receptor for MHC class I (110). Nowadays, a contemporary modification of the “missing-self” hypothesis might state that “NK cells patrol for abnormal cells that lack MHC class I or overexpress ligands for activating NK cell receptors” (107). This way, if a cell does not express MHC class I molecules but they also do not express activating ligands, NK cells have no response towards that cell. This situation results in the same outcome as when a target cell expresses MHC class I molecule and no activating ligands. When a target cell expresses activating ligands and does not express MHC class I molecules on their surface, NK cells attack the target cell. A more complex situation arises when a target cell expresses both MHC class I molecules and activating ligands. In this case, the outcome is determined by the balance of activating and inhibitory signals. In certain situations, NK cells can be activated simply by exposure to cytokines present in the surrounding environment without requiring engagement of their activating receptors (111). Additionally, because NK cells express the activating Fcγ receptor CD16 they have the ability to attack virus-infected cells that are coated with IgG (111).

Regulated by activating and inhibitory signals, NK cells are effector cells against pathogens and damaged cells while simultaneously “screening” and sparing cells expressing self-MHC class I. Mature NK cells express granzymes and perforin, and their lytic response can be triggered within minutes, without requiring transcription, translation, or cell proliferation. In addition, the NK cell receptors are invariant and constitutively present on a large proportion of cells within the population, which make these cells a key component of early immune defense (107). However, NK cells still require type I IFNs (IFNα and IFNβ), predominantly produced by DCs, and/or pro-inflammatory cytokines (IL-15, IL-12 and IL-18) to become activated and totally functional as effector cells. Type I IFNs induce IL-15 production, which increase NK cytotoxicity and cytokine production (111). Activated NK cells produce IFNγ that can promote DC maturation as well as the effector function of other immune cells, such as macrophages, granulocytes and other lymphocytes at the site of infection (111). NK cells also secrete TNFα, which can recruit neutrophils and monocytes to the site of infection, and GM-CSF, which activates macrophages. Thus, production of IFNγ and, perhaps, TNFα by activated NK cells is critical for the control of several viral infections (112). Indeed, a large variety of DNA viruses, such as mouse cytomegalovirus (MCMV) (113, 114), human cytomegalovirus (HCMV) (115), herpes simplex virus (HSV) (116), and adenovirus (117) as well as RNA viruses, e.g. influenza (118), vaccinia virus, ectromelia virus and coxsackievirus, are sensitive to the cytokine-dependent antiviral activity of NK

cells. NK cells display at least two effector functions that can contribute to contain and control viral spread during the initial hours and days of the infection. First, they can directly kill infected cells; second, they are a rich source of inflammatory cytokines with antiviral activity, in particular IFNγ (119).

Notably, in humans, the inflamed liver in chronic HBV infection is characterized by a few HBV-specific $CD8^+$ T cells among a large infiltrate of NK cells ($CD3^-CD56^+$). Furthermore, it was recently demonstrated that these NK cells express the pro-apoptotic ligand TRAIL and can mediate liver injury through engagement of a TRAIL death-inducing receptor on hepatocytes. It is thought that this pathway was turned on by cytokines (IFNα and IL-8), produced during active HBV infection (120). An early rise in circulating NK cells has also been documented in the incubation phase of HBV infection in humans (36).

NKG2D receptor

Initially discovered as a NK cell receptor, NKG2D is a type II transmembrane-anchored glycoprotein expressed as a disulfide-linked homodimer on the surface of virtually all human and mouse NK cells, two thirds of NKT cells, a subset of activated γδ T cells, as well as human $CD8^+$ T cells and activated mouse $CD8^+$ T cells. Since two thirds of mouse resident intra-hepatic immune cells are NK, NKT and $CD8^+$ T cells, NKG2D-expressing lymphocytes are particularly enriched in the liver. Despite its name, NKG2D does not belong to the same family of the other NKG2 receptors. NKG2D has different functions and it recognizes a distinct set of ligands. The human NKG2D receptor gene was first identified in 1991 (121), and since then its orthologues have been described in mice (122), rats (123) and chimpanzees (124), among other species. NKG2D is encoded by a single monomorphic gene (121, 124, 125), which is in the NK cell gene complex on human chromosome 12p12.3-p.13.2 and on mouse chromosome 6. Surface expression of NKG2D can be up or down regulated by the presence of certain cytokines. Several independent studies have shown that expression of NKG2D can be increased on human and mouse $CD8^+$ T lymphocytes by IL-2 stimulation (126-128) and on mouse $CD8^+$ T and NK cells by culture with IL-15 or TNFα (129, 130). It has also been demonstrated that the presence of TGFβ significantly down-regulates NKG2D surface expression both on NK and T cells (131, 132). Additionally, NKG2D is internalized after engagement with its ligands (107, 133). In fact,

chronic exposure to cell surface bound NKG2D ligands is able to promote NKG2D downregulation and a reduction in NKG2D-mediated cytotoxicity (134, 135).

In mice, two isoforms of NKG2D generated by alternative RNA splicing have been described: the long form (NKG2D-L) and the short form (NKG2D-S) (Figure 1.3). These NKG2D proteins differ in the presence (NKG2D-L) or absence (NKG2D-S) of 13 amino acids at the N terminus in the cytoplasmic domain (136).

NKG2D contains an extracellular C-type lectin domain that binds to ligands and a relatively short intracellular tail with no known signaling ability. Thus, in order to propagate signals through NKG2D, this receptor requires coupling to a transmembrane adapter protein for which a charged Arg or Lys in the transmembrane region is critical. The adapter molecules associated with NKG2D are different in humans and mice (Fig. 1.4). In mice, NKG2D pairs with two distinct signaling adapter proteins, namely DAP10 and DAP12/KARAP. Specifically, NKG2D-L exclusively associates with the DAP10 adapter protein, whereas NKG2D-S has been shown to pair with either DAP12 or DAP10 (136, 137). In contrast, humans only express the long form of NKG2D protein, which exclusively pairs with DAP10 for signaling. In resting mouse NK cells, mouse T cells and human NK and T cells, the isoform NKG2D-L forms a multi-subunit complex with the transmembrane adapter protein DAP10 (Figure 1.4). DAP10 has an YxxM motif in its cytoplasmic tail, which upon phosphorylation binds to the p85 subunit of phosphatidylinositol (PI)-3 kinase and initiates a signaling cascade that results in Akt phosphorylation, similar to the pathway used by the co-stimulatory molecules CD28 and ICOS (138). In activated mouse NK cells, the short isoform of NKG2D can also associate with the DAP12 adapter protein (Fig. 1.4). DAP12 has immunoreceptor tyrosine-based activation motifs (ITAMs) in its cytoplasmic domain. Phosphorylation of the ITAMs of DAP12 leads to recruitment and activation of Syk and/or ZAP70 tyrosine kinases (139). This difference in signaling capacity between DAP10 and DAP12 adapter molecules has been proposed to correlate with an ability of NKG2D to function as a primary stimulatory molecule in mouse cells, or as a co-stimulatory molecule in mouse and human cells (107). Surface expression of NKG2D on resting cells requires its co-association with DAP10, both in mice and humans, since NKG2D is retained in the cytoplasm and partially degraded in the absence of DAP10 (138, 140).

Signaling mediated through DAP12, in association with a variety of other receptors expressed in different cell types (NK and myeloid cells), has been described to mediate activating effector responses (141). Surprisingly, more recent data suggests that DAP12 can also mediate inhibitory responses (142-144). Thus, it appears that DAP12 is an adapter

protein with dual functionality (145). However, more experiments need to be performed to better understand the regulation of activation versus inhibition mediated by the same adapter molecule DAP12.

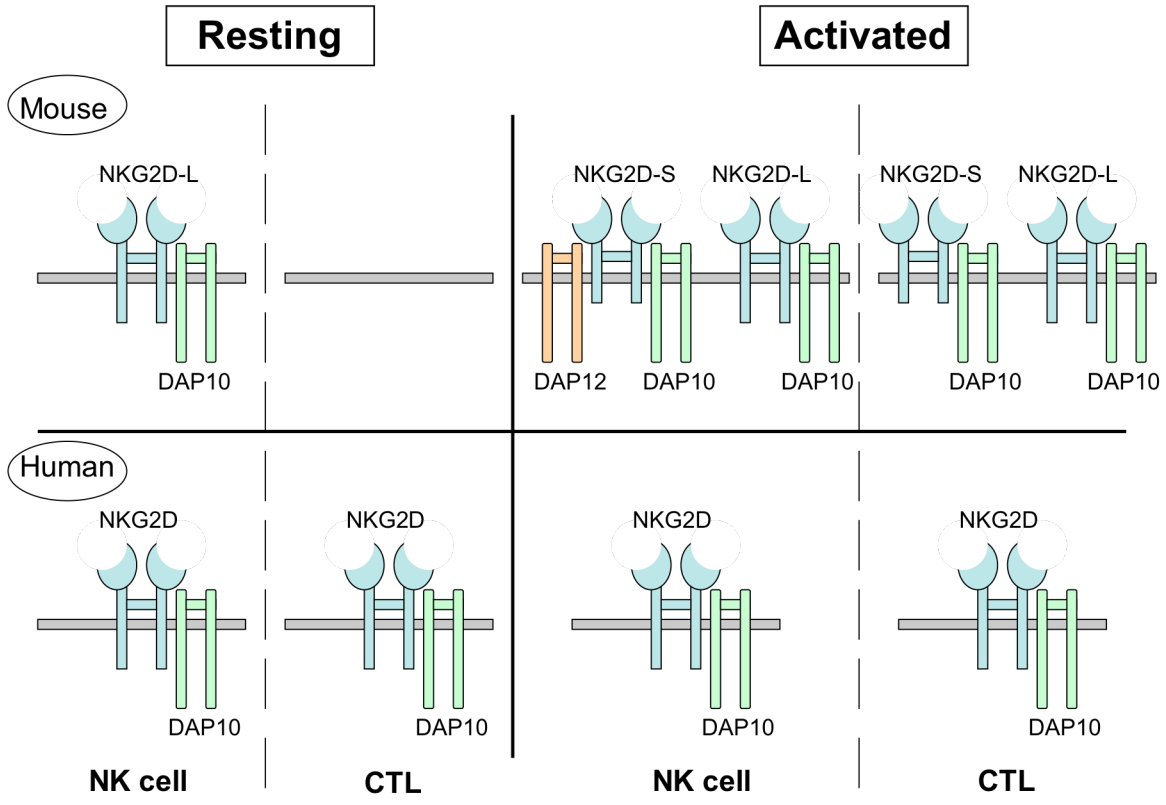

Figure 1.4. NKG2D expression and respective transmembrane adapter association in mouse (top row) and human (bottom row) NK cells and CTLs, both in resting (left columns) and activated (right columns) status. Adapted from Upshaw J.L. & Leibson P.J., Seminars in Immunology (2006) 18 (3):167-75.

Until recently, NKG2D was the only receptor described to associate with DAP10. However, it was known for quite some time that DAP10 expression does not overlap completely with NKG2D expression, suggesting that other receptors might associate with this adapter. Coudert *et al* demonstrated that Ly49D and Ly49H associate with the DAP10 adapter, in addition to DAP12 (146).

As discussed above, mouse NK cell activating receptors can associate with more than one adapter. Likewise, each signaling adapter can pair with multiple receptors, existing some redundancy in terms of NK cell receptors and adapters functions.

In summary, NKG2D$^+$ lymphocytes can be unaffected, co-stimulated or fully activated by NKG2D-ligand interactions, since NKG2D-mediated responses can be modulated by the cytokine milieu and/or by the NKG2D ligand expressed (147).

NKG2D ligands

The human and mouse NKG2D receptor recognizes cell surface glycoproteins structurally related to MHC class I molecules (148-150) (Fig. 1.5). However, they do not associate with β2-microglobulin (148) and they do not present antigen peptides to T cells (149-152). Human ligands are the MHC class I-related chain A and B, MICA and MICB, and the UL-16-binding proteins (ULBP, also called RAET1), ULBP1, ULBP2, ULBP3 and ULBP4 (Fig. 1.5). MICA and MICB, which have more than 100 different alleles are encoded by genes within the human MHC (153), whereas all the other known mouse and human NKG2D ligands are not encoded by genes in the MHC complex. The mouse NKG2D ligands described so far are the five members of the retinoic acid early inducible-1, RAE-1 family (Rae-1α, β, γ, δ, ε), three minor histocompatibility antigen called H-60 (or H-60a) (154, 155), H60b and H60c (156), and the murine UL16-binding protein-like transcript-1 (MULT-1) glycoprotein (157) (Fig. 1.5).

Although MULT1 mRNA (but not protein) was detected ubiquitously (157), other NKG2D ligands are mostly silent in normal, healthy adult tissues. In mice, *RAE-1* genes, which are abundantly expressed during embryonic development but silent in adult mice, were initially discovered and named because they were induced in the F9 embryonic carcinoma cell line after retinoic acid treatment (158, 159). RAE-1 glycoproteins were found to bind to NKG2D (154). It was also discovered that the tumor cell line RMA (that expresses MHC class I-proteins) also expresses RAE-1γ and δ, which allowed rejection of these tumors in vivo, indicating that the RAE-1-NKG2D interaction could override MHC class I inhibition, and demonstrating the importance (dominance) of the NKG2D receptor in activating NK cell effector functions (160, 161). It is now well established that the NKG2D ligands are, in general, “stress-inducible” molecules. Interestingly, however, RAE-1 is transcribed preferentially in the liver of healthy, adult mice (http://source.stanford.edu/cgi-

bin/source/sourceSearch), and undetectable in most other tissues. Cell surface expression of MICA and MICB, which are under the control of a heat shock promoter, can be induced by cellular stress, including heat shock, transformation and viral or bacterial infections (162-165). In addition, it has been shown that the *RAE-1* and *ULBP* genes can be induced upon DNA damage (166).

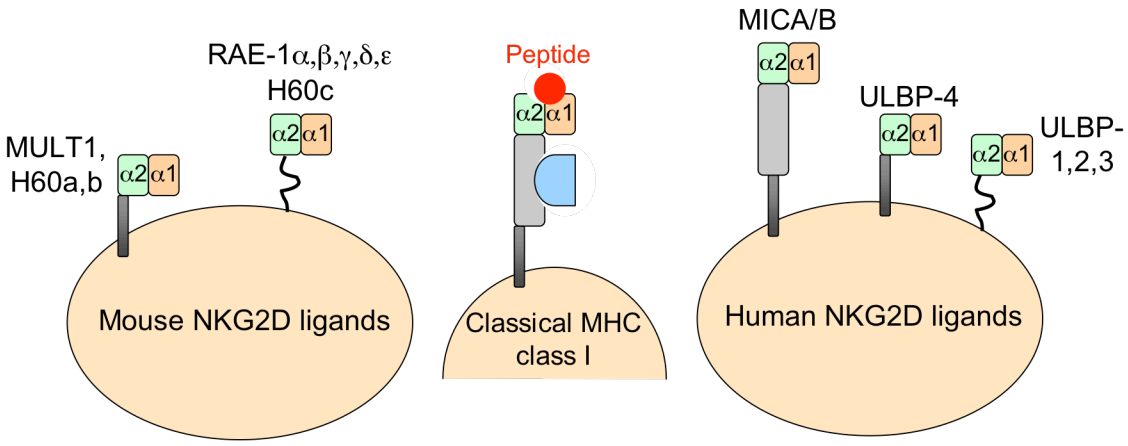

Figure 1.5. Mouse and human NKG2D ligands. H60a mouse ligands is exclusively expressed in BALB/C mice and not in C56BL/6 animals. Adapted from Ogasawara K. & Lanier L.L., J. Clin. Immunol. (2005) 25:6, 534-540.

The RAE-1 proteins are highly related (>85%), whereas the H60 and MULT1 proteins show less than 25% homology to each other or with RAE-1 proteins (107). The five RAE-1 proteins are also differentially expressed in different mouse strains. For instance, RAE-1 α, β and γ are expressed in BALB/C mice, whereas RAE-1δ and ε are expressed in C57BL/6 and B10D2 mice. The human counterparts of the mouse *RAE-1* genes are the *ULBP* genes. A comparison of the ULBP and MIC proteins indicates that they share less than 20% homology with each other or with any of the mouse ligands (107). Thus, it is remarkable that all these proteins, with low homology, bind with relatively high affinity to NKG2D. Interestingly, structural analyses of NKG2D-ULBP3, NKG2D-MICA, and NKG2D-RAE-1β complexes demonstrates that the receptor and ligand interfaces are similar, despite their use of totally different residues for binding (149-151).

Taking all these findings together, it remains intriguing why there are so many human and mouse genes encoding ligands for a single invariant receptor such as NKG2D. One possibility is that the diverse expression patterns of different ligands might be specialized to respond to distinct indications of cellular distress (167).

NKG2D related immune diseases

The NKG2D receptor has been shown in both mice and humans to play a role in the recognition of viral infections, tumors, transplanted cells and organs, as well as in autoimmune disorders.

In mice and humans, the role of NKG2D in immune responses against CMV is perhaps the best example known of the importance of this activating receptor in antiviral immunity. Notably, MCMV has evolved by devoting considerable resources to block the expression of NKG2D ligands on the cell surface of infected cells. Specifically, m152 inhibits expression of RAE-1 (168), m155 and m138 down-regulate H60 (169, 170), while MULT1 is targeted by m145 and m138 (170, 171). In MCMV-infected cells, genes that encode NKG2D ligands are transcribed, but viral proteins interact with and cause degradation of NKG2D ligand proteins, thus inhibiting detection by NKG2D, in this case on NK cells (111). Like MCMV, HCMV has developed strategies to evade NKG2D, by evolving unique genes that prevent expression of the human NKG2D ligands, such as MICA (172), MICB and ULBPs (173, 174). Notably, the importance of the NKG2D activating pathway is highlighted by the down-regulation of its ligands by diverse viruses, such as the HCMV (172, 175) and MCMV (170, 171, 176), zoonotic orthopoxviruses (177), HIV (178) and Kaposi's sarcoma-associated herpesvirus (KSHV) (179). On the other hand, MICB has been detected on human macrophages infected with influenza A or Sendai virus, by a mechanism which is partially dependent on virus-induced IFNα production (180).

In mouse hepatitis virus (MHV) infection, Dandekar *et al* have shown evidence that the pathology of the central nervous system caused by MHV infection is partially due to a NKG2D-dependent mechanism mediated by $\gamma\delta$ T cells (181). Moreover, Walsh *et al* recently suggested differential roles for NKG2D signaling in innate host defense against coronavirus-induced neurological and liver disease. Specifically, they demonstrated that NKG2D neutralization in vivo increased viral titers, exclusively in the liver, suggesting a protective role for NKG2D signaling in this organ (182).

Recent studies on ectromelia virus, an Orthopoxvirus that causes mousepox and is the mouse equivalent of human smallpox, demonstrated that this virus leads to up-regulation of RAE-1. Following this viral infection, NK cells proliferate and up-regulate NKG2D on their surface, contributing to the prevention of mousepox dissemination (183). Furthermore, blocking NKG2D function during infection leads to uncontrolled early virus dissemination and impaired cytotoxicity (183).

Before we began our studies, very little was known about the role of NKG2D and its ligands during HBV infection. Carrington and colleagues (184) hypothesized that MICA heterogeneity would affect recovery from HBV infection since the human NKG2D ligand, MICA, is highly polymorphic and its various allotypes bind to NKG2D with different affinities (185). However, their data suggested that differential distribution of MICA does not significantly affect HBV infection outcome.

Tumors, in general, are poorly immunogenic since they develop from self-cells. However, NKG2D ligands are frequently expressed on a substantial number of human and mouse tumors. A role for NKG2D in surveillance against primary tumorigenesis was described when mice exposed to chemical carcinogens developed fibrosarcomas at a higher incidence post-treatment with an anti-NKG2D monoclonal antibody (186), and when RAE-1 was constitutively overexpressed as a transgene in the carcinogen-treated mice (134). A positive association has been demonstrated between human tumor immunotherapy and the development of antibodies targeting secreted MICA (187).

Despite its host protection against infectious diseases and tumors, NK cells can reject bone marrow grafts. Interestingly, it was shown that mouse NK cells are able to reject syngeneic C57BL/6 bone marrow cells when these otherwise normal bone marrow cells expressed sufficient amounts of a NKG2D ligand (188). Moreover, in humans, it has been recently suggested that antibodies generated against MICA have prognostic value in renal graft rejection (189).

NKG2D-mediated responses are beneficial in the majority of immune responses against tumors and pathogens. However, there has been some evidence that this pathway can cause aberrant activation of the immune system leading to autoimmunity. In human patients with rheumatoid arthritis, MICA was detected on synoviocytes in joints accompanied by the presence of an unusual subset of $CD4^+$ T cells that do not express CD28 but do express NKG2D on their surface (190). Although an abundance of soluble MICA is also found in these patients, they do not down-modulate NKG2D, probably due to the activity of the pro-inflammatory cytokines, TNFα and IL-15. The inflammation associated

with celiac disease (a gluten-induced intestinal inflammatory disorder) also results in elevated levels of the pro-inflammatory cytokine IL-15, which in turn induces NKG2D expression on intra-epithelial lymphocytes (IELs) (191). Moreover, MICA, constitutively expressed at low levels on epithelial cells, was also shown to be significantly increased in celiac patients. Several independent studies support that NKG2D/MICA interaction triggers activation and costimulation of IELs, leading to an innate-like cytotoxicity toward epithelial targets, which corresponds to one of the major complications of celiac disease (192-194). In mice, RAE-1 was detected on the surface of pancreatic islet cells in NOD mice, and treatment with a neutralizing, non-depleting anti-NKG2D mAb, seemed to prevent diabetes (195).

