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博士論文

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小鼠模式中表現無唾液酸神經節甘脂GM1的肝臟佇留CD8 T細胞對HBV的清除扮演必要的角色

Asialo GM1-positive liver-resident CD8 T cells are essential for immune clearance of hepatitis B virus in a mouse model

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博士生涯一路上最重要的是家人的支持，尤其是慧茹，一肩扛起家中的大小事，小孩仕元和芸希也非常懂事，讓我無後顧之憂。非常感謝許秉寧老師這些年來的指導，我非常喜歡老師使用因勢利導的方式，培養我們邏輯思考的能力。對老師而言，符合科學的推理、流暢地呈現實驗結果，是學生成長不可或缺的一環。但老師最重視的，還是學生是否以最大的努力，將實驗做到儘可能無懈可擊的地步，對凡事只求及格的我而言，實是幕鼓晨鐘，讓我脫胎換骨到我以前無法企及的境界。謝謝口試委員朱清良老師、楊宏志老師、陳培哲老師和謝世良老師，不僅在論文上給我指導，更在關鍵時間適時伸出援手，朱老師是我在困頓時的避風港；楊老師在我需要特別難以取得的材料時，提供支援；陳老師在我實驗遇到瓶頸時，鼓勵我繼續深入，試著發表論文，因此才有我最後兩年的突破；謝老師談笑間就能給我新穎正確的方向，讓我的研究有更大的願景。

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中文摘要

HBV 感染引起與慢性肝炎、肝硬化以及肝癌，造成全球性的健康問題。免疫系統如何控制病毒的機制知之甚少。以前的研究顯示，注射抗 AsialoGM1（ASGM1）抗體可阻斷小鼠動物模型中 HBV 清除的能力，並且一般認為這是由於抗 ASGM1 導致 NK 細胞消失。為了進一步驗證 NK 細胞在 HBV 清除中的作用，在本研究中，我們使用 NFIL3 敲除（KO）小鼠（NK 細胞缺乏的小鼠）來支持 NK 細胞對 HBV 的清除作用。意外的是，在 NFIL3 KO 小鼠中，清除 HBV 的能力沒有受到影響，表明 NK 細胞在 HBV 清除中不起關鍵作用。此外，用抗 ASGM1 處理的 NFIL3 KO 小鼠仍導致抗 HBV 抗性喪失，暗示存在其他非 NK 的 ASGM1 表達免疫細胞參與 HBV 清除。為了探討這個問題，我們對 NFIL3 KO 小鼠的肝內 ASGM1 +細胞進行了分選，並分析了這些細胞的免疫表型，發現共表達 CD44 和 LFA-1 的肝臟駐留 CD8 T 細胞在這些細胞中佔多數。我們的研究表明， NK 細胞在 HBV 清除中不是必需的。相反地，表達 CD44 和 LFA-1 的 ASGM1 陽性 CD8 T 細胞是參與抗 HBV 免疫的主要效應免疫細胞。重要的是，基因轉錄體分析，顯示 ASGM1 陽性 CD8 T 細胞相對其他細胞具有獨特的基因轉錄模式，比較像其他研究中所述的組織佇留 T 細胞(TRM)。我們進一步用過繼性細胞轉移的實驗證明，唯有來自肝臟的 ASGM1 陽性 CD8 T 細胞可以順利進入接受小鼠的肝臟中並有接近半數可存活超過二週，證明肝臟的 ASGM1 陽性 CD8 T 細胞確實擁有組織佇留的能力。總結以上幾點，我們認為 NK 細胞在 HBV 清除中並非關鍵的細胞，相對地，具有組織佇留能力的 ASGM1 陽性 CD8 T 細胞，同時表現 CD44 和 LFA-1，在 HBV 清除中扮演重要的角色。根據本篇的研究，肝細胞上是何種接受子幫助 ASGM1 的結合，是未來可以進一步的探討的方向。

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關鍵詞: B 型肝炎病毒 HBV; 高壓尾靜脈注射法小鼠模式;無唾液酸神經節甘脂 GM1; NFIL3 基因缺失小鼠; 肝臟 CD8 T 細胞; 去唾液酸糖蛋白受體

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Abstract

Hepatitis B virus (HBV) infection causes chronic liver diseases that may progress to chronic hepatitis, liver cirrhosis, and subsequent hepatocellular carcinoma. Previous studies demonstrated that CD8 T cells are critical in HBV elimination. However, whether there is a distinct subtype of CD8 T cells response for the clearance of HBV remains unclear. In this study, we showed that the treatment with anti-asialo GM1(ASGM1) antibody (Ab), an NK cell-depleting Ab for ablating the function of NK cells in vivo, led to impairment of the ability to clear HBV in a mouse animal model. Unexpectedly, the ability to clear HBV was not significantly impaired in NFIL3 KO mice, which are deficient in NK cells, indicating that conventional NK cells do not play a critical role in HBV clearance. Moreover, the ability to clear HBV was abolished when NFIL3 KO mice were further treated with anti-ASGM1 Ab, implying that other non-NK ASGM1-positive immune cells mediate HBV clearance. We isolated intrahepatic ASGM1^{+} cells from the liver of NFIL3 KO mice and analyzed the immune phenotype of these cells. Our results demonstrated a distinct CD44^{+} LFA-1^{hi} CD8 T cells that were the major intrahepatic ASGM1-positive immune cells. Importantly, transcriptome analysis revealed that ASGM1-positive CD8 T cells existed substantial differences from others and have similar

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core gene signature of tissue resident memory cells (TRM). Regarding ASGM1-positive CD8 T cells showed both transcriptional and phenotypic liver residency, we further proved that these cells indeed homed to and remained long-lasting in the liver after adoptive transferring. Collectively, our study indicates that conventional NK cells are not essential for HBV clearance in this mouse model. Instead, ASGM1-positive liver-resident CD8 T cells which express CD44 and LFA-1 are the major effector immune cells mediating anti-HBV immunity. The hepatic receptor which mediates the binding of ASGM1 will be investigated.

Key word: Hepatitis B virus; Hydrodynamic injection mouse model; Asialo GM1; Liver resident CD8 T cells; Asialoglycoprotein receptor

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Chapter 1 Introduction

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1.1 Hepatitis B virus (HBV)

Hepatitis B virus (HBV) is a noncytopathic, hepatotropic and partially double-strand DNA virus belong to a member of the family Hepadnaviridae. Hepatitis B surface antigen (HBsAg) was found as AuAg in 1963 by Baruch Blumberg. Prophylactic vaccine is available since 1984. Therefore, the risk of developing cirrhosis or even the liver cancer due to HBV is also prevented. However, HBV vaccine has no effect on existing infections. Estimated 257 million people afflicted. More than 880,000 deaths annually worldwide from HBV related liver disease, including cirrhosis and liver cancer (Shin et al. 2016; Yang H. I. et al. 2002). 2.4% vaccinated infants born from HBsAg-seropositive mothers will develop hepatocellular carcinoma in the future, which is 30 times higher than from HBsAg-seronegative ones (Chang et al. 2009). In Taiwan, there are 15% adults HBV seropositive, and about 5,000 people dead due to the HBV relative diseases. It is ranks number night among ten leading causes of death.

HB virions, or ‘Dane particles’, comprise an outer envelope of the lipid-embedded small (S), middle (M) and large (L) surface proteins (HBsAg in serology) and an inner nucleocapsid (core particle; hepatitis B core antigen in serology). The most remarkable features of the genome are its tiny size (~3 kb) and extremely compact organization, in

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where the four overlapping open reading frames (ORFs) are responsible for the transcription and expression of seven different hepatitis B proteins (preS1/preS2/S (S), precore/core (C), polymerase (P), and X antigen genes). It is known that one of the key HBV receptors is sodium taurocholate cotransporting polypeptide (NTCP, Yan et al., 2012)). After entry into the hepatocytes, the nucleocapsids move to nuclear membrane and bind to Nup153 in nuclear pore complexes (Schmitz et al., 2010), where the capsids release relaxed circular (RC) DNA into nucleus. HBV then forms covalently closed circular (ccc) DNA in the host cell's nucleus before transcribing viral RNAs. Current therapies rarely achieve a cure due to this intracellular viral intermediate (Alter et al., 2018; Yeh et al., 2015). After expressing HBV genes, the components of HBV reassemble, the HBV Pol reverse transcribes the RNA pregenome (pg) RNA to produce new RC-DNA, and new HBV leave infected hepatocytes for healthy ones. In general, cccDNA can persist throughout the lifespan of quiescent hepatocytes without affecting their viability, and HBV Pol inhibitors cannot directly affect the nuclear viral cccDNA.

Unlike Hepatitis C virus (HCV) infection can be cured by Harvoni, which is introduced by Gilead and comprised of sofosbuvir and ledipasvir, the current recommended first-line antiviral therapies for CHB, including pegylated-interferon (Peg-IFN) α-2a, entecavir (ETV) and tenofovir disoproxil fumarate (TDF), are rooms for

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improvement. Nucleos(t)ide analogue (NA) therapy such as long-term ETV therapy, in most nucleoside-naive patients, including those with advanced fibrosis or cirrhosis at the baseline, leads to potent suppression of HBV DNA, normalization of ALT, and improvement in liver histology with accompanying regression of fibrosis (Chang et al. 2010). However, it can only inhibit the replication and reinfection of the HBV, but cannot trigger the apoptosis of infected hepatocytes. Clearing cccDNA, as well as killing infected hepatocytes, rely on host's own immune system. On the other hand, Peg-IFN therapy has the disadvantages of having a moderate antiviral effect, inferior tolerability and a risk of adverse events (Yeh et al. 2015).

1.2 Host immunity against HBV

Regarding the effective long-term cancer prevention by recombinant HBsAg vaccination (Chang et al. 2009), adaptive immune responses, especially plasma cells and helper T (Th) cells, have been shown to contribute to HBV elimination (Guidotti, et al.1999; Schmidt et al. 2013; Yeo et al., 2009). Plasma cells are thought to play a critical role in viral clearance. The antibody response to HBsAg is a T cell-dependent process. Because anti-HBsAg antibodies are readily detectable in patients who clear the virus and recover from acute hepatitis, and they are usually undetectable in patients with chronic HBV infection, they are thought to play a critical role in viral clearance by complexing

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with viral particles and removing them from circulation or possibly by preventing their attachment and uptake by susceptible cells (Malice, et al. 1986). Moreover, the risk of HBV reactivation is >10% for both HBsAg- positive and HBsAg-negative, anti-HBc–positive patients, highest among all immunosuppressive therapy, indicating B cell immune response is essential in HBV clearance (Perrillo, et al. 2015).

On the other hand, a vigorous, polycloneal and multispecific cytotoxic (CTL) and Th cell response to HBV is readily detectable in the peripheral blood of patients with acute self limited hepatitis B but is weak, antigenically restricted or undetectable in patients with chronic infection (Chisari, et al. 1995). HBV-specific CTLs profoundly suppress hepatocellular HBV gene expression in HBV transgenic mice by a non cytolytic process, the strength of which greatly exceeds the cytopathic effect of the CTLs in magnitude and duration. IFN-γ and TNF-α mediate the regulatory effect of the CTLs (Guidotti et al. 1994). Besides, a report monitored the course of HBV infection in control, CD4-depleted, and CD8-depleted chimpanzees. The researchers found that the reappearance of $CD8^+$ cells between weeks 11 and 17 was associated with the onset of a mild liver disease and with a 50-fold reduction in total liver HBV DNA in the entire liver, even though the number of HBcAg-positive hepatocytes decreased only 18% during the same time period, suggesting that HBV replication was inhibited noncytopathically at this stage of the infection (Thimme et al. 2003). However, the exactly noncytopathic

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mechanisms *in vivo* are not clear. They might be the inhibiting hepatitis B virus replication through a common molecular mechanism (Pagliaccetti et al. 2010) and via activation of the NF-κB signaling pathway (Watashi et al. 2013). The critical role of both CD4 and CD8 T cells have been further confirmed by using a hydrodynamic injection-based mouse model (Yang, P.L., et al. 2010). Clearance of HBcAg- positive hepatocytes from the liver in $CD4^{-/-}$ and $CD8^{-/-}$ mouse strains occurred with kinetics that closely paralleled the clearance of NOD scid mice, comparing to the fast disappeared from wild-type mouse between 10 and 20 days aftertransfection.

