The development of novel therapeutic vaccines for chronic Hepatitis B infection

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ABSTRACT

Chronic infection with Hepatitis B virus (HBV) remains a significant health problem, infecting millions of people worldwide. Cirrhosis and hepatocellular carcinoma, the sequalae of longstanding HBV infection, have a high mortality. Current therapy controls HBV replication but does not eradicate the virus, and therefore most people require life-long treatment. A robust adaptive immune response is required to control acute HBV but is dysfunctional in individuals that go onto develop chronic infection. The immune response is an attractive target for novel HBV therapies, which aim to induce functional cure of HBV in finite treatment courses by restoring HBV specific immunity. Therapeutic vaccination, which describes the process of delivering non-infective HBV antigen into the body in order to induce or boost cellular immune responses to HBV, is one strategy under investigation for chronic HBV treatment.

In this thesis I evaluate the novel vaccines ChAdOx1-HBV and MVA-HBV as candidates for HBV therapeutic vaccination. I investigate their immunogenicity in heterologous prime-boost strategies in healthy mice, describing in detail the fine specificity, functionality and phenotype of vaccine induced T cell responses in the blood and the liver. I also describe the safety and immunogenicity ChAdOx1-HBV in a phase-1 open label clinical trial in healthy volunteers and patients with chronic HBV infection. This work is important to inform the development of therapeutic strategies using ChAdOx1-HBV and MVA-HBV in patients with chronic HBV going forward.

THESIS CONTRIBUTION BY OTHERS

Contributions to the experiments or analysis in this thesis by others are as follows:

Chapters 2:

The HBV immunogen used in the ChAdOx1-HBV and MVA-HBV vaccines was designed by Senthil Chinnakannan. Analysis to generate genotype C and genotype D HBV consensus sequences was performed by Azim Ansari and Anna McNaughton. Vaccines for use in mice were made by Senthil Chinnakannan, Oxford Viral Vector Core (Oxford, UK) or Sarah Sebastian at Vaccitech (UK).

Chapter 3:

Claire Hutchings and Tim Donnison assisted with vaccination of mice for experiments presented in Figures 3.2-3.13.

Senthil Chinnakannan assisted me with the ELISA assays for experiments presented in Figures 3.13.

Undergraduate student Theo Cooke assisted me with the ELISpot assays for the experiments presented in Figure 3.14.

Claire Hutchings and Senthil Chinnakannan performed the vaccination of mice and Senthil Chinnakannan and Tim Donnison performed the ELISpot assays for the experiments presented in Figure 3.32.

Chapter 4:

Undergraduate student Sanjana Narayan assisted me with ELISpot assays and flow cytometry experiments over a 2-month period, contributing to data for Figures 4.2-4.4, Figures 4.6, 4.8-4.13, 4.15-4.20 and 4.21-4.22.

Tim Donnison and Wenqin Li kindly performed the flow cytometry assays of one experiment (of a total of 4), contributing to Figures 4.6, 4.8-4.13, 4.15-4.20 and 4.21-4.22 whilst I was seconded back to the NHS during the first wave of Covid-19.

Chapter 5:

Vaccitech (Ltd) were the study sponsor of HBV001 and provide recruitment and operational oversight, led by Benaka Karanth. At Oxford University Hospitals NHS Foundation Trust, recruitment, vaccination and study visits for HBV001 cohorts 3 and 4 were conducted by Paola Cicconi and Louise Holland. Antony Brown assisted with fresh ELISpot assays for cohorts 1 and 2 of HBV001 at a single timepoint and conducted processing of fresh blood and fresh ELISpot assays for approximately $1/8^{th}$ of the ELISpot assays for cohorts 3 and 4.

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DEDICATION

This thesis is dedicated to my mother Janet.

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ABBREVIATIONS

Ad – Adenovirus

Ab – Antibody

Ag – Antigen

ALT - Alanine aminotransferase

anti-HBc Ab - anti-Hepatitis B core antibody

anti-HBe Ab - anti-Hepatitis B e antibody

anti-HBs Ab - anti-Hepatitis B surface antibody

cccDNA - covalently close circular deoxyribonucleic acid

CD – Cluster of differentiation

ChAd – Chimpanzee adenovirus

CMV - Cytomegalovirus

ConA - Concanavalin A

DMSO - Dimethyl suphoxide

DNA - Deoxyribonucleic acid

EASL - European Association for the Study of the Liver

EDTA - Ethylenediaminetetraacetic acid

FCS - Foetal calf serum

FNA – Fine needle aspirate

GFP - Green fluorescent protein

HBcAg - Hepatitis B core antigen

HBeAg - Hepatitis B e antigen

HBsAg - Hepatitis B surface antigen

HBV - Hepatitis B virus

HCC – Hepatocellular carcinoma

HCV - Hepatitis C virus

HDV - Hepatitis D virus

HIV - Human Immunodeficiency Virus

IFNα - Interferon-alpha

IFNγ - Interferon-gamma

IL - Interleukin

i.m. – intramuscular

IQR – interquartile range

IU – International units

i.v. - intravenous

mL - millilitre

MVA - Modified vaccinia virus Ankara

NaN_3 - sodium azide

ORF - Open reading frame

PBS - Phosphate buffered saline

PegIFNα - pegylated interferon-alpha

PBMC - Peripheral blood mononuclear cells

PD-1 - Programmed cell death protein 1

pfu - Plaque forming units

pgRNA - Pre-genomic ribonucleic acid

PMA - Phorbol myristate acetate

rcDNA – relaxed circular deoxyribonucleic acid

RNA - Ribonucleic acid

SFU - Spot forming units

T_{CM} - Central memory T cells

T_{EM} - Effector memory T cells

T_{RM} - Resident memory T cells

TLR - Toll-like receptor

TNFα - Tumour necrosis factor- alpha

μm – Micrometre

vp - Viral particles

PUBLICATIONS RELATING TO THIS THESIS

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Chinnakannan S*, **Cargill T*** et al. The Design and Development of a Multi-HBV Antigen Encoded in Chimpanzee Adenoviral and Modified Vaccinia Ankara Viral Vectors, A Novel Therapeutic Vaccine Strategy against HBV. (2020) Vaccines 8(2). doi10.3390/vaccines8020184. * Authors contributed equally

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CHAPTER 1: Introduction

1.1 THERAPEUTIC VACCINE DEVELOPMENT FOR HEPATITIS B IN THE CONTEXT OF THE GLOBAL BURDEN OF VIRAL HEPATITIS

Chronic infection with hepatitis B (HBV) is a serious public health issue with an estimated prevalence of 257 million people globally in 2015 (1). Viral hepatitis is responsible for in excess of 1 million deaths a year, comparable to mortality rates of other important communicable diseases such as tuberculosis and Human Immunodeficiency Virus (HIV) (1,2). Together Hepatitis B and C account for the majority of viral hepatitis related deaths, the burden of which is disproportionately felt in the in the Asian region where over 70% of deaths occur (2).

The United Nations Agenda for Sustainable Development, identifies hepatitis B elimination by 2030 as a key target towards Development Goal 3, 'achieving healthy lives for all' (3). The World Health Organisation (WHO) Viral Hepatitis Strategy set out a blueprint towards achieving this, outlining a multipronged approach to prevent new infections through widespread prophylactic vaccination and identification and treatment of existing cases (4). Achieving such a goal will rely on economic and political will and significant investment from major stakeholders (5). Improvements have been reported in some regions (6), however, disruption caused by the COVID-19 pandemic threatens to negatively impact on progress making it all the more important to renew efforts into research and programmes to tackle HBV (7).

Widespread access to treatment is an important strand of strategies to eradicate HBV (4). Current antiviral therapy suppresses HBV replication but does not eliminate the virus and

lifelong therapy is necessary in the majority (8). This poses significant long-term costs and necessitates the development of systems to facilitate undisrupted access to medication, both of which make the realisation of therapy for all who need it more difficult to achieve (2). The development of novel therapies that control HBV in finite courses are a priority for research (9–11). On an individual level, such therapies should offer the chance of long-term viral control and reduce the risk of liver cirrhosis and hepatocellular carcinoma after just a short course of treatment (9). On a population level, finite treatment courses might reduce overall cost and improve treatment availability, facilitating more widespread access to therapy.

It is with this backdrop that therapeutic vaccination as a potential novel treatment strategy for chronic HBV is being developed (12). Therapeutic vaccination describes the process of delivering antigen in a non-infective form in order to induce new or boost existing immune responses towards HBV infection, the ultimate goal being long term immune control of HBV after a course of vaccination (12,13). HBV specific T cell immunity is robust in individuals that clear acute HBV infection but in those that go onto develop chronic infection the immune response is dysfunctional (14). A small proportion of chronically infected individuals go onto control HBV infection either spontaneously or after treatment with peginterferon or nucleos(t)ide therapy (15,16). In such persons, HBV specific T cell immune responses are restored and have comparable functionality to T cells in individuals that clear acute HBV infection (17). This demonstrates that T cell dysfunction in chronic HBV infection is not irreversible and restoring T cell immunity is a laudable goal of HBV therapy. Therapeutic vaccination is one strategy under development for HBV treatment, aiming to restore HBV specific immune responses (12–14).

In this thesis I describe the immunogenicity of novel HBV therapeutic vaccine candidates ChAdOx1-HBV and MVA-HBV, used in heterologous prime-boost strategies in mice (chapter 3). I explore different vaccination strategies to maximise vaccine responses in the liver (chapter 4), where the local cellular immune response is key to the immunological control of HBV (18). Finally, I report the findings form the first in-human study of ChAdOx1-HBV in both healthy participants and in individuals with chronic HBV infection (chapter 5) and outline how these results will inform future development of the vaccines.

1.2 HEPATITIS B VIROLOGY

1.2.1 Genome organisation and virus structure

Hepatitis B virus is a partially double stranded DNA virus belonging to the *Hepadnaviridae* family, with a relatively small genome of 3.2 kilobase pairs (reviewed in (19)). Four overlapping open reading frames (ORF) encode the major viral proteins, which form the infective virion or Dane particle (Figure 1.1, (20,21)). In this form the viral genome exists in a relaxed circular conformation with a complete negative strand and incomplete positive strand. Covalently attached to the genome is the viral DNA polymerase, encoded by the polymerase ORF (22). Surrounding this is the nucleocapsid, formed of multiple hepatitis B core protein (HBc) subunits encoded by the pre-core/core ORF (23–25). The outer layer is an envelope containing host derived lipids together with the small (S), middle (M) and large (L) surface proteins, collectively known as hepatitis B surface antigen (HBsAg) (20,26). These proteins encoded by the pre-S1/pre-S2/surface ORF share a common C-terminus, with progressive additions at the N-terminus to form the longer M and L proteins (27).

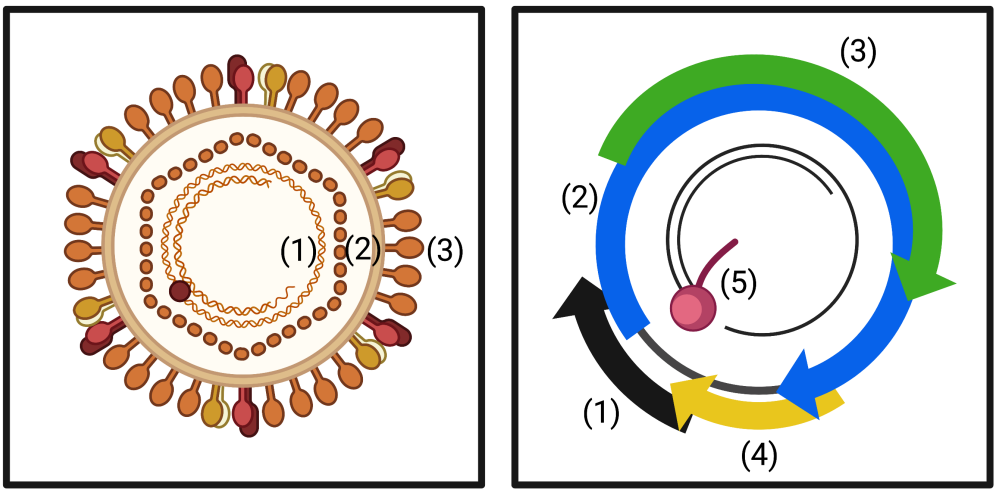


Figure 1.1: Cartoon of the HBV infective virion

Dane particle (left panel): (1) Partially double stranded DNA HBV genome, (2) viral capsid (HBV core antigens), (3) viral envelope (HBV surface antigens). Hepatitis B virus genome and open reading frames (ORF) (right panel): (1) Pre-core/core ORF, (2) Polymerase ORF, (3) Pre-S1/Pre-S2/Surface ORF, (4) X ORF (5) Partially double stranded DNA that forms the HBV genome, together with the covalently attached viral polymerase. Images created using BioRender.com

Non-structural proteins encoded by HBV include the Hepatitis B e antigen (HBeAg), encoded by the pre-core/core ORF, which is secreted into the blood stream and acts as an immune decoy mechanism (reviewed in (28)). Finally the X ORF encodes the X-protein, which is essential for viral protein transcription (29) and is associated with the development of hepatocellular carcinoma (HCC) (30–33).

1.2.2 Viral Replication

Viral replication involves several stages (reviewed in (19), Figure 1.2). HBV exclusively infects hepatocytes via the bile acid transporter sodium taurocholate co-transporting polypeptide (NTCP) (34). Internalised nucleocapsids move to the nucleus and disassemble, releasing relaxed circular DNA (rcDNA). Here the positive strand of the rcDNA is completed by the viral polymerase or host cellular enzymes, forming covalently closed circular DNA (cccDNA) (reviewed in (35)). The cccDNA localises with histones to form a mini-chromosome (36,37). This acts as a template for the transcription of pre-genomic RNA

(pgRNA) and HBV proteins using cellular enzymes. Newly formed pgRNA is enclosed by nucleocapsid and reverse transcribed by viral polymerase to rcDNA (38). Nucleocapsids released into the cytoplasm are either directed back to the nucleus to increase the cccDNA reservoir (39) or encapsulated by surface proteins to form new virions. Secretion of infective viral particles into the blood stream occurs via multivesicular bodies (40). In addition to infective virions, empty subviral particles composed of surface antigen are also secreted into the blood stream. HBV infection can be highly efficient, with a single HBV virion able to infect of 100% of hepatocytes in non-human primate studies (41).

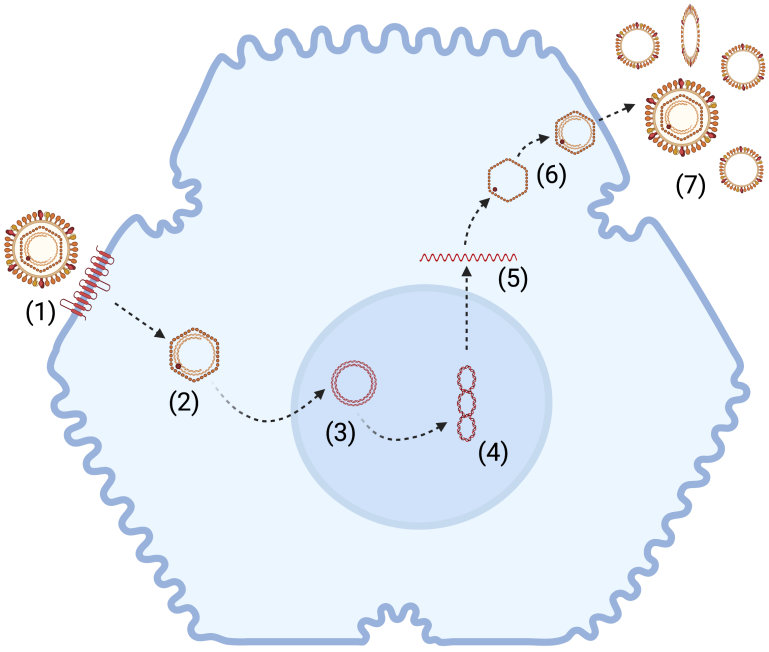


Figure 1.2: Cartoon of the replication stages of HBV within the hepatocyte

(1) Viral entry through the bile acid transporter sodium taurocholate cotransporting polypeptide (NTCP), (2) internalised nucleocapsid, (3) HBV genome as relaxed circular DNA within the hepatocyte nucleus, (4) HBV genome as covalently closed circular DNA (cccDNA) in the nucleus, forms a template for viral replication, (5) Pre-genomic RNA transcribed from cccDNA, (6) capsid assembly, (7) encapsulation by HBV surface antigen and secretion alongside empty subviral particles composed of surface antigen. Images created using BioRender.com

1.2.3 Genetic diversity

The overlapping structure of the HBV genome restricts the ability of the virus to mutate without fitness cost and its genome is therefore relatively stable across geological time (reviewed in (42)). Conversely within an infected individual, mutations arising during viral replication are frequent due to the lack of proof-reading ability of the viral polymerase (42,43). This explains the curious observation that the between-host evolutionary rate is slower than the within-host evolutionary rate. Whilst quasispecies with high sequence homology to the infecting virus are readily able to infect other hosts, those with higher sequence diversity give the virus an advantage in maintaining chronicity in the face of immune pressure.