Natural Killer T cells and hepatitis

Although it was known for quite some time that NKT cells are enriched in the liver, their role in HBV infection has not been thoroughly addressed. Chisari and collaborators demonstrated that classical NKT cells, when activated by the injection of α-GalCer into HBV-transgenic mice contributed to inhibition of viral replication, through the production of IFNγ (196). The same group also showed that IL-18 produced by activated APCs rapidly, reversibly, and noncytopathically inhibited HBV replication (197). They further demonstrated that the antiviral effect of IL-18 was mediated by its ability to activate resident intra-hepatic NK and NKT cells to produce IFNγ and also by its ability to induce IFNα/β production in the liver (197).

Baron *et al* were the first group to demonstrate in vivo that NKT cells are activated by a naturally occurring hepatotropic virus, namely HBV, and can play a role in liver injury mediated by such pathogen. Furthermore, this study narrowed the NKT cell population to a non-classical NKT cell subset, known as CD1d-restricted type II NKT cells.

The liver injury seen in the autoimmune hepatitis model of concanavalin (ConA)-induced hepatitis, extensively used by immunologists, has also been described to be NKT cell-mediated (198). Using this model, Swain and colleagues demonstrated that CCR5 deficiency promotes development of acute fulminant liver failure in ConA-injected mice by preventing activated CD1d-restricted NKT cells (but not conventional T cells) from dying by activation-induced apoptosis (199). Further evidence supported that fulminant liver failure in CCR5-deficient mice was correlated with increased IL-4 production, but not IFNγ. Intra-

hepatic CCR5-deficient NKT cells secreted significantly higher amounts of IL-4 as compared to wild-type NKT cells (199).

In humans, where immunological studies are significantly more demanding and restricted, an intra-hepatic enrichment of classical NKT cells was found in biopsies from patients with chronic HBV and HCV hepatitis (200). These classical NKT cells exhibit a modification in their effector potential, consisting in the capacity to produce IL-4 and IL-13 in addition to IFNγ, which characterizes the cirrhotic state. Since these functional changes in classical NKT cells are present in both chronic HBV and HCV infections, it suggests that these cells respond to factors which are not viral specific but perhaps that are released during hepatocyte necrosis. In the same study, it was also demonstrated that CD1d is strongly expressed in cirrhotic liver, mainly by APCs infiltrating the liver parenchyma and on cells juxtaposed to hepatic stellate cells (200).

Objectives

The work presented in this thesis is aimed at understanding the molecular and cellular mechanism(s) of early immune recognition of HBV-expressing hepatocytes in a mouse model of primary human HBV infection.

The results of this dissertation are divided in two main parts.

The **first part**, composed by chapters II and III, focuses on:

I. the study of the molecular mechanism of non-classical NKT cell activation in this transgenic mouse model of primary hepatitis B virus infection after adoptive transfer of syngeneic naïve wild-type splenocytes.

The **second part**, comprised by chapter IV, investigates:

II. other intra-hepatic innate immune cells, in particular NK cells, that might be involved in HBV recognition, potentially generating signals that could contribute to the hepatic immunological environment in which immune responses are primed.

CHAPTER II

Blockade of NKG2D on NKT cells prevents hepatitis and the acute immune response to hepatitis B virus

Chapter II of this dissertation is based on material published in *Proceedings of the National Academy of Sciences U.S.A*. (2007), 104:46, 18187-18192, entitled “Blockade of NKG2D on NKT cells prevents hepatitis and the acute immune response to hepatitis B virus”. Kouetsu Ogasawara, Stephen Nishimura, Lewis L. Lanier and Jody L. Baron are the co-authors of this work, listed in the same order as appears in the published paper.

INTRODUCTION

The transgenic mouse model of primary human HBV infection developed by our laboratory (described in chapter I) allows uniquely the study of innate and adaptive immune responses to HBV. It was previously demonstrated that the acute liver injury observed in these mice is mediated by non-classical type II NKT cells, which do not recognize the classic NKT cell ligand, α-GalCer–CD1d and do not express the canonical TCR Vα14 (71). However, activation of these cells in this model is dependent on the expression of CD1d and HBV (71).

Innate immune effector cells mediate the acute hepatitis in our model, although the mechanism of activation of these cells in response to the presence of HBV in the liver is not known. Our previous data suggested that the presence of HBV leads to alterations in the class I-like molecule CD1d, and subsequently to the activation of non-classical NKT cells and hepatitis (71). NKT and NK cells share many of the same activating and inhibitory receptors. One such receptor is NKG2D, a type II transmembrane-anchored glycoprotein, which has been shown to be an activating or co-stimulatory receptor expressed on the surface of all NK cells, activated $CD8^+$ T lymphocytes, and most γ/δ T cells, both in mice and humans (136, 138, 201). Although NKG2D is known to also be expressed on the surface of NKT cells (167, 202), a role for NKG2D in NKT cell activation has not yet been demonstrated. NKG2D binds to a family of ligands with structural homology to MHC class I molecules. In mice, NKG2D ligands include the RAE-1 family of proteins (RAE-1α, β, γ, δ, ε), H60, and MULT1 (154, 155, 157).

In this study, we addressed the question of whether NKG2D and its ligands play a role in the non-classical NKT cell-mediated immune response to HBV and the subsequent acute hepatitis that ensues. Our results demonstrate that NKG2D is modulated on NK and NKT cells at the time of acute hepatitis; and the presence of HBV in the livers of our transgenic mice can lead to an increase in RAE-1 surface expression on hepatocytes. Furthermore, blockade of an NKG2D–ligand interaction completely prevents the HBV-specific and CD1d-dependent, non-classical NKT cell-mediated acute hepatitis and liver injury.

RESULTS

NKG2D expression is modulated on intra-hepatic immune cells from HBV-Env$^+$*Rag-1*$^{-/-}$ mice with acute hepatitis

Our previous studies have demonstrated that activation of non-classical NKT cells is necessary for the acute hepatitis to develop and that NK cells or conventional T cells alone cannot initiate the acute hepatitis (71). NK1.1$^+$ cells from the livers of HBV-Env$^+$ *Rag-1*$^{-/-}$ and *Rag-1*$^{-/-}$ mice before adoptive transfer of syngeneic naïve splenocytes expressed equivalent amounts of NKG2D on their surface (Fig. 2.1A). However, when we analyzed the expression of NKG2D on liver lymphoid cells during the acute immune response and hepatitis seen in the livers of HBV-expressing mice with reconstituted immunity (Fig. 2.1B), we found that NK1.1$^+$ cells from HBV-Env$^+$ *Rag-1*$^{-/-}$ mice (which include both resident and donor NK cells and donor NKT cells) expressed higher levels of NKG2D than the same population eluted from *Rag-1*$^{-/-}$ mice that also had reconstituted immunity (Fig. 2.1C).

We next analyzed the surface expression of NKG2D on the NKT and NK populations in the liver at the peak of acute hepatitis. We found that NK cells eluted from the livers of HBV-Env$^+$ *Rag-1*$^{-/-}$ mice with acute hepatitis expressed high levels of NKG2D (Fig. 2.1D), but the majority of activated NKT cells expressed very low levels of NKG2D on their cell surface (Fig. 2.1D). Because the majority of NKT cells in the spleen (the cells adoptively transferred) and liver of wild-type mice expressed high levels of NKG2D (Fig. 2.1E), this result suggests that NKT cells eluted from the livers of the HBV-Env$^+$ *Rag-1*$^{-/-}$ mice have down-regulated the surface expression of NKG2D. This is consistent with the fact that NKG2D is known to be internalized after interaction with its ligands (71, 107, 133). Taken together, these results suggest that NKG2D expression is up-regulated on the NK cells, and down-regulated on the NKT cells, during acute hepatitis.

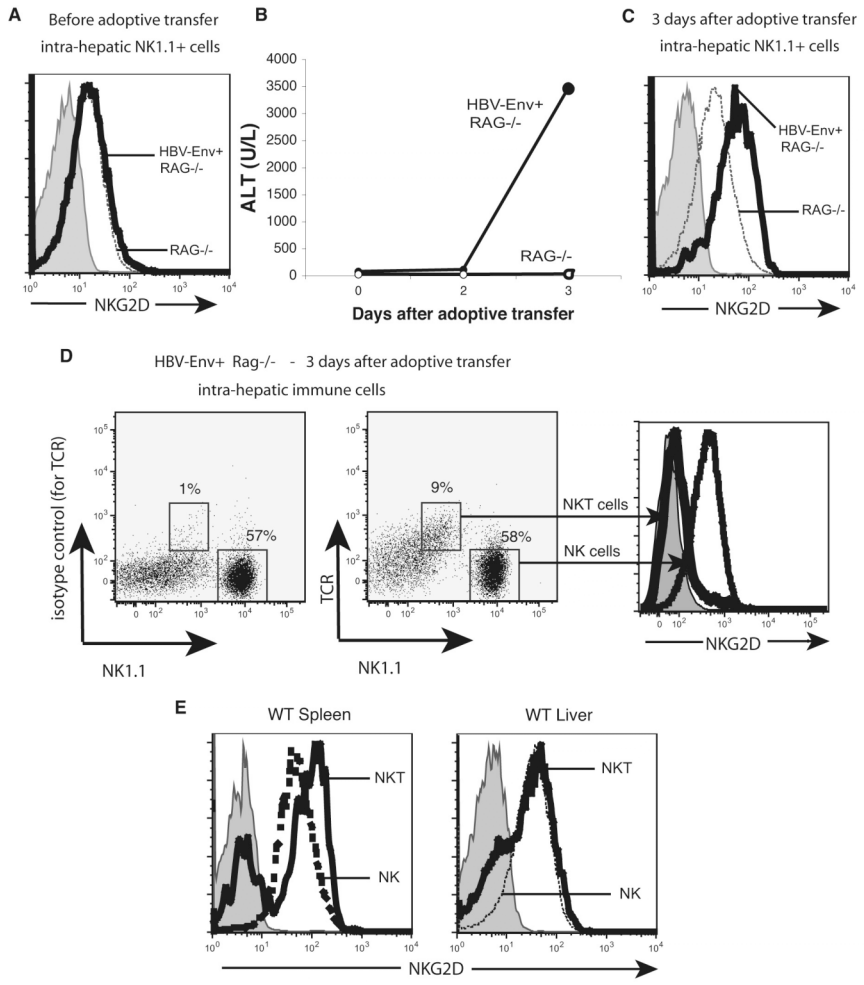

Figure 2.1. Modulation of the NKG2D receptor during acute hepatitis. NKG2D expression on the surface of NK1.1^{+} cells (forward light scatter (FSC) x side light scatter (SSC) lymphocyte gate plus NK1.1 gate) from HBV-Env^{+} *Rag-1*$^{-/-}$ (——) and *Rag-1*$^{-/-}$ (-----) before (panel **A**) and 3 days after (panel **C**) adoptive transfer of 1x10^{8} splenocytes. Tinted histograms depict staining using an isotype-matched control rat IgG1. Hepatic necrosis in these animals was assessed by the measurement of ALT in the sera of RAG$^{-/-}$ (○) or HBV-Env^{+} *Rag-1*$^{-/-}$ mice (●). ALT values are shown as mean ± SEM (panel **B**). Surface expression of NKG2D on intra-hepatic NKT (——) and NK cells (-----) from HBV-Env^{+} *Rag-1*$^{-/-}$ mice 3 days after adoptive transfer (panel **D**). The left dot plot depicts the isotype-matched control Ig staining of TCR on NKT cells. Surface expression of NKG2D on NKT cells (——) and NK cells (-----) from the spleen and liver of wild-type mice (panel **E**). The percentages (64% and 57%) refer to NKG2D^{+} NKT cells in the spleen and liver, respectively. The tinted histograms depict staining using an isotype-matched control rat IgG1 both on NK and NKT cells. All experiments were repeated at least three times, and representative data are shown.

Constitutive surface expression of RAE-1 on hepatocytes is elevated specifically on HBV-Env$^+$ hepatocytes

In light of these data, we examined the expression of NKG2D ligands on wild-type non-transgenic hepatocytes and on HBV-Env$^+$ hepatocytes. In the genetic background of the HBV-transgenic mice (B10.D2 and C57BL/6), the NKG2D ligands expressed are *RAE-1*δ, *RAE-1*ε, and MULT1 (107). Although RAE-1 is not expressed in most tissues isolated from healthy, adult mice, RAE-1 is transcribed preferentially in the liver of healthy, adult mice (http://source.stanford.edu/cgi-bin/source/sourceSearch). We examined the expression of these NKG2D ligand proteins on primary hepatocytes and intra-hepatic immune cells of HBV-Env$^+$ *Rag-1*$^{-/-}$ and wild-type non-transgenic *Rag-1*$^{-/-}$ mice before adoptive transfer. We found constitutive low-level surface expression of RAE-1 on hepatocytes from *Rag-1*$^{-/-}$ mice, which was increased specifically on the surface of HBV-Env$^+$ hepatocytes (Fig. 2.2A). This constitutive expression of RAE-1 on hepatocytes, and increased expression in the HBV-Env$^+$ transgenic mice, was also found in wild-type mice that were not crossed to *Rag-1*$^{-/-}$ mice (data not shown). We did not find expression of RAE-1 on splenocytes or on intra-hepatic immune cells from HBV-Env$^+$ *Rag-1*$^{-/-}$ mice, *Rag-1*$^{-/-}$ mice, or wild-type mice (Fig. 2.2B, and data not shown). The constitutive surface expression of RAE-1 on hepatocytes is an interesting finding since the expression of RAE-1 family members is strictly regulated in normal cells, and little expression is found on healthy adult tissue (107). Increased RAE-1 expression on hepatocytes from HBV-Env$^+$ *Rag-1*$^{-/-}$ mice demonstrates that RAE-1 can be modulated on hepatocytes in a HBV-specific manner. We did not detect MULT1 expression, or a change in either MHC class I or CD1d expression, on primary hepatocytes derived from either HBV-Env$^+$ *Rag-1*$^{-/-}$ or *Rag-1*$^{-/-}$ mice (Fig. 2.2C) or on intra-hepatic immune cells (data not shown).

Constitutive expression of RAE-1 on primary hepatocytes from RAG$^{-/-}$ mice was confirmed by quantitative PCR of reverse transcribed RAE-1 mRNA normalized to the expression of *Hprt* transcripts (Fig. 2.2B). Increased RAE-1 expression on hepatocytes from HBV-Env$^+$ *Rag-1*$^{-/-}$ mice, as compared to *Rag-1*$^{-/-}$ mice, was also confirmed by quantitative RT-PCR. There was an almost 7-fold increase in RAE-1 mRNA from HBV-Env$^+$ *Rag-1*$^{-/-}$ hepatocytes, as compared to *Rag-1*$^{-/-}$ hepatocytes (Fig. 2.2B).

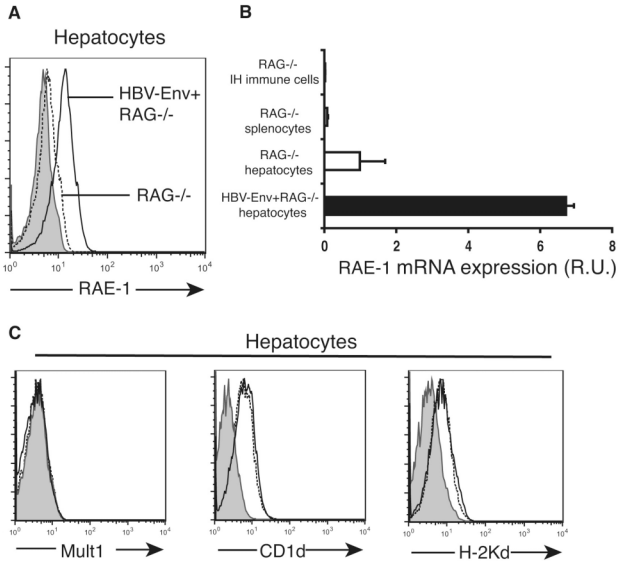

Figure 2.2. **Constitutive surface expression of the NKG2D ligand RAE-1 on hepatocytes is up-regulated specifically on HBV-Env-expressing hepatocytes, before adoptive transfer of syngeneic naïve splenocytes.** Surface expression of RAE-1 (panel **A**), MULT1, CD1d, and H-2K^{d} (panel **C**) on hepatocytes from HBV-Env^{+} *Rag-1*$^{-/-}$ (——) and *Rag-1*$^{-/-}$ mice (-----). Tinted histograms depict staining using the appropriate isotype-matched control Ig. (**B**) RAE-1 mRNA expression in hepatocytes from HBV-Env^{+} *Rag-1*$^{-/-}$ (▮) and *Rag-1*$^{-/-}$ (□) mice and from intra-hepatic (IH) immune cells and splenocytes from *Rag-1*$^{-/-}$mice (▯) in comparison with HPRT expression. All data are representative of at least three independent experiments.

Blocking of an NKG2D-ligand interaction in vivo prevents the acute immune response to human hepatitis B virus

In view of the fact that NKG2D and one of its ligands are modulated during the acute immune response to HBV, we studied the effects of blocking this interaction on the onset of the acute hepatitis by using an anti-mouse NKG2D monoclonal antibody (CX5), which efficiently blocks the binding of NKG2D to its ligands and does not deplete NKG2D-bearing cells *in vivo* (195). HBV-Env^{+} *Rag-1*$^{-/-}$ recipient mice were treated with 200 μg of anti-NKG2D mAb (CX5) or control rat IgG the day before and 4 days after adoptive transfer of syngeneic

naive splenocytes. Blocking the NKG2D receptor completely prevented acute liver injury in all HBV-Env^{+} *Rag-1*$^{-/-}$ mice, whereas the control antibody had no effect as all mice showed signs of massive acute hepatitis, as revealed by the elevated serum ALT values at days 3 and 4 after adoptive transfer (Fig. 2.3A). Histological analyses of liver sections also showed that mice treated with control IgG developed a severe hepatitis, pathologically characterized by parenchymal inflammation, hepatocellular damage, and portal inflammation and necrotic hepatocytes at day 4 after adoptive transfer (Fig. 2.3B). These histological abnormalities were absent at the same time point in all of the mice treated with anti-NKG2D mAb (Fig. 2.3B). These results demonstrate a fundamental role played by NKG2D in the acute immune response to HBV-expressing hepatocytes, and the consequent development of hepatitis and hepatic necrosis.

HBV-Env^{+} *Rag-1*$^{-/-}$ mice have an HBV-dependent increase in the frequency of IFN-γ and IL-4-producing cells in their livers 3 days after adoptive transfer (71). Because NKT cells mediate this cytokine burst detected at the time of acute hepatitis, we investigated the cytokine profile of lymphoid cells in anti-NKG2D or control IgG-treated HBV-Env^{+} *Rag-1*$^{-/-}$ mice. We quantified the number of IFN-γ and IL-4-producing intra-hepatic immune cells by Elispot at day 3 and 4 after adoptive transfer of syngeneic wild-type splenocytes. Three days after the adoptive transfer, the number of IFN-γ and IL-4-producing cells increased by 8- and 7-fold, respectively, in mice that received control IgG (and developed hepatitis) as compared to NKG2D-blocked mice (Fig. 2.3C). A similar difference was observed on day 4 after adoptive transfer. These data demonstrate that blocking of NKG2D also severely impaired the production of cytokines by intra-hepatic immune cells in mice expressing HBV antigens. Flow cytometric analysis of the intra-hepatic immune cells derived from the anti-NKG2D or control IgG-treated HBV-Env^{+} *Rag-1*$^{-/-}$ mice revealed that the absolute number of NK cells eluted from both groups of mice was similar, whereas the absolute number of NKT cells was reduced by 2- and 3-fold in mice that received the anti-NKG2D treatment and did not develop hepatitis (Table 2.1). This specific reduction in the number of NKT cells, but not NK cells, in the livers of the anti-NKG2D treated mice suggests that the antibody is specifically affecting the NKT cells.

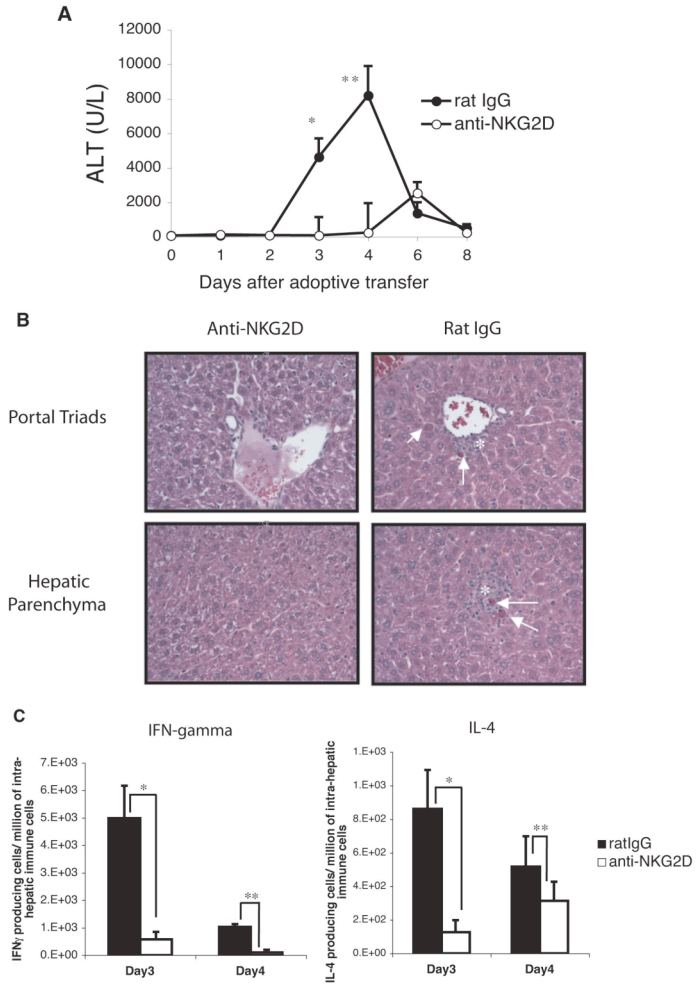

Figure 2.3. Blocking NKG2D in vivo prevents the liver injury caused by the acute immune response to hepatitis B virus (HBV). (**A**) Serum ALT levels of HBV-Env^{+} *Rag-1*$^{-/-}$ mice treated with anti-NKG2D mAb (○) or rat IgG (●). The ALT values as mean ± SEM are shown. Student's t test analyses: *p<0.02 **p<0.01. Hematoxylin and eosin stained section (20x) of portal triads (panel **B,** upper pictures) and hepatic lobular parenchyma (panel **B**, bottom pictures) from HBV-Env^{+} *Rag-1*$^{-/-}$ mice treated with anti-NKG2D mAb (left pictures) or rat IgG (right pictures), 4 days after the adoptive transfer of 1x10^{8} splenocytes. Arrows point to necrotic hepatocytes and asterisks indicate inflammatory infiltrate. Elispot analyses of IFN-g and IL4-producing intra-hepatic immune cells (panel **C**) from HBV-Env^{+} *Rag-1*$^{-/-}$ mice treated with control rat IgG (▮) or anti-NKG2D mAb (□) at days 3 and 4 after adoptive transfer. Representative data are shown as mean ± SD. Student's t test analyses: *p<0.005 **p<0.02. All data are representative of at least three independent experiments.