Nevertheless, the role of innate immunity during HBV infection still remains unknown. HBV does not trigger innate immune responses readily measurable *in vivo* (Wieland et al., 2004). Recent studies revealed that virus-derived nucleic acids (both RNA and DNA) sensing pattern recognition receptors (PRRs), such as retinoic acid-inducible gene-I (RIG-I, Sato et al. 2015) and melanoma differentiation–associated gene 5 (MDA5, Lu et al. 2013), might be transiently induced by virus infection, but not crucial for HBV clearance. A cell line base research suggest that HBV infection induced ISG56 through the cyclic GMP-AMP synthetase (cGAS)–STING signaling pathway (Dansako et al. 2016). Moreover, by using a hydrodynamic injection-based mouse model (HDI, Huang et al., 2006), our previous study demonstrated that HBV viral clearance rate of IFN-α/β receptor (IFNAR), RIG-I, MDA5, MYD88, NLRP3, ASC, and IL-1R KO mice was not

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different from WT mice (Tzeng et al., 2014). On the other hand, WT mice treated with anti-TNF-α neutralization antibody or TNF-α KO mice showed delayed HBV clearance or HBV reactivation, indicated that TNF-α leads to the clearance of HBV (Ebert et al., 2015; Tzeng et al., 2014; Chyuan, I. T. et al. 2015). Kupffer (KC) and Natural killer cells (NK) are the major TNF-α producing cells among intrahepatic leukocytes (KC, NKs, natural killer like T (NKT) cells, Dendritic (DC) cells and some other rare cells such as basophil and innate lymphoid cells (ILC) (Ravanelli et al. 2006)). The role of both cell type in HBV clearance needs to be elucidated.

1.3 Kupffer Cells and inflammatory monocytes plays opposite roles in HBV clearance.

The occupied of IHL of the mice are Kupffer cells, NKT, NK, T cells, B cells and others sequentially (Crispe 2003; Freitas-Lopes et al. Cells. 2017 6(4): 48). Liver contains a large KC population as the first-line phagocyte against pathogen or contributing in immune tolerance. To explore the role of KC, mice were transiently depleted of KCs by clodronate liposome treatment on day -2 preceding hydrodynamic injection of HBV plasmid, resulted in almost complete HBV elimination within 4 weeks, whereas control mice failed to eliminate HBV at the same time-point (Xu et al., 2014). One the other hand,

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HBV viral clearance was found to be strongly associated with a sharp induction of TNF-α-secreting Ly6C$^+$ monocytes accompanied by a severe reduction in KC number at D3 in the liver. Besides, CCR2 antagonist administration hampered Ly6C$^+$ monocyte recruitment and delayed HBV clearance, implying the Ly6C$^+$ monocytes play critical in HBV clearance (Wu, et al. 2019). However, whether anti-HBV TNF-α was secreted by Ly6C$^+$ monocytes needs to be further confirmed.

1.4 Nature killer cells produce TNF-α, suggesting its role to control hepatitis B virus.

NK cells, the large granular lymphoid-like cells and found in 1975 (Kiessling et al., 1975), play important roles in controlling viral infections and elimination of tumor cells through both cytolysis and noncytopathic mechanisms (Vivier et al., 2011, Wang et al., 2012). NK cells occupy less than 5% among peripheral immune cells but are enriched in the liver. As Kupffer cells, NK cells induce cytopathic effect through producing cytokines such as interferon-γ and TNF-α. Otherwise, NK cells perform cytotoxic effect through perforin/granzyme axis, TRAIL and FasL to eliminate infected or transformed cells (Zhang Q., et al. 2018). Accordingly, NK cells are important to early resistance to murine cytomegalovirus (MCMV) infection (Forbes et al., 2014) and herpes simplex virus infection (Biron et al., 1989). Nowadays, NK cells were classified into class I innate

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lymphoid cells (Spits et al. 2013). Most common way to elucidate the role of NK cells is treating mice i.p. with NK depletion antibody – anti-asialoGM1 (α-ASGM1, Habu et al. 1979).

ASGM1 is one of the glycosphingolipids (GSLs) which consist of glycans conjugated to a lipid core. GSLs pack densely together with cholesterol to form lipid raft which serve as cell surface signaling platforms. Ganglioside deficient mice have been generated and showed mild degeneration of nerve system (Takamiya et al. 1996). The GSL repertoire of different immune cells varies per cell type under physiological conditions. β1,4-N-acetylgalactosaminyltransferase 1 (B4GALNT1) catalyzes the generation of asialo GM2, initializing the synthesis of o-series gangliosides including ASGM1. Otherwise, GM1 is catalyzed to generate asialo-GM1 by NEU-1 sialidase (Chen et al. 1997; Zhang et al. 2019). NK cells one of the few cells are known to express lots of ASGM1 on the cell surface and hence can be depleted by anti-asialoGM1 treatment. Since NK are enriched in the liver microenvironment, whether there is any liver specific receptor of ASGM1 on hepatocytes is interesting.

ASGPR is abundantly expressed on the surface of hepatocytes but minimally expressed by extrahepatic cells (Ashwell et al. 1982). It is involved in the clearance of circulating desialylated glycoproteins and in the elimination of activated lymphocytes

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(Guy et al. 2011; McVicker et al. 2013). Previous study demonstrated that ASGPR binds ASGM1 as well (Saunier et al. J Virol. 2003 77(1):546-59), suggesting ASGPR might be the liver specific receptor of ASGM1.

Though the expression of ASGM1 is not strictly confined to NK cells but a subpopulation of NKT, CD8 T and other hematopoietic cells (Nishikado et al. 2011), anti-ASGM1 treatment is considered as a specific NK cell depletion (André et al. 2018). However, α-ASGM1 treatment deplete not only NK cells but some other cells such as basophil or partial of CD8 T cells. Thus, any suggestion supported by α-ASGM1 depleting studies needs to be confirmed by genetic NK deficient mice.

There was no appropriately NK deficient mice until 2009. Four teams generated NFIL3 KO mice (Kamizono et al. 2009) indepently between 2009 to 2011. NFIL3 protein binds to the 5'-flanking region of the human IL-3 promoter (Zhang et al. 1995). Nevertheless, the most predominant phenotype of the knockout mice is the loss of NK cells while the functions and numbers of T cells, B cells, and NKT cells are normal. Though that scientists later found the $CD8^+$ dendritic cells (Kashiwada et al. 2011) and all kinds of innate lymphoid cell are impaired in NFIL3 KO mice (Seillet et al. 2014), the mice are considered as the best NK deficient mic.

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1.5 Hydrodynamically transfection animal models are used to explore the role of immune response after HBV transfection.

Human being is the only natural host of hepatitis B virus. It leads to the experimental limitation that there is no suitable HBV animal model yet to date, especially the inbred mouse model. The first one, HBV transgenic mice (Chisari et al., 1985) are centrally tolerant to viral antigens. Adenovirus base infection mice (Sprinzl et al., 2001) might infect cells other than hepatocytes and induce unwanted immune response. Humanized mice (Dandri et al., 2001) have deficient immune system. A novel animal model harboring both a human immune system (HIS) and human hepatocytes (HUHEP) in BALB/c $Rag2^{-/-}$ IL-$2R\gamma c^{-/-}$ NOD.sirpa $uPA^{tg/tg}$ mice has be develop (Dusséaux et al., 2017; Strick-Marchand et al., 2015;). It might be a good choice to study the immune response of HBV infection, but is still not available yet for us. Hydrodynamically transfected mice (Huang et al., 2006) cannot mimic the response to reinfection of HBV, but be used to explore acute immune response after HBV transfection. We used hydrodynamically transfected system developed by Dr. Chen's lab. A pAAV/HBV1.2 plasmid kindly provided by Dr. Chen contains the HBV fragment spanning from nucleotides 1400~3182/1-1987 flanking by inverted terminal repeats (ITRs) of AAV. Ten micrograms of plasmid DNA/DPBS in a volume equivalent to 8% of the mouse body

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weight will be injected via a tail vein in 5s. The volume exceeds the cardiac output and form a backflow in inferior vena cava into liver vein. Most injected solution will be uptake by liver and about 15~20% hepatocytes will be transfected.

1.6 Tissue resident memory CD8 T cells (TRM) play important role in controlling intracellular pathogens.

In addition to innate immune cells, recent studies in viral models have now revealed a distinct tissue-localized T cell lineage, tissue resident memory (TRM) cells, that resides in peripheral tissues that is crucial for protective immunity in peripheral tissue (Mueller S. N., et al. 2016). The lack of recirculation via the bloodstream is key to defining TRMs . Moreover, TRMs in many non-lymphoid tissues persist for long periods of time after formation, at least in mice. In patients with leukaemic cutaneous T cell lymphoma who were treated with a CD52-specific antibody to deplete T cells showed that, although T cells were eliminated from the skin, a proportion of T cells in skin were spared, indicating that these cells were truly resident in the tissue (Clark et al. 2012).

As a distinct T cell lineage, CD8 tissue resident memory T cells can be distinguished from other T cell subsets by immune markers including CD44, CD103, CD69, and CD49a

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(Topham et al., 2018). It is now recognized that peripheral tissues including liver are surveyed by TRM cells that vastly outnumber their recirculating memory T cells. However, several observations suggest that the liver may be an exception to the conventional leukocyte migration paradigm involving rolling, adhesion, and extravasation from postcapillary venules. In contrast to vascular beds in most organs, LSEC lack tight junctions as well as a basal membrane. Thus, the fenestrated endothelial barrier of sinusoids provides the opportunity for direct interaction of circulating cells with underlying hepatocytes (Guidotti et al., 2015). How TRMs residing in the liver may differ from those in other vascular districts.

Accumulating evidences suggested that TRMs play important role in controlling intracellular pathogens (McNamara et al. 2017, Fernandez-Ruiz et al. 2016) in mice. Instead, a study characterized a population expressing liver-homing/retention markers $CD69^{+}CD103^{+}$ found within the intrahepatic but not the circulating memory CD8 T cell pool. These TRM-like cells are preferentially expanded in patients with partial immune control of HBV infection and can remain in the liver after the resolution of infection (Pallett et al. 2017). The expansion and retention were not limit to $CD103^{+}$ but all $CD69^{+}$ CD8 T cells, suggesting the role of TRM in controlling HBV. How to elucidate the role of TRM in mouse model is needed.

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Recently, a study demonstrated that anti-ASGM1 treated mice failed to eliminated HBV and implicated that NK cells can enhance the generation of HBV-specific CD8 T cells through producing interferon gamma (Zheng et al., 2016). In contrast, another study demonstrated that NK cells, consistent with B cells, perforin, and IFN-α/βR, do not contribute to suppress HBV replication (Yang et al., 2010). Thus, the function of NK cells in controlling HBV has remained controversial. Here we employed a NK deficient mice, NFIL3 knockout mice, to explore the role of NK cells in HBV clearance.

Besides, CD8 T cells are known to play critical role in HBV clearance. Accumulating evidences implicated that TRM are a distinct cell subset and critical for protective immunity in peripheral organs. Our preliminary data showed that ASGM1$^+$ CD8 T are phenotypically similar to TRM. The second aim of this study was to investigate whether ASGM1$^+$ CD8 T are genetically and functionally similar to TRM as well.

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Chapter 2 Materials and Methods

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2.1 Mice

All mice used in this study are from the C57BL/6 (B6) background and at 6-8 weeks of age. C57BL/6 WT mice are purchased from the National Laboratory Animal Center (Taipei, Taiwan). CD45.1/45.2^{+} congenic and NK-deficient (NFIL3 KO) mice will be kindly provided by Dr. Chien-Kuo Lee and Dr. Tak Mak (The Campbell Family Institute for Breast Cancer Research), respectively. All mice are bred and kept in specific pathogen–free (SPF) facility at College of Medicine, National Taiwan University. Procedures and the use of the animals are reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

2.2 Hydrodynamics-based transfection

pAAV/HBV plasmid DNA are purified by using Endotoxin-Free Maxi plasmid kit. C57BL/6 mice (male, 6-8 weeks old) will be warmed under pet lamp. Ten micrograms of plasmid DNA/DPBS in a volume equivalent to 8% of the mouse body weight will be injected via a tail vein in 5s. Facial vein blood will be collected weekly with lancet. Serum levels of HBsAg will be determined by using Roche's cobas e 411 Immunoassay Analyzer.

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The cutoff value for determining HBsAg positivity is a signal-to-cutoff (S/CO) ratio of ≥ 1 according to manufacturer's instructions.

2.3 Anti-ASGM1 treatment *in vivo*

The lyophilized anti-Asialo GM1 antisera (Wako, JP) was dissolved with distilled water according to the manufacturer's instructions. Mice (male, 6-8 weeks old) were intraperitoneal injected 20 μl antisera solution one day before hydrodynamically transfection and twice a week for 8-10 weeks. The intrahepatic immune cells population was determined by flow cytometry.

2.4 Isolation of intrahepatic lymphocytes

Mice are anesthetized by administrated 10 μl Zoletil/rompun premix intravenous and then perfused by 10ml DPBS. Suspend the cells in HBSS buffer by mincing the perfused liver with a plunger and passed through a 70 μm strainer. Hepatocytes and large cell clumps are removed by 50 g centrifugation 3 min twice. The supernatant containing intrahepatic leukocytes (IHLs) is pelleted by 300 g centrifugation at 4℃ for 10 min.

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Resuspend the cells to 40%/70% percoll (GE) gradient centrifugation, 1200 g, 20min. Interface cells are washed by HBSS once.