To date, 9 genotypes of hepatitis B virus (A-I) have been described, which differ by 7.5% or more in their nucleotide sequences (reviewed in (44)). A further genotype J has been proposed, but thus far has only been isolated in a single case (45). Sub-genotypes have also been described, which differ by between 4 and 7.5% in nucleotide sequence (44). Different genotypes are associated with clinical factors including transmission method, likelihood of development of chronic infection, response to treatment and progression of chronic disease to cirrhosis and hepatocellular carcinoma (reviewed in (46)). Genotypes have a distinct geographical distribution with A-E being most abundant worldwide (Figure 1.3). Genotypes A and D predominate in Europe, North America and North Africa, genotypes B and C in South-East Asia and genotype E in Western and Southern Africa (47).



Figure 1.3: Geographical distribution of HBV genotypes A-H

In Europe, genotypes A and D predominate. Genotype D is also dominant in Russia, Central Asia, Northern Africa and on the Indian subcontinent. Genotypes A and E predominated in Eastern and Western Africa respectively. In Southern and Eastern Asia and Australasia genotype B and C are most common. Genotype A and F predominate in Southern America. In Northern America there is a mixture of genotypes A, B C and D. Figure based on data from Velkov et al. 2018. Image created using BioRender.com

HBV serotypes describe nucleotide variation within the region coding for surface protein, HBsAg. The four major serotypes *adw*, *adr*, *ayw* and *ayr* are described by the combination of the *a* region on the surface of HBsAg (48), with either *d/y* or *w/r* regions (49–51), which determine reactivity towards reference antibodies. Serotype and genotype are associated, but there is variability of serotypes within genotypes (52). Also described are HBV viral variants, associating particular mutations in surface, polymerase or core encoding regions with clinical outcomes including treatment resistance and disease progression to hepatocellular carcinoma (reviewed in (53)). Of particular importance are resistance associated and vaccine escape mutations, which reduce the efficacy of current HBV treatment or prevention strategies (54). Recent analysis of worldwide HBV viral variant frequencies has revealed particular mutations can be associated with genotype or geographical region independently (52). These

findings may be biased towards genotypes A-D which are sequenced relative frequently compared to genotypes E-I. Further understanding of these relationships requires increased sequencing of the HBV genome, particularly for underrepresented genotypes.

1.3 CLINICAL INFECTION WITH HEPATITIS B

1.3.1 Geographical distribution

HBV infection has an unequal distribution globally. Over 3% of the general population worldwide is thought to be chronically infected with HBV. The percentage of infected people is estimated to be as high as 8.8% in the WHO African Region and 5.3% in the WHO Western Pacific Region but much lower at 0.8% in the WHO Americas region (55). Worldwide HBV prevalence is decreasing (56,57), driven in part by wider coverage of HBV prophylactic vaccination in infants leading to reductions in cumulative incidence in children aged under 5 (58).

Contemporary estimates of the prevalence of HBV infection by nation are available from a systematic review of Hepatitis B surface antigen (HBsAg) seroprevalence between 1965 and 2013 (55), the World Health Organisation (59), the Polaris Observatory Collaboration (60) and the Institute for Health Metrics and Evaluation Global Burden of Disease Study (61). Although these sources use different method to derive their estimates, a recent analysis has found they agree on the overall patten of HBV burden worldwide with the highest prevalence of disease in Western Africa, Oceania, and Eastern, Central and South-Eastern Asia (62).

1.3.2 Transmission

Transmission of HBV through infected blood or mucosal surfaces initially results in acute infection, which is then either controlled or goes onto persist as chronic disease. The risk of

developing chronic HBV after transmission is strongly associated with age of acquisition, with up to 90% of those infected as neonates going onto develop chronic infection (63,64), compared to less than 10% of individuals exposed as adults (65).

Vertical transmission from mother to child at birth or horizonal transmission during early childhood account for the majority of new infections worldwide, although the latter have been decreasing due to increasing childhood HBV vaccination programmes (58). The risk of perinatal transmission is hugely increased when mothers are positive for HBeAg (63,64,66–68), possibly due to the immature immune system of the child or tolerance induced in the child by HBeAg crossing the placenta (28).

In adults, unprotected sexual intercourse is one of the main risk factors for acquisition of infection. Regular sexual contacts of infected persons within a household, persons who have multiple sexual partners and men who have sex with men are all at increased risk of HBV infection (62,69–71). Healthcare associated transmission through infected blood transfusions and reuse of needles also occurs, although infection via this route is thought to have decreased in the early years of the 21st century (72). Adults who inject drugs are at particularly high risk of HBV transmission through the sharing of infected needles and syringes (73,74). Prisoners have a higher prevalence of HBV than in the general population and there is evidence of acquisition of HBV whilst in prison, through sharing contaminated razors, injecting drugs and unprotected sex (75,76).

1.3.3 Clinical presentation and diagnosis

Clinical presentation with Hepatitis B virus can be variable. The majority of infected persons have no clinical symptoms. In these cases, testing and diagnosis might only occur

opportunistically if an individual falls into a group at high risk of infection. For, example babies born to mothers with known hepatitis B infection or first-degree relatives and sexual partners of someone with HBV infection might be tested (8). Opportunistic diagnosis might also occur during screening of at-risk groups, such as intravenous drug users, prisoners or at anti-natal screening (8,77,78).

Although infection is asymptomatic in the majority, some people with acute infection develop liver inflammation and acute liver failure. Viral hepatitis is within the differential diagnosis for such a presentation and diagnosis is reliant on having a high enough clinical suspicion to test for HBV serological markers (79). In persons with chronic infection, symptoms of hepatitis B might only arise if liver cirrhosis or hepatocellular carcinoma develop. Clinical manifestations of decompensated chronic liver disease such as ascites, hepatic encephalopathy or bleeding of oesophageal varices might be the first indication of hepatitis B infection (80). In all cases, there is no set of symptoms and signs unique to hepatitis B infection and it can co-exist with other infectious and non-infectious risk factors for developing chronic liver disease.

The presence of HBsAg in the blood, persisting for over 6 months is diagnostic of chronic HBV infection and warrants further tests to diagnose the phase of disease (8). This relies on serum measurement of HBsAg, HBeAg and HBV DNA to assess viral activity in addition to the assessment of HBV induced liver damage (8). Current guidelines recommend the assessment of liver disease severity to be determined non-invasively in the first instance using a combination of physical examination, serum alanine aminotransferase (ALT) and liver ultrasound scan (8). The estimation of liver stiffness using transient elastography can be used to assess liver fibrosis in HBV, with the advantage that it is non-invasive and can be

monitored longitudinally (81). However liver biopsy remains the gold standard for the evaluation of HBV induced liver damage and it is useful to evaluate necroinflammation and fibrosis progression when results from non-invasive tests are not conclusive (8).

1.3.4 Clinical course

Once chronic Hepatitis B infection has been diagnosed, the disease phase can be determined through a combination of HBeAg and HBV DNA level to assess viral activity and ALT level, transient elastography or liver biopsy to assess liver damage (Table 1.1, (8)). The phases of chronic HBV infection reflect the complex interplay between the immune response to HBV and the virus itself.

Early in the course of infection viral replication is high, reflected by high HBV DNA titres and detectable HBeAg in the blood. During this 'HBeAg positive chronic infection' phase, there is little or no liver inflammation, and ALT is low. This stage is also known as the 'immune tolerant' phase, reflecting the relative tolerance of virus specific cellular immunity towards HBV (82). However evidence that T cell function is actual preserved in this period has in part lead to the change in nomenclature in Europe (83).

Individuals can then transition to the 'HBeAg positive chronic hepatitis' or 'immune active' phase, characterised by fluctuating ALT levels secondary to immune mediated liver damage. This usually precedes loss of HBeAg in the blood, seroconversion to anti-HBeAg and a reduction in HBV DNA titres (28). Some individuals that have lost HBeAg then normalise their ALT, have low HBV DNA titres and little liver inflammation, the so called 'HBeAg chronic infection' or 'inactive carrier' phase. A proportion go on to develop 'HBeAg negative chronic hepatitis', characterised by raised ALT levels or other indicators of liver

damage. Individuals can progress through these stages during the course of infection, but not necessarily in a sequential or unidirectional fashion (8).

Table 1.1: The clinical phases of chronic Hepatitis B infection, adapted from the EASL 2017 Practise Guidelines for the management of Hepatitis B infection (8)

	HBeAg positive chronic infection	**HBeAg positive chronic hepatitis**	**HBeAg negative chronic infection**	**HBeAg negative chronic hepatitis**
Also known as	Immune tolerant	Immune reactive	Inactive carrier	Immune escape
HBsAg	+	+	+	+
qHBsAg	+++	++	+	++
HBeAg	+	+	-	-
HBV DNA	$>10^7$ IU/mL	10^4-10^7 IU/mL	$< 2x10^3$ IU/mL	$> 2x10^3$ IU/mL
ALT	Normal	Raised	Normal	Raised
Liver disease	None	Moderate/severe	None	Moderate/severe

1.3.5 Prognosis

Liver cirrhosis and hepatocellular carcinoma (HCC), the major long term sequalae of chronic Hepatitis B infection, are responsible for the estimated 800,000 deaths from HBV globally in 2015 (84). The various phases of disease are associated with different risks of the development of these liver related complications.

1.3.5.1 Cirrhosis

In prospective cohorts of HBsAg positive individuals with persistent biochemical or histological liver inflammation (raised serum ALT or hepatitis on liver biopsy), annual

incidence of cirrhosis is estimated at between 1 and 6% (85,86). Such studies report a higher incidence of cirrhosis in persons with increased age, persistently raised HBV DNA and bridging hepatic necrosis on liver biopsy (85,86). In an unselected cohort of persons positive for HBsAg, persistent ALT elevation was associated with cirrhosis development (87). This data supports liver inflammation as a driving factor for progression to fibrosis and cirrhosis in chronic HBV. However, in the largest prospective study of unselected HBsAg positive individuals, elevated HBV DNA predicted the risk of cirrhosis development independently of HBeAg status and ALT level (88).

In individuals in the HBeAg positive chronic infection phase ('immune tolerant') who have raised HBV DNA but normal ALT, the risk of progression to cirrhosis appears to be low (89). A prospective study using transient elastography to estimate fibrosis found 4.1% of individuals with HBeAg positive chronic infection had progressive liver stiffness over a 4-year period (90). Retrospective histological examination of liver biopsies in this population have reported mixed results, with some reporting all participants having none or minimal fibrosis and other reporting an increase in significant fibrosis with age (91,92). However a prospective study of individuals with HBeAg positive chronic infection that remained in this phase of infection over the course of follow up, showed no significant progression of fibrosis stage on paired liver biopsies 5 years apart (93).

Overall, the evidence supports that several host and virus-related factors increase the risk cirrhosis. Risk scores to predict cirrhosis in persons with chronic HBV incorporate age (older), sex (male) and higher ALT, DNA and quantitative HBsAg levels, which all significantly increase the risk of cirrhosis development (94).

1.3.5.2 Hepatocellular Carcinoma

The risk of developing hepatocellular carcinoma (HCC) is raised in HBsAg positive compared to negative persons, with approximately 40% of males infected neonatally with HBV going on to die from HCC or cirrhosis (95). The incidence of HCC increases after the age of 30 years (95).

A large prospective population study of HBV infected individuals in Taiwan reported an association between HBeAg positivity and the development of HCC, although this may have reflected the higher levels of HBV DNA associated with the HBeAg positive disease phases (96). Subsequently it was reported that elevated HBV DNA predicts the risk of HCC development independently of HBeAg status and ALT level (97). Risk scores to predict HCC in persons with chronic HBV incorporate age (older), sex (male) and higher ALT, DNA and HBsAg levels which all significantly increase the risk of HCC (94).

The true risk of HCC development during the HBeAg positive chronic infection ('immune tolerant') phase is controversial. A retrospective cohort of individuals in the HBeAg positive chronic infection phase estimated a high HCC cumulative incidence of nearly 13% at 10 years (98). There is pathophysiological evidence that supports that carcinogenic events that promote HCC development occur during this phase of infection, including HBV DNA integration in to host chromosomes and clonal hepatocyte expansion (99,100). However a recently published retrospective cohort study reported a much lower cumulative incidence of HCC in the HBeAg positive chronic infection phase at approximately 1% and 2% at 5 and 9 years of follow up (101). The divergent findings in these two studies may be due to different definitions of HBeAg positive chronic infection phase, with the former study including individuals with HBV DNA levels >20,000 IU/mL, in accordance with the Asia-Pacific

guidelines (102), compared to >1,000,000 IU/mL in the latter, in accordance with European and American guidelines (8,77). The authors hypothesised that the lower HCC rates in their study with higher HBV levels for inclusion were more accurate, due to reduced chances of including persons who were beginning to transition to the HBeAg chronic hepatitis phase, heralded by lower DNA levels (101). Consistent definitions of disease phases and prospective studies are needed to better assess the risk of HCC development to inform treatment decisions going forward.

1.4 TREATMENT OF CHRONIC HEPATITIS B INFECTION

1.4.1 Functional cure as the goal of HBV treatment

The primary treatment goal of chronic HBV infection is to prevent morbidity and mortality associated with liver cirrhosis and HCC (8). Complete sterilising cure of HBV would be required to maximally reduce the risk of HCC development, where all HBV DNA including integrated DNA and cccDNA is eliminated and HBsAg is persistently undetectable in the serum (9). However even in persons who resolve acute HBV infection, HBV integrated DNA and cccDNA remain at low levels. Therefore complete sterilising cure is not a realistic goal for HBV therapy at present (9).

An alternative goal of treatment is functional cure, defined as a persistent loss of HBsAg, suppression of HBV DNA and the absence of liver inflammation (9). Spontaneous HBsAg loss occurs in only 1% of persons with chronic HBV infection per year during natural infection (16,103,104). HBsAg loss at aged 50 years or younger is associated with a lower risk of developing HCC and HBV DNA remains undetectable in the majority long term, despite the persistence of low levels of integrated HBV DNA and cccDNA in hepatocytes (105–108).

Sustained suppression of HBV DNA and normal ALT after a finite course of treatment in the absence of HBsAg loss (partial cure) is another possible treatment outcome, reflecting a lower HCC risk than in the natural course of infection (9). However, as HBsAg loss confers additional benefit to mitigating HCC risk, an international consensus agreed functional cure rather than partial cure is currently the most realistic and aspirational goal for HBV therapy (9,109).

1.4.2 Existing treatment options for HBV

Two therapeutic approaches are currently recommended for the treatment of chronic HBV infection: pegylated interferon-alpha (PegIFNα) and nucleos(t)ide analogues (8,77,102). Treatment is recommended for people with a higher risk of HBV disease progression, including all those with biochemical evidence of liver inflammation (raised ALT), histological evidence of moderate liver necroinflammation or fibrosis and all individuals with cirrhosis (8,77,102).

PegIFNα therapy is recommended for the treatment of HBeAg positive and negative persons with raised serum ALT or moderate necroinflammation on biopsy (8). It is thought to modulate the immune response to HBV and promote long term immune control of the virus (8). PegIFNα is given in a finite course of 48 weeks, however it has a high side effect burden that can limit its use and is contraindicated in several groups such as those with decompensated cirrhosis (8). In HBeAg positive individuals, functional cure with loss of HBsAg occurs in 3-7% at 24-26 weeks after completing treatment (110,111). Long term follow up of between 18 months to 5 years revealed cumulative incidence of HBsAg loss increases to up to 11% (112). In HBeAg negative individuals, HBsAg loss occurs in 1.4 - 4%

at 24-26 weeks after treatment completion (113,114). Extending the duration of PegIFNα to either 72 or 96 weeks can improve HBsAg loss to 6-11% at 24-28 weeks post treatment and up to 36% at 48 weeks post treatment (115,116). However, a sizable proportion of individuals both HBeAg positive and negative treated with PegIFNα do not achieve suppression of HBV DNA (partial cure) (8). These persons might subsequently require alternative therapy if their ALT remains or becomes raised.

Nuleos(t)ide analogue therapy is an alternative treatment for chronic HBV, where PegIFNα is contraindicated, not tolerated or has not induced a sustainable response (8). Nuleos(t)ide analogues with a high barrier to resistance such as Tenofovir and Entecavir effectively supress HBV replication in the majority and are generally well tolerated (8). Treatment reduces but does not eliminate the risk of cirrhosis and HCC, particularly for those with cirrhosis (117–120). If treatment is stopped viral load often rebounds, driven by cccDNA persistence in infected hepatocytes. Currently rules to predict when nucleos(t)side therapy can be withdrawn safely are lacking and therefore life-long therapy is often required (121,122).

There is still debate over whether individuals with normal ALT levels in the HBeAg positive chronic infection phase are at sufficient risk of cirrhosis and HCC to warrant treatment (123). In the largest prospective study of unselected HBsAg positive individuals, elevated HBV DNA predicted the risk of HCC development independently of HBeAg status and ALT level (97). However, many included in the study were not in the HBeAg positive chronic infection (‘immune tolerant’) phase and therefore this finding might not be applicable to this subgroup (97). Randomised trials assessing nucleos(t)ide therapy with or without PegIFNα in children and adults with HBeAg positive chronic infection do not support their routine use in this

population. In these trials HBV DNA suppression is achieved in most, although the rates of HBeAg loss are low and viral replication rebounds when therapy is stopped (124–126). It is argued the current evidence is insufficient to support that treatment changes the disease course in the HBeAg positive chronic infection phase. However, no study has assessed whether treatment reduces the long-term HCC risk in this group.

Currently the rates of functional cure in the majority of those treated with nuleos(t)ide analogues are low. Loss of HBsAg during treatment is rare, occurring at a rate of under 0.5% a year (127–129). The incidence of HBsAg loss can be increased using nuleos(t)ide analogues and PegIFNα in combination (130). If HBsAg loss is achieved, it is persistent in the majority and these individuals are at low risk for HCC development (15). However, in most cases functional cure on either PegIFNα or nuleos(t)ide analogues is not realised. New therapeutic approaches that lead to higher rates of functional cure with finite treatment courses are therefore urgently needed.