Experiment #1					
Experimental group [a)]	Total # cells	% IH NK cells	Total # IH NK cells	% IH NKT cells	Total # IH NKT cells
RatIgG	$3.35x10^6$	17.5	$5.87x10^5$	9.17	$3.07x10^5$
Anti-NKG2D	$2.03x10^6$	28.7	$5.83x10^5$	5.42	$1.1x10^5$
Experiment #2					
RatIgG	$11.25x10^6$	27.0	$3x10^6$	9.33	$1.05x10^6$
Anti-NKG2D	$13x10^6$	31.15	$4.05x10^6$	5.22	$6.78x10^5$

Table 2.1. Effects of blocking of NKG2D on the number of intra-hepatic immune cells during acute hepatitis.

[a)] Representative data of two independent experiments are shown. The total number of intra-hepatic (IH) immune cells corresponds to a pool of 3 livers in experiment #1 and of 4 livers in experiment #2.

Note: The total number of cells eluted from the livers of an HBV-Env$^+$ *Rag-1*$^{-/-}$ 3 days after adoptive transfer is variable. However, in both experiments the total number of IH NK cells is consistently similar between both experimental groups whereas the total number of IH NKT cells is consistently reduced by 2 to 3 fold in the mice that received the anti-NKG2D mAb treatment.

NKG2D is expressed on approximately 60% of NKT cells in the spleen (Fig. 2.1E). To determine whether donor NKT cells expressing NKG2D are responsible for the induction of acute hepatitis after transfer into HBV-Env$^+$ *Rag-1*$^{-/-}$ mice, splenocytes from wild-type mice were depleted of NKG2D$^+$ lymphocytes by flow cytometric cell sorting. NKG2D-depleted splenocytes were adoptively transferred into HBV-Env$^+$ *Rag-1*$^{-/-}$ recipients. In this way, the transferred donor NKT cells would not express surface NKG2D, but resident NK cells in the recipient mice would still express NKG2D. HBV-Env$^+$ *Rag-1*$^{-/-}$ mice received NKG2D-depleted splenocytes or appropriate controls for the total number of splenocytes, or total number of NK and NKT cells transferred. The HBV-Env$^+$ *Rag-1*$^{-/-}$ recipient mice received one of three different populations of donor splenocytes: 50 million NKG2D-depleted splenocytes (which included 1.25 x 10^5 NKG2D$^-$ NKT cells, and no NK cells); 50 million unsorted splenocytes (which included 2.5 x 10^5 unsorted NKT cells and 1.25 x 10^6 NK cells); or 33 million NK cell-depleted splenocytes (which included 1.25 x 10^5 unsorted NKT cells, and no NK cells). In this latter group, the NK cells were depleted from the donor mice by injection of anti-asialoGM1 antisera, which is known to deplete NK cells, but not NKT cells (203).

Depletion of NKG2D$^+$ NKT cells, but not depletion of NKG2D-bearing NK cells, from donor splenocytes significantly diminished the acute liver injury and cytokine burst seen in the HBV-Env$^+$ *Rag-1*$^{-/-}$ mice as compared to either control group (Fig. 2.4A and B). Thus, NKG2D receptor expression on NKT cells and not on NK cells is required for efficient disease induction. The finding that depletion of NKG2D-bearing cells did not completely eliminate all disease in all recipient mice could be accounted for the limitations of cell sorting, and expression of NKG2D on initially NKG2D$^-$ NKT cells after adoptive transfer (data not shown).

To control for the possibility that NKG2D expression on other cells types, e.g. CD8$^+$T cells, CD4$^+$ T cells or $\gamma\delta$ T cells, contribute to the induction of hepatitis, we selectively depleted only NKG2D$^+$ NKT cells from donor splenocytes, and transferred these sorted cells (which included all other cell types potentially expressing NKG2D) into HBV-Env$^+$ *Rag-1*$^{-/-}$ mice. As a control, unsorted splenocytes stained with the antibodies used for NKG2D NKT cells depletion were also transferred into HBV-Env$^+$ *Rag-1*$^{-/-}$ mice. Depletion of NKG2D$^+$ NKT cells from donor splenocytes completely prevented induction of acute hepatitis in the HBV-Env$^+$ *Rag-1*$^{-/-}$ mice (Fig. 2.5). By contrast, mice receiving an equivalent number of the stained, but unsorted splenocytes developed hepatitis (Fig. 2.5).

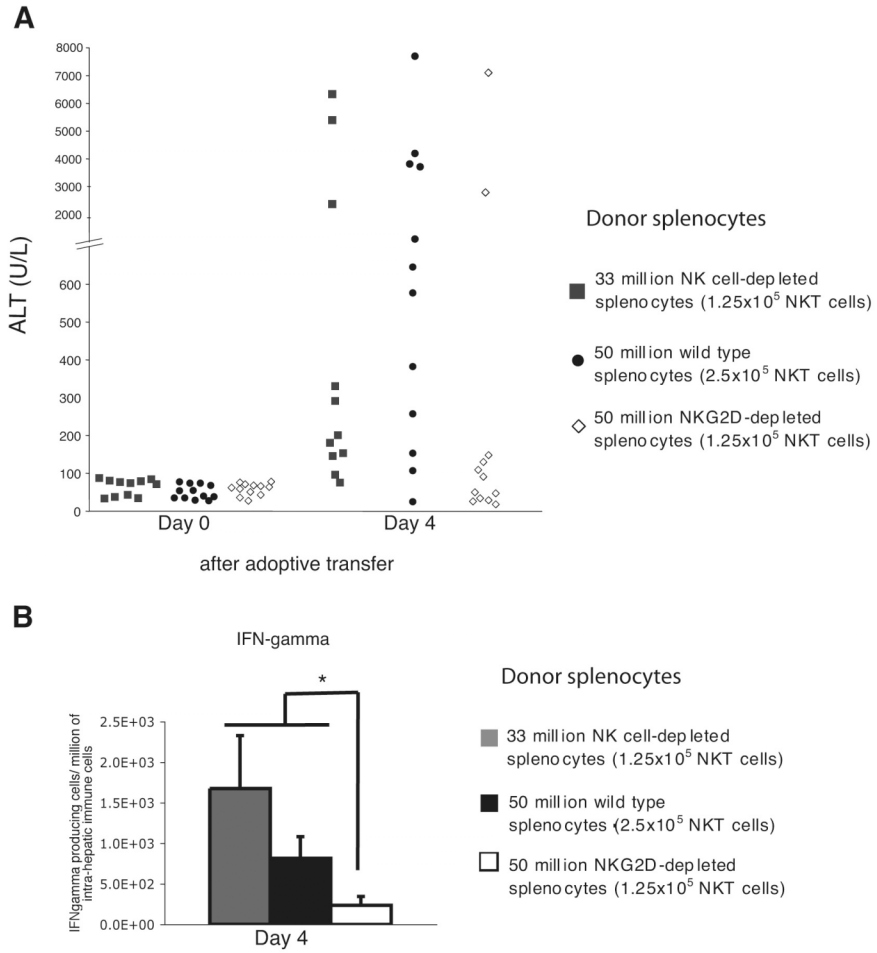

Figure 2.4. NKG2D$^+$ NKT cells are required for efficient disease induction and cytokine production during the acute immune response to HBV. (**A**) Hepatic injury as assessed by the measurement of ALT in the sera of HBV-Env$^+$ *Rag-1*$^{-/-}$ mice at day 4 after adoptive transfer of 50x10^6 NKG2D-depleted splenocytes (which included 1.25x10^5 NKG2D$^-$ NKT cells, and no NK cells) (◇) was compared with hepatic injury in HBV-Env$^+$ *Rag-1*$^{-/-}$ mice that received the same total number of unsorted wild-type splenocytes (50x10^6, which included 2.5x10^5 unsorted NKT cells, and 1.25x10^6 NK) (●) (Mann-Whitney test analyses: $p<0.02$); or that received the same total number of unsorted NKT cells and NK cells (33x10^6, which included 2.5x10^5 unsorted NKT cells, and no NK cells) (□). (Mann-Whitney test analyses: $p<0.03$). The NKG2D-depleted splenocytes were isolated by staining splenocytes with anti-NKG2D mAb and purifying by flow cytometry the NKG2D-negative fraction (purity >98%). The NK cell-depleted splenocytes were prepared by injecting donor mice with anti-asialo GM1 antisera two day before cell harvest, a procedure that depleted NK cells, but not NKT cells. (**B**) Elispot analyses of IFN-γ producing intra-hepatic immune cells from HBV-Env$^+$ *Rag-1*$^{-/-}$ mice depicted in panel A. 50x10^6 NKG2D-depleted splenocytes (□), 50x10^6 unsorted wild-type splenocytes (▮), or 33x10^6 NK cell-depleted splenocytes (▯). Representative data are shown as mean ± SD. Student's t test analyses: *$p<0.001$.

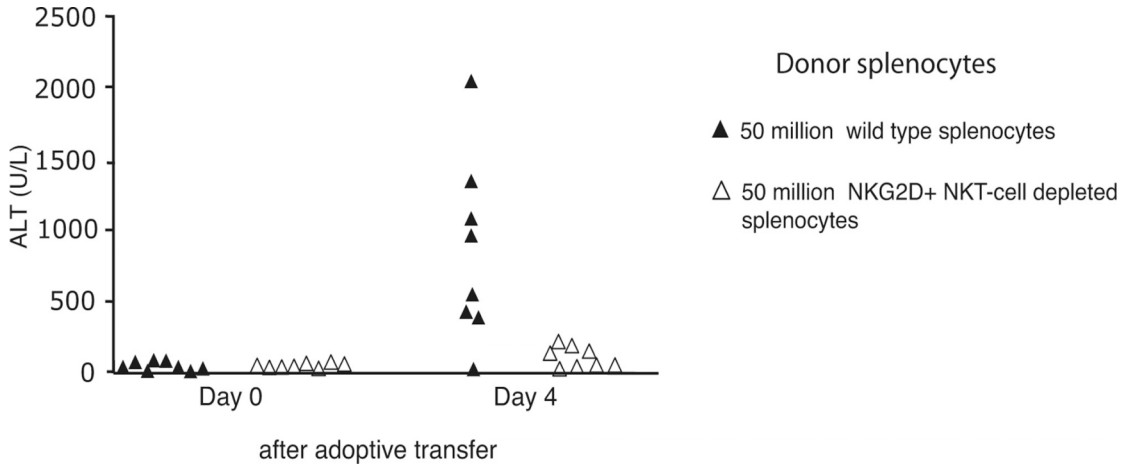

Figure 2.5. Depletion of NKG2D$^+$ NKT cells from donor splenocytes prevents the acute immune response to HBV. Hepatic injury as assessed by the measurement of ALT in the sera of HBV-Env$^+$ *Rag-1*$^{-/-}$ mice at day 4 after adoptive transfer of 50 x 10^6 NKG2D$^+$ NKT cell-depleted splenocytes (△) was compared with hepatic injury in HBV-Env$^+$ *Rag-1*$^{-/-}$ mice that received the same total number of unsorted, stained, wild-type splenocytes (▲)(Mann-Whitney test analyses: $p<0.0102$).

NKG2D blockade prevents acute hepatitis and the cytokine burst seen in HBV-Replication$^+$ *Rag-1*$^{-/-}$ transgenic mice

In order to assess the relevance of our observations to responses to authentic human HBV infection, we characterized the role of NKG2D in the acute immune response developed in HBV-Replication$^+$ *Rag-1*$^{-/-}$ mice, which display intra-hepatic HBV replication and produce infectious virions (64). Reminiscent of the usual initial presentation of human HBV infection, these mice develop a mild, sub-clinical hepatitis after adoptive transfer of naïve splenocytes. Analogous to our observations in the HBV-Env$^+$ *Rag-1*$^{-/-}$ mice, this hepatitis is mediated by non-classical NKT cells in an HBV-specific and CD1d-dependent manner, leading to cytokine production (71) (and Baron lab, unpublished data). Although the severity of hepatitis seen in the two lines of HBV-transgenic mice is different, owing to an increase in hepatocyte sensitivity to cytotoxic effects of IFN-γ in the HBV-Env$^+$ mice (204), a similar disease pattern is seen in both lines of HBV-transgenic mice. Specifically, a biphasic ALT rise that is seen in the HBV-Env$^+$ *Rag-1*$^{-/-}$ mice is also observed in the HBV-Replication$^+$ *Rag-1*$^{-/-}$ mice, but as expected, the ALT rise is much more modest than that seen in the

HBV-Env^{+} mice — typically, serum transaminases were elevated no more than 2-fold above background, which is a clinically significant finding in human HBV disease.

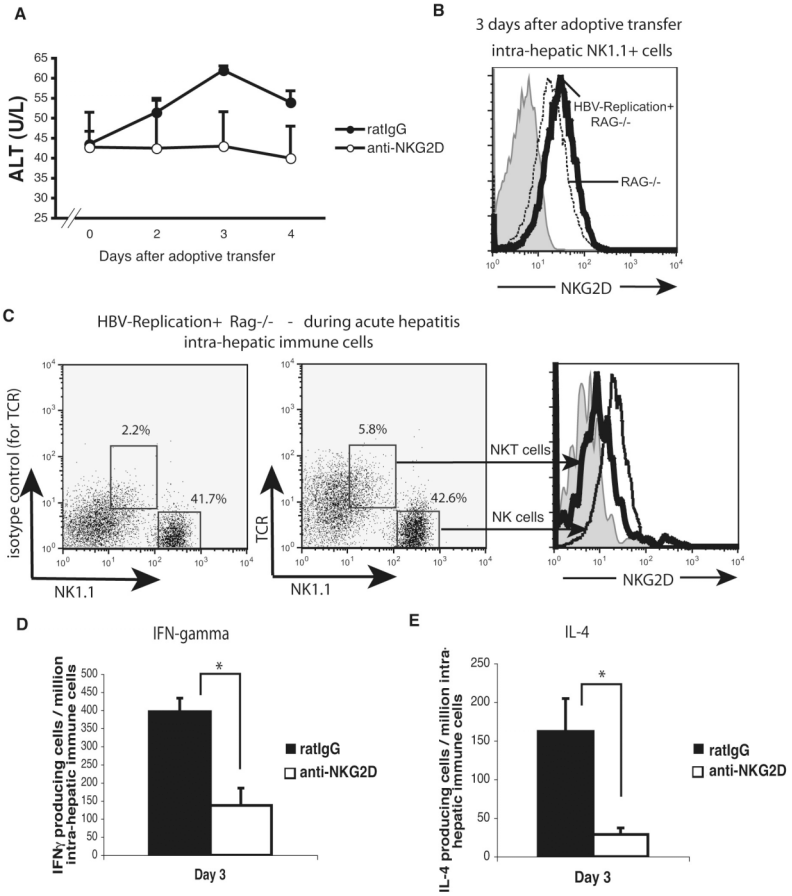

Figure 2.6. Blocking an NKG2D-ligand interaction in HBV-Replication^{+} *Rag-1*$^{-/-}$ mice prevents liver injury and cytokine production mediated by the acute immune response to HBV. (A) Serum ALT levels of HBV-Replication^{+} *Rag-1*$^{-/-}$ mice treated with anti-NKG2D mAb (○) or rat IgG (●) at 2, 3, and 4 days after adoptive transfer of 1x10^{8}syngeneic splenocytes are shown as mean ± SEM. (**B**) NKG2D surface expression on intra-hepatic NK1.1^{+} cells from HBV-Replication^{+} *Rag-1*$^{-/-}$ mice (——) as compared to *Rag-1*$^{-/-}$ mice (-----) at day 3 after the adoptive transfer of syngeneic naïve splenocytes. Tinted histogram depicts staining using the appropriate isotype-matched control Ig (rat IgG1).(**C**) Surface expression of NKG2D on intra-hepatic NKT (——) and NK cells (-----) from HBV-Replication^{+} *Rag-1*$^{-/-}$ mice 3 days after adoptive transfer. The left dot plot depicts the isotype-matched control Ig staining of TCR on NKT cells. Elispot analyses of IFNγ (**D**) and IL-4-producing (**E**) intra-hepatic immune cells from HBV-Replication^{+} *Rag-1*$^{-/-}$ mice treated with rat IgG (▮) or anti-NKG2D mAb (▯) at day 3 after adoptive transfer. Representative data are shown as mean ± SD. Student's t test analyses: *p<0.001. All data are representative of at least two independent experiments.

HBV-Replication^{+} *Rag-1*$^{-/-}$ mice were treated the day before the adoptive transfer of syngeneic naïve splenocytes with 200 μg of anti-NKG2D or control IgG and the serum ALT levels were monitored. We found that the modest rise in the serum ALT in the HBV-Replication^{+} *Rag-1*$^{-/-}$ mice treated with control IgG was not evident in mice treated with anti-NKG2D (Fig. 2.6A). Together with this modest rise of ALTs, we observed that 3 days after the adoptive transfer, the number of IFNγ and IL-4-producing cells increased by 3- and 6-fold, respectively, in mice that received control IgG as compared to NKG2D-blocked mice (Fig. 2.6D and E). In order to evaluate the role of NKG2D in acute hepatitis developed in HBV-Replication^{+} mice, we examined the expression of NKG2D on NK1.1^{+} cells from HBV-Replication^{+} *Rag-1*$^{-/-}$ mice 3 days after adoptive transfer. Just as we observed in the HBV-Env^{+} *Rag-1*$^{-/-}$ mice, NK cells from the livers of HBV-Replication^{+} *Rag-1*$^{-/-}$ mice had increased expression of NKG2D during acute hepatitis, as compared to *Rag-1*$^{-/-}$ mice; and the NKT cells had down-regulated NKG2D (Fig. 2.6B and C).

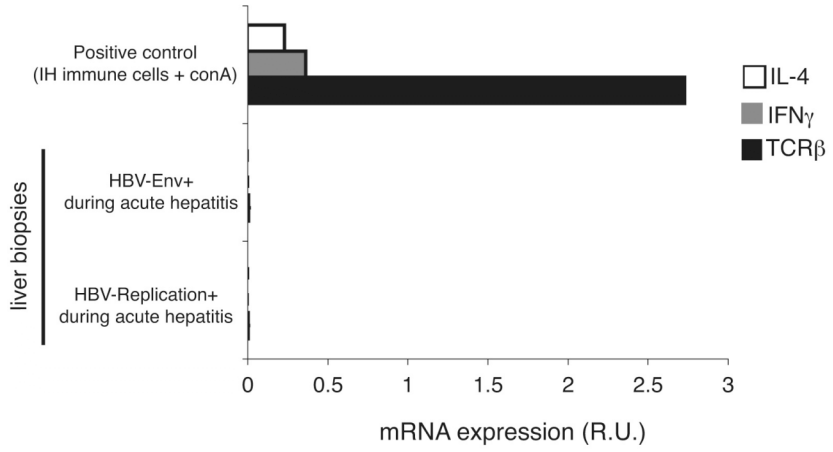

Figure 2.7. Taqman analyses of liver biopsies from HBV-Replication^{+} and HBV-Env^{+} *Rag-1*$^{-/-}$ mice during acute hepatitis. *IL-4* (□) ,*IFN-γ* (▯), and *TCR* (▮) mRNA expression in liver biopsies of HBV-Replication^{+} and HBV-Env^{+} *Rag-1*$^{-/-}$ mice 3 days after adoptive transfer of syngeneic naïve splenocytes in comparison to *Hprt* expression. Positive controls were intra-hepatic immune cells eluted from wild-type mice 12 h after i.v. injection of 10 mg/Kg of ConA. All data are representative of at least two independent experiments.

DISCUSSION

Collectively, our findings clearly establish a role for NKG2D in the HBV-specific, CD1d-restricted non-classical NKT cell-mediated acute hepatitis and cytokine production seen in both the HBV-Env^{+} *Rag-1*$^{-/-}$ and HBV-Replication^{+} *Rag-1*$^{-/-}$ mice. These results demonstrate, for the first time, a role for a NKG2D-ligand interaction in NKT cell activation. NKG2D is expressed on several cell types in the liver. However, NKG2D-bearing NK cells alone, or splenocytes depleted only of NKG2D^{+} NKT cells do not induce acute hepatitis in the HBV-Env^{+} *Rag-1*$^{-/-}$ and HBV-Replication^{+} *Rag-1*$^{-/-}$ mice (71).

A direct effect of NKG2D blockade on NKT cell activation in our studies is suggested by several lines of evidence. First, anti-NKG2D mAb treatment efficiently prevented production of IL-4, which is expressed by the HBV-activated NKT cells but not NK cells. Second, anti-NKG2D mAb treatment decreased the number of NKT cells, but not the number of NK cells, in the livers of mice with hepatitis. Finally, NKG2D receptor expression on NKT cells, and not on other cells types, is required for efficient disease induction in our transgenic mouse model of primary HBV infection.