2.5 Analysis of intrahepatic lymphocytes and splenocytes

Single cell suspensions were firstly stained with FC receptor blocker (BD Biosciences, San Jose, CA, USA) for 10 min on ice and then subjected to surface marker-specific antibody staining for 60 min. Cells were labeled with polyclonal antibody specific for ASGM1 and monoclonal antibodies specific for CD3ε(17A2), CD8α (53-6.7), CD25 (pc61.5), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD103 (M290), CD107a (1D4B), CD127 (SB/199), CD137 (17B5), CXCR3(CXCR3-173), ICOS (7E.17G9), KLRG1 (2F1), LFA-1 (H155-78), NK1.1 (PK126), PD-1 (29F-1A12) or RANKL (IK22/5) from BioLegend, San Diego, CA. After washed twice, the cells were resuspended in FACS buffer (2% FBS in PBS) and analyzed by flow cytometry (Canto II, BD Biosciences, San Jose, CA, USA). Regarding cell sorting, single cell suspensions were stained with FC receptor blocker and then subjected to anti-ASGM1, anti-CD44 or anti-LFA-1 antibody single-staining for 60 minutes. Cells were sorted under indicated criteria using the FACS Aria IIIu (BD Biosciences, San Jose, CA, USA).

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2.6 Immunohistochemistry (IHC)

Perfused livers were fixed in 10% formalin embedded in paraffin. Intrahepatic HBcAg was detected by IHC staining with rabbit anti-HBc and Envision System, horseradish peroxidase (DAB) (Dako, Glostrup, Denmark). Hematoxylin was used to stain liver section nuclei.

2.7 Adoptive transfer

CD45.1/45.2^{+} congenic mice were intravenous administrated of 1 μg KN-62. 30 minutes later, intrahepatic leukocytes were harvested and stained. A FACSAria cell sorter ((BD Biosciences, San Jose, CA) was used to purify ASGM1+ and ASGM1- CD8 T cells. Purity of sorted cells was more than 90%, as verified by flow cytometry. CD45.2+ C57BL/6 mice received intravenously 8x10^{4} donor cells.

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2.8 Gene expression profile analysis

3 x 10^4 ASGM1$^+$ and ASGM1$^-$ CD8 T cells from the liver of naive NFIL3 KO mice were sorted on a FACSAria III (BD Biosciences, San Jose, CA). For each sample, we extracted total RNA using Trizol (Invitrogen, Waltham, MA). After amplification and conversion to cDNA, samples were hybridized to an Affymetrix Transcriptome Array (Mouse Clariom D) (Affymetrix Inc. Santa Clara, CA) at NCFPB High-throughput Genome Analysis Core Facility. Image analysis was done using Affymetrix Transcriptome Analysis Suite (TAS) (Affymetrix Inc. Santa Clara, CA).

2.9 Enzyme-linked immunospot assay (ELISPOT)

Intrahepatic leukocytes cells or splenocytes were cultured and assayed for the frequency of antigen-specific IFN-γ-secreting cells using a Murine IFN-γ Single-Color Enzymatic ELISPOT Assay kit (Cellular Technology Limited, Shaker Heights, OH). Briefly, 1 × 10^5 mononuclear cells were cultured with $HBcAg_{18-27}$ and $HBcAg_{93-100}$ peptides (50 μg/ml, Mission Biotech, Taipei, TW) in 200 μl CTL-Test Medium and incubated for 18 h at 37°C. Spot-forming cells were revealed by biotin-conjugated

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detection Ab with alkaline phosphatase-conjugated streptavidin and substrates, BCIP and NBT.

2.10 Quantitative PCR analysis

For quantitative PCR, total RNA was isolated from indicated cells using Trizol (Invitrogen, Waltham, MA) and cDNA was synthesized with iScript (Bio-Rad, Hercules, CA) according to the manual. Quantitative amplification was performed with SYBR Premix ExTaq II (TaKaRa, Shiga, Japen) in an Thermo PIKOREAL 96 system (Thermo Fisher Scientific, Vantaa, Finland) with the primers: (Inpp4b: 5'- CAGTAGCAAGGATGGAGAGGCA-3' (forward), 5'- TTGCTCGGTTCACCACTT CTCC-3' (reverse); Itgae: 5'- GAAGTGGAACGGAG GGTCCTTT-3' (forward), 5'- GTCTGGAACTCGTAGGTGACCT-3' (reverse); Eomes: 5'- CCACTGGATGAG GCAGGAGATT-3' (forward), 5'- GTCCTCTGTCACTTCCACGATG-3' (reverse); Ly6C2: 5'- GCGCCTCTGATGGATTCTGCAT-.

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2.11 Materials

NucleoBond Xtra EF	Macherey-Nagel Düren DE
Fluorescent-conjugated antibodies	BD Biosciences Pharmingen San Diego, USA
MojoSort CD3 T cell isolation kit	BioLegend San Diego, CA, USA
Fixation/Permeabilization kit	Invitrogen, Carlsbad, CA, USA
ImmnoSPOT kit	Cellular Technology, OH, USA

2.12 Statistical analysis

Statistical analysis of experimental groups is performed by unpaired Student's t-tests using GraphPad Prism 6.0 software (GraphPad Software, San Diego USA). $P<0.05$ was considered significant.

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Chapter 3 Results

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3.1 Anti-AsialoGM1(ASGM1) treatment resulted in lack of sustained HBsAg clearance from serum.

To study the role of NK cells in HBV clearance, anti-ASGM1 was intraperitoneal (i.p.) injected to BALB/c or C57BL/6 mice to deplete NK cells. The percentages of NK cells in the liver were markedly decreased in mice treated with the antisera **(Figure 1a, e)**. After hydrodynamic injection (HDI) of a replication competent HBV DNA, the anti-ASGM1 treated mice were remained HBsAg seropositive until the end-point and led to significant higher serum-HBsAg titer in anti-ASGM1 treated mice than isotype-control treated mice **(Figure 1b, f)**. The appearance of anti-HBs antibodies in the serum was delayed in mice treated with anti-ASGM1 comparing to isotype control **(Figure 1c, g)**. Similarly, the immunohistochemistry analysis revealed that the staining for HBcAg remained detectable in the livers of anti-ASGM1-treated BALB/c and C57BL/6 mice on Day 42 post-transfection. However, the staining was much lower in untreated mice **(Figure 1e, h)**. Taken together, our results indicated that anti-ASGM1 treatment significantly impaired the ability of HBV clearance in both BALB/c and C57BL/6 mice, suggesting that some $ASGM1^+$ immune cells contribute in clearing virus.

3.2 NFIL3 KO mice remained immune-competent to eliminate HBV.

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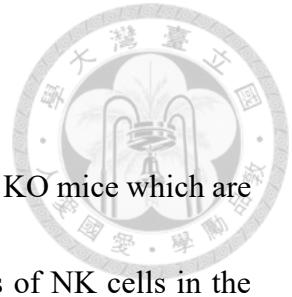

To further investigate the role of NK cells, we employed NFIL3 KO mice which are known to have the deficiency of NK development. The percentages of NK cells in the liver decreased in NFIL3 KO mice comparing to wild-type C57BL/6 mice **(Figure 2a)**. Unexpectedly, the average HBsAg level in the sera of NFIL3 KO mice was comparable to WT mice after HDI with HBV DNA **(Figure 2b)**. There was no significant difference of the ability of producing anti-HBs antibodies **(Figure 2c)**, and the expressing of HBcAg in the liver six weeks after HDI **(Figure 2d)** between NFIL3 KO mice and the WT mice. Our data showed that NFIL3 KO mice were functional competent in HBV clearance, suggesting that NK cells might be not the key effector cells whose depletion causing the impairment of HBV clearance when the mice were treated with anti-ASGM1 antisera.

3.3 The non-NK ASGM1 positive immune cells mediated HBV clearance.

Since anti-ASGM1 treatment significantly abolished the HBV clearance in WT mice, while the loss of conventional NK cells in NFIL3 KO mice had the normal ability to clear virus, it raised a question that is there a cell type which can be depleted by anti-ASGM1 treatment and is crucial in HBV clearance in NFIL3 KO mice? We treated NFIL3 KO mice with anti-ASGM1 or isotype control to see whether anti-ASGM1 treatment impairs

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the ability of HBV clearance in NFIL3 KO mice. As WT mice, the HBsAg level was much higher in anti-ASGM1 treated mice than in isotype control treated mice **(Figure 3a)** after HDI. None of anti-ASGM1 treated mice was found anti-HBsAg antibodies in the serum, while a significantly increasing of the anti-HBs antibodies in the sera after HDI in control mice **(Figure 3b)**. The expressing of HBcAg in the liver were still detectable in anti-ASGM1 treated mice six weeks after HDI **(Figure 3c)**. Our data indicated that there are the non-NK cells which can be depleted by anti-ASGM1 treatment and is critical in HBV clearance.

3.4 The majority of intrahepatic ASGM1 positive immune cells in NFIL3 KO mice were CD8 T cells.

To further analyze the possible cells which can be depleted by anti-ASGM1 treatment and is critical in HBV clearance, we sorted the intrahepatic $ASGM1^+$ cells from WT or NFIL3 KO mice and analyzed the immune phenotype of these cells. The percentage of $NK1.1^+CD3^-$ cells were significantly higher in sorted cells than in total intrahepatic leukocytes from WT mice **(Figure 4a)**, consistent with our knowledge that conventional NK cells expresses large amount of ASGM1. Our data showed that the percentage of $NK1.1^-$ $CD3^+$ cells were significantly higher in sorted cells than in total

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intrahepatic leukocytes as well from both WT mice and NFIL3 KO mice **(Figure 4a, b)**. Furthermore, the percentage of $CD8^+$ cells among $CD3^+$ cells were significantly higher in sorted cells than in total cells in both mice **(Figure 4c, d)**. In addition, anti-ASGM1 treatment depleted the intrahepatic HBcAg specific CD8 T cells in NFIL3 KO mice **(Figure 5)**. These data pointed out that the cell critical in HBV clearance in NFIL3 KO mice might be CD8 T cells.

3.5 The intrahepatic ASGM1 positive CD8 T cells in HBV carrier mice expressed CD44 and LFA-1.

CD8 T cells were considered as one of the key effector immune cells in HBV clearance. CD8 T depleted chimpanzee and CD8 KO mice were showed to lost the ability to against HBV (Thimme et al. 2003). The inactivation of $PD1^+$ CD8 T cells from HBV carrier mice can be reversed by PD1 blockage treatment (Tzeng, H.T., et al., 2012). Chronic HBV carrier mice regained the ability to clear HBV after treated with anti-PD1 antibodies. However, only partial of CD8 T cells express ASGM1. It raised a question that are the CD8 T cells which express ASGM1 and are critical in HBV clearance a sub-population of CD8 T cells. Therefore, we tested a panel of phenotypic markers of CD8 T cells from naïve NFIL3 KO mice. We found that among intrahepatic $ASGM1^+$ CD8 T

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cells, most of them expressed CD44 and LFA-1 **(Figure 6, 7a)** instead of naïve markers CD62L and CD127. The expression of other phenotypic markers (such as CD25, CD103, CD107, CD137, ICOS, RANKL and Tim-3 as shown in **Figure 6**) were low or absent. Among these markers, the expression patterns of LFA-1 and CXCR3 were significantly different between $ASGM1^{+}$ and $ASGM1^{-}$ CD8 T cells **(Figure 6b)**. Meanwhile, we noticed that almost all $ASGM1^{+}$ CD8 T cells expressed LFA-1 and can be divided into two group, $LFA\text{-}1^{int}$ and $LFA\text{-}1^{hi}$. Regarding $LFA\text{-}1^{hi}$ were reported to be one of the markers of a distinct liver resident CD8 T cells which are CD69 positive as well, we analyzed the expression of CD69 and LFA-1 among ASGM1 positive or negative CD8 T cells.

3.6 The intrahepatic ASGM1, CD44 and LFA-1 triple positive CD8 T cells were a distinct sub-population.

Figure 7a demonstrate that among intrahepatic ASGM1-positive CD8 T cells, most of them expressed CD44, CD69, and LFA-1. By comparing the CD44, LFA-1 and ASGM1 expression patterns of CD8 T cells, we found that the intersection occupied almost all the LFA-1 set and the majority of ASGM1 set, implicating that this triple-positive CD8 T cells belonged to a distinct sub-population **(Figure 7b)**. To further verify that ASGM1, CD44 and LFA-1 triple positive CD8 T cells is a distinct sub-population,

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One day after injecting anti-ASGM1 antibodies, the $CD44^{+}LFA^{hi}$ CD8 T cells of both WT or NFIL3 KO mice were depleted **(Figure 7c)**. Collectively, our data showed that ASGM1, CD44 and LFA-1 triple positive CD8 T cells was not only a distinct sub-population, but occupied most of the phenotypically liver resident CD8 T cells.

3.7 Intrahepatic ASGM1+ CD8 T cells had distinct transcriptional profile from ASGM1-CD8 T cells and showed similarity to core gene signature of Trm cells.

We next sought to determine whether differences between $ASGM1^{+}$ and $ASGM1^{-}$ CD8 T cells in not only the phenotypic marker, but also the transcriptional signature. Both cells were respectively sorted for gene expression microarray analysis. 1,129 expressed genes showed a fold change of 2 or greater **(Figure 8a)**, indicated that $ASGM1^{+}$ CD8 T cells were distinct from $ASGM1^{-}$ ones in transcriptional level. The differentially expressed genes included those encoding chemokine signaling pathway, T cell receptor signaling pathway, integrin-mediated cell adhesion, focal adhesion and XPodNet protein-protein interactions **(Figure 8b)**. This raised the question that $ASGM1^{+}$ CD8 T cells might have unique adhesive behavers in the liver. We found that many genes represented in the core gene signature of liver Trm cells (Mackay LK et al. 2016, Fernandez-Ruiz et al. 2016) were similarly up- or down-regulated in liver $ASGM1^{+}$ CD8 T cells **(Figure 8c)**.