1.4.3 Novel treatment approaches

A better understanding of the lifecycle of HBV alongside viral and host features that promote HBV chronicity have led to the development of several novel therapeutic strategies for HBV (reviewed in (131,132)).

Many strategies directly target the virus itself (Figure 1.4). These include: (i) entry inhibitors, which prevent HBV entry into hepatocytes through the NTCP receptor (133), (ii) core protein allosteric modulators which disrupt the viral capsid, preventing packaging of the HBV genome, viral replication and cccDNA synthesis (134), (iii) strategies that remove or silence the cccDNA HBV replication template through either genome disruption or epigenetic

modification (135), (iv) interference RNA (RNAi) or single-stranded oligonucleotides (SSO), which disrupt post translational HBV transcripts preventing the formation of new HBV viral particles (136), and (v) Nucleic acid polymers which inhibit the assembly and secretion of HBsAg in the form of HBV subviral particles (137).

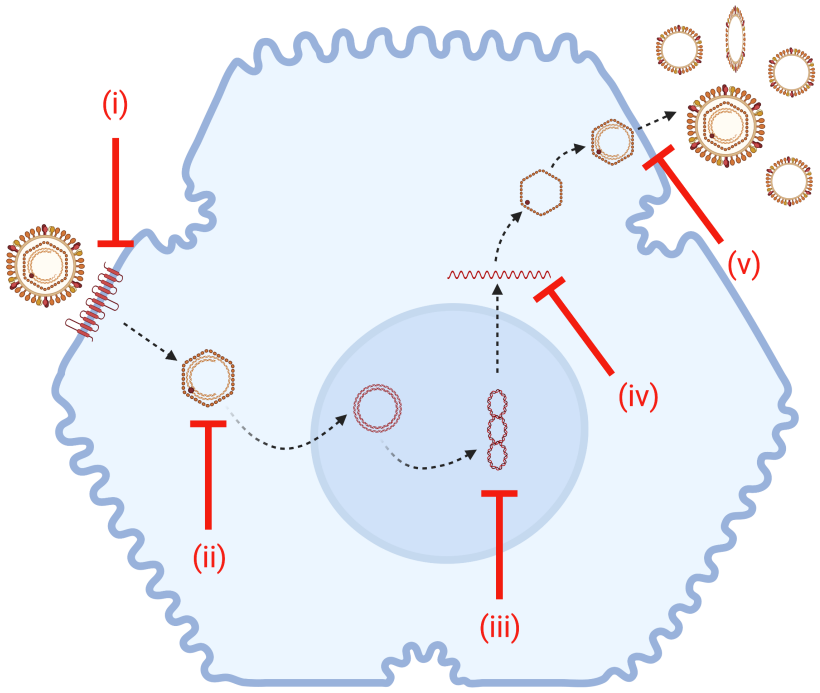


Figure 1.4: Novel treatment targets in the HBV viral lifecycle

(i) prevention of HBV entry into hepatocytes through the NTCP receptor, (ii) disruption of the viral capsid, (iii) direct cccDNA modulators (iv) disruption of post translational HBV transcripts and (v) inhibition of the assembly and secretion of viral and sub-viral particles. Image created using BioRender.com

Other therapeutic strategies target HBV indirectly through immune system modulation, with the aim of inducing sustained immune control of the virus ((12,138), Figure 1.5). These include: (i) agonists of toll-like receptors, which promote innate anti-viral immune responses (131,139), (ii) checkpoint inhibitors that prevent signalling through receptors such as programmed cell death protein 1 (PD-1), which is involved in HBV specific T cell dysfunction (140), (iii) transfer of modulated functional T cells, designed to recognise HBV

epitopes by either redirecting their T cell receptor (TCR, TCR-redirected T cells) or engineering chimeric antigen receptors (CAR, CAR T cells) (138,140) and (iv) therapeutic vaccination which aims to induce or boost HBV specific cellular immune responses through delivering non-infectious HBV antigen via vaccination (14,138).

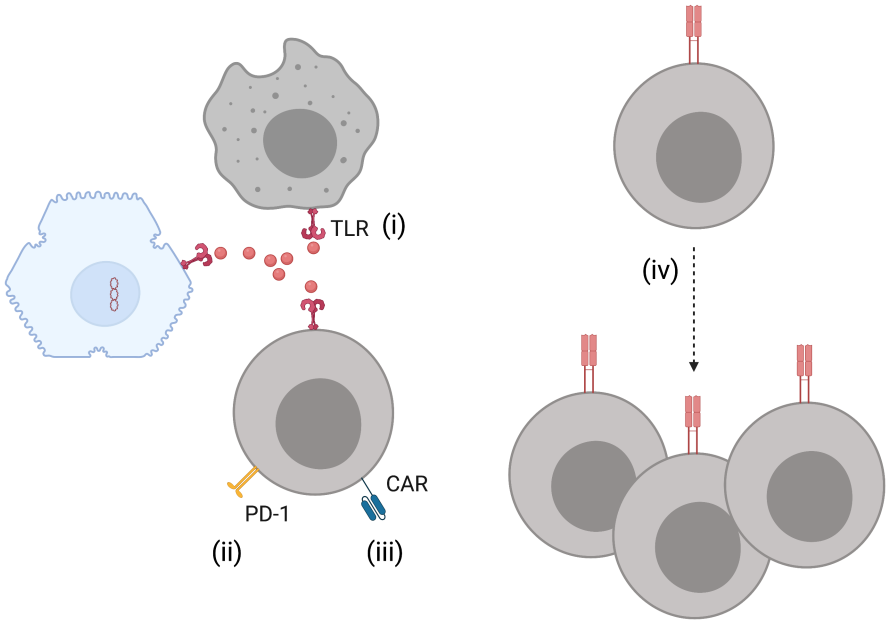


Figure 1.5: Novel treatment targets via immune modulation

(i) Toll-like receptor (TLR) agonists (ii) checkpoint inhibitors for example programmed cell death protein 1 (PD-1) inhibitors (iii) chimeric antigen receptor (CAR) engineered T cells (iv) therapeutic vaccination leading to expansion of HBV specific T cells. Image created using BioRender.com

The rationale for therapeutic vaccination as a treatment for HBV is underpinned by evidence that in chronic infection, the HBV specific immune response is highly dysfunctional (140). Dysfunction is not irreversible, as individuals that achieve sustained HBsAg loss spontaneously or after therapy have restored HBV specific cellular responses (17,141). The innate and adaptive immune responses to both acute and chronic HBV infection are discussed in detail in the following section, which inform the key immunological aims of therapeutic vaccination.

1.5 THE IMMUNE RESPONSE TO HEPATITIS B INFECTION

1.5.1 Innate immunity in acute infection

The role for the innate immune response in acute HBV infection in humans has been difficult to study, due to the challenge of identifying infected persons prior to symptom onset. However, evidence from *in-vitro* studies, animal models of infection and in human studies of early HBV infection have greatly improved our understanding of the innate immune response after HBV acquisition.

Initial evidence supported that acute HBV infection might not activate innate immune signalling pathways. *In-vitro* studies revealed pattern recognition receptor signalling was not altered in permissive human hepatocytes infected with HBV (142) and microarray studies of HBV infection in chimpanzees found no transcriptional upregulation of innate immune response genes in the liver (143). In early HBV infection in humans, circulating type I interferon levels were significantly lower compared to healthy controls (144) and at peak viraemia proinflammatory cytokine interferon-gamma (IFNγ) was supressed, whilst anti-inflammatory cytokine interleukin-10 (IL-10) was increased (144). In addition, persons with acute HBV infection appeared to have unchanged or low levels of circulating cytokines in comparison to individuals after acute infection with HIV, supporting that the innate immune system might not be activated by HBV infection (145).

As well as potentially evading sensing by the innate immune system, there is evidence that HBV directly suppresses innate immune responses. HBV polymerase and/or HBsAg have been found to suppress toll-like receptor (TLR) -3 signalling in liver sinusoidal endothelial cells and primary human hepatocytes (146–149) and inhibit TLR-9 signalling in

plasmacytoid dendritic cells (150). Physiological infection of HepaRG cells with HBV led to inhibition of innate immune responses through reduced TLR-3 signalling (151).

More recent studies support that innate immune pathways are activated in early HBV infection. *In-vitro* studies indicate that several cell types are able to sense HBV infection including hepatocytes, macrophages and dendritic cells through TLR-2, TLR-4, TLR-7 and TLR-9 (151–156). Several *in-vitro* studies demonstrate that interferons or products of interferon stimulated genes are able to inhibit HBV replication at various stages during its lifecycle (157,158). Although unable to upregulate type I interferons, *in-vitro* HBV infection has been found to activate type III interferons such as IFNγ in human hepatocytes (159,160). Some humans studies have found an elevation in pro-inflammatory cytokines such as IFNγ and interferon gamma-induced protein 10 (IP-10) during acute HBV infection (161,162). In chimpanzees infected with HBV, increases in several circulating cytokines were associated with early infection including interferon-alpha-2 (IFN-α2) and interleukins -5, -8 and -17A (163). Furthermore, chimpanzees infected with pre-core mutant HBV had raised IP-10 levels and developed a more severe clinical hepatitis than animals infected with usual strain HBV (163), indicating that the cytokine profile induced by infection could be associated with clinical outcome.

The role Natural Killer (NK) cells play in the response to acute HBV infection has also been investigated. Consistently NK cells have been found to be enriched in the liver, with maximal activation and cytotoxicity during early HBV infection (144,161,162,164,165). The expression of HLA-DR on NK cells in HBV infection has been observed to be negatively correlated with HBV DNA and positively correlated with serum ALT, suggesting NK cells might be involved in both viral control and liver inflammation (162). NK cells from acutely

infected individuals were able to produce more IFNγ and CD107a on restimulation compared to NK cells derived from healthy controls (162). However, other studies have found NK cell function during acute HBV infection to be impaired with reduced macrophage inflammatory protein 1β (MIP-1β) production (166). More recently distinct phenotypic signatures within the $CD56^{dim}$ NK cell compartment have been described, that predict the temporal dynamics of HBsAg clearance in acute HBV infection (167). This study found the expression of CD158a, CD94, NKp30 and CD161 on $CD56^{dim}$ NK cells predicted time to resolution of infection in individuals with early HBsAg clearance, and was associated with stronger antibody dependent cytotoxic responses and increased CD107a expression (167). After resolution of infection, activation markers on NK cells decrease (162). These results suggest NK cells play an important role in the resolution of acute infection via antibody dependent cytotoxic responses and cytokine production.

1.5.2 Adaptive immunity in acute infection

1.5.2.1 T cells

The adaptive immune response to acute HBV has been more extensively studied. Early reports of lymphocytes extracted from the blood of persons with acute HBV infection revealed CD4 and CD8 T cell responses were present towards epitopes in HBV regions encoding core, polymerase and surface proteins (168–175). HBV specific CD8 T cells were cytotoxic and produced IFNγ in response to cognate peptide stimulation. Over the course of acute infection their magnitude followed the trajectory of markers of clinical infection, peaking with liver transaminase levels and waning as HBeAg and HBsAg levels became undetectable (176), associating a robust cellular immune response with clearance of infection.

The dynamics of the adaptive immune response towards acute HBV infection in the liver itself were first studied in chimpanzees inoculated with human HBV (177). This work confirmed that HBV itself was not directly cytopathic to hepatocytes, as liver transaminases were within the normal range at time of peak viral replication. It was not until after HBV DNA levels began to decline and intrahepatic CD3 and CD8 RNA levels increased that histological evidence of liver inflammation appeared, implying liver damage might be immune mediated. However, because the number of infected hepatocytes at maximal infection far outweighed those necrosed during the inflammatory stage, the authors concluded that viral clearance must largely be mediated via non-cytopathic mechanisms (177). This was confirmed in people with acute HBV studied prior to symptom onset, where HBV DNA levels began to fall prior to the onset of liver inflammation (164). Circulating CD8 T lymphocytes specific to HBV core and polymerase antigens were present before symptomatic hepatitis, peaked in association with high transaminase levels and reduced as HBsAg was cleared (164). The integral role of CD8 T cells for viral elimination in acute HBV was confirmed in depletion studies in non-human primates (178). HBV specific CD4 and CD8 cells specific to HBV surface, polymerase and core antigens all accumulated in the liver prior to viral clearance. Depletion of CD8 but not CD4 T cells at viraemia onset disrupted the clearance of HBV infection, which did not occur until CD8 levels had recovered to normal levels (178).

Together these data support the hypothesis that strong immune responses during acute HBV infection are necessary for viral elimination and that HBV specific CD8 T cells with several specificities are the key players in this process (178).

1.5.2.2 B cells

After infection with hepatitis B virus, cells of the B cell lineage produce antibodies towards several HBV antigens including Hepatitis B core, e and surface antigens (HBcAg, HBeAg and HBsAg (179)). Alongside this, B cells perform several other functions including antigen presentation to T cells and cytokine production, although precisely how these contribute to the successful resolution of acute HBV infection is not yet clear (180). Treatment with B cell depleting agents such as Rituximab in persons that have successfully resolved HBV can lead to virological relapse, suggesting B cells are actively involved in the long term immunological control of the virus (181,182).

Early in HBV infection IgM anti-hepatitis B core antibodies (anti-HBc Ab) are detectable. Subsequently IgG anti-HBc Abs predominate, remaining positive during and after resolution of infection (179). Anti-HBc Ab can be produced by both T cell dependent and independent mechanisms, unlike anti-hepatitis B e antibodies (anti-HBe Ab) which are dependent on T cell help (183). In acute infection it is not clear whether anti-HBc Abs are necessary for the resolution of infection. However in HBV associated acute fulminant liver failure anti-HBc Abs appear to play a central role in pathogenesis, characteristic features of which include an intrahepatic infiltration of plasma cells secreting anti-HBc Ab and complement deposition (184,185).

A functional HBV specific B cell response is important for the resolution of acute HBV infection, which is characterised by the loss of HBsAg and the development of anti-hepatitis B surface antibody (anti-HBs Ab) in the serum (179). Anti-HBs Ab is neutralising to HBsAg, preventing entry of HBV virions into hepatocytes. This is evidenced by both monoclonal antibodies specific to HBsAg which prevent viral entry (186) and anti-HBs Abs induced by

recombinant HBsAg vaccination, which successfully prevent infection (187). Polyclonal hepatitis B immunoglobulin (HBIG) is useful in preventing vertical transmission of Hepatitis B (188) and to reduce the risk of reinfection after liver transplantation (189,190) by neutralising HBsAg and reducing HBsAg secretion from infected hepatocytes (191).

HBV specific B cells during acute HBV infection have not been studied extensively. The frequencies of circulating HBs specific B cells appear to be lower in acute compared to chronic HBV and are not associated with HBV viral load nor liver inflammation over the course of infection (192). However, the proportion of HBs specific B cells with a dysfunctional atypical memory phenotype appears to decrease after ALT flare and infection resolution, suggesting a functional HBs B cell response is associated with HBV immune control (192). The mechanisms that support functional HBV specific B cell responses and the precise roles B cells play in the resolution of acute HBV infection need to be further studied.

1.5.3 Innate immunity in chronic infection

Innate immune signalling through TLRs have been found to be impaired during chronic HBV infection. Several studies have observed that TLR-2 and TLR-8 expression is associated with a successful responses to PegIFNα treatment, but expression on monocytes is decreased in chronic infection (193–197). TLR-3 expression on hepatocytes, Kupffer cells and peripheral blood mononuclear cells (PBMC) and TLR-9 expression on plasmacytoid dendritic cells has also been found to be impaired (198,199). Decreased TLR signalling in chronic HBV might permit unchecked HBV replication, which in HBV transgenic mice is inhibited by TLR-ligand administration (200). Reduced TLR receptor signalling might also promote T cell dysfunction. In murine models of chronic HBV infection, TLR signalling promotes HBV specific immune responses and cytokine production (201–204). Treatment with oral TLR-7

agonists induced sustained viral suppression in chimpanzees infected with HBV, however human trials have not shown superiority of HBV DNA suppression nor HBsAg loss by adding a TLR-7 agonist to nucleos(t)ide treatment (205,206). TLR-8 has also been identified as a therapeutic target for chronic HBV infection, and early phase I trials have shown administration of TLR-8 induces transient increases in IL-12p40 and IL-1RA (207,208). TLR agonists might prove a useful adjunct in novel HBV treatment strategies.

Differences in NK cell frequency, phenotype and function have also been described in chronic HBV infection which might contribute to HBV persistence. Several studies have reported that NK cells maintain their cytotoxic functions during chronic HBV but their capacity to produce cytokines is impaired (209–214). This is in contrast to acute HBV, where resolution of infection is associated with effective NK cytotoxicity and cytokine production (162).

NK cell function is controlled by activating and inhibitory signals received through surface receptors, which are differentially expressed by NK cells during chronic HBV. Some activating receptors are downregulated on NK cells in chronic HBV, including the natural cytotoxicity receptor NKp3 and the Fc gamma receptor III (FcγRIII or CD16), which mediates antibody dependent cellular cytotoxicity (211,212,214). Other studies have found activating natural cytotoxicity receptor NKp46 and the inhibitory c-type lectin receptor NKG2A to be upregulated on NK cells in chronic HBV (211,215,216). However, there is disagreement as to whether these changes are associated with an increase or a decrease in HBV DNA levels (211,215,216), or even if these markers are upregulated in all cases (214). The inhibitory receptor siglec-9 has also been reported to be upregulated on NK cells in chronic HBV infection and is negatively correlated with HBV DNA levels (217). These data

support that the balance of several NK cell receptors is disrupted in chronic HBV infection and might be associated with viral replication.