For these reasons, we propose a model in which non-classical NKT cells are first activated in a HBV-specific, CD1d-restricted and NKG2D-dependent manner, leading to the production of cytokines, which in turn activate other intra-hepatic immune cells. NKG2D ligand interaction can function to directly activate cells or function as a co-stimulatory molecule (136, 138, 201). That the activation of non-classical NKT cells in our mouse model of HBV infection requires expression of HBV and CD1d, in addition to an NKG2D-ligand interaction, suggests that non-classical NKT cell activation requires a CD1d-dependent signal through its T cell receptor, and that NKG2D may function as a co-stimulatory molecule. Further studies will be required to address whether NKG2D is also important in the subsequent NK cell activation. While our studies were under review, Chen *et al* also reported the ability of NKG2D blockade to prevent hepatitis in HBV-transgenic mice; however, in these experiments induction of the disease required the injection of the mitogen Con A, which polyclonally activates all T cells and possibly other cell types such as NK cells in the host (205).

We detected the NKG2D ligand, RAE-1, on the surface of all hepatocytes in normal, wild-type as well as HBV transgenic mice. This constitutive expression of RAE-1 was increased on hepatocytes from the HBV-Env^{+} *Rag-1*$^{-/-}$ mice. Unlike the HBV-Env^{+} *Rag-1*$^{-/-}$

mice, we could not detect an increase in the amount of RAE-1 on hepatocytes from the HBV-Replication^{+} *Rag-1*$^{-/-}$ mice when compared with non-transgenic hepatocytes (data not shown). NKG2D is nonetheless necessary for the non-classical NKT cell activation and onset of hepatitis in both the HBV-Env^{+} *Rag-1*$^{-/-}$ mice and HBV-Replication^{+} *Rag-1*$^{-/-}$ mice because disease was completely prevented and cytokine production was greatly diminished by anti-NKG2D blockade. Therefore, we hypothesize that the constitutive, basal levels of RAE-1 on the hepatocytes are sufficient to trigger the HBV-specific, CD1d-restricted, NKG2D-dependent, non-classical NKT cell-mediated hepatitis. This constitutive surface expression of RAE-1 on hepatocytes is also an interesting finding, since the expression of RAE-1 family members is strictly regulated in normal cells, and little expression is found on healthy adult tissue.

Because the HBV-Env^{+} *Rag-1*$^{-/-}$ mice have increased expression of one of the three isoforms of HBV envelope protein (large or L protein) that is retained in the endoplasmic reticulum, these mice display increased sensitivity to the cytotoxic effects of IFN-γ (204). The up-regulation of RAE-1 in the liver of these mice may be a direct or indirect consequence of increased large envelope expression. Increased expression and accumulation of envelope proteins is also one of the pathophysiologic consequences of HBV infection in humans (206).

Our present findings reveal a mechanism by which human HBV activates the innate immune system, and sets up the cytokine milieu in which the subsequent adaptive immune response develops. The question of whether HBV alerts the innate immune system, and what role the innate immune system plays in HBV pathogenesis is controversial. Studies of acute HBV infection in primates and humans reveal an initial quiescent phase of about 4-7 weeks before HBV starts to replicate vigorously, reaching levels of 10^{9} to 10^{10} copies/mL (31, 207, 208). Activation of components of the innate immune system are likely to play a central role in control of this initial HBV burst because HBV-DNA quantity decreases by almost 90% well in advance of the appearance of an antigen-specific CD8^{+} T cell response and hepatopathology (36, 207-210). However, identification of the individual components of the innate immune system responsible for this rapid down-regulation of viral replication, and the mechanism of activation, has been elusive. NK cells have been implicated in this process, since there is an increase in the number of peripheral NK cells before the peak of viral replication (210). However, Northern blot and gene expression analysis of total liver RNA derived from core liver biopsies during this period have failed to reveal evidence of

activation of innate immune effector pathways, leading to the hypothesis that HBV does not alert the innate immune system (120, 211).

Our current data demonstrating that non-classical NKT cells are activated to produce cytokines in an HBV-specific, CD1d-restricted, and NKG2D-dependent manner is consistent with a role for these cells in the initial response to HBV. The finding that activation of these non-classical NKT cells leads to a cytokine burst in the absence of overt hepatocellular injury in the HBV-Replication^{+} mice is consistent with the usual initial sub-clinical presentation of HBV infection. Using real-time PCR analysis on whole liver biopsies, we, like others, cannot detect an innate immune response (the presence of TCR, IL-4, or IFN-γ transcripts) in the HBV-transgenic *Rag-1*$^{-/-}$ mice three days after adoptive transfer of syngeneic splenocytes (Fig. 2.7). In contrast, we clearly demonstrate the presence of NKT cells (using flow cytometry) and the production of IL-4 and IFN-γ (using ELispot assays) in the eluted lymphocytes from the same livers used in the real-time PCR experiments, as depicted in Fig. 2.1, 2.3, and 2.5 (data not shown). Thus, our data suggests that innate immune responses to HBV infection exist, and likely have been previously unappreciated because NKT cells only represent a small fraction of the total cell mass of the liver, thus any NKT cell transcripts are diluted by the overwhelming abundance of hepatocyte RNA.

These mouse models of HBV infection lay the foundation for directed studies analyzing the role of NKT cells, NK cells, NKG2D, and its ligands in human HBV infection. In addition, since the activation of innate effector cells has also been implicated in hepatic flares in chronic HBV infection (120), our models offer the opportunity to examine the role of NKG2D and its ligands in chronic HBV infection, and suggest possible new strategies for therapeutic intervention in this disease.

CHAPTER III

The role of DAP10 and DAP12 in NKG2D-mediated NKT cell activation

INTRODUCTION

Mice express two different isoforms for NKG2D generated by alternative splicing. A long form (NKG2D-L), which associates only with DAP10; and a short form (NKG2D-S) that pairs with both DAP10 and DAP12 (136). The pairing of NKG2D with either DAP10 and DAP12 adapter proteins is an unique feature for mouse NKG2D, as there is no evidence for the short form of human NKG2D. NKG2D-S expression is very low on resting mouse NK cells but it is increased upon activation both in vivo and in vitro. In mice, NKG2D surface expression on resting NK cells and activated $CD8^+$ T cells (through TCR stimulation) only requires association with DAP10, whereas NKG2D expression on activated mouse NK cells requires both adapters, DAP10 and DAP12 (136). Although a great deal is known about DAP10 and DAP12 function in NKG2D signaling both on NK and $CD8^+$ T cells (107, 167), the role of these adapters in NKG2D signal transduction in NKT cells still remains to be determined.

In chapter II, we showed that the acute immune response observed in our mouse model of primary HBV infection is $NKG2D^+$ non-classical NKT cell-mediated. This finding raises an interesting experimental opportunity to dissect the contribution of both DAP10 and/or DAP12 adapters in NKT cell activation via NKG2D.

In this study we demonstrated, for the first time, that $NKG2D^+$ NKT cells express higher levels of DAP12 mRNA and similar amounts of DAP10 mRNA as compared to $NKG2D^-$ NKT cells. Furthermore, adoptive transfer of *Dap12*$^{-/-}$ splenocytes into HBV-$Replication^+$ *Rag-1*$^{-/-}$ recipient mice resulted in impaired acute hepatitis when compared to HBV-$Replication^+$ *Rag-1*$^{-/-}$ animals that received wild type splenocytes. However, adoptive transfer of *Dap10*$^{-/-}$ splenocytes into HBV-$Replication^+$ *Rag-1*$^{-/-}$ recipient mice led to a more severe acute hepatitis as measured by intra-hepatic IFNγ and IL-4 production three days post-transfer. These opposite outcomes led us to hypothesize that perhaps a balance between the signal transduction mediated by DAP10 and DAP12 determines the severity of the inflammatory response.

RESULTS

NKG2D$^+$ NKT cells express higher levels of DAP12 mRNA than NKG2D$^-$ NKT cells

The role of DAP10 and DAP12 adapter molecules in NKG2D signal transduction has been well studied in mouse NK cells (136). Here, we sought to determine the role of these two adapters in NKG2D signaling in mouse NKT cells. NKG2D is expressed on approximately 60% of all NKT cells (Fig. 2.1E). First, we analyzed the gene expression of *Dap10*, *Dap12*, *NKG2D-S* and *NKG2D-L* in sorted NKG2D$^+$ and NKG2D$^-$ NKT cell subsets and in NK cells by RT-PCR. We found that NKG2D$^+$ and NKG2D$^-$ NKT cell populations express similar amounts of *Dap10* mRNA and about 2-fold less than NK cells (Fig.3.1A). *Dap12* mRNA expression in NKG2D$^+$ NKT cells was approximately 3-fold more than in NKG2D$^-$ NKT cells, but 50-fold less than in NK cells (Fig.3.1B). Nevertheless, the amount of *Dap12* mRNA on NKG2D$^+$ NKT cells (1.13 R.U.) is abundant and thus highly significant (Fig.3.1B).

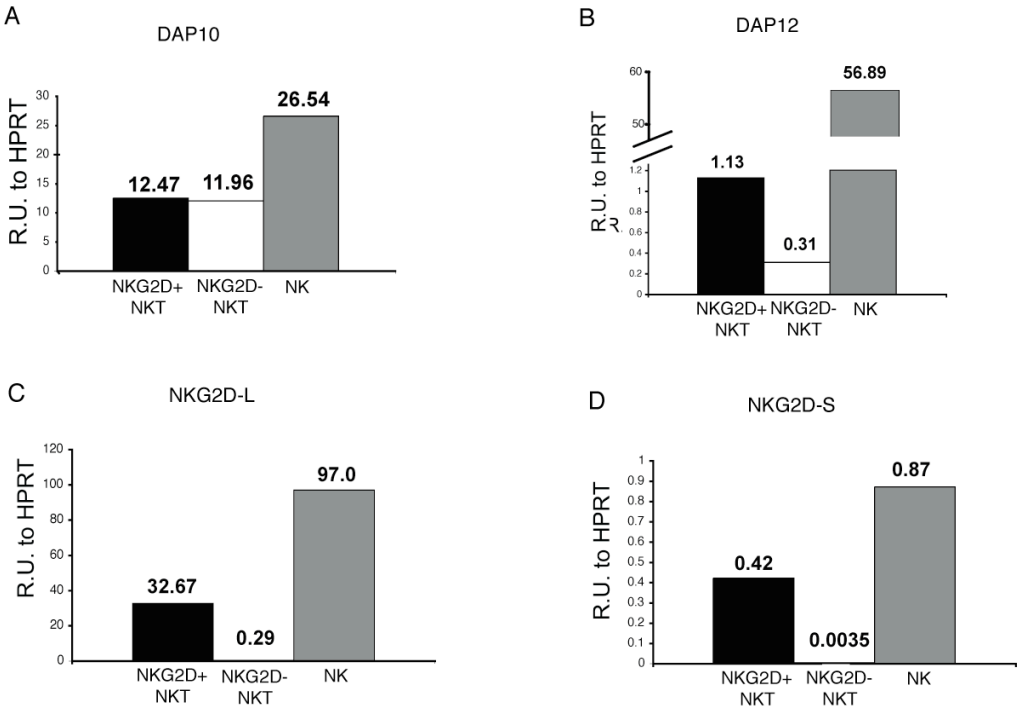

Figure 3.1. Expression analysis of NKG2D isoforms, *DAP10* and *DAP12* in mouse NK and NKT cells. *DAP10* (**A**), *DAP12* (**B**), *NKG2D-L* (**C**), *NKG2D-S* (**D**) mRNA expression in FACS sorted intra-hepatic NKG2D$^+$ NKT cells (▮), NKG2D$^-$ NKT cells (▯) and NK cells (□) isolated from wild type C57/BL6 mice. Data are representative of two independent experiments.

As expected, the long isoform of NKG2D is abundantly expressed in resting NKG2D$^+$ NKT cells and NK cells, whereas the short isoform is expressed at a much lower magnitude (Fig.3.1C and D).

Surface NKG2D expression on *Dap10*$^{-/-}$ and *Dap12*$^{-/-}$ NKT cells

We further analyzed the NKG2D surface expression on NKT cells isolated from livers of *Dap10*$^{-/-}$ and *Dap12*$^{-/-}$ mice. As described in the literature (137), we found that resting *Dap10*$^{-/-}$ NKT cells barely express NKG2D on their surface (Fig.3.2A), whereas resting *Dap12*$^{-/-}$ NKT cells show the same bimodal pattern of NKG2D expression as wild type NKT cells (Fig.3.2B and 2.1E). However, DAP10 deficiency did not influence the basal expression of *NKG2D* genes at the mRNA level, as assessed by Taqman analysis of lymphoid tissues isolated from naïve mice (212).

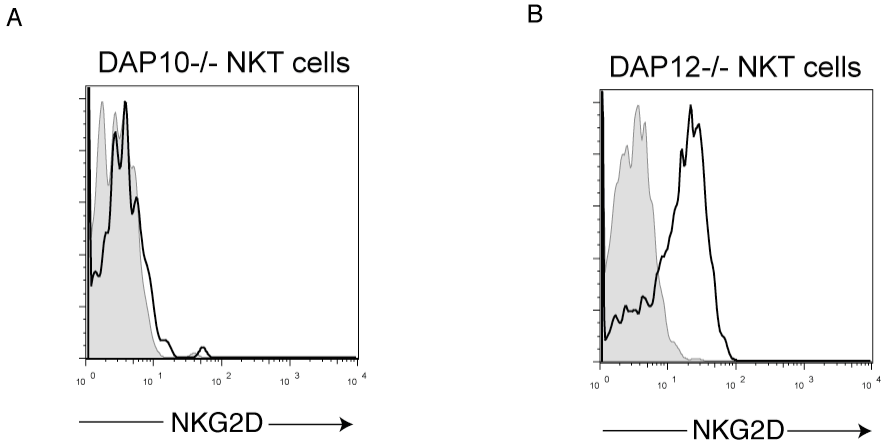

Figure 3.2. **NKG2D surface expression on *Dap10*$^{-/-}$ and *Dap12*$^{-/-}$ NKT cells.** Surface expression of NKG2D on *Dap10*$^{-/-}$ NKT cells (**A**) and *Dap12*$^{-/-}$ NKT cells (**B**). The tinted histograms depict staining using an isotype-matched control rat IgG1. Data are representative of at least three independent experiments.

NKG2D expression is increased on wild type NKT cells 20 hours after ConA stimulation in vivo, and *Dap10*$^{-/-}$ NKT cells show NKG2D surface expression (Ogasawara K & Lanier LL, unpublished data) under the same stimuli. In contrast, NKG2D surface expression on *Dap12*$^{-/-}$ NKT cells seems unaffected 20 hours after ConA injection in vivo (Ogasawara K & Lanier LL, unpublished data), suggesting that DAP12 is important for NKG2D induction in NKT cells in ConA-hepatitis.

Adoptive transfer of *Dap10*$^{-/-}$ or *Dap12*$^{-/-}$ splenocytes into HBV-Replication^{+} Rag-1$^{-/-}$ recipient mice results in opposite disease outcome

Blocking NKG2D receptor function completely prevented acute liver injury in all HBV-Replication^{+} *Rag-1*$^{-/-}$ mice, whereas the control antibody had no effect on disease prevention. Thus, we examined the disease outcome in HBV-Replication^{+} *Rag-1*$^{-/-}$ recipient mice three days after the adoptive transfer of either *Dap10*$^{-/-}$ or *Dap12*$^{-/-}$ splenocytes. Interestingly, our results showed that the adoptive transfer of *Dap10*$^{-/-}$ splenocytes led to an increased intra-hepatic cytokine burst three days after adoptive transfer as compared to HBV-transgenic mice that received wild type immune cells (Fig.3.3A and B).

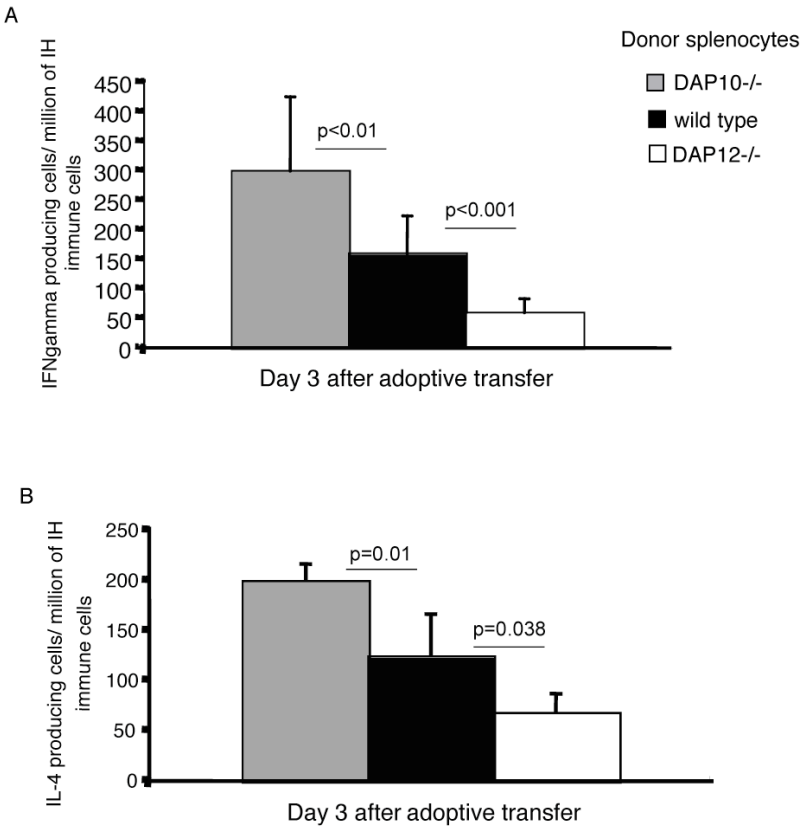

Figure 3.3. Intra-hepatic cytokine production three days after adoptive transfer of *Dap10*$^{-/-}$ or *Dap12*$^{-/-}$ NKT cells into HBV-Replication^{+} *Rag-1*$^{-/-}$ recipient mice. Elispot analyses of IFNγ (**A**) and IL-4-producing (**B**) intra-hepatic immune cells from HBV-Replication^{+} *Rag-1*$^{-/-}$ mice three days after adoptive transfer of *Dap10*$^{-/-}$ (▯), *Dap12*$^{-/-}$ (□) or wild type splenocytes (▮).Representative data are shown as mean ± SD. All data are representative of at least two independent experiments.

In contrast, adoptive transfer of *Dap12*$^{-/-}$ splenocytes resulted in impaired acute immune response as measured by decreased levels of intra-hepatic cytokines at the same time point (Fig.3.3A and B) in comparison to the same control group. These latter findings are in agreement with results presented in chapter II that demonstrate a decrease in disease in animals which received anti-NKG2D blocking antibody.

DISCUSSION

As shown in chapter II, NKG2D receptor expression on NKT cells, and not on other cell types, is required for efficient disease induction in our transgenic mouse model of primary HBV infection. Adoptive transfer of NKG2D-bearing NK cells alone, or NKG2D^{+} NKT cell-depleted splenocytes do not induce acute hepatitis in the HBV-transgenic *Rag-1*$^{-/-}$ recipient mice (71, 213). NK cell receptors, such as NKG2D, can associate with more than one adapter. Likewise, each adapter molecule can pair with multiple receptors (145). Thus, NK cell receptors and adapters exhibit some functional redundancy. Nevertheless, here, we sought to investigate which NKG2D adapter molecule(s), DAP10 and/or DAP12, are involved in NKG2D signal transduction at the time of acute hepatitis in our model of primary HBV infection. Our data demonstrated that intra-hepatic wild type NKT cells that express NKG2D on their surface express three times more *DAP12* mRNA than NKG2D^{-} NKT cells. Moreover, adoptive transfer of *DAP12*$^{-/-}$ donor splenocytes resulted in impaired liver injury in the HBV- Replication^{+} *Rag-1*$^{-/-}$mice, suggesting that NKG2D may require DAP12 for signal transduction.

On the other hand, both intra-hepatic NKG2D^{+} and NKG2D^{-} NKT cell populations express *DAP10* mRNA abundantly and in identical amounts. In fact, this is an example where the mRNA expression patterns of *NKG2D* and *DAP10* do not completely overlap reinforcing the possibility that DAP10 associates with other receptors than NKG2D. Recently, Coudert *et al* demonstrated that Ly49D and Ly49H can also associate with DAP10 adapter, even if to a lesser extent than to DAP12 (146). Surprisingly, adoptive transfer of *Dap10*$^{-/-}$ donor splenocytes into HBV-Replication^{+} *Rag-1*$^{-/-}$ recipient mice led to a more severe acute liver injury as measured by intra-hepatic cytokine burst when compared with HBV-transgenic mice that received wild type syngeneic splenocytes. This result is consistent with data from Philips and colleagues (214) that showed that activated *Dap10*$^{-/-}$ NKT cells produce significantly higher levels of cytokines, including IFNγ, as compared to wild type

NKT cells. They further demonstrated that *Dap10*$^{-/-}$ NKT cells had a significant increase in turnover proliferation rate compared with wild type NKT cells, which led the authors to suggest that they might be constitutively hyperactive (214). This study also showed that although IL-2 activated *Dap10*$^{-/-}$ NKT cells killed less efficiently NKG2D ligand expressing YAC-1 tumor cells when compared with wild type NKT cells, anti-NKG2D mAb partially inhibited the killing in both cases. The authors suggested that activated *Dap10*$^{-/-}$ NKT cells express a partially functional NKG2D receptor (214).