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We confirmed several of these findings by qPCR **(Figure 8d)**, suggesting that these cells are liver resident cells.

3.8 Intrahepatic ASGM1+ CD8 T cells homed to and persisted in the liver after transplantation.

Recent evidence indicates that large numbers of resident CD8 T cells are harbored within liver for protection against pathogen challenges (Keating R, et al. 2007). Nevertheless, whether liver resident CD8 T cells contribute the major role among total CD8 T cells in host protection against HBV is still unclear. Our results indicate that the major intrahepatic ASGM1-positive immune cells in NFIL3-KO mice highly co-expressed LFA-1, which is recently reported as a phenotypic marker of liver-resident T cells (Beura et al. 2018; McNamara et al. 2017). To further address the liver resident role of intrahepatic ASGM1^{+} CD8 T cells, we compared the expression of LFA-1 between intrahepatic and splenic CD8 T cells first. As showed in **Figure 9a**, LFA-1 was highly expressed by intrahepatic ASGM1^{+} CD8 T cells but not by splenic T cells. Moreover, only ASGM1^{+} CD8 cells sorted from liver but not spleen homed to the livers of recipients after adoptive transferring, and these liver-homing donor cells persisted for more than two weeks **(Figure 9b, c).** Besides, CD69^{+}LFA-1^{hi} CD8 T cells with the liver-resident

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phenotype were also depleted after anti-ASGM1 Ab treatment (**Figure 9 d)**. Collectively, ASGM1-positive CD8 T cells not only phenotypically but also functionally showed liver residency.

3.9 Blocking of ASGPR1 dampened the liver homing of ASGM1$^+$ CD8 T cells.

The liver resident CD8 T cells express high level of ASGM1. Meanwhile, NK cells, which are known to express high level ASGM1, are especially enriched in the liver. Those implied that the expression of ASGM1 might have role in adhesion and there might be some ASGM1 receptors enriched in the liver. Trm of intestinal intraepithelial leukocytes (IEL) are known to express CD103. Neither CD103 positive nor negative IEL expressed certain ASGM1 in naive mice, indicating that the expression of ASGM1 was not universal to the tissue resident CD8 T cells in different organs.

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Chapter 4 Discussion

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4.1 Contribution of this work.

We showed that HBV clearance of mice was abolished by α-ASGM1 treatment on both BALB/c, C57BL/6 and NFIL3 KO mice, while NFIL3 KO mice without α-ASGM1 treatment were immune competent against HBV as wild type mice. This result suggested there is a non-cNK ASGM1^{+} cell critical in eliminating HBV. We further characterize a distinct CD44^{+}LFA^{hi} CD8 T cells highly expressing ASGM1 among intrahepatic leukocytes and can be depleted by α-ASGM1 treatment. Next, we compare the gene expression profile of this distinct subset of CD8 T cells with core gene signature of TRM, and employed adoptively transferring to explore that intrahepatic ASGM1^{+} CD8 T cells were phenotypically and functionally liver-resident. Finally, we test the possibility that ASGPR might be the hepatic receptor of ASGM1^{+} cells with ASGPR1 blocking antibody.

4.2 Deficient of conventional NK cells is not enough to impair the ability of HBV clearance.

A study consisted with our data to demonstrate that anti-ASGM1 treatment abolished the HBV elimination. However, they further showed that the ability of HBV clearance was impaired in NFIL3 KO mice (Zheng et al., 2016). We notice that the kinetic of the serum HBsAg in their NFIL3 KO mice work was different from the anti-ASGM1 one. Same as our work that HBV clearance looks more like totally abolished by anti-

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ASGM1 treatment. Nevertheless, HBV clearance in NFIL3 KO mice looks slightly different from wild type mice. Our results didn't rule out the possibility that NK cells can help to enhance the quantity of HBV-specific CD8 T cells. Instead, we strengthen the important role of CD8 T cells since the depletion the liver-resident CD8 T led to the abolish of HBV clearance in NFIL3 KO mice.

4.3 The role of distinct CD8 T subset in the immune response needs to be explore.

Current antiviral therapies such as reverse transcriptase inhibitors-nucleotide analogue suppress viral load but fail to achieve HBV cure. Combination of antiviral and immune modulatory therapies is thought to cure hepatitis B virus infection. There were several clinical trials of immune checkpoint blockade about activating HBV specific T cells. However, the safety and efficacy of anti-PD-1 therapy for HBV clearance remain concerned (Johnson et al. 2016; El-Khoueiry et al 2017; Gane et al. 2019). New approaches are needed to help HBV specific T cells to eliminate HBV.

Anti-ASGM1–mediated NK cell depletion was thought to be a powerful tool to analyze in vivo functions of NK cells, nevertheless, the expression of ASGM1 is found on not only NK cells, but a subpopulation of NKT, CD8 T and other hematopoietic cells under certain experimental conditions (Nishikado et al. 2011). It has been documented

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that about 10%~30% naïve CD8 T cells (Kosaka et al. 2007) expressed ASGM1, but from where these T cells come and what is the biological role of them are remain undetermined. It has been also documented that most of the LCMV-specific CD8 T cells expressed ASGM1 (Slifka et al. 2000), while what these cells are and whether they are functionally different from ASGM1 negative T cells remain unknowns as well. Herein we provided the first evidence that ASGM1 is expressed by a distinct effector CD8 T cells expressing CD44 and LFA-1. It has been documented that activated T cells expressing specific activation profile have their own distinct character (Wei et al. 2017). The authors defined five distinct MC38 tumor-infiltrating T cell clusters phenotypically, only two of them were CD44, CD69 double positive. Different clusters showed unique immune response under the treatment of immune-checkpoint blockade, implied that CD44 and CD69 might not merely represent the activation of T cell. Soon after activation, T cells expressed CD44 which binds to hyaluronan (HA) for T cells rolling and extravasation. Though that CD44 was considered a memory marker as well, it has been documented that the engagement of CD44 reduces the formation of memory CD8 T cells (Lee-Sayer et al. 2018). The role of CD69, a C-type lectin, have remained elusive for a long time though that it is rapidly expressed after T cell activation.

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4.4 TRM or liver resident CD8 T will be the potential target of immune therapy.

TRM cells are thought to reside in peripheral tissues and is crucial for protective immunity in peripheral tissue. TRM is a functional term raised from the study of CD8 T cells from infected skins. Conventional TRM highly expressed CD44, CD49a, CD103, and CD69. The former three binds Hyaluronic acid, type IV collagen and E-cadherin, respectively. The last inhibits S1PR1 and further stop the egress of the cells to blood. Those molecules coordinately help cells to reside in peripheral tissues. However, accumulating evidences pointed out that liver-resident CD8 T cells are atypical TRM since they don't express CD49a and CD103 (Fernandez-Ruiz et al. 2016; McNamara et al. 2017). Accordingly, the study employed CD69, CD103 double positive markers as the premise of human liver TRM without other validations became no significance (Pallett et al. 2017). Thus, other molecule must take place to provide enough power for residency and be considered as a reliable liver-resident marker..

it has been demonstrated that effector CD8 T cells which arrested within liver sinusoids contributed to recognize hepatocellular antigens in HBV mouse model (Guidotti et al. 2015). These mouse liver-resident T cells were reported recently to express CD69 and highly express LFA-1, which binds ICAM-1/4 (McNamara et al. 2017). However, LFA-1 is expressed by all activated immune cells. Technically scientists cannot

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study the role of liver TRM by depleting with anti-LFA-1 antibodies or LFA-1 knockout mice. The CD69 expressing liver-resident CD8 T cells can be found in naïve mice (Fernandez-Ruiz et al. 2016; Holz et al 2018), indicated that the so-called TRM might be not really a subset of memory cells but the CD8 T cells with liver-resident functions. Our data showed that the intrahepatic $CD69^{+}LFA\text{-}1^{hi}$ CD8 T cells were depleted by anti-ASGM1 treatment, suggesting that this treatment led to the abolish of HBV clearance due to the depletion of liver resident CD8 T cells. It suggests that ASGM1 is a marker of liver-resident CD8 T cells. Whether ASGM1 helps CD8 T cells to stay in the liver through ASGPR will be further study in the future. Furthermore, we found that there are ASGM1 positive CD8 T cells in human livers (**Figure 12**), indicating that ASGM1 as the liver resident marker is not only in mice but also human-being.

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Figures

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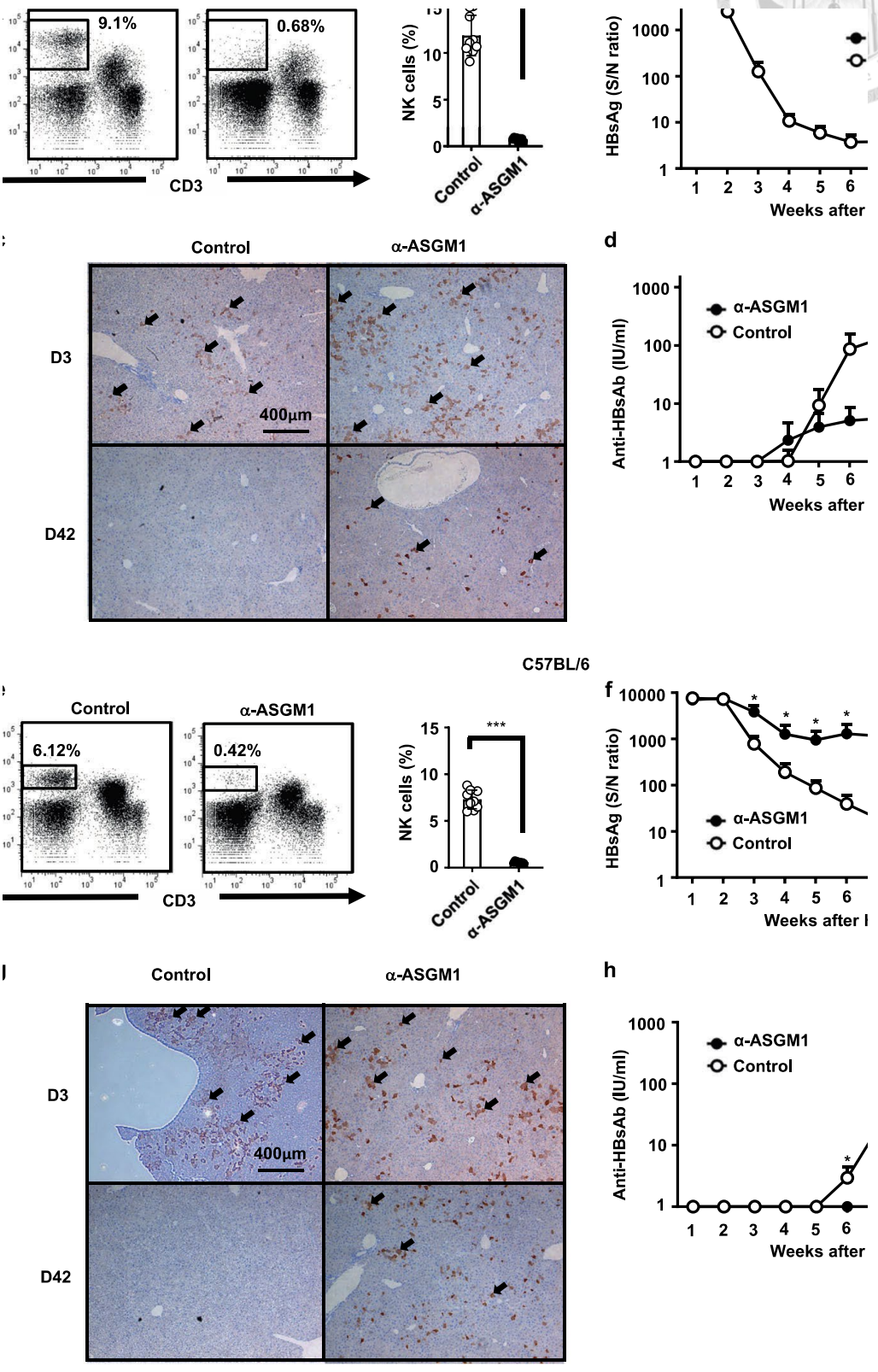

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Figure 1. α-ASGM1 treatment abolished HBV viral clearance. (a) 6~7 weeks old BALB/c mice were treated with anti-ASGM1 antisera or isotype control one day before sacrificed. Intrahepatic leukocytes were isolated and the NK cell frequency were analyzed by flow cytometry. BALB/c mice were hydrodynamically injected(HDI) with pAAV/HBV1.2 plasmids in the presence of isotype control or anti-ASGM1 treatment. Anti-ASGM1 treatment was performed twice a week over the detection period. Serum titer of HBsAg(b) and anti-HBsAb(d) in the indicated time were determined by an ELISA. Immunohistochemical staining for expressing HBcAg in the livers of anti-ASGM1-treated BALB/c mice compared to control mice on Days 3 and 42 after pAAV/HBV1.2 injection(c). (e) Intrahepatic leukocytes from C57BL/6 mice treated with anti-ASGM1 antisera or isotype control were isolated and the NK cell frequency were analyzed by flow cytometry. C57BL/6 mice were HDI with pAAV/HBV1.2 plasmids in the presence of isotype control or anti-ASGM1 treatment. Anti-ASGM1 treatment was performed twice a week over the detection period. Serum titer of HBsAg(f) and anti-HBsAb(h) in the indicated time. Immunohistochemical staining for expressing HBcAg in the livers on Days 3 and 42 after pAAV/HBV1.2 injection (g). Data represent at least two independent experiments with each group comprising ten mice and are shown as the mean ± standard deviation (SD). B-D, F, G, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between selected relevant comparisons by Student's t-test.