NK cell phenotype also appears to vary depending on the phase of chronic HBV infection. In the HBeAg positive chronic infection phase where liver inflammation is low, reduced expression of CD122 has been found to mediate NK cell dysfunction which can be reversed *in-vitro* by IL-2 or IL-15 (218). NK cell activation might also be related to HBeAg status, as in both the HBeAg positive chronic infection and chronic hepatitis phases NKp46 expression was increased (219). One study has investigated NK cells at the transcriptional level, which found very little differential gene expression across the phases of HBV infection. However immune related genes were differentially expressed during the HBeAg positive chronic hepatitis phase, supporting the hypothesis that NK cells might play a role in the immune activation characteristic of this stage of infection (220).

Several lines of evidence support NK cells might directly mediate liver damage in chronic HBV. During clinical phases of HBV associated with liver inflammation, NK cells appear to express higher activation makers such as NKp44 and CD69 and degranulation is increased (213,221,222). Activation of NK cells in the liver and periphery have been associated with ALT and histological inflammatory activity (221). The expression of tumour necrosis factor related apoptosis inducing ligand (TRAIL) might be a key regulator of NK induced liver damage in chronic HBV. TRAIL upregulation on NK cells and hepatocytes is associated with ALT flares, and liver resident NK cells in chronic HBV express high TRAIL levels (210,222–224). There is also evidence that NK cells kill HBV specific T cells through TRAIL and perforin dependent mechanisms (222,225). NK cells might also negatively regulate T cells through NKG2D, which has been found to be upregulated on NK cells in the

liver in chronic HBV infection together with increased NKG2D-ligand expression on CD4 HBV specific T cells (214,226). Thus, NK cells might simultaneously induce liver damage and impair HBV specific T cell mediated immunity during chronic HBV infection.

Until recently, the molecular mechanisms underpinning NK cell dysfunction in chronic HBV had not been described. A recent study of circulating NK cells from individuals in the chronic infection phase of HBV found $CD56^{dim}$ NK cells had altered activation marker expression (increased NKG2D but decreased NKp30 and CD16), preserved cytotoxity but decreased antibody mediated cytokine production (214). This was associated with a transcriptional signature similar to dysfunctional exhausted CD8 T cells in chronic HBV infection, characterised by increased expression of the transcription factor *TOX* (214). Hyporesponsive CD8 T cells in chronic HBV infection express high levels of the checkpoint inhibitor PD-1 (227), and recently its ligand PD-L1 has been found to be upregulated on liver NK cells in chronic HBV which inhibit HBV specific CD8 T cells (Mariana Diniz and Mala Maini, personal correspondence and manuscript in preparation). In HBV transgenic mice activated NK cells can inhibit HBV replication (228), an effect that can be enhanced by *in-vivo* blockade of the inhibitory PD-1 – PDL1 signalling pathway (229). Together these data suggest the dysfunction of both NK cells and T cells in chronic HBV infection might have shared molecular mechanisms which might be reversed by preventing the PD-1 - PDL1 signalling.

1.5.4 Adaptive immunity in chronic infection

1.5.4.1 T cells

In comparison with the robust T cell response necessary for the clearance of acute HBV, T cells are dysfunctional and depleted in chronic infection (Figure 1.6). Circulating HBV

specific T cells are detectable in much lower frequencies in chronic compared to acute infection and often require *in-vitro* expansion with cognate peptides to have sufficient numbers for functional analysis. These T cells are less able to proliferate, less cytotoxic and less able to produce proinflammatory cytokines such as IFNγ on restimulation with HBV peptides (230–236).

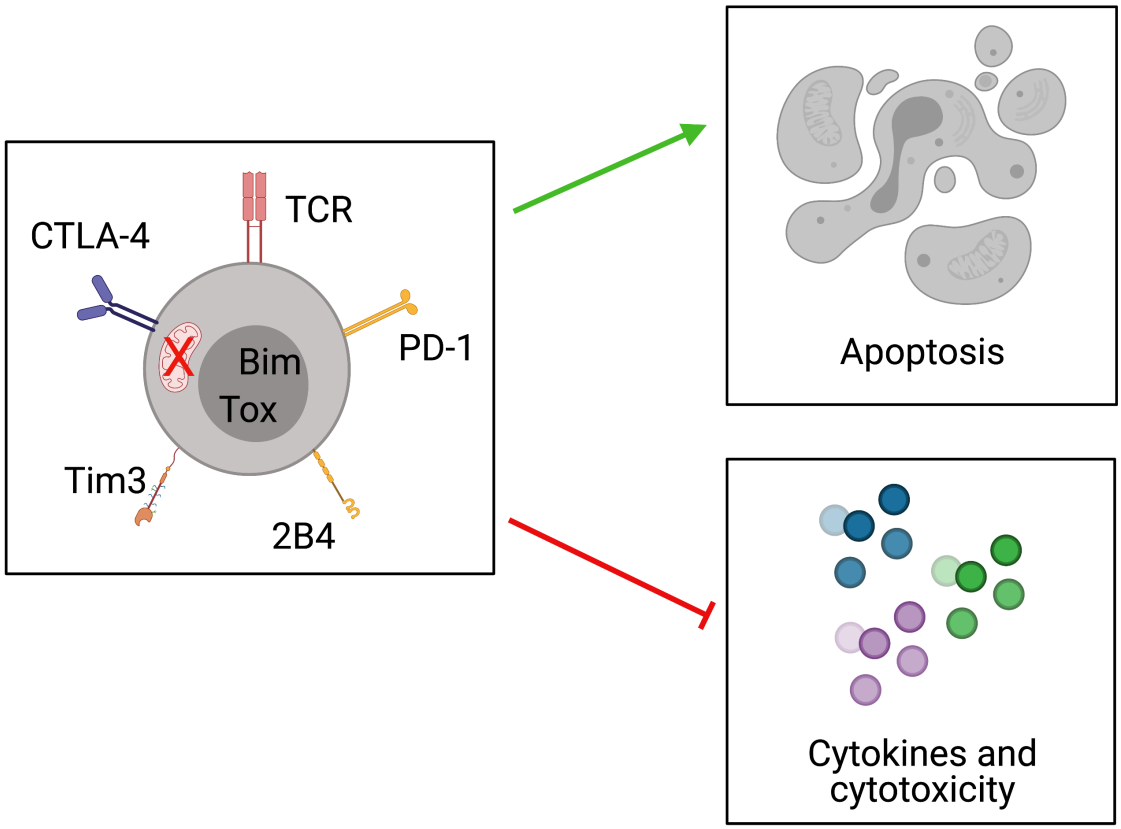


Figure 1.6: Mechanisms of T cell dysfunction in chronic HBV

HBV specific T cells develop mitochondrial dysfunction and upregulate transcription factors such as *Bim* and *TOX*. The surface expression of immune checkpoints such as PD-1, 2B4, Tim3 and CTLA-4 is increased. These changes lead to the decreased function of HBV specific T cells including reduced cytokine production and cytotoxicity. HBV specific T cells are more prone to deletion by apoptosis. Image created using BioRender.com

The unique tolerogenic microenvironment of the liver contributes to the HBV specific T cell hyporesponsiveness associated with chronic HBV infection (reviewed in (237)). The liver receives constant high antigenic loads from food and gut microorganisms via the portal vein

and has developed several mechanisms to restrict immune responses primed in the liver to such antigens. For example antigen presentation by liver sinusoidal epithelial cells, plasmacytoid dendritic cells and hepatocytes can downregulate antigen specific T cell activation and lead to T cell apoptosis (238). As HBV is a hepatotropic organism, much of the adaptive immune response will be primed in this anti-inflammatory liver environment. Individuals in the HBeAg positive chronic infection stage of chronic HBV have low immune mediated liver damage in the face of high HBV antigens (8), indicating the immune response is tolerant of HBV infection. There is evidence that immunoproteasome and antigen presentation related transport proteins are downregulated in chronic HBV infection, which might promote T cell anergy (239). The development of HBV chronicity as opposed to resolution after initial infection, might be due to an imbalance of tolerogenic mechanisms favouring the development of unreactive HBV specific T cells (82).

T cell dysfunction has been described in the context of other chronic viral infections and cancer as well as HBV (240). Increased immune checkpoint mediated inhibition as a response to repetitive antigen stimulation over long periods is one mechanism that might drive this phenomenon. Surface expression of immune checkpoint receptors including PD-1, 2B4, CTLA-4 and Tim-3 are increased on HBV specific T cells in chronic infection (230,241–245) and their inhibition *in-vitro* improves T cell function (230,243,246,247). Liver derived T cells, which by their locality have the highest exposure to HBV antigens, have higher PD-1 expression than circulating T cells, supporting that repetitive stimulation by HBV antigens is associated with immune checkpoint expression (18,227,241). Of the various immune checkpoints described, the PD-1 – PDL1 signalling pathway has been found to contribute the most to the dysfunction of HBV specific T cells, making it an attractive therapeutic target for interventions to restore HBV specific cellular immunity (227).

As well as phenotypic changes on their cell surface, HBV specific T cells also have an altered transcriptional program which parallels T cell exhaustion in other settings. Recently the transcription factor *TOX* has been found to be upregulated on HBV specific T cells in chronic infection and correlates with PD-1 expression and T cell dysfunction (141). The pro-apoptotic transcription factor *Bim* is also upregulated, which has led to the hypothesis that apoptotic deletion of exhausted cells might be the reason for low *ex-vivo* frequencies of HBV specific T during chronic infection (243,248). Transcriptional downregulation of genes associated with mitochondrial metabolism (249) and the pro-inflammatory transcription factor *T-bet* (250) have also been described in HBV specific T cells. Overall, this evidence suggests that transcriptional disruption in HBV specific T cells results in metabolic dysregulation, reduced effector functions and apoptotic deletion.

Recent evidence suggests that T cell exhaustion associated with HBV infection is heterogeneous, with T cells of different specificities appearing differentially affected. Circulating T cells specific to HBV core or polymerase are more abundant than surface specific T cells, which are often only detectable after *in-vitro* expansion with cognate peptides (227,230,232–234,251,252). Surface specific T cells have the highest PD-1 expression (252) and their frequencies correlate negatively with the number of years an individual has been exposed to HBsAg during chronic infection (253). This implies they have the most severely exhausted phenotype and are preferentially deleted by apoptosis. Marked differences have also been observed between core and polymerase specific T cells, with a subset of polymerase specific cells having a naïve T cell phenotype (251,252). These observations suggest core specific T cells may be the most functionally recoverable with immunotherapy.

Differences in HBV specific T cell function and phenotype in chronic HBV infection also exist depending on location, particularly between T cells derived from the circulation and liver compartments (234,254). The majority of liver derived HBV specific T cells have a phenotype consistent with resident memory T cells, which permanently occupy the liver niche without recirculating in the bloodstream (18). Despite expressing high levels of PD-1, they readily produce proinflammatory cytokines on restimulation with cognate HBV peptides, suggesting they are active in the local control of HBV during chronic infection (18). These intrahepatic cells might represent the best target for immunotherapies aiming to boost existing HBV cellular immunity to eradicate infection. Future studies should prioritise intrahepatic sampling and specimens from biopsy or liver transplant, to further evaluate the interaction between intrahepatic T cells and infected hepatocytes in HBV infection.

1.5.4.2 B cells

Although less well studied than their T cell counterparts, B cells play several roles during chronic Hepatitis B infection including antibody production, antigen presentation to T cells and cytokine production (reviewed in (180)).

Approximately 15% of the intrahepatic inflammatory infiltrate in chronic HBV is comprised of B cells, and CD20 expression in the liver has been observed to be positively correlated with histological activity (255,256). The frequency of circulating B cells is increased in chronic HBV compared to healthy persons (257–259) and global B cell populations have phenotypic and transcriptional signatures consistent with B cell activation (260,261). The HBeAg positive or negative chronic hepatitis phases of HBV are associated with increased expression of B cell associated genes in the circulation and liver (262,263). Together these

observations suggest B cells might play a role in liver inflammation during chronic HBV infection.

Circulating regulatory B cells (Breg), characterised by a high surface expression of CD24 and CD38 and the production of anti-inflammatory cytokine IL-10, are also expanded in chronic HBV (264–266). The frequency of Bregs negatively correlated with Th1 helper T cells and led to reduced CD4 T cell production of proinflammatory cytokines such as IFNγ and IL-17 in *ex-vivo* co-culture (265,266). In addition, co-culture with Bregs led to CD4 T cells differentiating towards a regulatory T cell phenotype (Treg) (266). This suggests Bregs might mediate the suppression of pro-inflammatory helper T cell responses during chronic infection.

The contribution of Bregs or Breg derived IL-10 towards immune mediate liver damage during chronic HBV is less clear. One study found a positive correlation between Breg frequency and hepatic flares, and IL-10 with serum ALT levels (264). However, a larger study found Breg frequencies negatively correlated with serum ALT (266). Further studies to understand the functions of Bregs *in-vivo* during chronic HBV infection are required to resolve these discrepancies.

Unlike T cell responses which are globally supressed, the production of anti-HBc Ab and anti-HBe Ab in chronic HBV is preserved, whilst anti-HBs Ab is undetectable (267). This appears in part to be a consequence of differences in the phenotype and function of HBc and HBs specific B cells (268), of which the latter are comparatively depleted and dysfunctional (192,253,269).

HBc specific B cells are abundant in chronic HBV infection, have a classical B memory phenotype and can differentiate into antibody secreting cells (270,268). Over the course of chronic infection, serum anti-HBc Ab levels differ between the various clinical phases of disease. Quantitative anti-HBc Ab levels are higher in HBeAg positive or negative chronic hepatitis where liver damage is evident, and positively correlate with serum ALT levels (271,272). On liver biopsy, the localisation of HBcAg expression in infected hepatocytes is associated with histological activity index. During the HBeAg positive chronic infection phase, characterised by low liver inflammation and high HBV DNA, HBcAg is expressed in hepatocyte nuclei but during chronic hepatitis when liver inflammation is evident, HBcAg expression switches to the cytoplasm (273–276). In persons with HBeAg negative chronic infection or when HBV DNA is suppressed by successful nucleos(t)ide treatment, HBcAg can become undetectable in hepatocytes (273,274,277,278). Together these data support that cytoplasmic HBcAg expression and serum anti-HBc Ab levels are associated with liver damage in chronic HBV infection, although whether the antigen or antibody is directly responsible is not yet clear.

The disappearance of HBcAg expression in hepatocytes during the HBeAg negative chronic infection phase of HBV might be a result of anti-HBc Ab mediated effects or alternatively might reflect a more generalised immune control of HBV. In a cohort of untreated HBeAg negative HBsAg positive individuals followed up for over a decade, anti-HBc Ab levels were a significant predictor of HBV DNA suppression and HBsAg loss (279). Several studies of persons with HBeAg positive chronic HBV have found higher baseline anti-HBc Ab levels predict HBeAg seroconversion in response to treatment with either PegIFNα or nucleos(t)ide analogues (280–283). Higher anti-HBc Ab levels were also associated with reduced relapse after withdrawal of nucleos(t)ide analogue therapy (284). These associations support that

anti-HBc Abs play an active role in HBV immunological control but are not yet sufficient to prove causality.

In contrast to HBc specific B cells, HBs specific B cells are less frequent in chronic HBV (268). HBs specific B cells derived from persons with chronic HBV infection produce less HBsAg than HBs specific B cells derived from healthy individuals after vaccination with recombinant HBV vaccine (192). They are ineffective at differentiating from memory B cells into antibody secreting plasma cells and produce less proinflammatory cytokines such as IL-6 or TNFα after B cell receptor dependent or independent stimulation (192,269). In chronic HBV infection, HBs specific B cells have an atypical memory phenotype, characterised by low CD21 and CD27 expression and express inhibitory markers such as Fc receptor IIb and PD1 (192,269). In those with acute HBV the proportion HBs specific B cells with an atypical phenotype is reduced (192), implying restoration of HBs specific B cell function might be important to achieve HBsAg loss. The function of HBs specific B cells can be partially restored by *in-vitro* PD1 blockade (192,269), suggesting that inhibiting PD1 signalling might be an effective therapeutic target.

It is unclear why HBs B cells seem to be selectively dysfunctional with an atypical memory phenotype, when HBc B cell functions are preserved. Atypical memory B cells have been described in other chronic viral infections in response to chronic antigen stimulation (285). Exposure to the persistently high levels of HBsAg across decades of HBV infection might drive selective HBs B cell dysfunction. This parallels the observations in T cells, where HBV surface specific T cells are less frequent and more exhausted in chronic HBV, and length of infection and HBsAg level is associated with HBV surface T cell dysfunction (253).

Overall, although global B cells are expanded and activated in chronic HBV, there is selective dysfunction of HBs specific B cells, which are less able to produce neutralising HBsAg to contribute to immunological control of the virus. Common mechanisms of B and T cell dysfunction such as increased expression of PD-1, imply PD-1 might be a useful therapeutic target to restore immunity across both arms of the adaptive immune system in chronic HBV.