In light of our results, it is possible that DAP10 and DAP12 adapters work as inhibitory and activating molecules, respectively, regulating the signal transduction threshold of the activating receptor NKG2D. For instance, in the absence of DAP10 (which hypothetically might dampen NKG2D signaling), the NKT cell is constantly activated and secreting elevated amounts of cytokines (Fig. 3.4A). Consistent with this is the fact that NKG2D engagement with its ligands leads to receptor internalization (110, 133), and NKG2D seems not to be expressed on the surface of *Dap10*$^{-/-}$ NKT cells (137). On the other hand, in the absence of the "positive" adapter DAP12, NKT cells produce significantly less cytokines upon stimulation through the NKG2D receptor (Fig. 3.4B). We speculate that this latter hypothetical model (Fig. 3.4A and B) could explain the divergent disease outcomes detected by the adoptive transfer of *Dap12*$^{-/-}$ or *Dap10*$^{-/-}$ splenocytes, in the specific liver immunological environment of the acute immune response observed in our transgenic mouse model of primary HBV infection. Consistent with this model were the recent findings of Medzhitov and colleagues using a new transgenic mouse that overexpresses a DAP10-ubiquitin fusion protein. In these mice, DAP10 and its associated proteins are dominantly targeted to lysossomal degradation in NK cells, including NKG2D (215). This way, any potentially compensatory association of NKG2D with DAP12 is thought to be abrogated and results in impaired NKG2D-mediated IFNγ production and cytotoxicity (215). It would be interesting to know if NKG2D function is similarly affected on NKT cells.

Nevertheless, we cannot exclude the possibility that *Dap10*$^{-/-}$ NKT cells are constitutively hyperactive in a NKG2D-independent manner. In fact, preliminary experiment of blocking NKG2D function in vivo in HBV-Replication^{+}*Rag-1*$^{-/-}$ mice, which received *Dap10*$^{-/-}$ splenocytes, suggested no effect in the robust cytokine burst detected at time of acute hepatitis. However, since NKG2D is nearly absent from the surface of NKT cells it is possible that this approach did not directly test if the hyperresponsiveness of *Dap10*$^{-/-}$ NKT cells is NKG2D dependent. Although *NKG2D*$^{-/-}$ mice are not currently available, a definitive experiment would be to cross these hypothetical knockout mice with *DAP10*$^{-/-}$ animals and

then adoptively transfer *DAP10 NKG2D* DKO splenocytes into HBV-Replication^{+}*Rag-1*$^{-/-}$ recipient mice. This way, it would be possible to determine if the hyperactive status of *DAP10*$^{-/-}$ NKT cells is NKG2D dependent or independent.

Collectively, our findings contribute to the knowledge of the interplay between DAP10 and DAP12 signaling in NKT cell activation/co-stimulation via NKG2D, in a very well defined in vivo system of NKG2D^{+} NKT cell-mediated CD1d-dependent acute immune response against HBV-expressing cells.

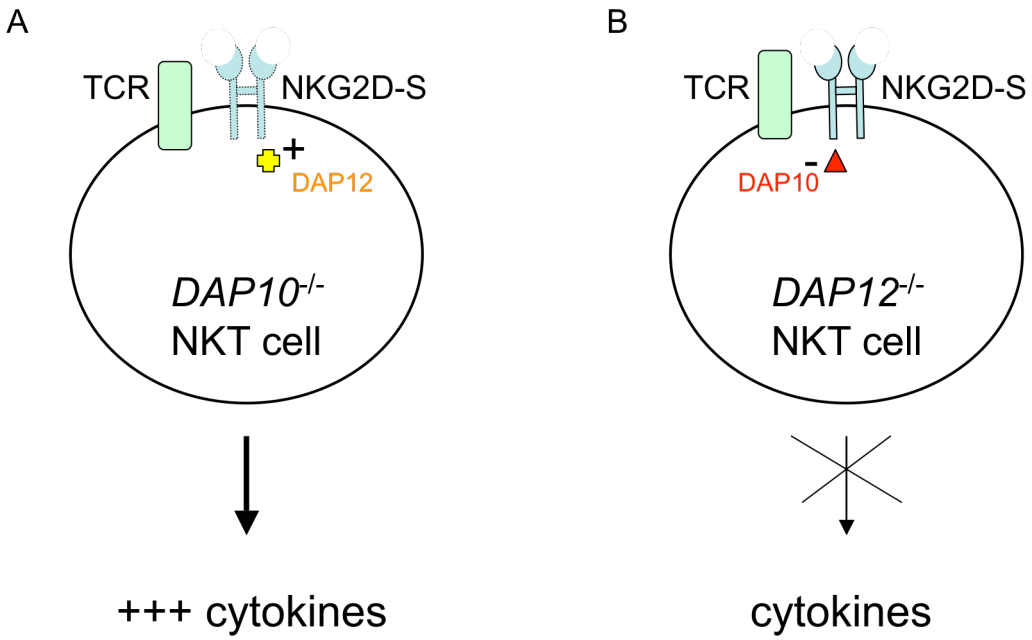

Figure 3.4. Hypothetical model for the role of DAP10 (A) and DAP12 (B) in NKG2D signal transduction in activated NKG2D^{+} NKT cells at the time of acute hepatitis in our transgenic mouse model of primary HBV infection. Note: Despite *Dap10*$^{-/-}$ NKT cells express very low amounts of NKG2D on their surface, its expression might be detectable upon activation.

CHAPTER IV

Mild hepatic necrosis detected in HBV-transgenic *Rag-1*$^{-/-}$ mice is IFNγ and NK cell-independent

INTRODUCTION

HBV itself is non-cytopathic (6) and it is the immune response against the HBV-expressing cells that causes the hepatic necrosis and liver damage (45).

It is well established that HBV immunopathogenesis is mainly caused by the adaptive immune system, in particular by a strong CTL response (13, 45, 216). On the other hand, the role of the innate immune system during this infection has not been thoroughly addressed over the past decades and remains unappreciated (211). The study of the innate immune response in the control of early HBV replication should not be dismissed, since most of the HBV DNA can be cleared from the serum and the livers of experimentally infected chimpanzees before an adaptive immune response is detected within the liver (31). In addition, it was reported that the inflamed livers of chronic HBV-infected patients showed a few HBV-specific $CD8^+$ T cells among a high proportion of activated NK cells ($CD3^-CD56^+$) (120).

In this chapter, we investigated whether other intra-hepatic innate immune cells might be involved in HBV recognition, potentially generating signals that could contribute to the non-classical NKT cell-mediated acute hepatitis observed in our HBV-Transgenic (HBV-Tg) *Rag-1*$^{-/-}$ mice three to four days post-transfer. Our results demonstrated a mild hepatic necrosis in the HBV-Tg *Rag-1*$^{-/-}$ mice pre-adoptive transfer, which is IFNγ and NK-cell-independent. Furthermore, our data indicate that the basal amount of IFNγ and the NK cell frequency in the livers of HBV-Tg *Rag-1*$^{-/-}$ recipient mice do not affect the non-classical type II NKT cell-mediated acute hepatitis.

RESULTS

HBV-Transgenic *Rag-1*$^{-/-}$ mice show mild hepatic necrosis even before adoptive transfer of syngeneic naïve wild-type splenocytes

By compiling dozens of baseline values of serum ALT from HBV-Tg and non-Tg *Rag-1*$^{-/-}$ mice before adoptive transfer of naïve splenocytes, it became evident that the HBV-Tg *Rag-1*$^{-/-}$ mice have significantly higher serum ALTs than the non-Tg *Rag-1*$^{-/-}$ (Fig. 4.1). In fact, both lines of HBV-Tg mice, HBV-Env^{+} and HBV-Replication^{+}, regardless of CD1d expression, showed significantly higher baseline ALT values as compared to non-Tg mice or to OVA-Tg mice (Fig.4.1). This latter group of transgenic mice was used as a control since these animals express ovalbumin (OVA) under the control of the albumin promoter. The mild rise of ALTs was also detected in HBV-Tg *Rag-1*$^{+/+}$ mice (data not shown).

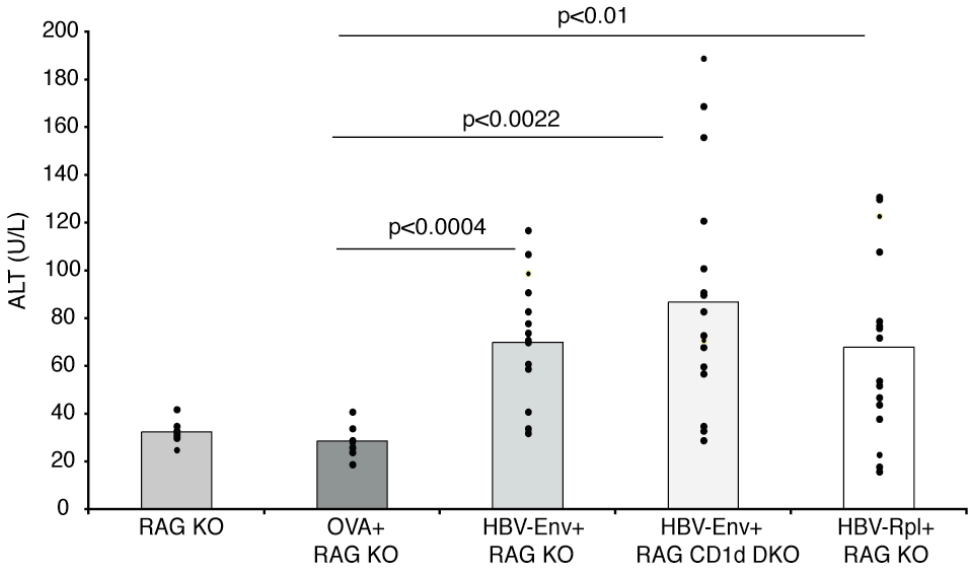

Figure 4.1. HBV-Tg mice show mild hepatic necrosis even before the NKT cell-mediated early immune activation. Baseline liver injury in HBV-Tg *Rag-1*$^{-/-}$ and HBV-Tg *Rag-1*$^{-/-}$ *CD1d*$^{-/-}$ mice in comparison to non-Tg or OVA-Tg *Rag-1*$^{-/-}$ animals were measured by sera ALT values, before adoptive transfer of syngeneic naïve wild type splenocytes. Data are pooled from two independent experiments.

IFNγ and IL-4 production is mildly increased in the livers of HBV-Transgenic *Rag-1*$^{-/-}$ mice before adoptive transfer of syngeneic naïve wild type splenocytes

In light of the previous data, we examined the frequency of IFNγ and IL-4-producing cells in the livers of HBV-Tg *Rag-1*$^{-/-}$ mice in comparison to non-Tg or OVA-Tg *Rag-1*$^{-/-}$ animals before adoptive transfer. We consistently found a 2 to 3 fold increase in intra-hepatic production of IL-4 and IFNγ in HBV-Tg *Rag-1*$^{-/-}$ mice compared to either non-Tg or OVA-Tg animals (Fig. 4.2A and B).

Since it is well established that HBV itself is non-cytopathic, we hypothesized that the mild hepatocyte necrosis and cytokine production (IFNγ and IL-4) detected in HBV-Tg mice is innate immune-cell dependent. Therefore, we sought to determine which innate immune cell population(s) could recognize or sense HBV-expressing cells.

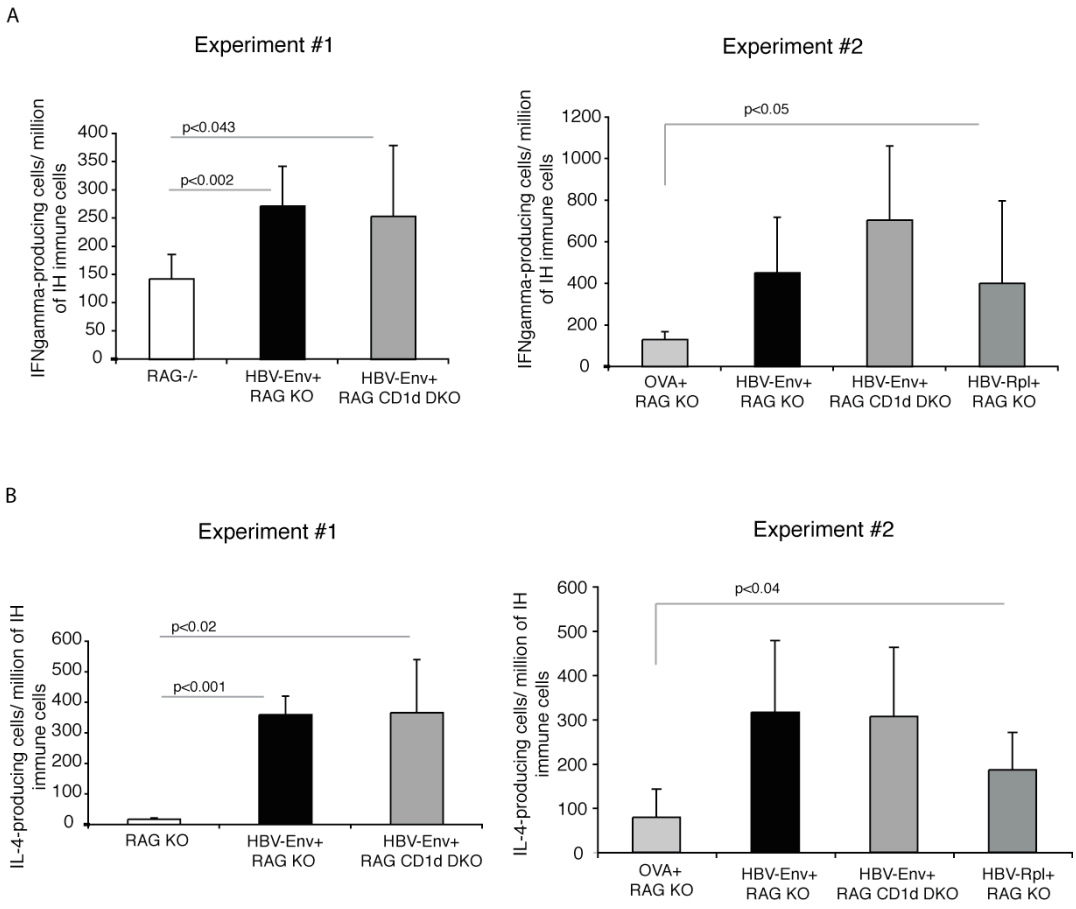

Figure 4.2. **Baseline cytokine production by intra-hepatic immune cells derived from HBV-Tg *Rag-1*$^{-/-}$ mice.** Elispot analyses of IFNγ (**A**) and IL-4-producing (**B**) intra-hepatic immune cells eluted from HBV-Tg *Rag-1*$^{-/-}$ mice before adoptive transfer of syngeneic naïve wild type splenocytes.

NK cells are key IFNγ-producing cells in the livers of HBV-Transgenic *Rag-1*$^{-/-}$ mice before adoptive transfer of syngeneic naïve wild-type splenocytes

In order to address which cell subset(s) are producing IFNγ and IL-4 in the livers of HBV-Tg *Rag-1*$^{-/-}$ mice, we first performed FACS cell sorting analysis of three distinct populations based on their size (FSC) and granularity (SSC), by grouping the cells into a lymphocyte gate, a SSC high gate and a FSC high gate (Fig. 4.3A). This method avoided direct cell staining with antibodies, which might potentially interfere with the level of activation and cytokine production of these cells. The sorted populations were immediately plated in ELIspot plates previously coated with anti-IFNγ or anti-IL-4 mouse antibody. Two independent sorting experiments demonstrated an increase in IFNγ production by intra-hepatic immune cells from the lymphocyte gate derived from HBV-Tg mice, independent of CD1d expression (Fig. 4.3B). No statistical difference was found in IFNγ production by either SSC high or FSC high cell sorted populations between HBV-Tg and non-Tg mice (Fig. 4.3C). On the other hand, we were unable to detect IL-4 secretion from any of the intra-hepatic immune cell subsets after sorting (data not shown). This result could have two possible interpretations: either the number of IL-4-producing cells in each sorted population is below the ELIspot detection limit or the manipulation of the IL-4-producing cells during sorting affected its production.

Since these animals have neither B nor T lymphocytes, the designated lymphocyte gate should be enriched in NK cells. Thus, we further examined whether intra-hepatic NK cells might be involved in HBV sensing or recognition. For that purpose, we injected intravenously 100μg of anti-NK1.1 depleting antibody (clone PK136) one or three times, resulting in total ablation of NK cells in HBV-Env^{+} *Rag-1*$^{-/-}$ mice for a period of four or twelve days, respectively (Fig. 4.4A and B). We found that depletion of NK cells diminished the intra-hepatic IFNγ production by two-fold, independent of the length of antibody treatment, and IL-4 production was only significantly reduced in mice treated with anti-NK1.1 depleting antibody (clone PK136) for a period of twelve days (Fig. 4.4B). However, there was no statistical difference in the baseline serum ALTs (data not shown).

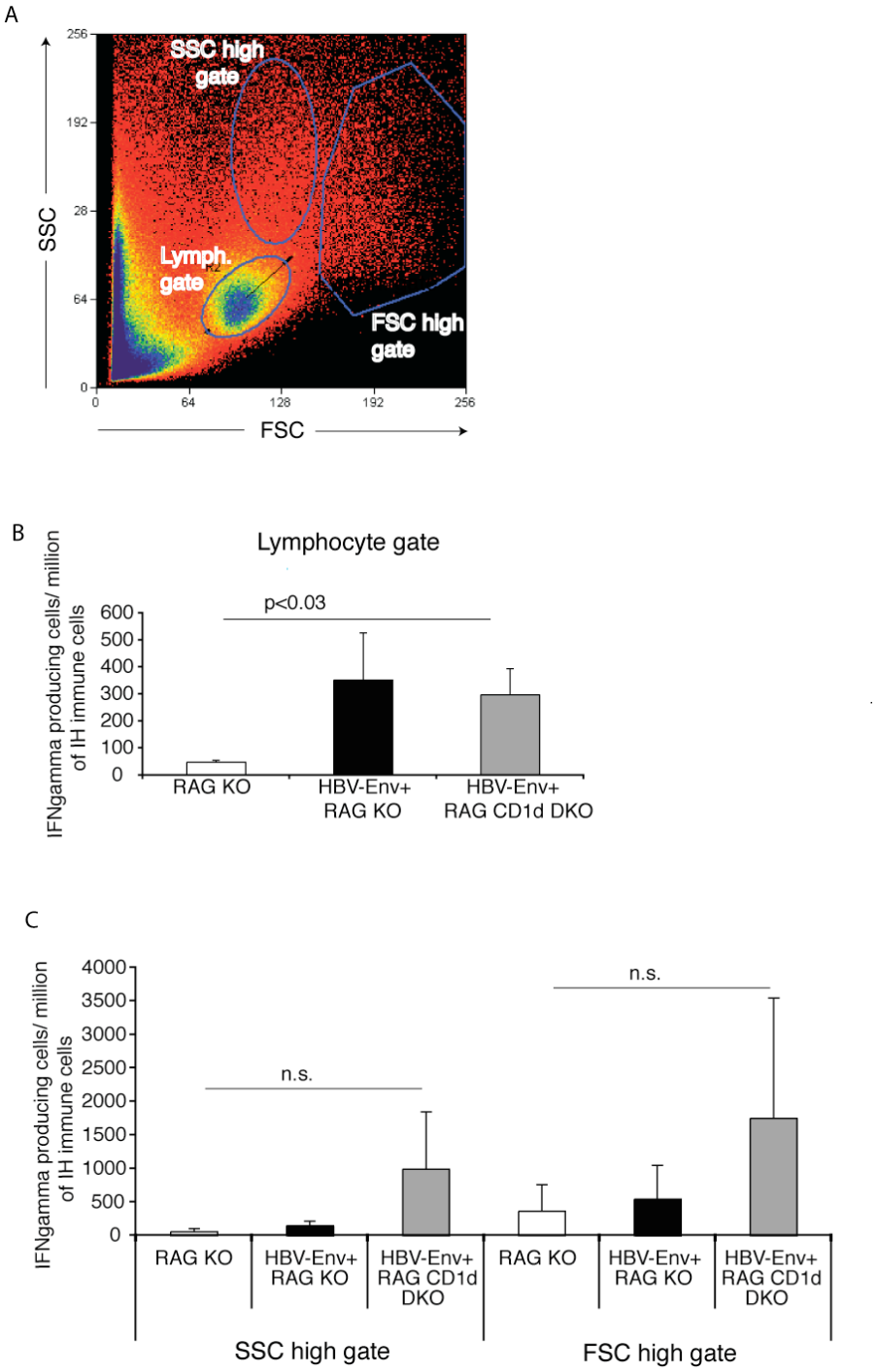

Figure 4.3. Baseline cytokine production of FACS cell sorted intra-hepatic immune cell populations derived from HBV-Tg *Rag-1*$^{-/-}$ mice. (**A**) FACS plot depicts the sorted three intra-hepatic immune cell populations, separated by size (FSC) and granularity (SSC). (**B**) The intra-hepatic immune cells sorted from the lymphocyte gate were plated in elispot plates coated with IFNγ**.** (**C**) Elispot analysis of IFNγ-producing intra-hepatic immune cells from the SSC high and FSC high sorted populations. Data are representative of two independent experiments.