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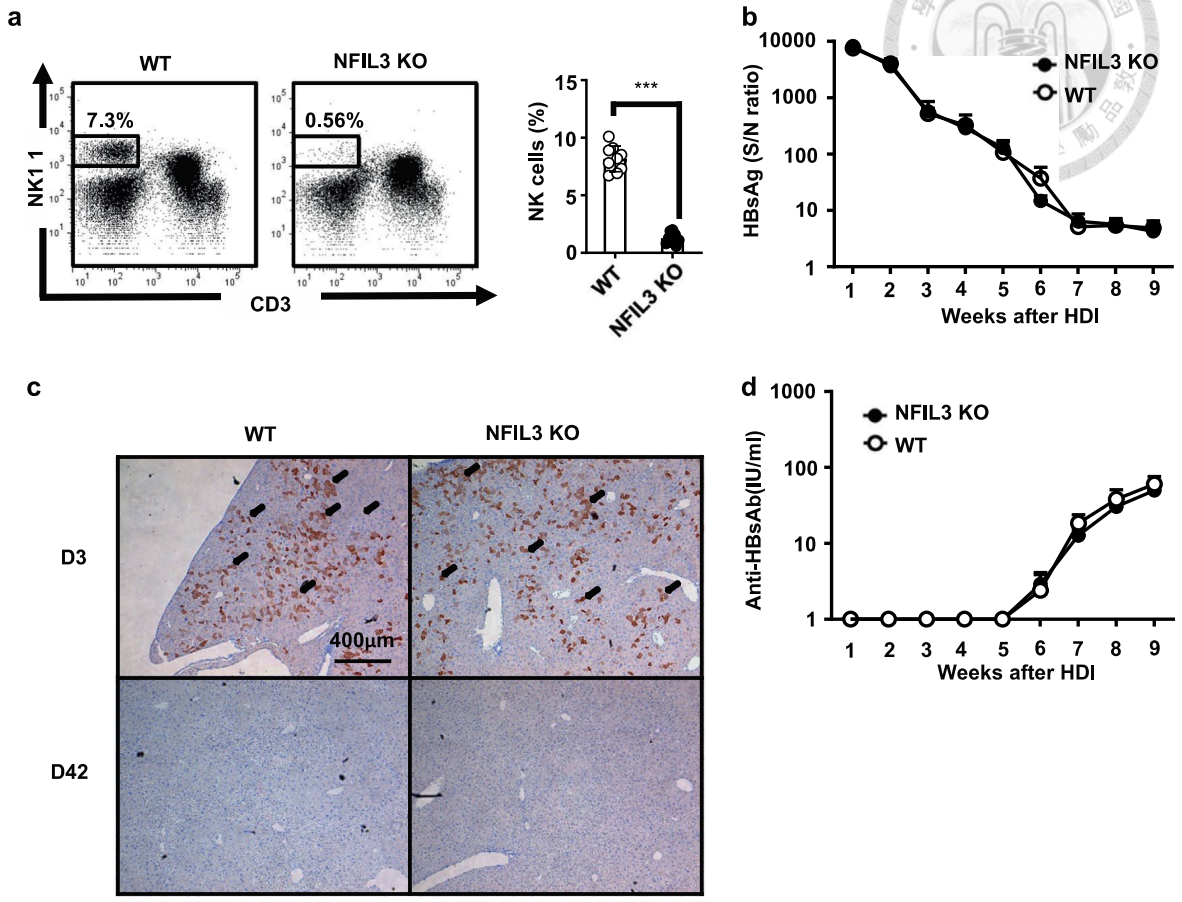

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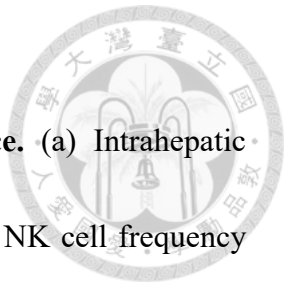

Figure 2. Conventional NK is not essential to HBV clearance. (a) Intrahepatic leukocytes of C57BL/6 and NFIL3 KO mice were isolated and the NK cell frequency were analyzed by flow cytometry. 6~7 weeks old C57BL/6 and NFIL3 KO mice were HDI with pAAV/HBV1.2 plasmids. Serum titer of HBsAg(b) and anti-HBsAb(d) in the indicated time after HDI were determined by an ELISA. IHC staining for expressing HBcAg in the livers of α-ASGM1-treated and control mice on Days 3 and 42 after HDI (d). Data represent at least two independent experiments with each group comprising eight mice and are shown as the mean ± SD. Statistics were calculated by non-parametric two-tailed Mann-Whitney U Test. WT, wild-type mice; NFIL3 KO, NFIL3 knockout mice; HBsAg, HBV surface antigen; anti-HBsAb, anti- HBV surface antigen antibody.

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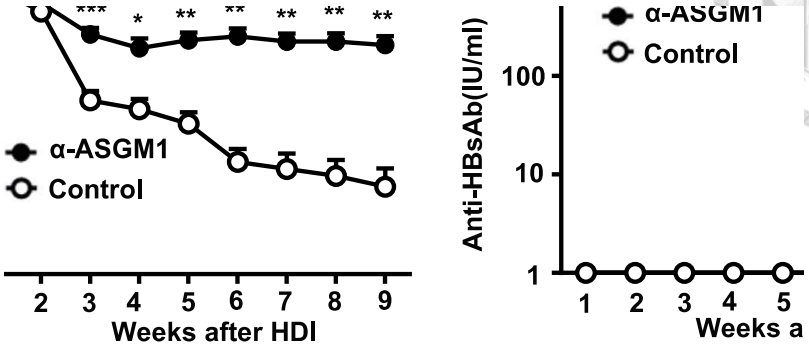

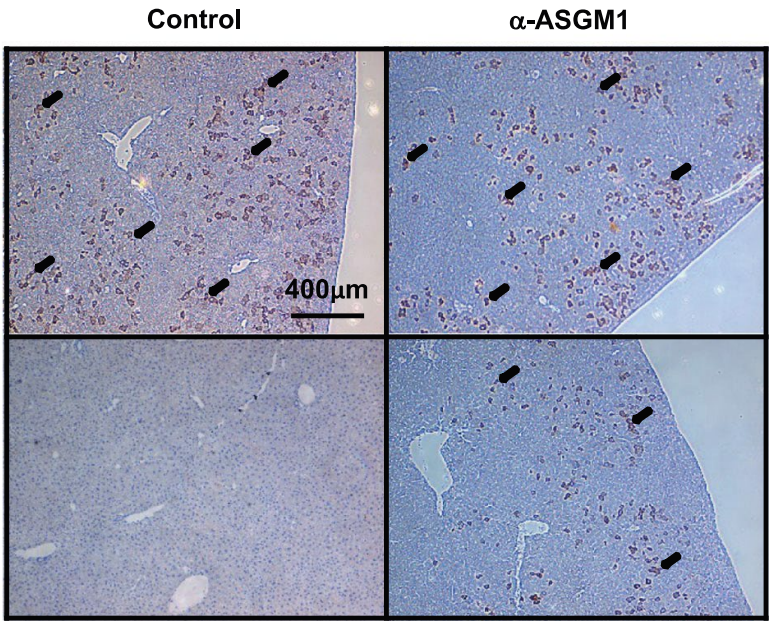

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Figure 3. Anti-ASGM1 treatment impaired the ability of HBV clearance in NFIL3 KO mice. 6~7 weeks old NFIL3 KO mice were HDI with pAAV/HBV1.2 plasmids in the presence of isotype control or anti-ASGM1 treatment. Anti-ASGM1 treatment was performed twice a week over the detection period. Serum titer of HBsAg(a) and anti-HBsAb (b) the indicated time were determined by an ELISA. IHC staining for expressing HBcAg in the livers of NFIL3 KO mice in the presence of anti-ASGM1 treatment on Days 3 and 42 after HDI were shown (c). Data represent at least two independent experiments with each group comprising eight to ten mice and are shown as the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between selected relevant comparisons by Student's t-test.

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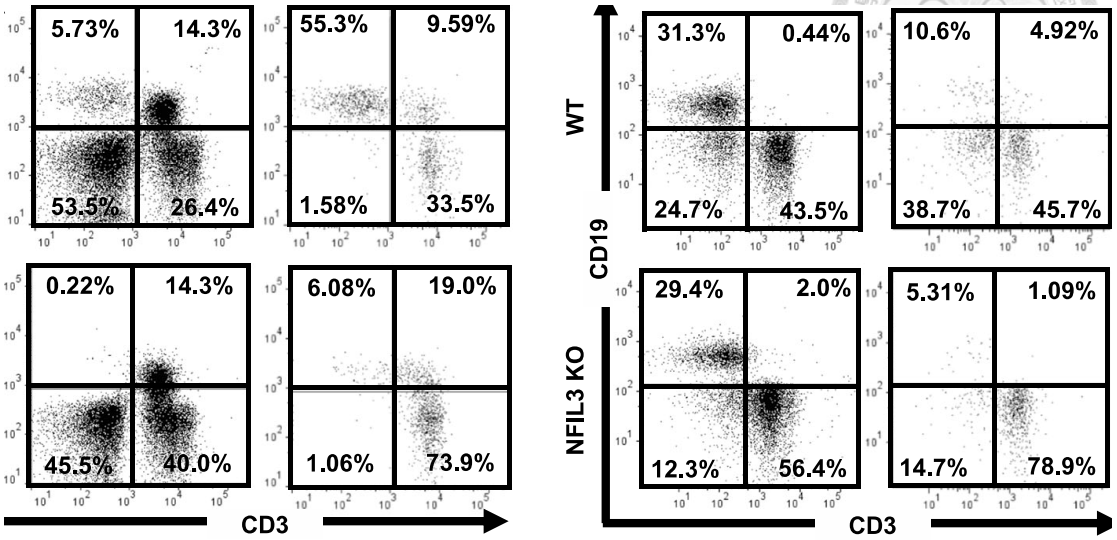

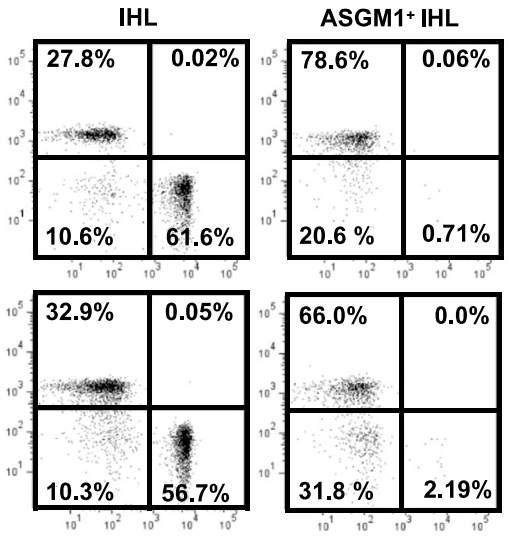

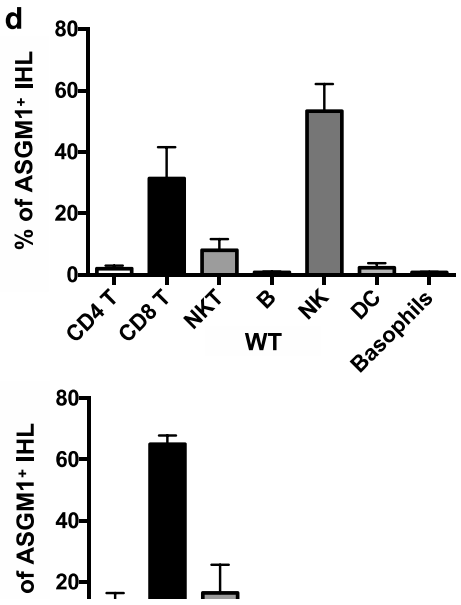

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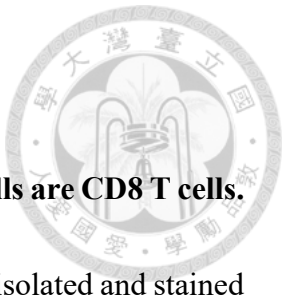

Figure 4. In NFIL3 KO mice, most of the intrahepatic ASGM1$^+$ cells are CD8 T cells.