1.6 IMMUNOLOGICAL AIMS OF THERAPEUTIC VACCINATION STRATEGIES

HBV therapeutic vaccination aims to induce new or restore existing HBV specific immune responses, by administrating non-infective HBV antigens for presentation to cognate T and B cells. The recovery of HBV specific responses occurs in a minority of individuals with chronic infection, in whom HBsAg becomes undetectable signifying functional cure (16). In such persons cellular immune responses are functionally restored, akin to HBV specific T cell responses in people that have resolved acute HBV infection (17). A better understanding of cellular immune responses in both acute and chronic HBV infection has enabled the rational design of HBV therapeutic vaccination strategies.

T cells are integral for immune control of HBV but are dysfunctional in chronic infection. Both CD8 and CD4 T cell responses towards multiple HBV antigens are necessary for resolution of infection (178). In particular CD8 T cells in the liver with a resident memory phenotype contribute to the containment of HBV (18) and non-hepatotoxic mechanisms of control such as production of proinflammatory cytokines including IFNγ are important to minimised immune induced liver injury (164,177). In chronic infection, T cell responses are hampered by the tolerogenic liver environment and repetitive antigen exposure, which fosters the development of molecular and functional T cell exhaustion (141,227). Therefore,

therapeutic vaccines should aim to induce a balance of CD8 and CD4 responses directed at multiple HBV antigens including core, polymerase and surface. The magnitude of CD8 T cell responses within the liver itself are likely to be crucial and should be maximised to overcome the tolerogenic signals of chronic HBV infection. The immunogen design, choice of vaccine platform and route of vaccination should all aim to induce CD8 and CD4 HBV specific T cells of high magnitude, wide breadth and high cytokine production in the liver.

The production of anti-HBs Ab to neutralise HBsAg also plays an important contribution to the development of HBV functional cure (179). HBs specific B cells are less efficient at differentiating into antibody secreting plasma cells in chronic HBV, which might contribute to the persistence of infection (192). HBV therapeutic vaccine strategies should therefore aim to induce high titres of anti-HBs Ab alongside high magnitude T cell responses to achieve the highest chances of success.

1.7 PREVIOUS CLINICAL TRIALS OF THERAPEUTIC VACCINATION FOR CHRONIC HBV TREATMENT

There have been numerous clinical trials evaluating various vaccine candidates for therapy in chronic HBV over the last 20 years (Appendix 1). As yet, none have been successful in inducing functional cure in participants, however the results of these studies are important to inform the design of future vaccine candidates and vaccine trials, to maximise their chances of success.

1.7.1 Recombinant vaccines

The earliest vaccination studies for the treatment of HBV investigated recombinant HBsAg as a therapeutic agent (Appendix 1, Table 1). These vaccines contain the small or middle

HBV surface protein, comprising the surface or surface/pre-S2 regions. Recombinant HBsAg vaccines are one of the cornerstone interventions recommended by the WHO to prevent new HBV infections (1), as they induce protective anti-HBsAg and CD4 T cell responses in uninfected persons (286–288). Their more widespread use in areas with HBV endemicity is thought to be responsible for the recently reduced incidence of new HBV infections in young children (58).

The first report describing the experience of recombinant vaccines for the treatment of chronic HBV was published in 1994 (289). In this cohort of 32 patients vaccinated with recombinant vaccine Gen Hevac B, the cumulative incidence of DNA suppression was 10 of 32 participants (31%) at 6 months after vaccination (289). This led to studies comparing recombinant HBV vaccine to no treatment in adults or children with chronic HBV (290–294). Although one non-randomised study found a significant reduction in HBV DNA compared to baseline in the vaccination group (291), in all randomised studies there were no differences in virological parameters including HBV DNA suppression, HBeAg loss or HBsAg loss between the vaccination and no treatment arms (289,290,292–294). Immunological outcomes were investigated in a subset of participants in only one of these studies (295). HBV surface specific CD4 T cell responses were detectable in under 20% of vaccinated participants and anti-HBsAg titres increased from baseline in 25% of participants in the vaccination group compared to 0% in the untreated group (295). This suggests that the majority of individuals with chronic HBV infection do not mount an HBV surface specific immune response when vaccinated with recombinant HBsAg. It also implies that even in those that do have an immunological response to vaccination, it is not of sufficient magnitude or quality to alter HBV virological parameters.

Subsequent trials compared groups receiving vaccination with or without antivirals to groups treated with antivirals alone (nucleos(t)ide analogues and/or pegIFNα therapy) (296–302). In four of seven studies, no convincing differences in HBV DNA suppression, HBeAg loss or HBsAg loss were observed between groups (296,298,300,301). In regimes containing pegIFNα therapy, the addition of nucleos(t)ide analogues but not therapeutic vaccine increased HBV DNA suppression (297,298). Two of five trials comparing combination therapy with vaccine and nucleos(t)side analogues found vaccination added additional benefit to nucleos(t)side analogue monotherapy in HBeAg positive participants (296,299–302). In the two studies that measured anti-HBsAg levels, antibodies were induced in between 42 and 83% of those receiving recombinant HBV vaccine but remained undetectable in unvaccinated individuals (300,302). HBV surface specific T cells were detectable in vaccinated subjects in one study (302), but in another study the percentage of participants with detectable surface specific CD4 T cells did not increase after vaccination (296). These findings suggest that recombinant HBV vaccination can induce CD4 T cell and antibody responses towards HBV surface antigen in some participants, but this response does not reliably alter chronic HBV viral infection in comparison to standard treatment regimes.

The majority of these early studies of recombinant HBV vaccination recruited individuals with raised HBV DNA and high antigen loads, which might promote and maintain T cell tolerance to HBV and reduce therapeutic vaccine efficacy (13). Two more recent studies have addressed this by limiting recruitment to HBeAg negative individuals with low quantitative HBsAg titres and suppressed HBV DNA. In a retrospective study, vaccination was not associated with HBV relapse prevention after nucleos(t)ide analogue withdrawal (303). Another non-randomised study found vaccination was associated with reduction and loss of

HBsAg in some participants, although randomised trials will be necessary to provide more robust evidence of this association (304).

Recombinant HBsAg vaccines were modified to combine hepatitis B immunoglobulin with HBsAg, aiming to improve the efficacy of vaccination (Appendix 1, Table 2, (305–309)). Initial results of small non-randomised phase I studies using this approach were promising, with HBV DNA suppression and HBeAg seroconversion occurring in a proportion of vaccinated individuals (305,306). However, ultimately in larger trials no significant differences in HBV DNA suppression occurred after vaccination compared to placebo (307–309). Another recombinant vaccine CVI-HBV-002 delivering the large, medium and small HBV surface proteins (surface/pre-S2/Pre-s1 regions) with adjuvant (L-pampo) was shown to induce anti-HBsAg responses in HBV-transgenic mice, although T cell responses were not investigated (310). This vaccine is currently in phase II clinical trials in chronic HBV patients, although results are yet to be reported (311).

Expansion of the antigen repertoire of recombinant HBV protein vaccines has also been attempted, to better mirror successful immune responses towards acute HBV in which T cell responses to multiple HBV antigens occur (178). A vaccine containing recombinant HBcAg with HBsAg was superior to pegIFNα monotherapy at supressing HBV DNA levels at 24 weeks after treatment completion in persons with HBeAg positive or negative chronic hepatitis, although HBsAg loss was not investigated (312). Further studies evaluating this vaccine in combination with nucleos(t)ide analogues or pegIFNα will be necessary to assess its ability to induce functional cure and assess the immunological response it induces in individuals with chronic HBV infection.

Overall, recombinant vaccination is not efficacious in inducing either partial or functional cure in chronic HBV compared to no treatment or established antiviral therapy. The predominantly CD4 and anti-HBsAg humoral response induced by vaccination can block viral entry and prevent infection in healthy persons. However in chronic infection immune restoration is likely to require CD8 as well as CD4 T cell responses (178), which recombinant vaccination does not readily induce.

1.7.2 Lipopeptide epitope-based vaccines

As it was clear that CD8 cell responses were required for HBV control (178), a therapeutic vaccine candidate CY-1889 (Appendix 1, Table 3) was designed based on a CD8 epitope towards HBV core (Core $_{18\text{-}27}$), induced in controllers of acute HBV (176). Unfortunately, CY-1889 did not lead to HBV DNA suppression in persons with untreated HBeAg positive or negative chronic hepatitis after 52 weeks of follow up (313). Vaccine induced cytotoxic CD8 T cell responses were lower in individuals with chronic HBV infection (313) in comparison to the responses induced in healthy volunteers (314). The poor efficacy of this vaccine may have been in part due to the dysfunction and exhaustion of T cells in chronic HBV, rendering them less receptive to boosting by vaccination. However, there are several additional disadvantages to a single epitope vaccine such as CY-1889, which are able to induce T cells of limited specificity. Such responses are unlikely to be broad enough to induce the immune control of HBV, are more likely to select for immune escape variants and will only be suitable for use in persons with complimentary HLA haplotypes.

1.7.3 DNA vaccines

Advantages of plasmid DNA vectors including relative ease of immunogen manipulation, long term stability and cost effective manufacture, made plasmid DNA vaccine platforms

attractive for the generation of therapeutic vaccines (315). Therapeutic DNA vaccines for HBV are summarised in Appendix 1, Table 4.

Early plasmid DNA therapeutic vaccine candidates were limited to expressing the HBV surface/preS-2 regions and did not improve HBV virological serological markers when compared to placebo or nucleos(t)ide analogue therapy (316,317). HBV surface T cell responses were induced in only a proportion of participants (316,317). Furthermore, vaccination was unable to prevent relapse after withdrawal of nucleos(t)ide analogue therapy (318). Aiming to increase the magnitude of T cell responses induced by vaccination, a plasmid DNA vaccine was combined in heterologous prime-boost strategies with an MVA vectored vaccine encoding the HBV surface/preS-2 regions (319). However this strategy also failed to alter HBV virological parameters or induce strong T cell responses in persons with chronic infection (319).

It is likely these vaccines were not immunogenic in the tolerogenic environment engendered by chronic HBV infection and were further constrained by including only the HBV surface regions. However the use of plasmid DNA vectors has been limited in general, as they do not appear to be as immunogenic in large mammals including humans, compared to small animals such as mice (315). In order to overcome these issues, HBV therapeutic vaccine candidates HB-100 and its later iteration HB-110 encoded HBV surface, core and polymerase regions within the immunogen together with human IL-12 as an adjuvant to improve vaccine induced T cell responses (320,321). However, in a randomised controlled trial of DNA vaccine HB-110 with nucleos(t)ide therapy compared to nucleos(t)ide monotherapy, there was no significant difference in HBV DNA suppression between arms (321). Polyfunctional

CD8 T cell responses were induced, but only in approximately a quarter of those vaccinated (321).

Other modifications have also been employed to enhance the immunogenicity of DNA plasmid vaccination for chronic HBV. A short electrical pulse, termed electroporation, at the time of vaccination has been shown to enhance plasmid DNA transfection and increase vaccine induced cellular immune responses (315). Two randomised trials have investigated combined nucleos(t)ide therapy with plasmid DNA vaccine encoding HBV surface/pre-S2 and IL-2/IFN-γ adjuvant, administered with electroporation in comparison with nucleos(t)ide monotherapy (322,323). Although increased IFNγ HBV specific CD8 T cells were detectable in the vaccination compared to control group after *in-vitro* expansion, there was no significant difference in HBV DNA suppression between groups and no individuals lost HBsAg (323). A trial of HBV vaccine candidate JNJ-64300535 using electroporation for delivery is ongoing (NCT03463369) and results from this will provide further evidence of the utility of using this strategy in chronic HBV (324).

Despite the optimisation of DNA plasmid vaccines, they have been unable to induce high magnitude HBV specific T cell responses in individuals with chronic HBV infection to date, suggesting these vaccines are unable to overcome the HBV specific T cell dysfunction characteristic of chronic infection. Vaccine platforms with greater immunogenicity and established success in generating high magnitude immune responses in healthy persons may be required to overcome HBV related T cell exhaustion.

1.7.4 Yeast derived vaccines

Recent HBV therapeutic vaccine designs have focused on the inclusion of a several HBV antigens with relatively conserved sequences across different HBV genotypes (Appendix 1, Table 5). The yeast derived vaccine GS-4774, encoding HBsAg, HBcAg and hepatitis B X within its immunogen, has been shown to induce HBV specific T cell responses in healthy volunteers (325). However in subsequent randomised trials in patients with chronic HBV infection, vaccination has not induced large declines in HBsAg levels or led to HBsAg loss (326,327).

T cell responses induced by GS-4774 vaccination in persons with chronic HBV were lower than those in healthy persons (325,326), likely due to the T cell exhaustion of HBV specific T cells in chronic infection. However in healthy persons the majority of vaccine induced responses were of low-moderate magnitude (below 200 spot forming units per million cells in IFNγ ELISpot assays (325)) and might not be sufficient to generate efficacy in the setting of chronic HBV infection.

1.7.5 Adenoviral vectored vaccines

Adenoviral vector vaccines are able to generate high magnitude cellular immune responses in humans (328). TG-1050 is human adenoviral vectored vaccine encoding HBV core, polymerase and surface antigens, has been evaluated in a phase I clinical trial of individuals with chronic HBV (Appendix 1, Table 6, (329)). TG-1050 administered either as a single dose or in homologous prime-boost regimes with nucleos(t)ide therapy was compared to nucleos(t)ide monotherapy. HBsAg loss of over 0.2 $\log_{10}$ IU/mL was achieved in 19% of vaccinated (n=7) compared to 8% of unvaccinated (n=1) participants and, similarly to other therapeutic vaccine trials, induced T cell responses in only a proportion of participants (329).

As the immunogenicity of TG-1050 has not been tested in healthy persons, it is difficult to tell whether this vaccine is not immunogenic in general or only in the context of chronic HBV infection.

Thus far therapeutic vaccines trialled in chronic HBV therapy have suffered from poor immunogenicity either due to the vaccine itself or due to host and viral factors relating to chronic HBV infection. It is therefore highly likely that different strategies will be required to optimise the effectiveness of HBV therapeutic vaccination going forward.

1.8 OPTIMISING THERAPEUTIC VACCINATION STRATEGIES

Previous trials of therapeutic vaccines for the treatment of chronic HBV have not led to improvements in partial or functional cure rates. HBV vaccine candidates do not reliably induce strong cellular or humoral immune responses in persons with chronic HBV infection, despite being able to induce immune responses in healthy persons in some cases (286–288,314,325,330–333). Several strategies to improve vaccine immunogenicity in the context of chronic HBV infection are under development and investigation. These aim to either maximise the ability of the vaccine itself to induce immune responses of the best magnitude and quality possible or minimise the inhibitory effects of chronic HBV infection on immune response generation.

1.8.1 Maximising the immunogenicity of vaccination strategies

1.8.1.1 Vaccine platform and regime

Therapeutic vaccines for HBV therapy need to use vaccine platforms that induce immune responses of the highest quality and magnitude possible, to have a chance of generating responses that are sufficient to control HBV infection and induce functional cure.

Virus like particles (VLP) are composed of structural protein(s) of a virus but lack viral genetic material and when used as vaccines induce strong cellular and humoral immune responses (334). The vaccine candidate VBI-2601 utilises a VLP platform and is currently being evaluated in a phase Ia/IIb randomised trial in persons with chronic HBV (ACTRN12619001210167) (335). Unofficial interim results suggest both T cell and antibody responses were induced in trial participants but further detailed information is yet to be released (336).

An alternative platform for vaccine delivery might be mRNA, which has recently been successful in inducing immune responses that protect against severe infection with SARS-CoV-2 (reviewed in (337)). The mRNA platform has not been used for HBV therapeutic vaccine development as yet but is an appealing option for future development.

Another way to induce strong cellular and humoral responses is to administer vaccines using different platforms in heterologous prime-boost strategies. In murine models of chronic HBV heterologous vaccination has been shown to induce HBV functional cure (338–340). This approach delivers particulate HBsAg and HBcAg as the prime vaccine which is later followed by a boost vaccine using a modified vaccinia virus Ankara (MVA) vector (338).

Viral vector vaccination using adenoviral and/or MVA vectors in heterologous combination are another promising option for HBV vaccine development. The combination of delivering the initial vaccine using a chimpanzee adenoviral (ChAd) vector (prime) followed by an MVA vectored vaccine (boost), has been shown to induce high magnitude cellular and humoral responses in humans (328,341). Chimpanzee adenoviral vectors in particular have

come to the fore during the development of Covid-19 vaccines, which has demonstrated they can be manufactured rapidly at scale and have minimal side effects in humans (342–345). Large immunogen sequences can be encoded using these vectors, which increases the capacity for HBV antigen delivery. An HBV therapeutic vaccine regime using ChAd vector ChAdOx1-HBV and MVA vector MVA-HBV were highly immunogenic in healthy mice and induced responses to all major HBV antigens (Chapter 3, (346)). ChAdOx1-HBV is being tested in healthy participants and persons with chronic HBV in a phase I clinical trial (NCT04297917, Chapter 5, (347)) and together with MVA-HBV and check-point inhibitor therapy in a phase II clinical trial (NCT04778904, (348)). Another clinical trial assessing heterologous combinations of ChAd vector, MVA vector and HBsAg and HBcAg protein vaccines for chronic HBV therapy is underway (GSK3528869A, NCT03866187) but has yet to report results (349).