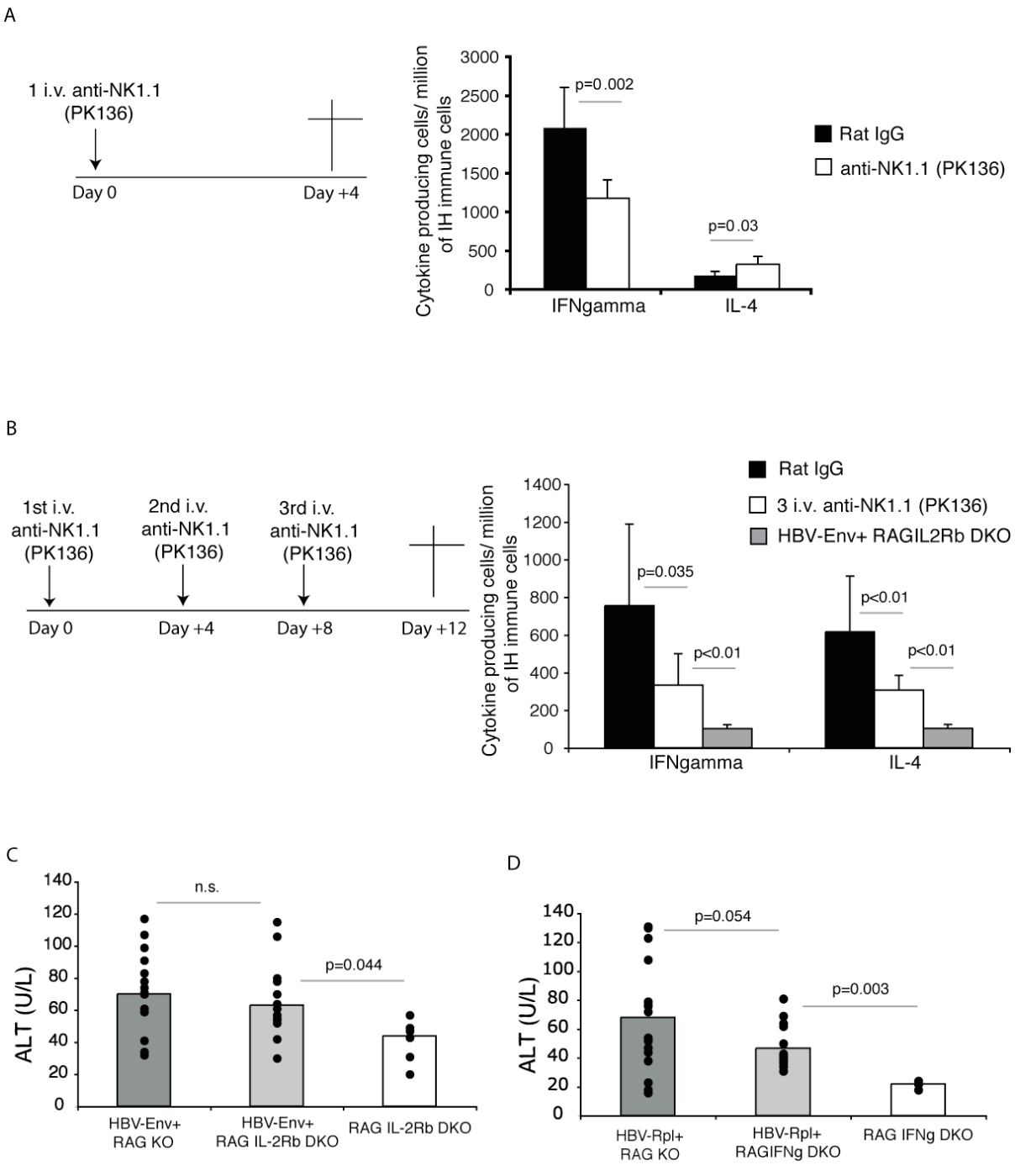

Figure 4.4. Depletion of NK cells from HBV-Env^{+} *Rag-1*$^{-/-}$ mice significantly decreases the intra-hepatic IFNγ production but has no effect on the baseline mild hepatic necrosis. Elispot analyses of IFNγ and IL-4-producing intra-hepatic immune cells derived from HBV-Env^{+} *Rag*$^{-/-}$ mice after one (**A**) or three (**B**) i.v. injections of 100μg of anti-NK1.1 depleting antibody (clone PK136) as compared to the ratIgG treated mice. Baseline liver injury in HBV-Env^{+} *Rag-1*$^{-/-}$ *IL-2Rβ*$^{-/-}$ mice in comparison to non-Tg *Rag-1*$^{-/-}$ *IL-2Rβ*$^{-/-}$ (**C**) and in HBV-Replication^{+} *Rag-1*$^{-/-}$ *IFNγ*$^{-/-}$ mice in comparison to non-Tg *Rag-1*$^{-/-}$ *IFNγ*$^{-/-}$ (**D**) were measured by serum ALT values, before adoptive transfer of syngeneic naïve wild type splenocytes.

To further explore whether NK cells participate in the innate immune response to HBV-expressing hepatocytes, we generated HBV-Env^{+} and HBV-Replication^{+} *Rag-1*$^{-/-}$*IL-2Rβ*$^{-/-}$ mice, which show a dramatic reduction in NK cell numbers (217) in addition to the absence of B and T lymphocytes. We found very low baseline levels of IFNγ and IL-4 in their livers (Fig. 4.4B and data not shown), suggesting that besides direct production of IFNγ, resident intra-hepatic NK cells should be indirectly involved in inducing IFNγ and IL-4 production from other liver cell types. However, this significant reduction in cytokine production did not correlate with a decrease in circulating ALTs, consistent with data from the NK1.1 depletion studies. In fact, HBV-Env^{+} *Rag-1*$^{-/-}$*IL-2Rβ*$^{-/-}$ mice showed a similar mild hepatic necrosis detected in all other HBV-Tg animals (Fig. 4.4C), indicating that the mild basal hepatocyte necrosis is not NK cell-mediated. Moreover, HBV-Replication^{+} *Rag-1*$^{-/-}$ *IFNγ*$^{-/-}$ mice also showed a mild rise of baseline serum ALTs when compared to non-Tg *Rag-1*$^{-/-}$*IFNγ*$^{-/-}$ mice (Fig. 4.4D), suggesting that the hepatocyte necrosis is also IFNγ-independent.

We also detected an increase in IFNγ secretion in both SSC high and FSC high populations in an HBV-dependent manner. However, due to its high variability in IFNγ secretion between mice, the difference was not statistically significant (Fig. 4.3C). Furthermore, we detected no difference in the levels of intra-hepatic IFNγ production or baseline serum ALTs between HBV-Env^{+} *Rag-1*$^{-/-}$ mice depleted of Gr-1^{high} cells for 24 and 48 hours and control animals (ratIgG treated) for the same period of time (data not shown).

Resident NK cells do not affect the subsequent acute hepatitis seen in HBV-Transgenic *Rag-1*$^{-/-}$ mice three to four days after adoptive transfer of syngeneic naïve wild-type splenocytes

We next addressed the question of whether the resident NK cells play a role in the non-classical NKT cell-mediated acute hepatitis observed in HBV-Tg animals three days after adoptive transfer, using HBV-Replication^{+} *Rag-1*$^{-/-}$*IL-2Rβ*$^{-/-}$ mice. Reminiscent of the clinical presentation of true human HBV infection, HBV-Replication^{+} mice develop a mild, sub-clinical hepatitis, characterized by a discrete rise of ALTs but accompanied with a significant intra-hepatic cytokine burst (71). To test this hypothesis, HBV-Replication^{+} *Rag-1*$^{-/-}$*IL-2Rβ*$^{-/-}$ mice received an adoptive transfer of syngeneic naïve wild type splenocytes. Unexpectedly, we detected a significantly stronger acute cytokine burst, HBV-independent, in the mice lacking B, T and NK cells as compared to HBV-Replication^{+} *Rag-1*$^{-/-}$ mice (Fig.

4.5). It is possible that donor NK cells aberrantly expanded while repopulating the HBV-Replication^{+} *Rag-1*$^{-/-}$*IL-2Rβ*$^{-/-}$ recipient mice, thus explaining the increase in cytokine production. Due to this limitation, we decided to address the question of whether NK cells are involved in the activation of non-classical NKT cells using HBV-Env^{+} *Rag-1*$^{-/-}$ mice, in B10.D2 background. These mice are characterized by a more severe liver injury four days after adoptive transfer of syngeneic naïve wild type splenocytes (71). NK cells were depleted by intravenous administration of anti-asialo GM1 (polyclonal antibody) from both donor and recipient mice one day before adoptive transfer. As a control group, untreated HBV-Env^{+} *Rag-1*$^{-/-}$ recipient mice received wild type splenocytes. Four days after adoptive transfer, serum ALTs and the intra-hepatic cytokine production were evaluated. There was no statistical difference between the acute immune response to HBV-expressing cells in HBV-Env^{+} mice with or without NK cells as measured by the rise of serum ALTs and the intra-hepatic cytokine burst four days post-transfer (Fig. 4.6A and B).

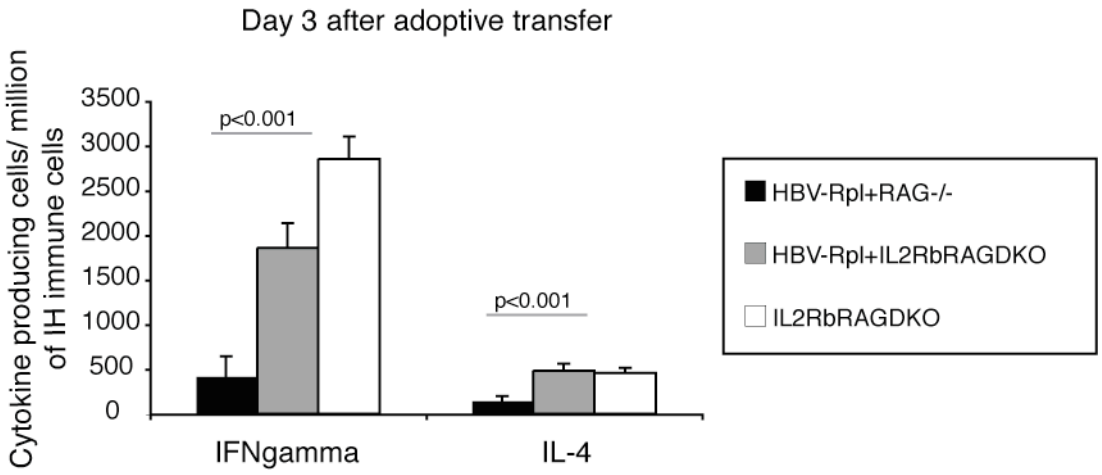

Figure 4.5. Ablation of resident intra-hepatic NK cells have no effect in the subsequent acute immune response seen in HBV-Replication^{+} *Rag-1*$^{-/-}$ mice three days after adoptive transfer of syngeneic naïve wild type splenocytes. Elispot analyses of IFNɣ and IL-4-producing intra-hepatic immune cells derived from HBV-Replication^{+} *Rag-1*$^{-/-}$ (▮), HBV-Replication^{+} *Rag-1*$^{-/-}$ *IL-2Rβ*$^{-/-}$ (▯) and non-Tg *Rag-1*$^{-/-}$ *IL-2Rβ*$^{-/-}$ (□) three days after adoptive transfer of wild type splenocytes. or wild type splenocytes.

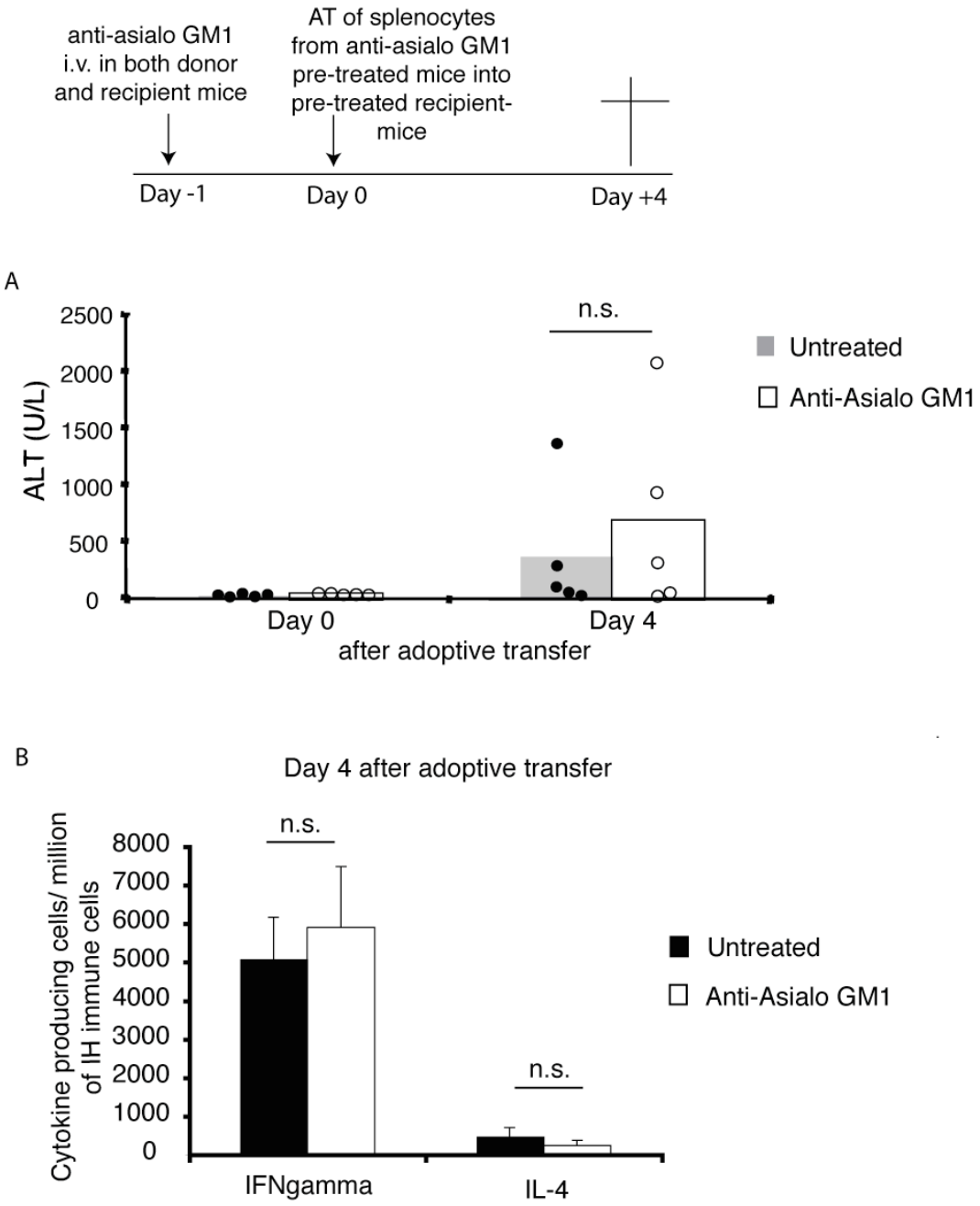

Figure 4.6. Ablation of NK cells have no effect in the subsequent acute immune response seen in HBV-Env^{+} *Rag-1*$^{-/-}$ mice four days after adoptive tranfer of syngeneic naïve wild type splenocytes. (**A**) Hepatic injury as assessed by the measurement of ALT in the sera of HBV-Env^{+} *Rag-1*$^{-/-}$ mice and elispot analyses (**B**) of IFNγ and IL-4-producing intra-hepatic immune cells isolated from HBV-Env^{+} *Rag-1*$^{-/-}$ mice at day four after adoptive transfer. HBV-Env^{+} *Rag-1*$^{-/-}$ recipient mice were either pre-treated with anti-asialo GM1 polyclonal antibody and received NK cell-depleted donor splenocytes (□) or were untreated and received wild type splenocytes (▮), Data are representative of two independent experiments.

DISCUSSION

Our data demonstrated that two distinct lines of HBV-Tg mice showed a basal mild hepatic necrosis as measured by serum levels of ALTs. These results strongly suggest that HBV is recognized by the innate immune system present in *Rag-1*$^{-/-}$ mice, since HBV itself is thought to be non-cytopathic (6). This is a surprising finding because it is widely accepted that HBV-Tg mice have a tolerant immune system, incapable of recognizing HBV antigens (45). In addition to this mild increase of serum ALTs, HBV-Tg mice also showed increased levels of baseline intra-hepatic IFNγ and IL-4-producing cells, before adoptive transfer. We further investigated which cell subsets were producing IFNγ in an HBV-dependent manner and whether a particular innate immune cell population could sense HBV antigens. NK cells were our main candidate as they are widely known to play a role in antiviral defense (218, 219), and they are main producers of IFNγ. Moreover, all NK cells express the activating receptor, NKG2D (167), whose corresponding ligand, RAE-1, is constitutively expressed in the liver (and not in other healthy organs) (http://source.stanford.edu/cgi-bin/source/sourceSearch), and up-regulated in HBV-expressing hepatocytes (chapter II).

Although resident intra-hepatic NK cells produce significant amounts of IFNγ in an HBV-dependent manner before adoptive transfer, our data demonstrated that the mild hepatic necrosis detected in HBV-Tg mice as measured by a two-fold rise of baseline ALTs is neither dependent on the presence of NK cells nor on the levels of intra-hepatic IFNγ. To test a potential involvement of resident intra-hepatic NK cells in HBV recognition, we generated HBV-Tg *Rag-1*$^{-/-}$*IL-2Rβ*$^{-/-}$ mice. These animals showed a dramatic reduction in total NK cell numbers in addition to the absence of B and T lymphocytes (217). Therefore, the immune cells present in these animals are restricted to granulocytes, macrophages and dendritic cells among other cells. Initially this model seemed an elegant system to investigate the role of NK cells before and after adoptive transfer as well as their interaction with other cell types. However, an increased intra-hepatic cytokine burst, non-HBV-specific, observed in these mice, during acute hepatitis, turned out to be a potential limitation on using these animals to study the immunopathogenesis of a hepatotropic infectious agent, such as HBV. Nevertheless, experiments using depleting antibodies strongly suggested that resident intra-hepatic NK cells do not significantly contribute to the non-classical NKT cell-mediated acute immune response to HBV-expressing cells in our mouse model of primary HBV infection.

Collectively, our findings demonstrated that HBV-Tg mice show a mild hepatic necrosis that is IFNγ, NK cell and Gr-1^{high} cell-independent. This HBV-specific mild rise of baseline serum ALTs might be either immune or non-immune mediated. If immune-mediated, the innate immune sensing machinery of the HBV-expressing cells might recognize viral particles and trigger an innate immune response; or innate immune cells other than NK and Gr-1^{high} cells might recognize HBV antigens. On the other hand, we cannot fully exclude the possibility that hepatocyte necrosis detected in these mice is non-immune mediated and HBV itself could be mildly cytopathic under certain specific conditions. Despite the body of evidence in support of HBV as a non-cytopathic virus (45), a more recent study in mice suggested that HBV may be directly cytopathic in conditions of severe immune suppression (220). These authors further correlated this observation in mice with human data that described suppression of the immune system, by immunosuppressive agents or progressive immune failure, in the context of acquired immune deficiencies, may lead to reactivation of seemingly recovered or “silent” HBV infections (221).

In summary, our finding that HBV-Tg mice showed mild hepatic necrosis, as detected by a mild rise of baseline serum ALTs, highlights the potential role of the innate immune system during HBV infection. Understanding the role of innate effector cells during immune activation both in acute phase as well as in hepatic flares would help to further elucidate our understanding of disease pathogenesis. Future experiments are needed to address the origin of this mild but consistent hepatic necrosis seen exclusively in the HBV-Tg mice.

CHAPTER V

DISCUSSION and CONCLUSIONS

Since the experimental results presented here have been previously discussed in chapters II, III and IV, the discussion that follows aims to bring all the data together, highlighting what is new, what remains to be answered, and what challenges arose from this study in the understanding of HBV immunopathogenesis and NKT cell biology. In this chapter, I will guide you through the unappreciated innate and "innate-like" immune responses to HBV, with particular emphasis on the potential role of intra-hepatic NKT cells, the NKG2D receptor and its ligands during this hepatotropic infection. I will critically analyze the current available HBV models, focusing on the advantages and limitations of our transgenic mouse model of primary human HBV infection; and I will then briefly discuss the potential implications of this work in HBV-related diseases.

Innate and "innate-like" immune responses to HBV-expressing cells

Human HBV immunopathogenesis is generally attributed to a strong polyclonal CTL response weeks after inoculation. In fact, by the time symptomatic HBV-infected patients seek medical attention and samples (blood and/or liver biopsies) are collected, they usually show a large intra-hepatic infiltrate of $CD8^+$ T lymphocytes. Therefore, during the past decades, many groups have focused their efforts on characterizing in detail these HBV-specific $CD8^+$ T lymphocyte-mediated immune responses. Important contributions have also been made in characterizing the humoral immune responses to the diverse HBV antigens. These investigations had a significant impact on identifying HBV-specific antigens and antibodies, which have currently been used either as diagnostic or follow-up analytical markers of HBV infection. On the other hand, the role of the innate immune system against HBV-expressing cells has not been thoroughly addressed over the past years. Thus, my graduate work presented in this dissertation aimed at contributing to the knowledge of the cellular and molecular mechanisms involved in the early immune recognition of HBV-expressing cells.

Although a great deal has been uncovered about the role and characteristics of the adaptive immune responses to HBV-infected cells, the factors that determine whether a given individual will mount an effective $CD8^+$ cytotoxic T cell response and antibody production to HBV are still poorly understood. Understanding this process represents a great current challenge in the HBV field.

Microarray analysis of multiple liver biopsies of experimentally infected chimpanzees

revealed striking differences in intra-hepatic gene expression in the early immune responses to HBV and HCV (including genes involved in a type I IFN response) (222-224). Early after inoculation, HCV induces vigorous intra-hepatic type I IFN responses, which can be detected by gene expression, while HBV does not induce any detectable changes in the expression of genes involved in type I IFN response in the first weeks of infection (210, 211). Based exclusively on these studies, the authors suggested that HBV behaves as a "stealth" virus that efficiently establishes itself without inducing an innate immune response in the cell it infects (211).

Using our transgenic mouse model of primary HBV infection we were able to study in detail the contribution of resident and donor innate immune cells in disease induction. We found that HBV-transgenic mice show a mild hepatocyte necrosis, as measured by serum ALTs, of yet unclear origin (chapter IV). This result suggests a HBV-specific immune-mediated response in the absence of an adaptive immune system. Despite this mild hepatocyte necrosis exclusively detected in HBV-transgenic mice being NK cell and IFNγ-independent, the resident NK cells from these mice secrete significant amounts of IFNγ in an HBV-specific manner (chapter IV). In addition, our current data demonstrated that a subset of non-classical type II NKT cells are activated in an HBV-specific, CD1d-restricted (71) and NKG2D-dependent manner (chapter II) as measured by IFNγ and IL-4 production, revealing a mechanism by which the human pathogen HBV activates the intra-hepatic innate immune system (Fig. 5.1). It has been well established that NKT cells function as antigen-responsive T cells. And evidence that these cells are an integral part of innate immunity is constantly growing. In fact, it seems that NKT cells occupy a unique niche as effector cells that behave as a bridge between innate and adaptive immune functions (225).