Intrahepatic leukocytes (IHL) of C57BL/6 and NFIL3 KO mice were isolated and stained with anti-ASGM1 antibodies. ASGM1$^+$ cells were sorted. The frequency of NK cell(a), B and T cells(b), and CD4 and CD8 T cells (c, gated on NK1.1$^-$CD3$^+$) were analyzed by flow cytometry. (d) Quantification of the percentages of indicated cells in the liver from WT and NFIL3 KO mice was shown.

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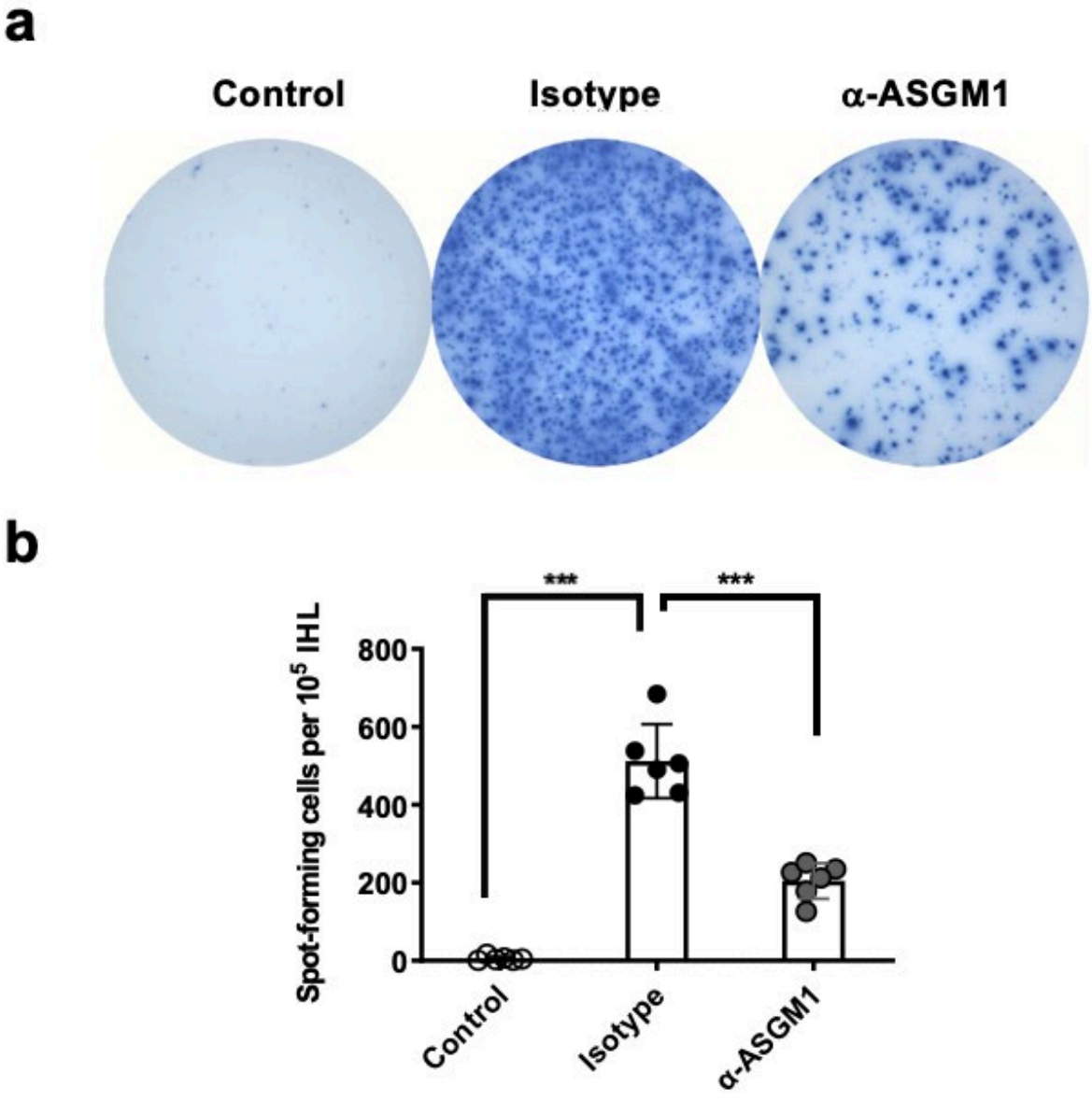

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Figure 5. In NFIL3 KO mice, α-ASGM1 treatment led to decrease of HBcAg specific CD8 T cells. (a) IHL were isolated from naïve (Control) and HBV transfected NFIL3 KO mice treated with isotype (Isotype) or anti-ASGM1 (a-ASGM1) for 24 hours on day 10 after hydrodynamic injection with HBV DNA. CD8 T cells of each group from HBV transfected mice were enriched by magnetic beads and mixed with naïve IHL in a ratio of 1:7. Cells were restimulated with $HBcAg_{18\text{-}27}$ and $HBcAg_{93\text{-}100}$ peptides for 18 hours. Peptide-activated interferon gamma (IFN-γ) responses were analyzed by a ELISPOT assay. (b) The frequency of peptide-activated IFN-γ secretion was quantified as spot-forming cells per 1×10^5 cells. Data are representative of at least two experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between selected relevant comparisons by Student's t-test.

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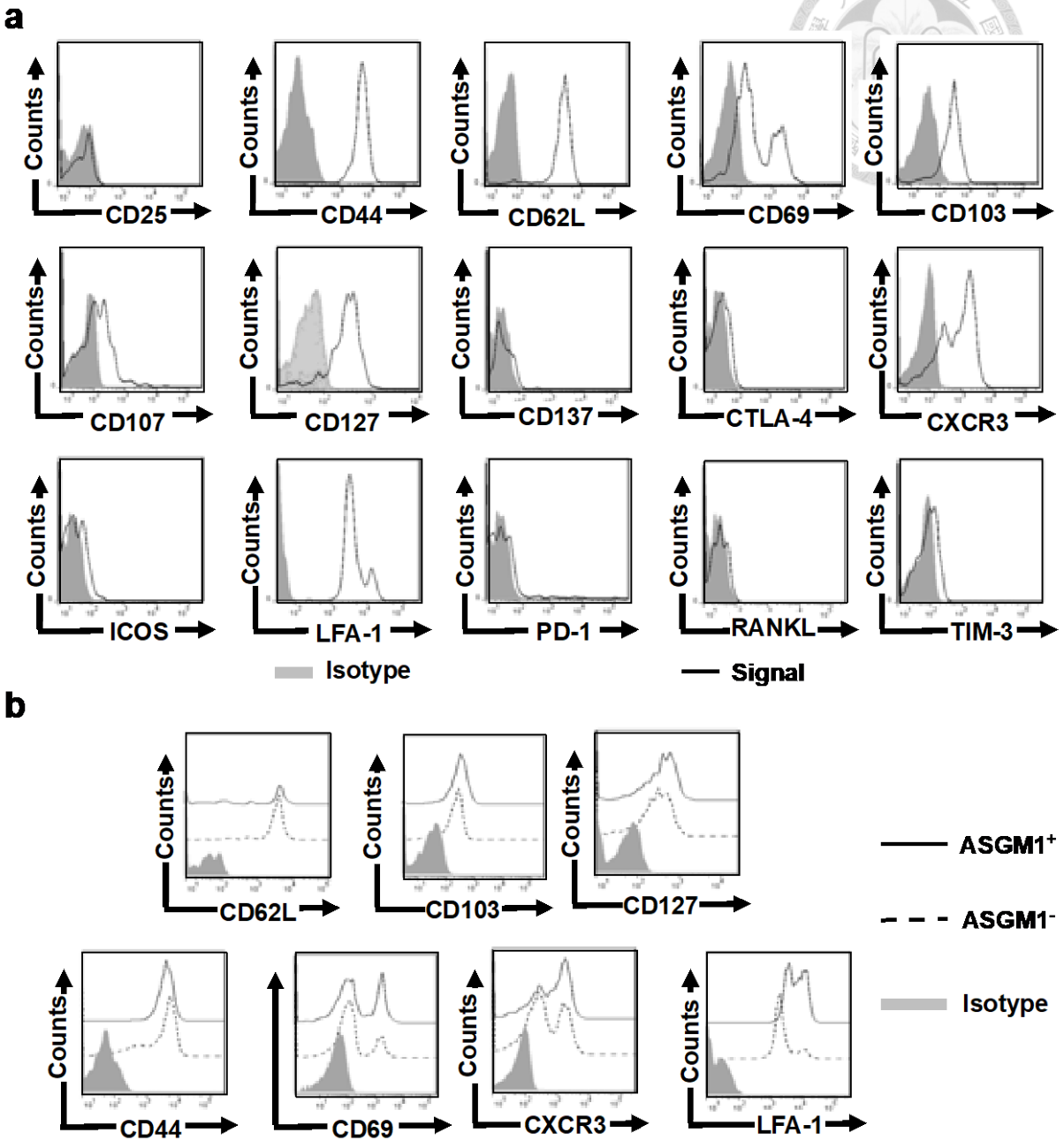

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Figure 6. There were more ASGM1$^+$ than ASGM1$^-$ CD8 T cells expressing CD44, CD69, CXCR3 and LFA-1 but not other phenotypic markers. Intrahepatic leukocytes from naïve NFIL3 KO mice were isolated and (a) a panel of phenotypical markers of ASGM1$^+$ CD8 T lymphocytes were analyzed by flow cytometry. (b) The comparison of CD44, CD62L, CD69, CD103, CD107, CD127, CXCR3 and LFA-1 expression between ASGM1$^-$ and ASGM1$^+$ CD8 T cells were analyzed.

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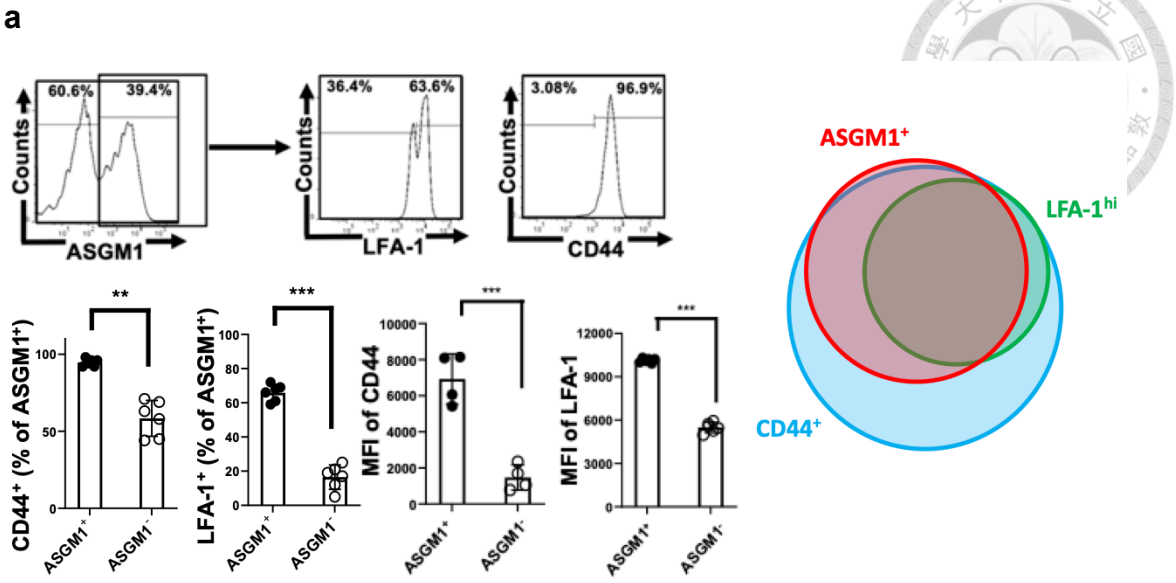