1.8.1.2 Immunogen design

Ideally, HBV vaccine immunogens would induce strong T cell responses to multiple HBV antigens, to emulate the successful immune response in those that clear acute infection (178). In addition, an ideal immunogen would induce effective responses in people with different HLA haplotypes or infected with different HBV genotypes to maximise efficacy across different populations. One approach to achieve this is to include large immunogens containing largely unaltered viral sequence, as in the ChAdOx1-HBV and MVA-HBV vaccines (346). Alternatively, conserved regions of the HBV genome can be used, which are more likely to induce responses across multiple HBV genotypes and HLA haplotypes. Peptide vaccine FP-02.2 (HepTcell) uses this approach, combining 9 HBV T cell epitopes within conserved regions of the HBV genome. This vaccine has been shown to be safe in a phase I study of persons with chronic HBV NCT02496897) and a phase II trial is ongoing

(NCT04684914) (350–352). Further work to understand protective T cell epitopes induced by acute HBV infection in the context of a variety of HLA backgrounds will further inform vaccine design going forward.

1.8.1.3 Route of vaccination

The magnitude and quality of the immune response induced by vaccination can be altered by using different routes of immunisation. In the context of chronic HBV, T cell responses in the liver have been shown to be important for local immune control at the site of infection (18). One strategy is to 'pull' T cells primed in the periphery into the liver. This can be done by administering ChAd vaccine (prime) intramuscularly, followed with an MVA (boost) vaccination delivered intravenously. In mice this regime improved the prevention of liver stage malaria after vaccination (353) and increased the magnitude of intrahepatic HBV specific T cell responses to vaccination with ChAdOx1-HBV and MVA-HBV (Chapter 2, (354)). Although this strategy requires further evaluation in murine models of chronic HBV to assess its efficacy, the use of intravenous vaccination might be another way of improving the efficacy of HBV therapeutic vaccination. Future vaccine trials in chronic HBV should evaluate the immune response induced by vaccination in the liver itself for example by fine needle aspiration (254), to better inform vaccine design.

1.8.2 Minimising the inhibitory effects of chronic HBV infection on immune response generation

1.8.2.1 Antigen reduction

Using vaccines with the highest immunogenic potential might not be enough to induce successful functional cure in persons with chronic HBV, in whom HBV specific immune responses are dysfunctional. Additional interventions might be required to overcome this.

Reducing quantitative HBsAg levels is one approach that has been shown to improve the efficacy of therapeutic vaccination in animal models of chronic HBV infection (338,355,356). In persons infected with chronic HBV, lower titres of HBsAg at the time of nucleos(t)ide therapy withdrawal are associated with HBsAg loss during follow up (357). Limiting recruitment of vaccine studies to persons with low HBsAg levels, might increase the chances of therapeutic success. Therapies that directly target HBsAg to reduce levels prior to vaccination such as small interference RNA (siRNA) or nucleic acid polymers (NAPs) (358–360) might also be useful in combination therapy. This approach could also enable groups with high HBsAg but relatively persevered HBV specific T cell responses (those in the HBeAg positive chronic infection phase) to be treated with therapeutic vaccine after HBsAg reduction therapy (253).

1.8.2.2 Immune checkpoint inhibition

Reversal of the HBV specific immune dysfunction in individuals with chronic HBV infection might be required before a therapeutic vaccination can be effective. T cell function can be rescued *in-vitro* using check point inhibitor blockage particularly towards the PD1 - PDL1 signalling pathway (227). In the woodchuck model of chronic woodchuck hepatitis virus infection, which shares 80% homology with Hepatitis B virus, HBsAg loss was induced by a combination of PD1 blockade with checkpoint inhibitor Nivolumab together with therapeutic vaccination (361). A small pilot study showed low dose Nivolumab alone or in combination with therapeutic vaccine GS-4774 was safe and one participant, who received Nivolumab alone, lost HBsAg (362). Recently a phase II clinical trial evaluating therapeutic HBV vaccines ChAdOx1-HBV and MVA-HBV in combination with Nivolumab has commenced recruitment (348). Results from this trial will be pivotal in our understanding of whether

immune checkpoint inhibition in combination with therapeutic vaccination might be an effective strategy to induce HBV functional cure.

1.9 DESIGN AND ENDPOINTS FOR CLINICAL TRIALS OF THERAPEUTIC VACCINATION

Developments in the definition of HBV cure have led to international discussion about the design of clinical trials for novel HBV therapies, to ensure results can be interpreted consistently (9,109). This has resulted in consensus recommendations endorsed by the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) covering participant selection, assessment of endpoints and the interpretation of ALT fluctuations in the clinical trial setting (9,109).

1.9.1 Participant selection

As chronic HBV is a heterogeneous disease, there are several aspects to consider when specifying clinical trial study populations (109). As the phases of chronic HBV infection reflect the interaction of the immune system with HBV and infected hepatocytes, the phase of infection is particularly important when considering populations to enrol for trials of immunotherapies.

In the HBeAg positive chronic infection phase, current treatment is not beneficial and therefore not recommended (8). However there remains a risk of progression through other stages of HBV infection and a lifetime risk of HCC development. Therefore this population arguably have most to gain long-term from novel treatment that induces functional cure (109). However, individuals in this phase might not be the optimal population to trial immunotherapeutic strategies in the first instance, as they have high HBsAg levels which

might impede recovery of HBV specific immunity (13). On the other hand, T cells during this phase of infection are less exhausted than at later phases and therefore might have a lower barrier to functional restoration (253).

Individuals in the HBeAg positive or negative chronic hepatitis phases are at highest risk for adverse liver outcomes and therapy is recommended by current guidelines (8). These populations have the highest need for effective therapy that induces functional cure in the short to medium term (109). However, because individuals in these groups have liver inflammation by definition, they might also be more likely to have on-therapy ALT increases which can be challenging to interpret (363).

In the case of clinical trials of immunotherapies, a lower risk strategy might be to conduct initial studies in persons with established HBV DNA suppression and ALT normalisation on nucleos(t)ide analogue therapy. In these circumstances, on therapy ALT flares are more easily interpreted as being immune mediated as a direct result of successful therapy, rather than as a consequence of viral reactivation (109). An additional advantage of conducting trials in this population is that many of them will have lower HBsAg levels at entry, which might increase the chances of functional cure being achieved, as has been shown in animal studies (355,356).

The HBV genotype of individuals is another important consideration of trial design, as it may impact on response to treatment (109,363). Immunotherapies based on a particular HBV genotype, might be restricted to persons with a complementary genotype and/or HLA haplotype. Other approaches include the recruitment of allcomers irrespective of genotype, to improve the generalisability of trial results (109,363).

Finally, it has been recommended that initial clinical trials should not be conducted in certain populations for example in those with cirrhosis, in whom drug induced ALT flares could lead to hepatic decompensation (109). Novel treatments would need an established safety and efficacy record in lower risk populations first, to justify moving into higher risk populations.

1.9.2 Evaluation of responses to HBV therapy and clinical trial endpoints

As well as routinely used serological parameters including HBsAg, HBeAg and HBV DNA levels, several non-invasive biomarkers of HBV viral activity and HBV induced liver damage are under development. Recommendations on how these biomarkers should be used to assess end points of clinical trials aiming to induce functional cure have been published (9,109).

The sustained loss of HBsAg is central to the definition of functional cure. An international consensus agreed the primary end point for phase III clinical trials of HBV therapy should be functional cure, defined by undetectable HBsAg measured at least twice 6 months or more apart, alongside sustained HBV DNA suppression (109).

HBsAg kinetics as measured by quantification of serum HBsAg (qHBsAg) might also be useful as an exploratory endpoint in the clinical trial setting to predict subsequent functional cure after therapy (109). qHBsAg detects HBsAg in all its forms (infectious virions as well as subviral particles), and is transcribed by both HBV cccDNA and integrated DNA (364). In the clinic, qHBsAg kinetics have shown utility in predicting non-response to pegylated interferon-alpha treatment in certain settings (364–367). Recently baseline qHBsAg levels were shown to predicted subsequent HBsAg loss after therapy in a clinical trial of combined pegIFNα and nucleos(t)ide analogue treatment (368). However safe withdrawal of

nucleos(t)ide analogue therapy cannot be predicted reliably using qHBsAg alone (8,364,369,370) and whether qHBsAg kinetics will predict HBsAg loss after treatment with novel therapies remains to be determined (109). Therefore reductions in qHBsAg are recommended only as an exploratory end point for clinical trials at present (109).

The elimination of cccDNA is another potential outcome of interest in response to therapy. Accurate estimates of hepatic cccDNA content can be made using liver biopsy, however this is an invasive test that is not often indicated in clinical practise (8). Several surrogate non-invasive biomarkers to estimate hepatic cccDNA have been evaluated including qHBsAg, HBV RNA and HBV core related antigen (HBcrAg). qHBsAg levels have been found to moderately correlate with cccDNA, however they do not accurately estimate cccDNA activity in HBeAg negative persons, in whom a large proportion of HBsAg transcription is driven by HBV integrated DNA (371,372). HBV RNA can be detected in the serum of persons with chronic HBV infection in the form of pregenomic RNA contained in virus-like particles (373) and varies with HBV disease phase reflecting the translational activity of cccDNA (374). HBcrAg is a composite of three antigens expressed by the precore/core region of the HBV genome; HBeAg, HBcAg and the 22kD precore fragment (375,376). A large cohort study and recent metanalysis have confirmed HBcrAg correlates well with quantitative intrahepatic cccDNA and its transcriptional activity (377,378). HBV RNA and HBcrAg are recommended as exploratory rather than primary endpoints for clinical trials at present, which aim for functional cure where, by definition, cccDNA persists at low levels (109). However, markers of cccDNA will be increasingly important when evaluating therapies directly targeting the elimination or silencing of cccDNA in future clinical trials, as a marker of drug activity.

1.9.3 Specific safety concerns - ALT flares

One concern in conducting clinical trials of any novel therapeutic for chronic HBV treatment is the interpretation of changes in serum ALT levels. ALT flares in the context of chronic HBV infection are acute increases in serum ALT levels of the magnitude of 5 or 10 times the upper limit of normal, depending on the definition used (379). Increases in ALT can occur when intrahepatic enzymes are released due to hepatocyte necrosis. HBV is non cytopathic and evidence suggests that spontaneous ALT flares during chronic infection are immune mediated (177). In some cases, ALT flares precede HBeAg loss and suppression of HBV DNA. However in other cases, viral reactivation can result in acute hepatic decompensation and repeated ALT flares are associated with cirrhosis development (379).

In the context of clinical trials, ALT flares could also represent drug induced liver injury as a result of the trial drug or other concomitant medications. Alternatively they could signify virological relapse as a result of drug resistance or ineffectiveness (109,363). With this in mind, guidance on how to interpret ALT flares in the context of clinical trials has been drawn up (Figure 1.7, (109,380)). Recommendations to stop treatment are dependent on the assessment of the baseline ALT level, the overall magnitude of the ALT flare during treatment or the increase in ALT during treatment in comparison to baseline. (380,109). However, additional parameters including the timing of the flare in relation to treatment, other biochemical indications of liver damage such as serum bilirubin, indicators of liver synthetic function including prothrombin time and indicators of virus activity, particularly the HBV DNA level, should be considered to determine the likely cause of an ALT flare. If any signs of liver decompensation are present, it is recommended that treatment should be immediately withdrawn (109).

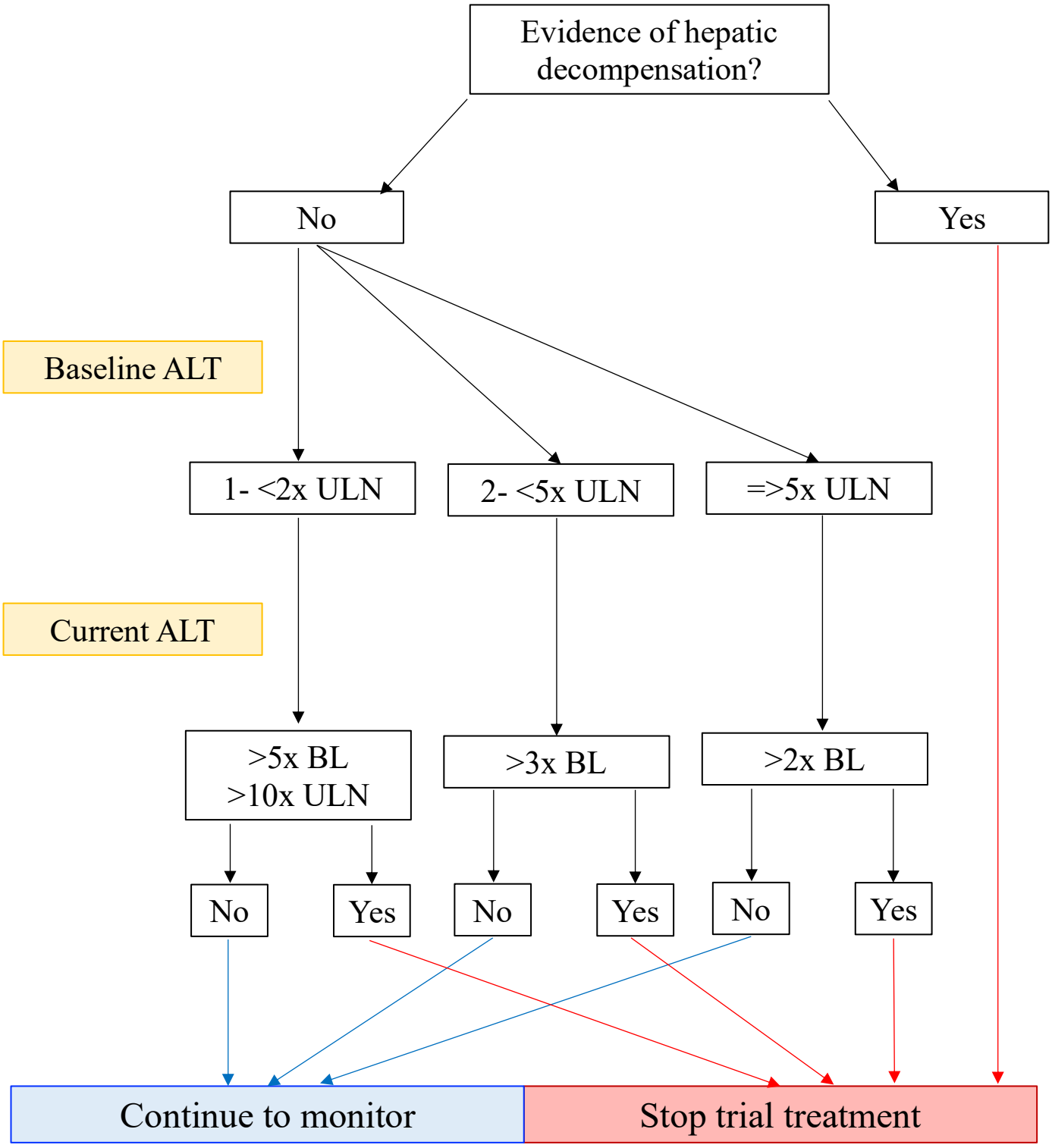


Figure 1.7: Stopping rules for ALT flares in HBV patients during clinical trials. Modified from Kullak-Ublick (2014) and Cornberg et al (2020)

Abbreviations: ALT = alanine aminotransferase, ULN = upper limit of normal, BL = baseline

1.10 THE DEVELOPMENT OF NOVEL HBV THERAPEUTIC VACCINES

ChAdOx1-HBV and MVA-HBV

Novel HBV therapeutic vaccine candidates ChAdOx1-HBV and MVA-HBV were designed to induce high magnitude CD8 and CD4 HBV specific responses to multiple HBV antigens and stimulate the production anti-HBs Ab (346).

Adenoviruses have been extensively studied for their use as vaccine vectors over recent decades (381). In nature, they infect multiple vertebrate species including humans and chimpanzees (reviewed in (382)). Human adenoviruses are classified into seven groups A to G (383). They have several properties that make them useful as vaccine platforms including the ability to insert large immunogens into their genome and their ability to transduce many cell types including antigen presenting cells, allowing their proteins to be effectively presented to the immune system as antigen (381). They are also able to be produced at high yields and do not require a cold supply chain (381).

The first investigation of the immunogenicity of a non-replicative human adenovirus (human adenovirus 5, Ad5) was published in 1995 (384). This showed intra-peritoneal immunisation of mice with a non-replicating Ad5 vector encoding beta-galactosidase induced cytotoxic CD8 T cells and to a lesser extent CD4 T cells and antibodies, specific to the vaccine immunogen (384). When compared head-to-head in rhesus monkeys, the Ad5 vectored HIV vaccine encoding HIV-1 gag induced higher magnitude CD8 T cell responses compared to DNA-plasmid or MVA vectored vaccines, which attenuated infectious challenge with simian-human immunodeficiency virus (SHIV) (385). Further studies showed antigen expression was sustained after vaccination with non-replicating adenoviral vector vaccines (386,387),

leading to durable and sometimes inflationary CD8 responses with an effector memory phenotype (387–389).

Unfortunately, human trials of Ad5 vectored vaccines were hampered by a high prevalence of neutralising antibodies towards Ad5 in some populations (390–393). This led to the investigation of other adenoviruses, both human and simian, as potential vaccine vectors that had lower pre-existing anti-vector antibodies in humans (394). Several chimpanzee adenoviruses (ChAd), including PanAd3, ChAd3 and ChAd63 were found to have comparable immunogenicity to Ad5 as vaccine vectors (394). These were subsequently shown to induce immunogen specific responses in human studies (395–397). Following this, ChAd vaccine strategies were optimised by using MVA-vectored vaccines in heterologous prime-boost strategies. Vaccine strategies for both hepatitis C virus, malaria and Ebola have shown MVA vectored boost vaccines increase the magnitude and breath of ChAd vaccine primed T cells and increase antibody responses (397–400).