We performed RT-PCR analysis on whole liver biopsies from HBV-transgenic mice at the time of acute hepatitis. In concordance with data from genomic wide analysis on liver biopsies from experimentally HBV-infected chimpanzees mentioned above, significant amounts of TCR, IFNγ or IL-4 mRNA expression were not detected (chapter II). This experiment indicates that intra-hepatic immune cellular component depicts an extremely small fraction of a whole liver biopsy, which is mainly constituted by parenchymal cells (hepatocytes). Thus, due to the difficulty of analyzing intra-hepatic immune cells in biopsies from humans and chimpanzees in general, and during early immune responses in particular, the role of innate immune system have been previously unappreciated. This way, our studies not only reveal a mechanism by which human HBV activates the intra-hepatic innate immune cells, but also provides a possible explanation to why the role of the innate

immunity in HBV immunopathogenesis has been unappreciated.

Since it is not possible to accurately control the time of HBV inoculation in humans to then study the early immune response to the virus-infected cells, some studies have been performed on chronic HBV-infected patients who develop recurrent, spontaneous “hepatic flares”. These “flares” are characterized by large, unexplained and uncontrolled fluctuations in liver inflammation accompanied by a propensity to progress to severe liver fibrosis. Therefore, “hepatic flares” provide an experimental opportunity to study in humans the mechanisms involved in the transition between immune tolerance and immune activation. Although immune-mediated liver injury in HBV-infected patients has conventionally been attributed to cytolytic killing of infected hepatocytes by virus-specific $CD8^+$ T lymphocytes, the presence of activated HBV-specific $CD8^+$ T cells in patients controlling HBV infection is not pathognomonic of liver inflammation (226). Moreover, in this same study, a large, non-antigen-specific lymphocytic infiltrate in the livers of patients with HBV-related chronic liver disease was described (226). Another independent investigation has further demonstrated that a large proportion of this non-antigen-specific lymphocytic infiltrate is NK cells (120). These cells express the pro-apoptotic ligand TRAIL and can mediate liver damage through engagement of a TRAIL death-inducing receptor on hepatocytes (120). In transgenic mouse models of HBV infection, reduction of recruitment and activation of this non-specific inflammatory infiltrate significantly diminished the severity of liver injury (227, 228). An early rise in circulating NK cells has also been documented in the incubation phase of HBV infection in humans (36).

Understanding the innate immune responses may also have insight into understanding disease outcome (resolution versus chronic hepatitis), since the quality of early immune activation might be a major player in shaping the subsequent adaptive immune response. On the other hand, two independent human studies (48, 49) reported that the frequency of regulatory T cells ($CD4^+CD25^+CD45RO^+$, foxp3 mRNA +) and the ability of these cells to suppress HBV-specific T cell proliferation and secrete IFNγ was variable among HBV-infected patients. The data suggests that circulating suppressor T regulatory cells, due to their anti-inflammatory ability, might promote inadequate HBV-specific immune responses leading to persistent infection. Thus, the quality of the early immune response to HBV-expressing cells and the balance between CTLs and regulatory T cell suppression are two possible mechanisms that independently or in cooperation might dictate disease outcome.

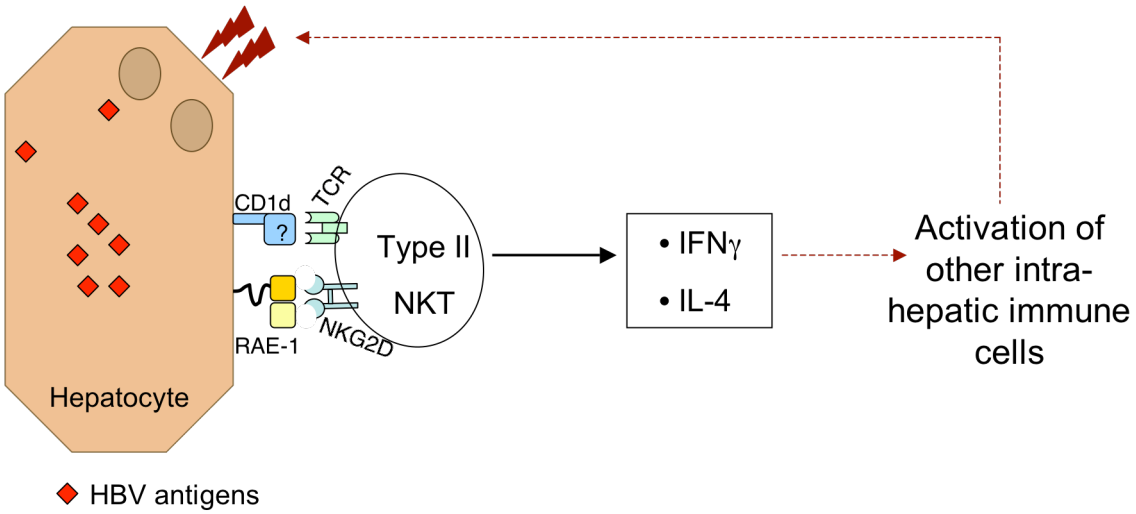

Figure 5.1. Schematic representation of the proposed model of non-classical type II NKT cell activation in our transgenic mouse model of primary human HBV infection, three days after adoptive transfer of syngeneic naïve wild type splenocytes.

HBV immunopathogenesis: lessons in NKT cell biology

NKT cells recognize highly conserved lipid antigens and respond rapidly to stimulation. These features have led them to be described for many years as a bridge between innate and adaptive immunity. There is evidence that NKT cells can amplify the innate immune response to many different microbes (225). The studies presented in this dissertation provide mechanistic evidence of how NKT cells have an important function in the initiation of pro-inflammatory responses to HBV-expressing cells, thus leading to potential implications in HBV clearance. It is not surprising that one of the most abundant cell population in the liver is involved in the first line of defense against a hepatotropic pathogen such as HBV. However, the NKT cell frequency in human livers is considerably lower when compared to mice. Thus, there is a debate on how realistic it is to translate advances in NKT cell biology from mice to humans. It is still not known whether there is only a NKT cell number difference between livers from mice and humans or whether these cells are also functionally different. Nevertheless, the difficulty to experimentally address these questions in humans is understandably high.

The diversity of clinical symptoms and disease manifestations associated to HBV infection strongly suggests that disease outcome is determined by the quality and vigor of the antiviral immune response produced by each infected individual. All arms of the immune response must cooperate to terminate this viral infection. Individual differences in the efficiency of viral antigen processing by hepatocytes and professional APCs as well as the responsiveness by B and T lymphocytes play an important role. These individual differences might be modulated by the level of antigen recognition and by the amount of IFNγ present in the liver at the time of T cell migration. The rapid mobilization and activation of non-classical NKT cells are compatible with studies of acute HBV infection in primates, which show a rapid drop in viral replication (HBV-DNA quantity decreases by almost 90%) in the presence of IFNγ production before the peak of an antigen-specific $CD8^+$ T cell response and liver damage (31, 32, 36). Based on our data, we propose that early and robust activation of resident NKT cells might contribute to a more effective immune response to HBV-infected cells accompanied with a decreased risk for chronicity.

The liver injury seen in the autoimmune hepatitis model of ConA-induced hepatitis, extensively used by immunologists, has also been described to be type I NKT cell and IL-4-mediated (198). Using this model, it was shown that activation of sulfatide-reactive type II NKT cells and plasmacytoid DCs caused IL-12 and MIP-2-dependent recruitment of type I NKT cells to the mouse livers. In this circumstance where type II NKT cells are first activated artificially, the type I NKT cells recruited to the livers were anergic and ConA-induced hepatitis was prevented by blocking the cytokine burst and neutrophil recruitment that follow ConA injection. This was one of the first studies that investigated the interactions between distinct NKT cell subsets as well as the subsequent immune consequences. Their data suggested that invariant type I NKT cells have a pathogenic role whereas the sulfatide-restricted type II NKT cells showed a protective role in liver injury induced by ConA administration (229).

In contrast, in our transgenic mouse model of primary human HBV infection, type II NKT cells are pathogenic, whereas type I NKT cells are not required for disease induction (71). Type II NKT cells were also found to play a proinflammatory role in the induction of human ulcerative colitis through their production of IL-13 (106) and to be enriched in livers of patients with chronic hepatitis C infection (230).

Moreover, type I and type II NKT cells have been described to play distinct and possibly opposite roles during infection with different parasites. In *Trypanosoma cruzi* infection, $J\alpha18^{-/-}$ mice (which are only deficient in type I NKT cells) showed much greater

morbidity and mortality than CD1d$^{-/-}$ animals (deficient in both type I and II NKT cells). This finding led the authors to suggest that during *Trypanosoma cruzi* infection, type II NKT cells were pro-inflammatory whereas type I NKT cells might counteract the effect of type II NKT cells when both types were present (231). In acute mouse schistosomiasis, these two types of NKT cells seem to have opposite effects on subsequent development of Th1/Th2 (232).

Type II NKT cells are a heterogeneous population of cells with or without surface expression of NK1.1 (233, 234) and which may or may not recognize sulfatide (100). Thus, it is possible that those type II NKT cells mediating proinflammatory responses might be a distinct subset from the type II NKT cells that mediate immunossupressive responses, contributing to tolerance (235). Additional experiments have to be performed to better address this possibility.

As I see in medical practice (bedside) a link to bench-orientated questions, I found in the study of mouse models of human diseases a link to fundamental biology. In fact, the work presented here is an example of how studying early immune responses to HBV-expressing cells in a transgenic mouse model provided the first evidence that NKT cells can be activated in vivo via its activating receptor NKG2D (chapter II). This finding automatically opened an experimental opportunity to investigate the role of both NKG2D adapter molecules, DAP10 and DAP12, in NKG2D signal transduction in this in vivo system (chapter III). It is well established that, in mice, NKG2D pairs with both adapters while, in humans, NKG2D associates only with DAP10. Additionally, in mice, it is also known that NKG2D signaling on NK cells requires both adapters, whereas on activated CD8$^+$ T cells only require DAP10. Since NKT cells share many phenotypic markers and functions with either NK cells or T lymphocytes, we sought to determine the function of each adapter molecule upon NKT cell activation through NKG2D under the specific conditions of our in vivo model of primary HBV infection. We found opposite results in cytokine production when DAP10 or DAP12 deficient cells were transferred. Adoptive transfer of *Dap12*$^{-/-}$ donor splenocytes resulted in impaired liver injury in the HBV-Replication$^+$*Rag-1*$^{-/-}$ mice. These results suggest that DAP12 is required for signal transduction via NKG2D on NKT cells at the time of acute hepatitis in our model (chapter III). In contrast, adoptive transfer of *Dap10*$^{-/-}$ donor splenocytes into HBV-Replication$^+$*Rag-1*$^{-/-}$ recipient mice led to a more severe acute liver injury as compared to the HBV-transgenic mice, which received wild type syngenic splenocytes (chapter III). Although not much is known about the role of DAP10 in NKT cells, a recent study (214) reported that activated *Dap10*$^{-/-}$ NKT cells produce significantly higher levels of cytokines, including IFNγ, as compared to wild type NKT cells. They further demonstrated that the

turnover proliferation rate was significantly increased in *Dap10*$^{-/-}$ NKT cells when compared to wild type NKT cells, leading them to suggest that *Dap10*$^{-/-}$ NKT cells might be constitutively hyperactive (214).

The opposite outcome found with the adoptive transfer of *Dap12*$^{-/-}$ or *Dap10*$^{-/-}$ splenocytes in a NKT cell-mediated NKG2D-dependent experimental mouse model, brings up the idea of a balance of signals, which is usual in the NK field. Reminiscent of the balance between activating and inhibitory signals on NK cells, which dictate either cell activation or inhibition, we propose that the balance between DAP10 and DAP12 might set the threshold of signal propagation through NKG2D. For instance, DAP10 might be important in dampening the signal transduction in NKT cell activation via NKG2D. Therefore, the cell is more activated when DAP10 is absent. In contrast, DAP12 may function as a "positive" adapter and the loss of DAP12 causes NKT cells to produce significantly less cytokines upon stimulation through the NKG2D receptor (chapter III). Although more experiments are required to test our hypothetical model, these findings enhance our understanding about the role of DAP10 and DAP12, and perhaps their interplay, following NKT cell activation/co-stimulation via NKG2D in a very well-defined in vivo system of NKG2D^{+} NKT cell-mediated acute immune response to HBV-expressing cells (Fig. 5.2).

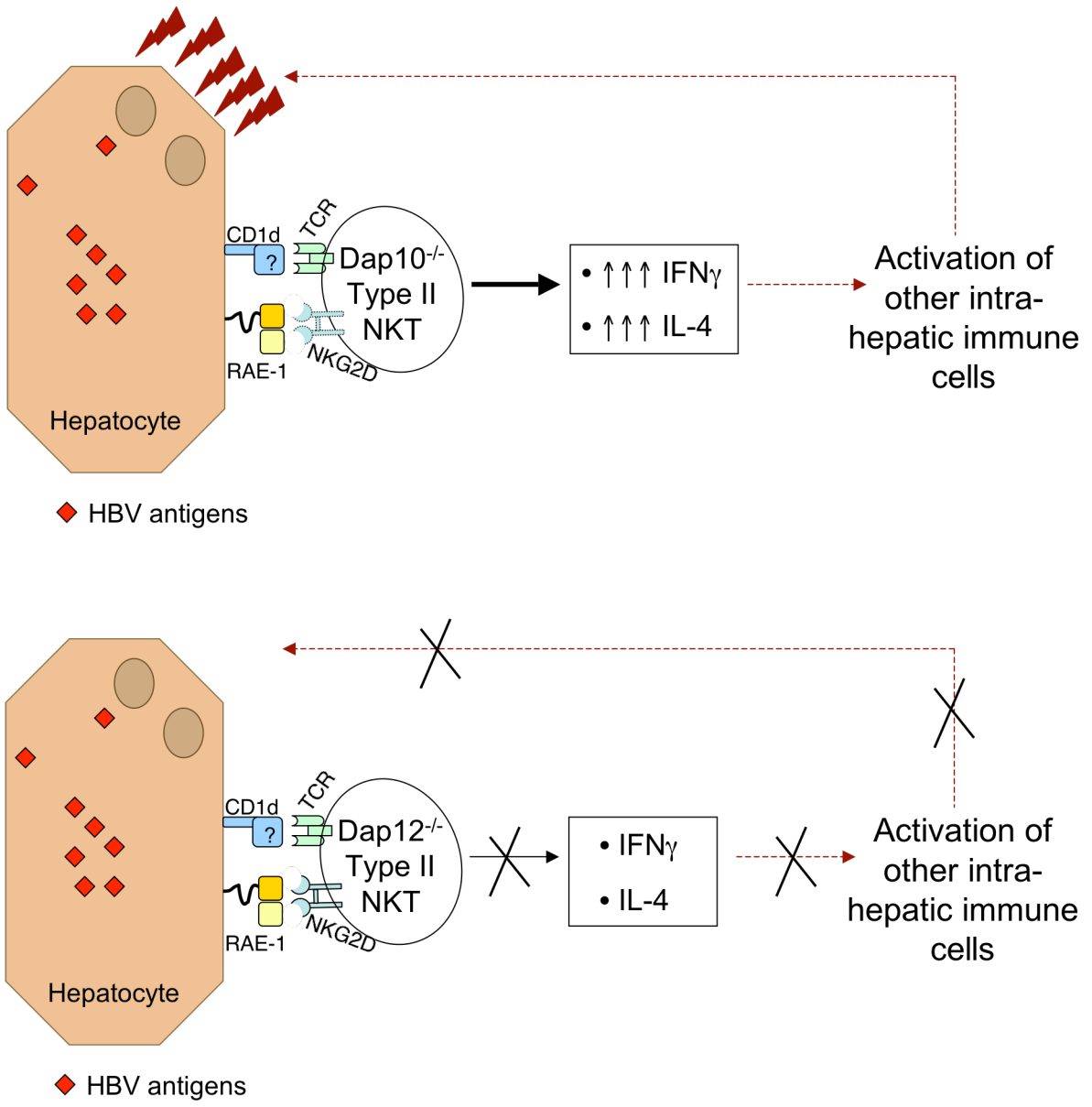

Figure 5.2. Schematic representation of the role of DAP10 and DAP12 adapter molecules in donor splenocytes transferred into HBV-transgenic *Rag-1*$^{-/-}$ mice. Note: Despite *Dap10*$^{-/-}$ NKT cells express very low amounts of NKG2D on their surface, its expression might be detectable upon activation.

Transgenic mouse model of primary human HBV infection

Ultimately, our goal is to mimic the immunological events that would normally occur during primary human HBV infection. Our transgenic mouse model allows manipulation of the immune system that is exposed to the virus, and identification of the cells and cytokines contributing to chronic hepatitis or disease resolution. This is a unique model to begin to address early events in immune activation to HBV and the spontaneous immune response and hepatitis that ensues. Mechanisms identified in these mouse models of primary HBV infection are the foundation for our current studies in the various clinical presentations of human HBV disease. For example, modest rises in ALT (1.5 to 2 fold increases above baseline) precede reactivation of hepatitis in chronic human HBV infection, known as "hepatic flares". Thus, a question that arises is whether the increase in ALT that anticipate the "hepatic flares" reflects early immune activation of NKT and/or NK cells, and whether this activation is NKG2D-dependent. It is also known that preceding "hepatic flares", patients usually experience an increase in HBV replication as detected by an increase in viral titers. It is possible that increased viral replication leads to large envelope protein retention in the ER. We hypothesize that accumulation of large envelope protein in hepatocytes might be involved in RAE-1 up-regulation, with NKT cell activation and consequent hepatic necrosis. In addition, this mechanism might also be involved in rare forms of fulminant hepatitis, since they are characterized by "ground glass" hepatocytes, which represents accumulation of large envelope protein within the hepatocytes.

The up-regulation of the stress-inducible molecule, RAE-1 (a distant homolog of MHC class I) on HBV-positive hepatocytes may be a mechanism by which the infected cell can present an activation signal alerting the immune system that an infection has occurred and mobilize an immediate immune response. Additionally, it is well established in our transgenic mouse model of primary HBV infection that hepatocytes and/or APCs also require CD1d on their surface to activate NKT cells (71). Therefore, a very important question remains unanswered: What is the nature of the molecule(s) being presented by CD1d and recognized by NKT cells in HBV-positive livers? The processing and presentation of lipid antigens by APCs is important for defense against infection, tumor immunosurveillance, and autoimmunity (236).

CD1 is a family of cell surface glycoproteins and is responsible for the binding and presentation of lipid antigens (237). In humans, five CD1 proteins have been identified and are divided in two groups, based on sequence identities in the α1 and α2 domains. Group 1

includes CD1a, CD1b, CD1c and CD1e and group 2 consists of CD1d (238-241). However, mice only contain CD1d orthologs. It is commonly believed that a chromosomal break event is the reason for the absence of group 1 CD1 from rodents (242-244). In contrast to MHC class I and II genes, polymorphisms of CD1 genes are extremely limited (245). The reasons for the lack of polymorphism of CD1 alleles are a matter of speculation. One possibility is that the opportunity of variation in the structure of lipids of microbial species may be considerably less than the potential for variation in the sequence of microbial antigenic peptides (236). Lipids are synthesized by multiple enzymatic steps, and the modifications that microorganisms might make are limited by the structural constraints needed for lipid organization in microbial membranes and cell walls (236). Thus, little polymorphism of CD1 grooves might be needed to accommodate lipid antigens binding (236). CD1d, known to present α-GalCer (a glycosphingolipid first discovered in marine sponge extracts), is recognized by the Vα14 invariant TCR on type I NKT cells (93), and leads to potent activation. In our model of hepatitis B infection, where type II NKT cells are activated through CD1d recognition, three possibilities should be considered. First, type II NKT cells could recognize a yet unidentified viral component in the context of CD1d. The candidates would include the myristate moiety of the large envelope protein or hydrophobic peptides from the HBV envelope protein. Second, CD1d might be presenting endogenous hydrophobic ligands (e.g., host lipids or glycolipids), which could be enhanced by HBV gene expression. Third, it is possible that HBV-specificity is given by RAE-1 up-regulation and not by what CD1d is presenting.

In summary, we propose a model in which non-classical type II NKT cells are first activated in a HBV-specific, CD1d-restricted and NKG2D-dependent manner within the liver, leading to IFNγ and IL-4 production (Fig. 5.1). Since activation of non-classical type II NKT cells requires HBV, CD1d and NKG2D, it is likely that NKT cells receive a CD1d – TCR interaction and NKG2D may function as a co-stimulatory molecule (Fig. 5.1). Signal one from CD1d-TCR interaction and signal two (co-stimulatory) from NKG2D, similarly to the requirements for activation of other T cell subsets (137).

Limitations of our transgenic mouse model of primary human HBV infection

Several criticisms have been raised concerning our transgenic mouse model of primary HBV infection. Most of them are inherent to any mouse model of a human pathogen. Specifically, since we cannot infect mice with human HBV, we cannot study viral clearance in this model. In a natural infection, the virus must recognize, bind and enter its target cells, and migrate to the appropriate cellular compartment, where its genome is transcribed, translated, and replicated to allow the assembly and export of new virions. The infection can then spread to additional susceptible cells and hosts. The hepatocytes of our transgenic mice are already expressing HBV antigens at the time of the adoptive transfer of syngeneic splenocytes, which account for the fact that HBV-Env$^+$ mice show a rise of ALTs as early as three to four days post-adoptive transfer of wild type splenocytes. This kinetics of disease progression are quicker as compared to studies of acute HBV infection in primates and humans, where there is an initial quiescent phase of four to seven weeks before HBV starts to replicate vigorously, leading to an ALT rise (31, 207, 246). It is also known that, in general, the mouse cellular metabolism is more accelerated than in humans, which might also account for the different kinetics and magnitude of ALT release.