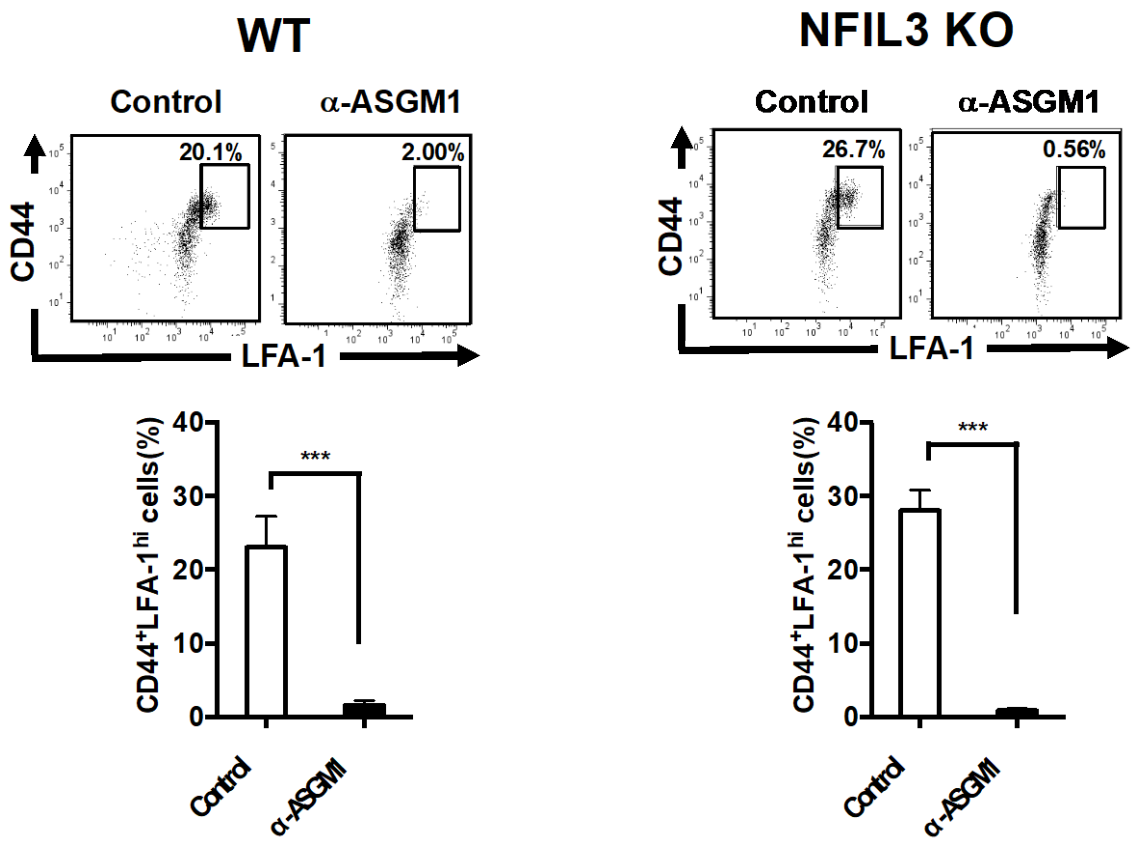

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Figure 7. ASGM1$^+$ CD8 T cells co-expressed CD44 and LFA-1, which was considered as a liver-resident marker. (a) Intrahepatic leukocytes from naïve NFIL3 KO mice were isolated. Among CD8$^+$ T cells, expression of ASGM1, CD44 and LFA-1 was examined. Left-top figure represented the expressing patterns of LFA-1 and ASGM1 among CD44$^+$ CD8 T cells. Left-bottom represented the ratio and MFI of CD44$^+$ and LFA-1 among ASGM1$^+$ CD8 T cells. Right figure represents Venn diagram showing the overlap among ASGM1, CD44, LFA-1 positive cells from each group. (b) C57BL/6 and NFIL3 KO mice were injected with 20 μl anti-ASGM1. One day later, IHLs were harvested, stained, and analyzed by flow cytometry. Most of the CD44+LFAhi CD8 T cells in both wild-type and NFIL3 KO mice were depleted after treatment with the anti-ASGM1 Ab. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between selected relevant comparisons by Student's t-test.

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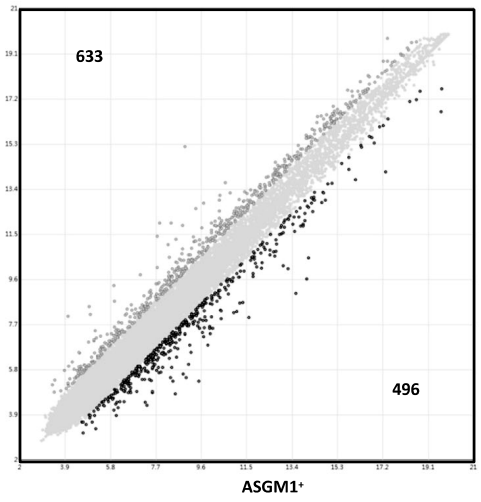

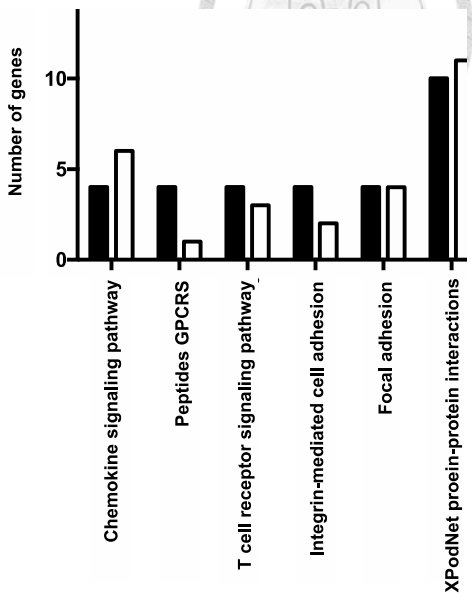

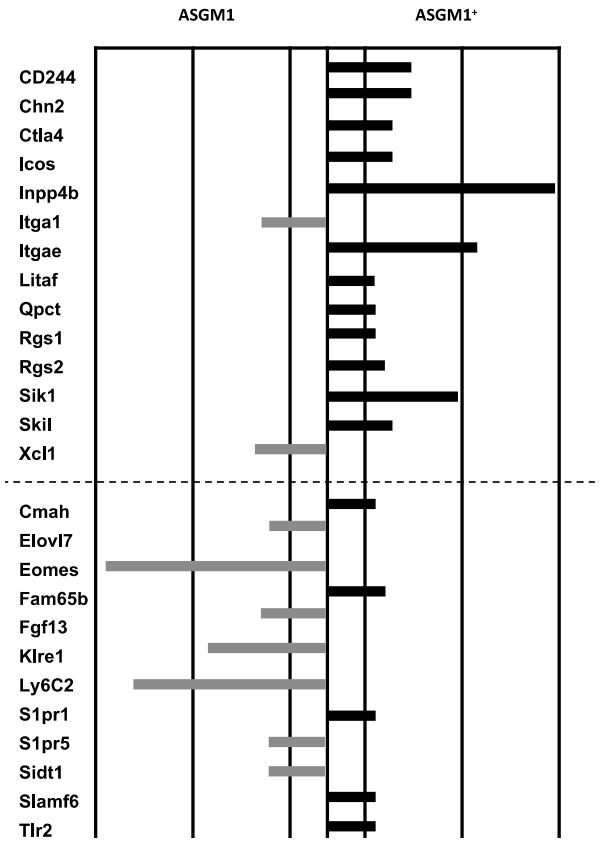

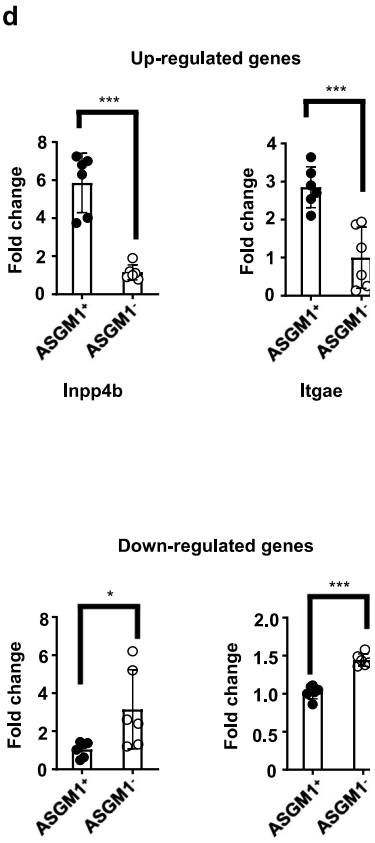

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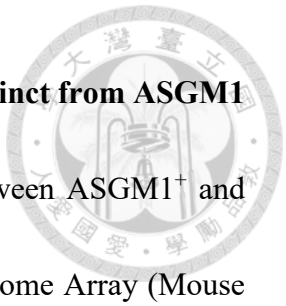

Figure 8. The intrahepatic ASGM1 positive CD8 T cells were distinct from ASGM1 negative CD8 T cells. (a) Comparative transcriptome analysis between ASGM1$^+$ and ASGM1$^-$ liver CD8 T cells was performed by Affymetrix Transcriptome Array (Mouse Clariom D). Differences in gene expression were analyzed for screening for the genes with a fold change > 2. The number of upregulated or downregulated genes is indicated. (b) Distribution by functional category of upregulated genes in intrahepatic ASGM1$^+$ and ASGM1$^-$ liver CD8 T cells. Genes with greater than or equal to 2-fold differences were included. (c) Fold change expression of tissue resident core signature genes in ASGM1$^+$ CD8 T cells relative to ASGM1$^-$ ones. (d) qPCR of genes represented in the core gene signature of liver resident CD8 T cells. Data are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between selected relevant comparisons by Student's t-test.

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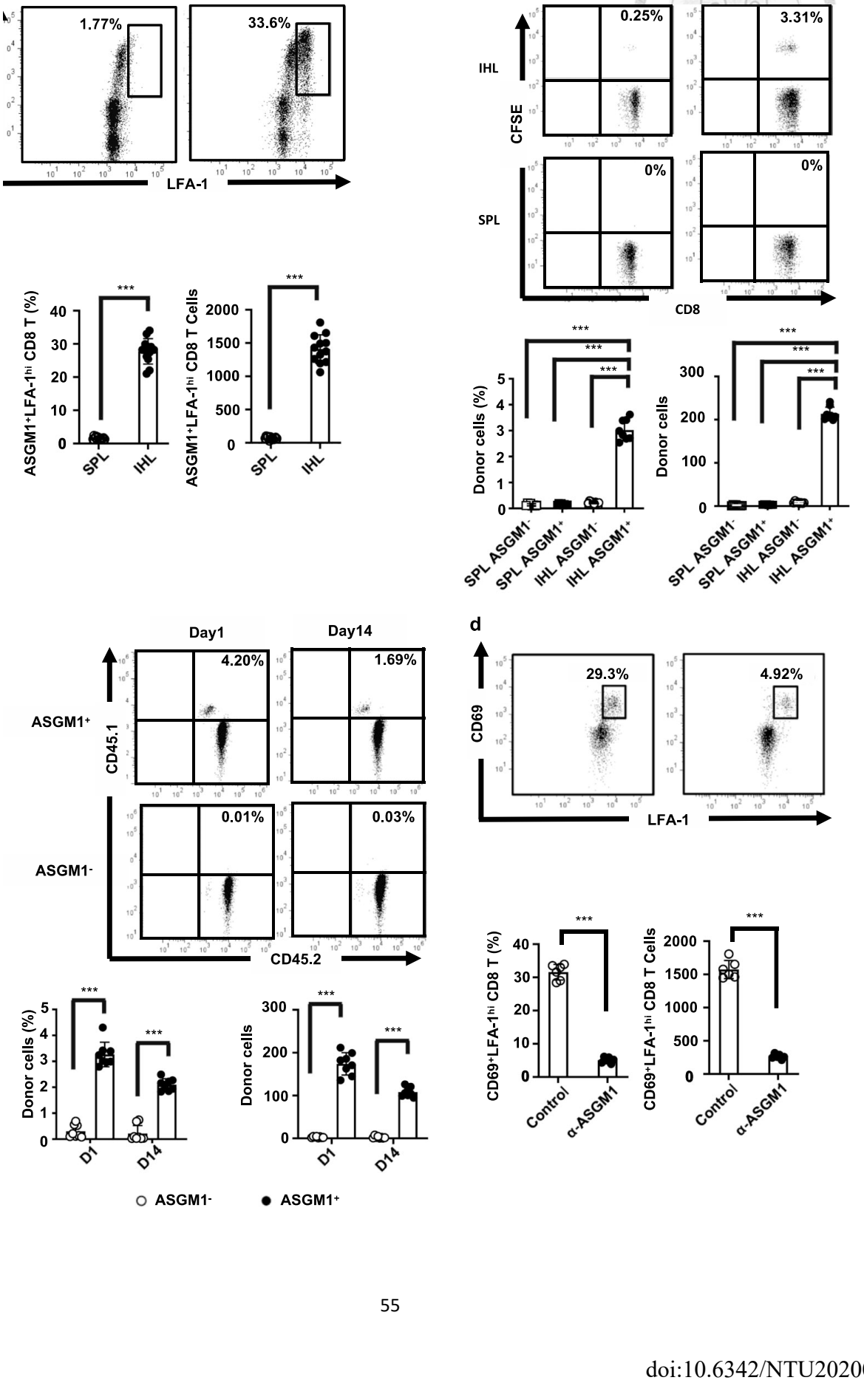

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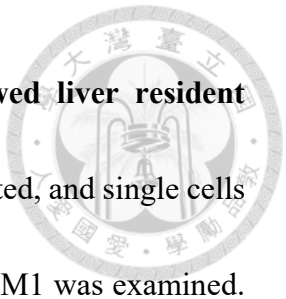

Figure 9 The intrahepatic ASGM1 positive CD8 T cells showed liver resident properties. (a) Spleens and livers from NFIL3-KO mice were harvested, and single cells were stained. Among CD8 T cells, expression of LFA-1 versus ASGM1 was examined. (b) Sorted ASGM1 positive or negative CD8 T cells from livers or spleens were stained with CFSE and adoptively transferred intravenously into C57BL/6 mice. One day later, intrahepatic leukocytes were analyzed were harvested, stained, and analyzed by flow cytometry. (c) Sorted ASGM1 positive or negative CD8 T cells from livers of congenic mice (CD45.1CD45.2) were adoptively transferred intravenously into C57BL/6 mice (CD45.2). One day and two weeks after transfer, intrahepatic leukocytes were analyzed were harvested, stained, and analyzed by flow cytometry. (d) NFIL3 KO mice were injected by 20 μl anti-ASGM1. One day later, livers were harvested and single cells stained. Among $CD8^+$ T cells, expression of LFA-1 versus CD69 was examined. Data are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between selected relevant comparisons by Student's t-test.