ChAdOx1 was developed as an alternative non-replicating ChAd vaccine vector, based on the group E chimpanzee adenovirus ChAdY25 (401). Pre-existing antibodies to ChAdY25 were rare in the human populations studied, and ChAdOx1 was found to induce comparable immune responses to another group E chimpanzee adenovirus, ChAd63 (401). Since then, ChAdOx1 has been used as the platform for several vaccine candidates alone or in combination with MVA-vectored vaccines for human and animal infections. These include rift valley fever (402–404), influenza (405,406), tuberculosis (407–409), human papilloma virus (HPV) (410), middle east respiratory syndrome (MERS) (411–415), zika virus (416–418) and HIV (419–422). ChAdOx1 is also the vector used in a therapeutic vaccine candidate for prostate cancer (423–425). It has a good safety record in human phase I studies

(405,406,409,413,423). Over the past 12 months, the ChAdOx1-nCoV-19 vaccine has been tested in thousands of humans in clinical trials. Although severe thrombotic events have been reported in a minority of people after vaccine roll out, overall the vaccine has proven safe, immunogenic and efficacious at preventing severe disease from SARS-CoV-2 infection (344,345,426–429).

During the initial development of the HBV therapeutic vaccinations by my research group, ChAd vectors ChAdOx1 and ChAdOx2 were explored, building on the accumulating evidence that ChAd vaccine platforms were safe in humans and induced high magnitude, durable T cell and antibody responses (346). In pre-clinical studies ChAdOx1 was more stable during in the manufacturing process and was therefore chosen to take forward for further clinical study (346). An MVA vaccine with a complementary immunogen was designed to use in combination with ChAdOx1-HBV, to maximise vaccine induced immune responses (346).

In this thesis, I explore the immunogenicity of heterologous prime-boost strategies of ChAdOx1-HBV and MVA-HBV vaccines in healthy mice to inform future human clinical studies (Chapter 3). I then investigate the phenotype of HBV specific T cells induced by vaccination in mice and explore different vaccination routes to optimise the magnitude of HBV specific T cells in the liver (Chapter 4). I also describe the safety and immunogenicity of ChAdOx1-HBV in a phase I clinical trial in healthy persons and individuals with chronic HBV infection.

1.11 KEY HYPOTHESIS TESTED IN THIS THESIS

1. Vaccination with ChAdOx1-HBV and/or MVA-HBV can induce HBV specific T cell and humoral responses in healthy mice (Chapter 3).
2. The magnitude of vaccine induced HBV specific T cells in the liver can be augmented by intravenous as opposed to intramuscular administration of MVA-HBV (boost) vaccination (Chapter 4).
3. Vaccination with ChAdOx1-HBV is safe and can induce HBV specific T cells in healthy volunteers and patients with chronic HBV infection (Chapter 5).

CHAPTER 2: Materials and Methods

2.1 VACCINE IMMUNOGEN AND VIRAL VECTORS

2.1.1 Immunogens

The HBV immunogen includes the entire HBV core, polymerase, pre-S1, pre-S2 and surface regions of HBV genotype C sequence accession number KJ173426 HBV isolate C2 (346,430). Eight point mutations were introduced in the HBV polymerase sequence, rendering it replication deficient (346). Truncated shark invariant chain (sIi), a molecular adjuvant and tissue plasminogen activator (TPA), a promotor for gene transcription were included in the immunogen cassette, as they have been shown to increase the immunogenicity of several chimpanzee adenoviral vectored vaccines (346,412,431,432). Part A of the immunogen included sIi, core, polymerase, the pre-S1 n terminus and pre-S2 and was generated under a CMV promotor (ChAdOx1, Figure 2.1.A) or F11 promotor (MVA, Figure 2.1.B). Part B of the immunogen included TPA, the pre-S1 c terminus and surface and was generated separately using the furin-2-A (F2A) cleavage protein (ChAdOx1, Figure 2.1.A) or mH5 promotor (MVA, Figure 2.1.B). The sequence order of part A and part B of the immunogen was reversed between ChAdOx1 and MVA vectors to minimise any responses to junctional regions when ChAdOx1-HBV and MVA-HBV were used in heterologous prime-boost strategies (346).

The HBV core immunogen genotype C includes the HBV core sequence only (Figure 2.1.C). The sequence is identical to the core sequence of accession number KJ173426 HBV isolate C2, the same sequence as in the full HBV immunogen (346,430). The HBV core immunogen genotype D includes the HBV core sequence only (Figure 2.1.D). The genotype D sequence is identical to the core sequence of accession number KC875277.1 isolate HBV7 genotype D

(433,434). This real patient sequence was also the consensus sequence identified by the recent analysis of all available HBV genotype D sequences (435). The rat hepacivirus (RHV) immunogen, used as a negative control, coded for non-structural 3 (NS3) to NS5 region of RHV-rn1 (436).

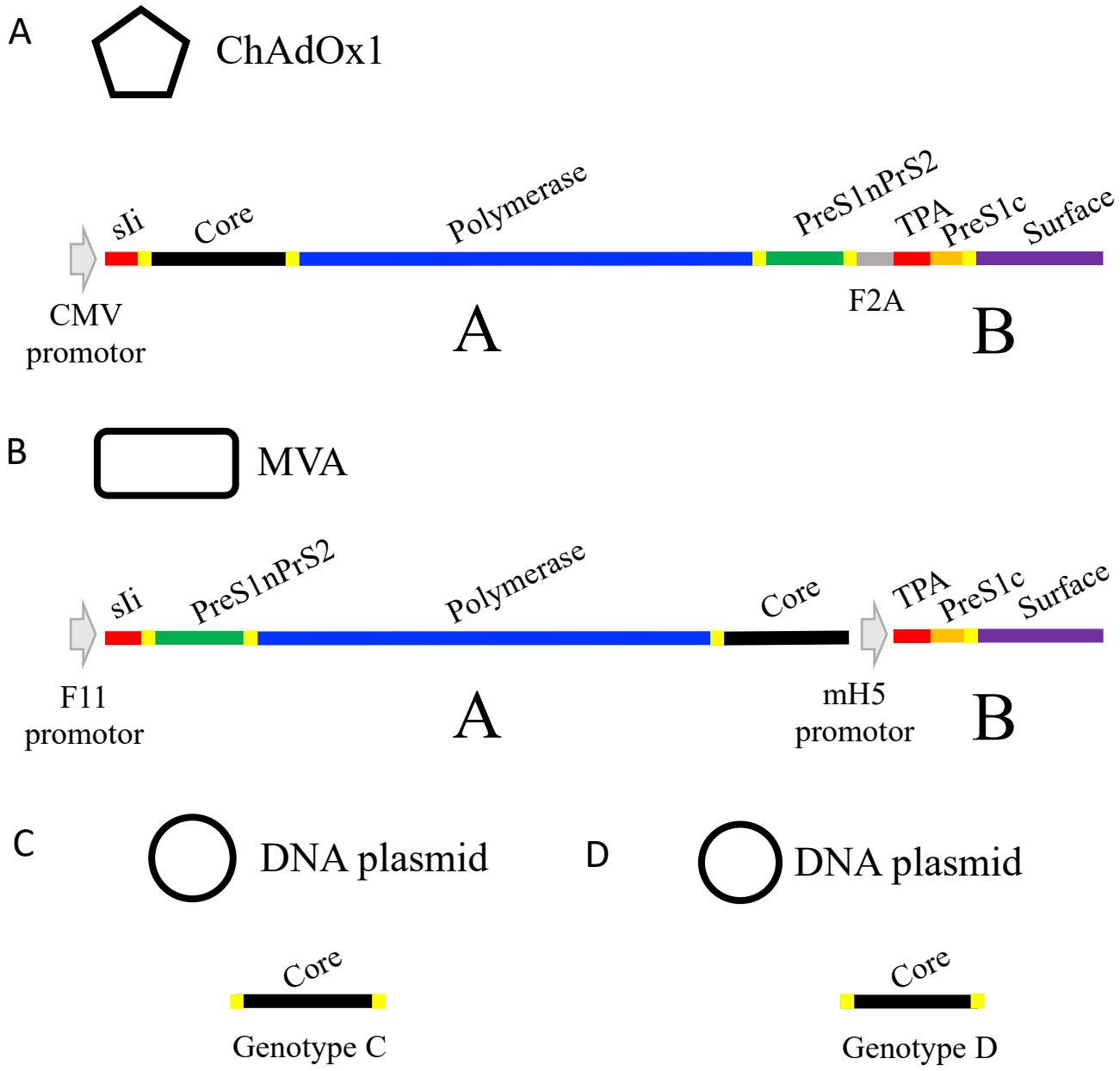


Figure 2.1: Immunogen design of the HBV vaccines

A: HBV immunogen in ChAdOx1 vectored HBV vaccine.
B: HBV immunogen in MVA vectored HBV vaccine.
C: HBV core immunogen (genotype C) used in DNA plasmid vaccine.
D: HBV core immunogen (genotype D) used in DNA plasmid vaccine.

Abbreviations: Chimpanzee adenoviral vector Oxford 1 (ChAdOx1), Cytomegalovirus (CMV), truncated shark invariant chain (sIi), HBV core (core), HBV polymerase (polymerase), HBV pre-S1 n terminus and pre-S2 (preS1nPreS2), tissue plasminogen activator (TPA), HBV pre-S1 c terminus (preS1c), HBV surface (surface), modified vaccinia Ankara (MVA).

2.1.2 DNA plasmid vaccines

The HBV core and RHV immunogens were generated using GeneArt (Thermo Fisher Scientific) and cloned into shuttle vector pENTR4. The immunogen coding sequences were then transferred to the destination plasmid, pChAdOx1 lacking the E1 and E3 regions, using LR gateway cloning (Thermo Fisher Scientific).

2.1.3 ChAdOx1 vaccines

The HBV immunogen was generated using GeneArt (Thermo Fisher Scientific) and cloned into shuttle vector pENTR4. LR gateway cloning (Thermo Fisher Scientific) was used to transfer the immunogen coding sequences to the destination plasmid, pChAdOx1 lacking the E1 and E3 regions rendering it replication deficient (401). This plasmid was linearised and transfected into T-REx-293 cells for production of ChAdOx1 vaccine at the Viral Vector Core Facility of the Jenner Institute, University of Oxford, UK. Manufacture to Good Manufacturing Practice standards for human use was conducted at the Advent Manufacturing Facility, Italy. ChAdOx1-GFP was generated at the Viral Vector Core Facility of the Jenner Institute, University of Oxford, UK.

2.1.4 MVA vaccines

The HBV immunogen was generated using GeneArt (Thermo Fisher Scientific) and cloned into vector p-MVA-shuttle. MVA vaccines were produced using the DF-1 cell line and purified using sucrose cushion centrifugation at the Viral Vector Core Facility of the Jenner Institute, University of Oxford, UK.

2.2 ANIMAL EXPERIMENTS

2.2.1 License

Animal experiments were conducted at the Biomedical Services Building, University of Oxford under Home Office project license numbers 30/3293 and PP3430109 and were approved by the University of Oxford Animal Care and Ethical Review Committee.

2.2.2 Experimental animals

Adult mice over 8-weeks old were housed in a containment level 2 facility in individually ventilated cages. Female outbred CD1, inbred BALBc and inbred C57BL/6J mice were purchased from Charles River, UK. Female and male HLA-A2.1 restricted HHD mice (437) were kindly donated by Uzi Gileadi, Weatherall Institute of Molecular Medicine, University of Oxford.

2.2.3 Experimental Procedures

Mice undergoing intramuscular vaccination into the hind leg received 5×10^5 or $5x10^7$ infectious units (iu) of ChAdOx1, 100μg of DNA plasmid or $2x10^6$ plaque forming units (pfu) of MVA diluted in 50μL phosphate buffered saline (PBS). Mice undergoing intravenous vaccination into the tail vein received $2x10^6$ pfu of MVA diluted in 200μL of PBS. For longitudinal experiments, mice were bled from the tail vain with a 26-gauge needle.

2.2.4 Experimental design

Where possible, experiments were designed with the aid of the NC3Rs Experimental Design Assistant and are reported below in accordance with the updated ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (438–440). A summary of the experimental

design of each experiment, alongside its figure number as reported in this thesis is detailed in Table 2.1.

- **Study design**: All experiments were designed prospectively with an intervention group (vaccinated animals) and a comparator group (alternative vaccination regime or unvaccinated animals).
- **Experimental unit:** For all studies the experimental unit was the individual animal.
- **Inclusion and exclusion criteria**: Inclusion and exclusion criteria were not set *a-priori*. In the experiments reported in this thesis, there were no instances in which an animal did not complete the intended protocol or were not included in the planned analysis.
- **Sample size:** The method of sample size calculation for each experiment is indicated in Table 2.1. Initially, sample sizes were chosen based on previous animal experiments in the research group, using similar vaccine vectors. A larger sample size (n=10 per group) was chosen for outbred mouse strains with higher genetic diversity (CD1 mice) than for inbred mouse strains (n=5 per group, BALBc and C57BL/6J mice). Later, sample sizes were calculated using the NC3Rs Experimental Design Assistant (439) and were based on the results from previous experimental data generated in C57BL6/J mice vaccinated with the same or similar interventional products.
- **Randomisation and blinding:** The randomisation and blinding status for each experiment is indicated in Table 2.1. Initially investigators were not blinded, and individual animals were not randomised to study group. This meant all mice sharing a particular cage received the intervention or control. Later, individual mice were randomised to intervention or control group using the NC3Rs Experimental Design Assistant computer generated algorithm (439). The investigator administering the vaccine

and conducting the immunoassays was blinded to the study intervention until after data analysis. Individual animals were identified by tail marks or ear clips.

- **Primary outcome measures:** The primary outcome measure for all experiments was the magnitude of T cell responses to vaccination. By ELISpot this was measured as total IFNγ spot forming units per million splenocytes towards all peptide pools corresponding to the HBV components of the vaccine immunogen (HBV core, polymerase, pre-S1, pre-S2 and surface) or to mini-pools or individual peptides, 15 amino-acids in length. By flow cytometry this was measured as pentamer positive cells as a percentage of total CD8 T cells in a sample.
- **Secondary outcome measures**: Secondary outcome measures included the magnitude of peptide specific T cell responses to vaccination, T cell polyfunctionality assessed by intracellular staining and T cell phenotype assessed by flow cytometry.

Table 2.1: Experimental design of animal experiments reported in this thesis

Corresponding Figures	Group 1	Group 2	Group 3	Mouse Strain(s)	Sample size calculation	Randomised	Blinded
3.1-3.13	ChAdOx1-HBV Or MVA-HBV	ChAdOx1-HBV + MVA-HBV	No vaccine	C57BL/6 BALBc CD1	no	no	no
3.14-3.17	ChAdOx1-HBV + MVA-HBV	No vaccine	-	C57BL/6 BALBc CD1	no	no	no
3.18-3.20	DNA-HBVc	DNA-HBVd	DNA-RHV	C57BL/6	yes	yes	yes
3.21-3.24	ChAdOx1-HBV + MVA-HBV	ChAdOx1-GFP + MVA-HBV	-	HHD	yes	yes	yes
3.32	ChAdOx1-sIi-HBV	ChAdOx1-hIi-HBV	ChAdOx1-GFP	HHD	yes	yes	yes
4.1-4.3	ChAdOx1-HBV i.m.	ChAdOx1-HBV i.m. + MVA-HBV i.m.	No vaccine	C57BL/6	no	no	no
4.4-4.25	ChAdOx1-HBV i.m. + MVA-HBV i.m.	ChAdOx1-HBV i.m. + MVA-HBV i.v.	No vaccine	C57BL/6	no	no	no

Abbreviations: Chimpanzee adenoviral vector Oxford 1 HBV vaccine (ChAdOx1-HBV), modified vaccinia Ankara HBV vaccine (MVA-HBV), chimpanzee adenoviral vector Oxford 1 green florescent protein (ChAd-GFP), DNA plasmid vectored genotype C HBV core vaccine (DNA-HBVc), DNA plasmid vectored genotype D HBV core vaccine (DNA-HBVd), intramuscular (i.m.), intravenous (i.v.).

2.2.5 Organ harvesting and processing

Animals were sacrificed prior to harvesting organs. Blood was collected via a heart bleed directly into FACS buffer on ice (PBS, 2% FCS, 0.1% sodium azide (NaN_3) and 5mM EDTA). Whole blood was used directly in immunoassays.

Spleens were collected into PBS on ice. Livers and lungs were collected after perfusion of 10ml PBS into the heart with the descending aorta ligated.

A single cell suspension of splenocytes was achieved through mechanical disruption of whole spleens through 40μm cell strainers (Argos Technologies). Red blood cells were lysed by exposure to ACK lysis buffer (Life Technologies) for one minute.

Intrahepatic lymphocytes were extracted via mechanical disruption through 40μm cell strainers (Argos Technologies) and isolated using 70% Percoll density gradient (GE healthcare). Red blood cells were lysed by exposure to ACK lysis buffer (Life Technologies) for two minutes.

To isolate lung lymphocytes, lungs were cut up using a scalpel blade and exposed to digestion solution (PBS with 170 units/mL collagenase (Gibco) and 60 units DNAse (Roche)) for 45 minutes at 37 degrees centigrade. They were then passed through 40μm cell strainers (Argos Technologies). Red blood cells were lysed by exposure to ACK lysis buffer (Life Technologies) for one minute.