Another implicit limitation of studying a human pathogen in a mouse model is that the co-evolution of the pathogen, in this case the HBV, together with its host(s) is neglected. Therefore, this limitation is not exclusively applicable to our model, but affects all HBV-transgenic mouse models, which, nevertheless contributed significantly for a better understanding of CTL responses in HBV infection. Thus, this limitation should not demote us to design improved mouse models for the study of HBV immunopathogenesis.

Significant variations in phenotype related to differences in genetic background constitute another general criticism of mouse models of human diseases. Therefore, rushed conclusions based on limited data or from observations made from mutations introduced into a single (pure or mixed) genetic background should be avoided because they can be misleading and slow the search for new findings (247). Thus, I would like to highlight the fact that the results presented here are reproducible in two independent lines of HBV-transgenic mice and in two close but distinct backgrounds, namely C57BL/6 (HBV-Replication$^+$ background) and B10.D2 (HBV-Env$^+$ background).

Despite the limitations described above, our transgenic mouse model of primary HBV infection should be seen as a valuable tool to identify novel candidate mechanisms and to

test their relevance in human disease. Our last discovery, uncovering a role for NKG2D and its ligand, RAE-1, in the acute immune response to HBV-expressing cells is one example.

HBV treatment and therapies: new insight

Hepatitis B is a preventable disease. The three possible but distinct read-outs of HBV control are inflammatory (ALT, biopsy), virologic (HBV DNA), and immunological (seroconversion: HBeAg to HBeAb and HBsAg to HBsAb). Current therapeutics to HBV consists of IFNα in combination with anti-viral drugs, such as lamivudine, adefovir, entecavir and telbivudine. Significant disadvantages exist among available therapeutics that include limited efficiency and promotion of drug-resistant viral strains. All these therapeutic approaches aim to eradicate the virus both indirectly by IFNα administration and directly with reverse transcriptase inhibitors (lamivudine and adefovir) or other anti-viral agents (entecavir and telbivudine). A combined effect of viral suppression and HBV-specific immune reconstitution is needed for effective long-term clearance of infection. Current therapeutics are effective in suppressing HBV viral replication but in most cases fail to clear the virus. The specific interaction between viral components and elements of the immune system may provide important clues to understand the mechanism of persistent infection and the basis of the “switch” from immune tolerance to immune activation phase in chronic HBV infections. Moreover, it is thought that the interaction between HBV and both the innate and adaptive immune responses determines the final outcome of the HBV infection. A better understanding of these events may potentially lead to novel therapeutics in the future (35).

Since HBV itself is non-cytopathic and liver injury is immune-mediated, new strategies for therapeutic intervention in this disease might aim at controlling the inflammatory/immunologic responses to HBV-infected cells in addition to targeting viral control and clearance. Our studies suggest that anti-NKG2D monoclonal antibody therapy might be a potential tool to control early immune responses to HBV-expressing cells, and prevent hepatocyte necrosis and liver damage. Early immune responses are thought to be important in clearing the infection, however it is known that immune re-activation during chronic hepatitis B infection leads to severe inflammation and liver fibrosis. Thus, in these latter situations controlling immune re-activation in addition to viral control may be beneficial for the host since immune-mediated hepatic damage might be reduced at the same time as viral replication which triggers the immune system is also controlled.

Fulminant hepatitis, which represents less than 1% of all hepatitis B infections, is a good example where the immune response of the host is responsible for the severe hepatic necrosis (hepatitis) with acute liver failure. In these cases, control of the immune-mediated response is instrumental in preventing hepatic failure and death. Thus, anti-NKG2D monoclonal antibody therapy could have an impact in these rare cases of fulminant hepatitis.

NKG2D and other immune-mediated liver diseases

There are several other causes of immune-mediated liver injury besides hepatotropic viral infections, such as autoimmune hepatitis, non-alcoholic steatohepatitis (NASH), drug-induced hepatitis, among others. Interestingly, recent work has suggested that inhibition of the innate immune system may attenuate liver injury from acetaminophen (APAP) toxicity in a mouse model without directly modifying the metabolism of the drug (248). Moreover, the observation that APAP-induced liver injury is NK and NKT cell-mediated led us to hypothesize that it could also be NKG2D-dependent. Overdose by APAP is the leading cause of acute liver failure in adults in the United States of America and Europe. However, anti-NKG2D monoclonal antibody treatment did not prevent acute liver injury induced by APAP overdose in mice (appendix 1), suggesting that the NKG2D-ligand system does not play a fundamental role in this acute liver disease.

Final considerations

"Human and mouse immunologists" are involved in a permanent debate about the best strategy for studying immunological mechanisms. While "human immunologists" generally question the relevance that findings in mouse models may have to human biology, "mouse immunologists" argue that studies in human systems are very limited and rarely extend beyond being descriptive. In fact, due to the relatively limited amount of human liver tissue available for research, especially at confidently definable disease stages, it seems that the "cross-talk" between mouse and human models will build the most coherent map of understanding. This may be particularly pertinent in mechanistic studies of liver immune regulation: tolerance versus activation. In fact, one important avenue towards understanding

HBV immunopathogenesis is to model hepatitis pathogenesis in mice, and unravel the role of the innate immune cell populations in the early immune recognition of HBV or/and in mastering a robust and effective adaptive immune response to HBV. It is very well established that $CD8^+$ T lymphocytes are the main actors in HBV infection, but it is essential to understand whether there are other (innate) cell populations that are primarily activated and orchestrate the adaptive immune system, resulting in different HBV infection outcomes.

The long-term goal of all my studies is to develop a more comprehensive understanding of the role of innate immunity and its interplay with adaptive immunity, in HBV clearance and virus-induced liver damage.

Note: While this thesis was being finalized Raulet and colleagues published their findings using $NKG2D^{-/-}$ mice in which NKG2D seems to be required for surveillance of primary tumors. In these mice, NK cells are defective in NKG2D function, but NK cell development is unaffected (Guerra et al., NKG2D-Deficient Mice Are Defective in Tumor Immunosurvaillance in Models of Spontaneous Malignancy, Immunity (2008), doi:10.1016/j.immuni.2008.02.016). The availability of these mice provides the opportunity to better understand the role of NKG2D on NKT cells, in general, and in HBV immunopathogenesis, in particular. Potential experiments using these mice are suggested in this dissertation.

CHAPTER VI

FUTURE PERSPECTIVES

Our findings raise provocative mechanistic questions and provide an experimental system in which they can be addressed. Three major lines of investigation arose from this work and are mentioned below.

RAE-1 regulation within the liver

The constitutive expression of RAE-1 on healthy hepatocytes and its up-regulation on HBV-expressing hepatocytes (chapter II) requires further investigation to determine which mechanism(s) are involved in RAE-1 expression. These findings raise two interesting questions. First is to understand why the constitutive expression of the NKG2D ligand, RAE-1, is exclusively in the liver and not in any other organs. Second is to identify the viral component responsible for RAE-1 up-regulation on hepatocytes and to assess its molecular mechanism. It is possible that the constitutive expression of this "stress-inducible molecule" in the liver, might be due to the permanent bath of antigens that come directly from the gut via the portal vein, in which the liver sits. Specifically, we have preliminary data that suggests that liver biopsies from germ-free mice as well as Myd88$^{-/-}$ mice have lower levels of RAE-1 mRNA expression than whole liver tissue from wild type mice with conventional gut flora. However, more experiments are required to confirm these results and to identify what compound is recognized by which pattern recognition receptor that might be involved in the mechanism of the up-regulation of RAE-1. On the other hand, we hypothesize that RAE-1 up-regulation on hepatocytes occurs in a HBV-specific manner and might be ER stress-related, since accumulation of large envelope proteins in the ER is thought to cause ER stress in those cells (204). However, preliminary data remains inconclusive and more analyses need to be performed to prove or disprove co-localization of large envelope proteins accumulation, overexpression of ER stress markers and RAE-1 up-regulation, in certain areas of HBV-positive livers. A potential interplay between CD1d and RAE-1 expression should also be explored both in vivo and in vitro. In the same sequence of ideas, it would be pertinent to identify what hydrophobic molecule, if any, is CD1d presenting to type II NKT cells during HBV infection. Since the myristate moiety of the large envelope protein is our first candidate as to what CD1d is presenting to NKT cell in our model, the first approach would be to introduce a point mutation in that region and analyze how it affects NKT cell activation either in vitro or in vivo, by hydrodynamic tail vein injection or by generating transgenic mice.

DAP10 and DAP12 in NKT cell activation

Our transgenic mouse model of primary human HBV infection can also be used as an experimental system to investigate in vivo the role of DAP10 and DAP12 adapter molecules on NKT cell activation via NKG2D receptor. We are currently in the process of investigating how these two adapters interact and signal in the specific conditions of our model. Despite the fact that *NKG2D*$^{-/-}$ mice are not currently available, a definitive experiment would be to cross these hypothetical knockout mice with *Dap10*$^{-/-}$ animals and then adoptively transfer *Dap10 NKG2D* DKO splenocytes into HBV-Replication^{+}*Rag-1*$^{-/-}$ recipient mice. This way, it would be possible to determine if the hyperactive status of *Dap10*$^{-/-}$ NKT cells is NKG2D dependent or independent.

Human studies

As a result of our findings described in chapter II of this dissertation, a patent is being issued on the use of an anti-NKG2D monoclonal antibody to treat HBV-related hepatitis. We are now set to extend our studies into human hepatitis B in collaboration with our hepatologist colleagues at UCSF and at California Pacific Medical Center, San Francisco. Since patients with acute HBV infection are normally asymptomatic and rarely seek medical attention, the goal of the human studies is to investigate whether the NKG2D-ligand system plays a role in immune reactivation in chronic HBV-positive patients. It is our plan to collect blood samples and liver biopsies from chronic HBV-infected patients, before, during and after “hepatic flares”. Initially, we will assess NKG2D ligand expression on hepatocytes and NKG2D modulation both on NK and NKT cells, isolated from peripheral blood and from liver tissue. Despite sample limitations, it would also be interesting to investigate the role of NKG2D receptor and its ligands in fulminant forms of human HBV infection, which is a rare although life-threatening medical condition.

These human studies aim to achieve not only a better mechanistic understanding of immune activation against HBV-infected cells, but also to identify a possible new strategy for therapeutic intervention in this disease.

CHAPTER VII

MATERIALS and METHODS

Mice and Disease Model

HBV-Env^{+} transgenic mice: lineage 107-5D [official designation Tg (Alb-1.HBV) Bri66; inbred B10.D2, H-2^{d}] (70) and HBV-Replication^{+} mice: lineage 1.3.46 [official designation Tg (HBV 1.3 genome) chi46; inbred C57BL/6, H-2^{b}] (64) were crossed to *Rag-1*$^{-/-}$ (249) animals (8-10 week-old). In experiments depicted in chapters II and III, these mice were intravenously injected with donor splenocytes from 6-10 week-old wild-type B10.D2 or C57BL/6 male mice (purchased from Jackson Laboratory, Bar Harbor, ME), respectively. The HBV-Replication^{+} mice contain a terminally redundant HBV DNA construct (64). These mice have high level viral replication in their hepatocytes and in the proximal convoluted tubules of their kidneys. The replication level seen in these mice is comparable to that observed in the infected livers of patients with chronic persistent HBV hepatitis, but the mice show no evidence of cytopathology (64). HBV-Replication^{+} *Rag-1*$^{-/-}$ mice were crossed to *IL-2Rβ*$^{-/-}$ (217) and *IFNγ*$^{-/-}$ (250) animals, and HBV-Env^{+} transgenic mice were also crossed to *IL-2Rβ*$^{-/-}$ animals. *Dap10*$^{-/-}$ (214) and *Dap12*$^{-/-}$ (251) mice backcrossed twelve generations to C57BL/6 were used. Mice were bled by tail vein at the described intervals and sera were collected. Other mice were sacrificed at the indicated time points, and livers were perfused or collected for histology. All mice were kept in a pathogen-free facility at UCSF.

Alanine-aminotransferase (ALT)

Over 100μL of blood per mouse was collected at the indicated time points into an eppendorf containing 10μL of 50mM EDTA. Blood samples were centrifuged and at least 50μL of sera were collected. Serum alanine aminotransferase (ALT) was measured by the standard photometric method using a COBAS MIRA plus auto-analyzer. Internal controls were used in all runs.

Isolation of intra-hepatic immune cells

Mice were anesthetized with 2.5% of isoflurane in 1.5L of oxygen (O_2), and livers were perfused via the thoracic portion of the inferior vein cava with digestion media (RPMI-1640 medium containing 0.2 mg/mL collagenase, 0.02 mg/mL DNAse, and 5% FCS at 37ºC) for 5 min at 5.5mL/min, and portal vein was cut. After perfusion, the livers were homogenized by forcing them through a 70μm cell strainer. The cell preparation was then centrifuged at 30xg for 3 minutes to remove hepatocytes. The supernatants were

centrifuged at 650xg for 10 minutes in order to spin down the lymphocytes. The pellets were resuspended in a 60%/40% percoll gradient and centrifuge at 2500 RPM for 20 min with no brake in order to enrich for intra-hepatic immune cells, which were collected from the interface. These immune cells were then washed in RPMI with 5% FCS for 10 minutes at 1800 RPM.

Isolation of hepatocytes

Mice were anesthetized with 2.5% of isoflurane in 1.5L of O_2, and livers were perfused via the thoracic portion of the inferior vein cava with a commercial liver perfusion medium (GIBCO) for 5 min at 5.5mL/min, followed by perfusion with a digestion media (DMEM Low Glucose 50% / F-12 50% mixture and 0.12-0.2mg/mL collagenase at 37ºC) for 8 min at 5.5mL/min. Livers were cut into small pieces and filtered through a 70 μm nylon cell strainer. Filtered cells were centrifuge at 30xg for 3 minutes, and viable cells counted by using trypan blue dye exclusion.

Immunofluorescent staining and flow cytometry

Fc-block (2.4G2 anti-CD16+32 mAb) and fluorochrome-conjugated antibodies against TCRβ (H57), NK1.1 (PK136), CD1d (1B1), H-2K^d (SF1-1.1), or the appropriate isotype-matched control Ig were purchased from BD. PE-labeled anti-NKG2D (CX5) antibody (rat IgG1 isotype) was purchased from eBioscience. Purified antibodies against RAE-1 (pan-RAE-1), which recognizes all known RAE-1 proteins (rat IgG2a isotype), and MULT1 were developed in collaboration with Dr. J. P. Houchins and are commercially available from R&D Systems, Minneapolis, MN (168).

For all stainings, approximately 1 x 10^6 cells were incubated with saturating amounts of antibody (previously titrated) on ice for at least 20 minutes.

DAPI was purchased from Roche. Viable cells were analyzed on a LSR II (BD) using FlowJo software.

Cell sorting

In chapter II, splenocytes were stained with anti-NKG2D mAb CX5 and the negative lymphocytes isolated. In some experiments, splenocytes were co-stained with anti-NKG2D

(CX5), anti-TCRβ (H57) and anti-NK1.1 (PK136). Cells expressing all three cell surface markers (NKG2D$^+$ NKT cells) were depleted. All flow cytometry sorting experiments showed >98% purity by using a FACS Aria cell sorter (BD).

In chapter III, intra-hepatic immune cells were co-stained with anti-NKG2D (CX5), anti-TCRβ (H57) and anti-NK1.1 (PK136). Cells expressing all three cell surface markers, NKG2D$^+$ NKT cells, as well as cells co-expressing TCRβ and NK1.1 but not NKG2D, NKG2D$^-$ NKT cells, were sorted. All flow cytometry sorting experiments showed >98% purity by using a FACS Aria cell sorter (BD).

In chapter IV, intra-hepatic immune cells were sorted by size and granularity. All flow cytometry sorting experiments showed >98% purity by using a FACS MoFlo cell sorter (Dako Cytomation).

TaqMan quantitative RT-PCR

Quantitative (real-time) RT-PCR was performed on cDNA generated from HBV-positive and negative hepatocytes, intra-hepatic immune cells and sorted intra-hepatic NKG2D$^+$ NKT cells, NKG2D$^-$ NKT cells and NK cells, by using an ABI 7300 according to the manufacture's instructions. Specific primers and probes were used for *Hprt* (133), *pan-RAE-1* (195), *IFNγ* (Primers: sense, ATGCATTCATGAGTATTGCCAAGT; anti-sense, GCTGGATTCCGGCACAG; Probe: FAM-CACAGGTCCAGCGCCAAGCATTC-TAM), TCRβ (Primers: sense, CACCCAAACCTGTCACACAGA; anti-sense, CATAGAGGATGGTTGCAGACAGAA;Probe:AGACTGTGGAATCACTTCAGCATCCTATCA TCAG); *IL-4* (Primers: sense, TCAACCCCCAGCTAGTTGTC; anti-sense, CGAGCTCACTCTCTGTGGTG, sybr green); *Dap10* (Primers: sense, GCGGTCATGTCACTCCTAATTG; anti-sense, ACCATCTTCTTGGGCAGGC; Probe from ABI, cat#185012327-6), *Dap12* (Primers: sense, TGGTCTCCCGAGGTCAAGG; anti-sense, GCGACTCAGTCTCAGCAATGTG; Probe from ABI, cat#185085152-4), *NKG2D-Long* (Primers: sense, CGGAAGTGGTGTCACATATCTTTAA; anti-sense,CCATGGCTACCTCAGATTTACAGA;Probe:CTCAGCATTCAGGAAGCAGAGGCAG ATT) and *NKG2D-Short* (Primers: sense, TGCACCCTCCACCAAATGAT; anti-sense, GTTCCTTGGCCCCACAGG; Probe: AGCTAAACACATTGTGTTCCCATAAAACA). Total RNA (isolated using Quiagen RNeasy kit) was DNase I-treated and cDNA was synthesized (using iScript kit, BIORAD). The cycling conditions for real-time PCR were: 2 min at 50°C,

10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 1 min at 60°C. All samples were normalized to the signal generated from the housekeeping gene mouse *Hprt*.

In vivo antibody treatment

A neutralizing, non-depleting rat anti-mouse NKG2D mAb, clone CX5 (rat IgG1), generated as described (133), recognizes the NKG2D extracellular domain and blocks the binding of NKG2D to its ligands. We injected i.p. 200μg of CX5 or control rat IgG (Sigma) per recipient mouse the day before and four days after the adoptive transfer of syngeneic naïve splenocytes.

To deplete NK cells from donor splenocytes and/or from HBV-transgenic recipient mice, a depleting rabbit anti-mouse/rat asialo GM1 polyclonal antibody (purchased from Cedarlane Laboratories) was used. These antisera depleted NK cells, but not NKT cells (21). We injected i.v. 100 μg of antibody per wild-type B10.D2 donor mouse one day before they were sacrificed. Depletion of NK cells (<0.1%) was verified by flow cytometry before the adoptive transfer was performed.

In the experiments performed before adoptive transfer depicted in chapter IV, anti-NK1.1 mAb, clone PK136, was used to deplete NK cells. Depletion of NK cells (<0.1%) was always verified by flow cytometry.

In experiments mentioned in chapter IV, anti-Gr1 monoclonal antibody, clone RB68C5, was injected intravenously, every 24 hours, to deplete neutrophils (maximum of 2 injections per mouse). Depletion of $CD11b^{+}$ $Gr\text{-}1^{high}$ cells from peripheral blood (<1%) and intra-hepatically (<3%) was assessed by flow cytometry.

Acetaminophen induced liver injury

C57BL/6 mice were matched by age (8-10 weeks of age) and gender (males), and fasted overnight prior to the administration of 400mg/Kg of acetaminophen intra-peritoneally. Mice were bled at 8, 24 and 48 hours after injection and sera were isolated and used to measure the ALT values in circulation.

ELISpot Assay

Intra-hepatic immune cells were eluted from HBV-transgenic mice, and at day 0, 3 and/or day 4 after adoptive transfer of syngeneic naive wild type splenocytes. Cells were counted and immediately plated in an anti-cytokine mAb coated 96-well microplate (BD, ELISpot mouse IFNγ and IL-4 kits). Eight serial 2- or 3-fold dilutions were done in duplicate, per condition. Spots were counted automatically by using an AID ELISpot Reader.

Histology

Liver was fixed, embedded in paraffin, and stained with hematoxylin and eosin. Liver sections were scored by an unbiased pathologist, according to the histopathologic standard scale for assessing viral hepatitis (252).

Statistical analysis

For all experiments, with the exception of studies depicted in figures 2.4 and 2.5, the Student's two-tailed T-test was used to test the difference between arithmetic mean values of each group (control versus experimental). Mann-Whitney two-tailed test was applied on experiments depicted in figures 2.4 and 2.5.

APPENDIX 1

Appendix 1. C57BL/6 mice were treated with 200μg anti-NKG2D monoclonal antibody (CX5) or its isotype control (ratIgG1) the day before of acetaminophen (400mg/Kg) intra-peritoneal administration. Experimental and control groups were bled at 8, 24 and 48 hours after injection in order to evaluate hepatocyte necrosis as measured by serum ALTs.

The chart above depicts one of three independent experiments and demonstrates no protective effect of anti-NKG2D monoclonal antibody treatment in acute liver injury caused by acetaminophen overdose. Mice were matched by age (8-10 weeks of age) and gender (males), and fasted overnight prior to acetaminophen dosing.

EPILOGUE

As I am finishing the writing of this dissertation, I realize that it has been a much more challenging task than I first expected, mainly due to all the scientific questions that still remain unanswered, the ones I was not able to answer even if I really propose myself to, and the numerous questions that arose during this project, directly or indirectly from our results. This is due not only to the fact that one result leads to more hypothesis and therefore experiments, but also because as we broad our scientific knowledge more questions we tend to ask and more questions we would like to be addressed. As we expand our knowledge we better understand what we know, what we do not know and what we really would like to pursue and be the first one to know.

I finish this thesis with enormous enthusiasm to continue to contribute to the advances towards a better understanding of liver immunology, both in health and disease. In fact, the immunology within the liver is yet poorly understood and this work is only a little step further in the beginning of a long journey, in which I envision myself for life.

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