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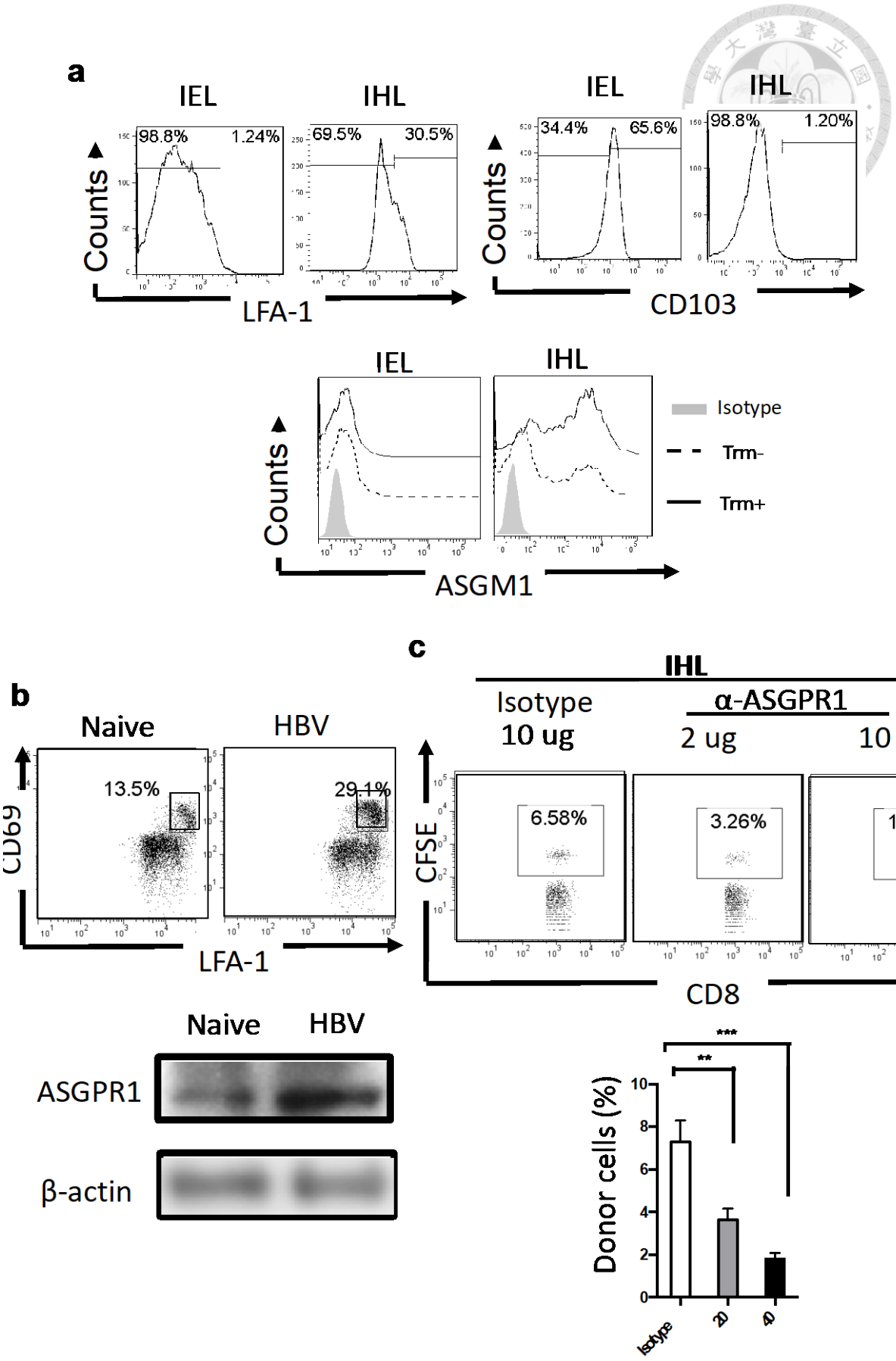

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Figure 10. Blocking of ASGPR1 dampened the liver homing of ASGM1^{+} CD8 T cells.

(a) Intestines and livers from NFIL3-KO mice were harvested, and single cells were stained. Among CD103^{+} or LFA-1^{hi} CD8 T cells, expression of ASGM1 was examined. (b) Livers of C57BL/6 mice HDI with PBS/HBV DNA were harvested. IHL were stained and analyzed by flow cytometry. The expression of ASGPR1 of isolated hepatocyes were analyzed by wester blot. (c) Sorted ASGM1 positive CD8 T cells from livers of congenic mice (CD45.1CD45.2) were adoptively transferred intravenously into C57BL/6 mice (CD45.2). One day and two weeks after transfer, intrahepatic leukocytes were analyzed were harvested, stained, and analyzed by flow cytometry. (d) NFIL3 KO mice were injected by 20 μl anti-ASGM1. One day later, livers were harvested and single cells stained. Among CD8 T cells, expression of LFA-1 versus CD69 was examined. Data are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between selected relevant comparisons by Student's t-test.

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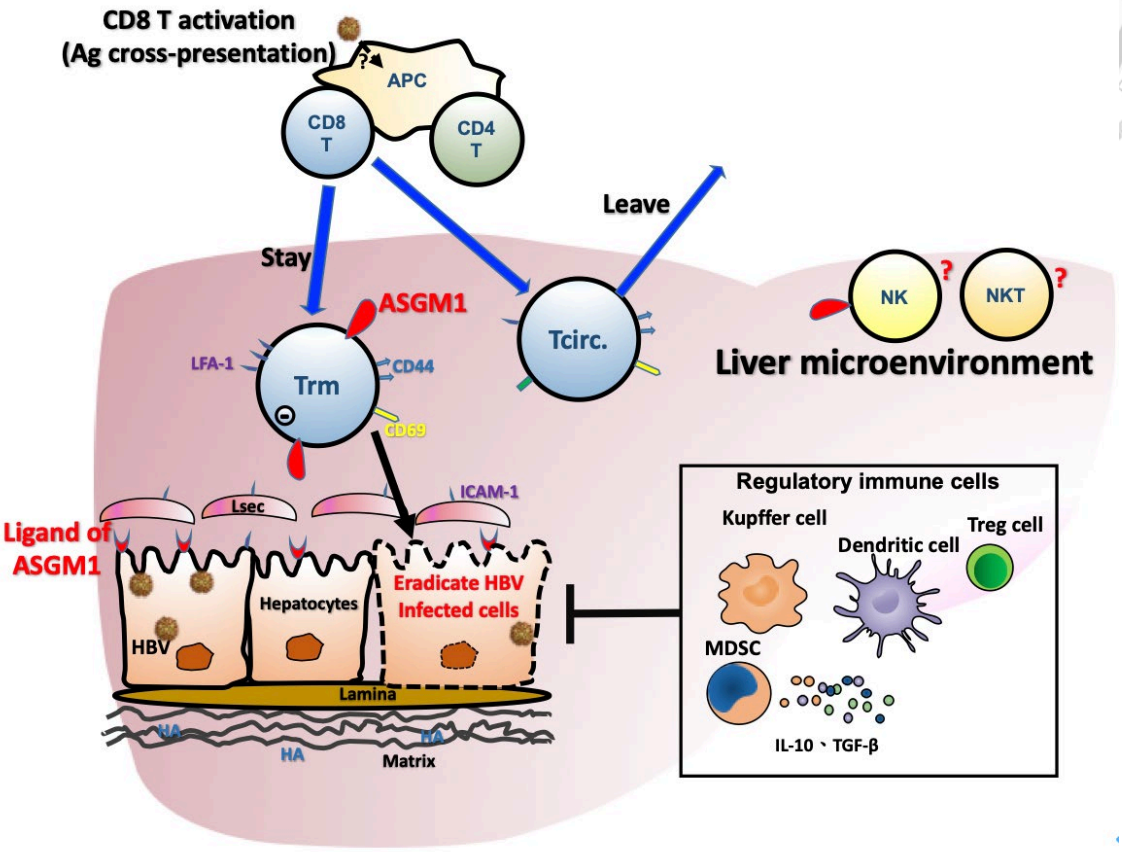

Figure 11 Working model. Unlike the regulatory immune cells such as Kupffer cells, DC, MDSC and Treg cells, ASGM1^{+} CD8 T cells are critical in HBV clearance. Under the help of APC and CD4 T helper cells, effector CD8 T cells expressed several adhesive molecules. Only the effector CD8 T cells gain enough power to reside in the liver will clear HBV infected hepatocytes. APC, antigen presenting cells; Trm, liver resident CD8 T cells; Tcirc, peripheral CD8 T cells; NK, natural killer cells; NKT, natural killer T cells; MDSC, myeloid derived suppresser cells.

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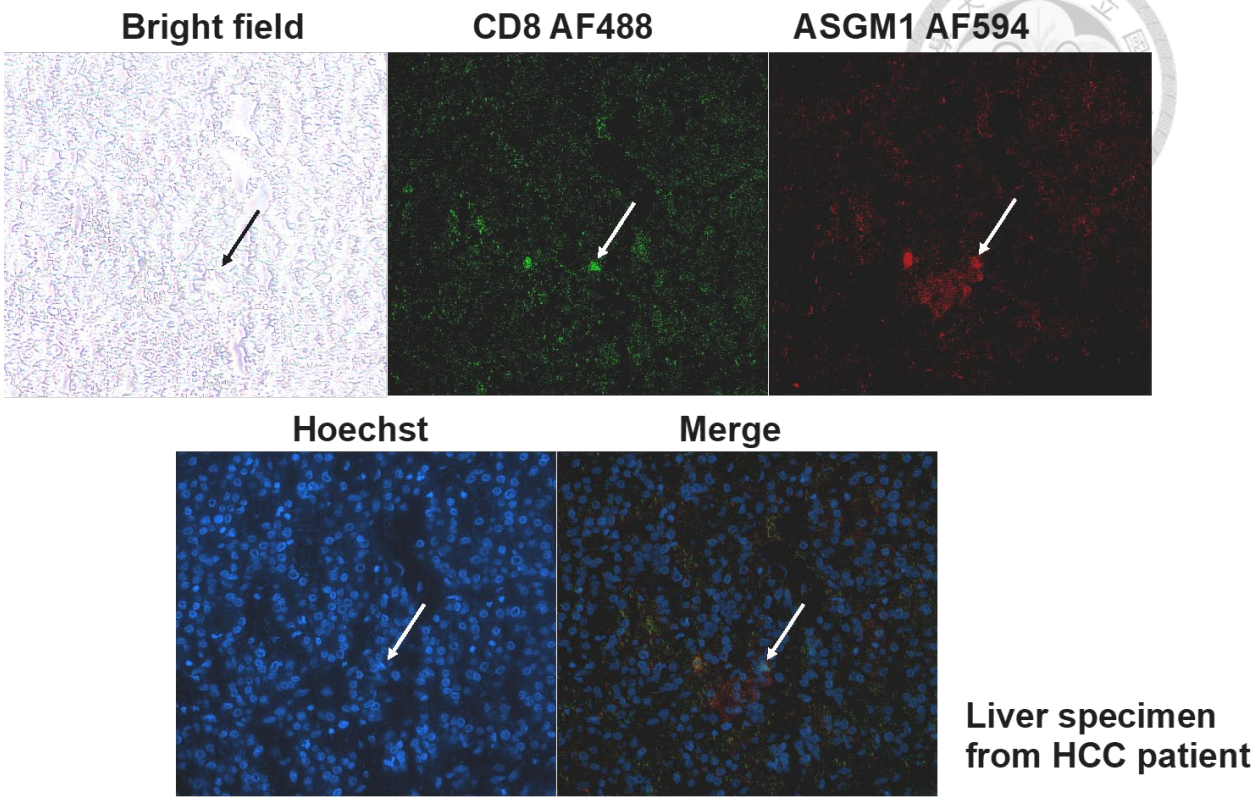

Figure 12 There were ASGM1 positive CD8 T cells in the livers of HCC patients.

HCC liver specimens were kindly provided by Department of Pathology, National Taiwan University Hospital. Slides were rehydrated then stained with mouse anti-CD8 and rabbit anti-ASGM1 antibodies overnight. After one hour secondary antibodies staining, slides were treated with Hoechst. Images were acquired with Nikon Eclipse TE2000-S.

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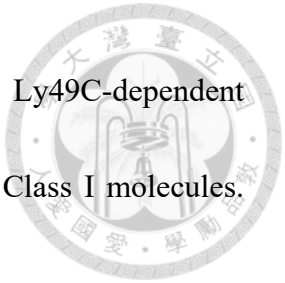

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