2.3 PEPTIDES FOR ANIMAL EXPERIMENTS

Custom made unpurified peptides, 15 amino acids in length (15-mer) and overlapping by 11 amino acids, covering the entire HBV immunogen were supplied by Mimotopes, Australia. The initial set of peptides manufactured in 2016 (Appendix 2) was designed for an earlier version of the HBV immunogen, which had 2 rather than 5 linker regions and did not include TPA. The set of peptides manufactured in 2019 (Appendix 3a) was designed for the working version of the HBV immunogen, which had 5 linker regions and included TPA. The sequences of the HBV regions did not change between the two peptide sets. For the HBV core region, peptides were also synthesised for regions corresponding to HBV genotype D, where the amino acid sequence differed to the genotype C sequence (Appendix 3b). Individual peptides were dissolved in dimethyl suphoxide (DMSO) at a concentration of 40mg/mL. Peptides were pooled together in pools and minipools for ELISpot or ICS at a concentration of 300 mg/mL (pooling strategy Table 2.2 and 2.3). All peptides were stored at -80 degrees centigrade.

Table 2.2: Pooling strategy for 2016 unpurified peptides

	Name	Peptide ID numbers	Number of peptides per pool
ELISpot Pool	sIi (Shark invariant chain)	1 – 4	4
	F2A (Furine-2-A)	273 – 281	9
	Linkers	5–8, 60-63, 380-385	14
	HBV Core	9 – 59	51
	HBV Polymerase 1	64 – 116	53
	HBV Polymerase 2	117 – 168	52
	HBV Polymerase 3	169 – 221	53
	HBV Polymerase 4	222 – 272	51
	HBV Pre-S1/pre-S2	282 – 324	43
	HBV Surface	325 – 379	55
ELISpot Minipool	C1	9 – 18	10
	C2	19 – 28	10
	C3	29 – 38	10
	C4	39 – 48	10
	C5	49 – 59	11
	P1	64 – 73	10
	P2	74 – 83	10
	P3	84 – 93	10
	P4	94 – 103	10
	P5	104 – 113	10
	P6	114 – 123	10
	P7	124 – 133	10
	P8	134 – 143	10
	P9	144 – 153	10
	P10	154 – 163	10
	P11	164 – 173	10

Table 2.2: Pooling strategy for 2016 unpurified peptides (continued)

	Name	Peptide ID numbers	Number of peptides per pool
ELISpot Minipool	P12	174 – 183	10
	P13	184 – 193	10
	P14	194 – 203	10
	P15	204 – 213	10
	P16	214 – 223	10
	P17	224 – 233	10
	P18	234 – 243	10
	P19	244 – 253	10
	P20	254 – 263	10
	P21	264 – 272	9
	S1	282 – 291	10
	S2	292 – 301	10
	S3	302 – 311	10
	S4	312 – 321	10
	S5	322 – 331	10
	S6	332 – 341	10
	S7	342 – 351	10
	S8	352 – 361	10
	S9	362 – 371	10
	S10	372 – 379	8
ICS Pool	sIi/F2A/linkers	1-4, 5–8, 60-63, 273-281, 380-385	27
	HBV Core	9 – 59	51
	Pol1+2 (HBV Polymerase 1+2)	64 – 168	105
	Pol3+4 (HBV Polymerase 3+4)	169 – 272	104
	HBV Pre-S1/pre-S2/surface	282 – 379	98

Table 2.3: Pooling strategy for 2019 unpurified peptides

	Name	Peptide ID numbers	Number of peptides per pool
ELISpot Pool	sIi (Shark invariant chain)	1-5	5
	F2A + TPA	006-016, 275-278, 022	15
	Linkers	4-10, 60-64, 001-007, 017-021	24
	HBV Core genotype C	9-61	53
	HBV Polymerase 1	61-118	58
	HBV Polymerase 2	117-171	55
	HBV Polymerase 3	169-224	56
	HBV Polymerase 4	222-271, 001-003	53
	HBV Pre-S1c/PreS1n	002-003, 291-321, 004-007	53
	HBV PreS2/Surface	018-021, 326-379	58
	HBV Core genotype D	400-408	9
ICS Pool	sIi/F2A/linkers	1 -5, 006-016, 275-278, 022, 4-10, 60-64, 001-007, 017-021	44
	HBV Core	9 - 61	53
	Pol1+2 (HBV Polymerase 1+2)	61-118, 117-171	111
	Pol3+4 (HBV Polymerase 3+4)	169 -224, 222-271, 001-003	109
	HBV Pre-S1/pre-S2/surface	002-003, 291-321, 004-007, 018-021, 326 - 379	111

2.4 IMMUNOASSAYS FOR ANIMAL EXPERIMENTS

2.4.1 Enzyme linked immunospot assay (ELISpot)

Enzyme linked immunospot (ELISpot) assays were conducted as described previously (346). ELISpot plates (Millipore, MSIP54510) were coated with mouse anti-IFNγ monoclonal antibody (MabTech, AN-18) and incubated overnight at 4 degrees centigrade. After washing four times with PBS, plates were blocked with R10 media (section 2.6) for 2 hours at 37

degrees centigrade. HBV peptide pools, minipools or peptides (section 2.3) diluted in R10 (final concentration in well 3μg/mL) were incubated with $2x10^5$ splenocytes plated in duplicate at 37 degrees centigrade overnight (16-20 hours). Cells were diluted in R10 media with DMSO diluted in R10 as a negative control and Concanavalin A (ConA, Sigma) diluted in R10 (final concentration10μg/mL) as a positive control. After washing 7 times with PBS, plates were incubated with biotinylated mouse anti-IFNγ (MabTech, R4-6A2) for 2 hours at room temperature. After washing a further 4 times with PBS, plates were incubated with alkaline phosphatase conjugated goat anti-biotin (Vector laboratories) for 2 hours at room temperature. After washing another 4 times with PBS, plates were developed with NBT/BCIP substrate (Thermo Fisher Scientific) for 5 minutes. Plates were washed with distilled water and air dried. Plates were counted using an AID ELISpot plate reader and final results were recorded as background subtracted values (background calculated by positive spots in the DMSO negative control well). A positive ELISpot well was defined as having a response equal to or exceeding 50 IFNγ spot forming units per million cells, 2 times greater than the background well and 2 times greater than baseline value.

2.4.2 Intracellular cytokine staining (ICS)

Intracellular cytokine staining assays were conducted as described previously (346). HBV peptide pools, minipools or peptides (section 2.3) diluted in R10 (final concentration in well 2μg/mL) were incubated with $1x10^6$ splenocytes at 37 degrees centigrade. As a positive control, cells were cultured with phorbol myristate acetate (PMA, Sigma, 500μg/mL) and ionomycin (Sigma, 500μg/mL diluted) made as a mixed PMA/ionomycin solution (8μL PMA and 20μL ionomycin diluted in 450 μL of R10, working solution diluted 1:10 in R10). As a negative control, cells were cultured with DMSO diluted in R10 media. After one hour of peptide stimulation, GolgiPlug (BD Bioscience) was added, and cells were incubated with

peptide for a further 4 hours. After washing, cells were surface stained with antibody cocktail diluted in PBS for 25 minutes at 4 degrees centigrade (Table 2.4).

Table 2.4: Surface staining antibody cocktail for murine ICS assay

Antibody target	Clone	Fluorochrome	Company
Anti-mouse CD8a	53-6.7	PerCp-Cy5.5	eBioscience
Anti-mouse CD4	GK 1.5	AF-700	eBioscience
Anti-mouse CD3	17A2	eF450	eBioscience
Near IR (live/dead)	-	APC Cy7	Invitrogen

Cells were fixed in fixation/permeabilization solution for 10 minutes at room temperature (BD Biosciences, Cytofix/Cytoperm) and stained with intracellular antibody cocktail diluted in perm/wash buffer (BD Biosciences, Cytofix/Cytoperm) for 25 minutes at 4 degrees centigrade (Table 2.5).

Table 2.5: Intracellular antibody cocktail for murine ICS assay

Antibody target	Clone	Fluorochrome	Company
Anti-mouse IFNγ	XMG1.2	PE	eBioscience
Anti-mouse TNFα	MP6-XT22	FITC	eBioscience
Anti-mouse IL-2	RMK-45	APC	BioLegend

After washing and resuspending in PBS, cells were acquired on a BD LSRII. FlowJo (TreeStar), Pestle and SPICEv6 (441) were used for flow cytometry analysis. Final results were recorded as background subtracted values (calculated by the percentage of positive cytokine responses in the DMSO negative control well).

2.4.3 Anti-HBs Ab Enzyme linked immunosorbent assay (ELISA)

Anti-HBs Ab ELISA were conducted as described previously (346). ELISA coating buffer (Biorad) was diluted in deionized water as per manufacturer's instructions. Recombinant hepatitis B surface antigen (Biorad, PIP002) was added to the coating buffer at 500ng per ml. Maxisorp 96 well plates (Nunc) were coated with 100µl per well of this solution and kept at 4 degrees centigrade overnight. Plates were blocked with 10% skimmed milk powder in 0.05% PBS-Tween (PBST) for an hour at 37 degrees centigrade. Mouse sera were diluted 1:3 in PBST. Hepatitis B surface antigen antibody (0.1mg/ml, GeneTex) was used a positive control to generate a standard curve. Samples were added in duplicate at 100µl per well and incubated for an hour at 37 degrees centigrade. After washing, anti-mouse alkaline phosphatase (Invitrogen) was diluted by a factor of 5000 in blocking solution, added at 100µl per well and incubated for an hour at 37 degrees centigrade. After washing, plates were incubated with 1-step ultra-TMB-ELISA substrate (34029, Thermo Fisher Scientific). Adding sodium hydroxide, when a colour change was detected in the positive control wells, stopped the reaction. Plates were read on a FLUOstar Omega microplate reader (BMG Labtech) at an absorbance of 405nm.

2.4.4 Pentamer staining and flow cytometry

Freshly isolated lymphocytes from the spleen, liver or lungs were plated at 0.5-1x10^6 cells per well in round bottom 96 well plates. Pentamer VWLSVIWM (ProImmune) conjugated to fluorochrome APC was microcentrifuged for 5 minutes at 13000xg. Cells were resuspended in 50µl/well of PBS with pentamer VWLSVIWM added at a 1:25 dilution for 10 minutes at room temperature. After washing, cells were surface stained with either the T cell memory antibody cocktail (Table 2.6) or the T cell residency antibody cocktail diluted in PBS (Table

2.7). After further washing and resuspending in PBS, cells were acquired on a BD LSRII. FlowJo (TreeStar) was used for used for flow cytometry analysis.

Whole blood diluted in FACS buffer in FACS tubes were stained directly. Pentamer VWLSVIWM (ProImmune) conjugated to fluorochrome APC was microcentrifuged for 5 minutes at 13000xg. Cells were washed and resuspended in 50ul/well PBS with pentamer VWLSVIWM added at a 1:25 dilution for 20 minutes at room temperature. After washing, cells were surface stained with either the T cell memory antibody cocktail (Table 2.6) or the T cell residency antibody cocktail diluted in PBS (Table 2.7). After further washing, 1ml 1x FACS lysing solution (BD) was added to each sample for 1-2 minutes until the solution was transparent. After further washing and resuspending in PBS, cells were acquired on a BD LSRII. FlowJo (TreeStar) was used for used for flow cytometry analysis.

Table 2.6: T cell memory surface staining antibody cocktail

Antibody target	Clone	Fluorochrome	Company
Anti-mouse CD8a	53-6.7	PerCp-Cy5.5	eBioscience
Anti-mouse CD62L	MEL-14	PE	BioLegend
Anti-mouse CD44	IM7	FITC	BioLegend
Anti-mouse KLRG1	2F1/KLRG1	PE Cy7	BioLegend
Anti-mouse CD127	A7R34	BV421	BioLegend
Anti-mouse CX3CR1	SA011F11	BV605	BioLegend
Near IR (live/dead)	-	APC Cy7	Invitrogen

Table 2.7: T cell residency surface staining antibody cocktail

Antibody target	Clone	Fluorochrome	Company
Anti-mouse CD8a	53-6.7	PerCp-Cy5.5	eBioscience
Anti-mouse CD69	H1.2F3	PE	BioLegend
Anti-mouse KLRG1	2F1/KLRG1	FITC	BioLegend
Anti-mouse CD11a	M17/4	PE Cy7	eBioscience
Anti-mouse CXCR6	SA051D1	BV421	BioLegend
Anti-mouse CX3CR1	SA011F11	BV605	BioLegend
Near IR (live/dead)	-	APC Cy7	Invitrogen

2.5 HBV001 STUDY DESIGN

HBV001 is a phase I clinical trial registered as NCT04297917 (347) entitled 'A Phase 1 Monotherapy Study to Evaluate the Safety, Tolerability & Immunogenicity of Vaccination With Candidate Chimpanzee Adenovirus-vectored Hepatitis B Virus Vaccine ChAdOx1 HBV in Healthy Participants & Participants With Chronic Hepatitis B Infection'.

2.5.1 Study aims

The primary outcome of the study is to assess the safety of a single dose of ChAdOx1-HBV when given to healthy participants or individuals with chronic HBV infection. Secondary outcomes include an assessment of vaccine T cell immunogenicity in both populations and changes in quantitative HBsAg levels in persons with chronic HBV.

2.5.2 Ethical approval

The study was approved by the South Central - Berkshire Research Ethics Committee

(REC: 19/SC/0419). Before enrolment, all participants gave informed written consent. All procedures were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice (GCP) guidelines (442).

2.5.3 Study design

The study is an open label, non-randomised phase I clinical trial. Recruitment is divided into 4 cohorts with participants sequentially enrolled (Table 2.8). Cohorts 1 and 2 recruited healthy volunteers to either low dose (2.5 $x10^9$ viral particles (vp), cohort 1) or high dose (2.5 $x10^{10}$ vp, cohort 2) intramuscular ChAdOx1-HBV. Cohorts 3 and 4 recruited patients with chronic HBV to either low dose (2.5 $x10^9$ vp, cohort 3) or high dose (2.5 $x10^{10}$ vp, cohort 4) intramuscular ChAdOx1-HBV.

Enrolment into each sequential cohort only began after a safety monitoring committee review of the safety data obtained up to 48 hours after vaccination of the previous cohort. Recruitment for cohorts 1 and 2 was undertaken at the Medicines Evaluation Unit, Manchester, UK. Recruitment for cohorts 3 and 4 was undertaken at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Churchill Hospital, Oxford, UK. Recruitment for cohort 4 is ongoing.

Table 2.8: Recruitment cohorts for HBV001

Cohort	1	2	3	4
Population	Healthy	Healthy	Chronic HBV	Chronic HBV
Participants (n)	5	5	6	6
Intervention	ChAdOx1-HBV Low dose	ChAdOx1-HBV High dose	ChAdOx1-HBV Low dose	ChAdOx1-HBV High dose
Dose	2.5 x10^9 vp	2.5 x10^{10} vp	2.5 x10^9 vp	2.5 x10^{10} vp

2.5.4 Inclusion criteria

Full inclusion criteria can be found in Appendix 4. In brief, all participants were required to be between 18 and 65 years of age, not pregnant and be able to provide informed consent.

For cohort 1 and 2 participants were required to have no pre-existing conditions that might influence either study safety or results.

For cohorts 3 and 4 participants were required to have controlled chronic HBV infection defined by:

- detectable HBsAg for at least 6 months
- on nucleos(t)ide analogue therapy for at least 12 months
- suppressed HBV DNA under 40 IU/mL for at least 6 months
- quantitative HBsAg titres of under 4000 IU/mL at recruitment (amended to under 10,000 IU/mL in a substantial amendment to the protocol in December 2020).

2.5.5 Exclusion criteria

Full exclusion criteria can be found in Appendix 4. In brief, participants were excluded if they had any significant uncontrolled acute or chronic medical condition, were Hepatitis C antibody positive or had received an adenoviral vectored vaccine in the past. For cohorts 1 and 2 participants were excluded if they had detectable HBsAg. For cohorts 3 and 4 participants were excluded if they were coinfected with Hepatitis D virus, had advanced fibrosis or cirrhosis, had previous or ongoing hepatocellular carcinoma or had alanine aminotransferase levels (ALT) exceeding 3 times the upper limit of normal (ULN).

2.5.6 Study procedures

A full list of study procedures can be found in Appendix 5. An overview of study visits, assessments and procedures is shown in Table 2.9. In brief, informed written consent was obtained during screening and prior to vaccination, which occurred at timepoint day 0. Baseline variables and baseline laboratory tests including full blood count, urea and electrolytes and liver function tests were taken at screening to assess inclusion and exclusion criteria. On the day of vaccination and at timepoints indicated during follow up, blood was drawn and sent to the John Radcliffe Hospital haematology and biochemistry laboratories for tests including full blood count, urea and electrolytes and liver function tests. Blood was also drawn and sent to the John Radcliffe Hospital microbiology laboratory to assess HBV disease markers including quantitative HBsAg, anti-HBc Ab, anti-HBs Ab and HBV DNA. Blood was also drawn for isolation of peripheral blood mononuclear cells (PBMCs) for immunology assessment. After vaccination, local and systemic reactogenicity was assessed for up to 7 days using an electronic diary. Serious adverse events were collected until the end of study.

Table 2.9: An overview of study visits, assessments and procedures

Visit	Screen	Vaccination	Follow up						End of study
Day	-42 to-1	0	1	7	14	28	56	84	168
Informed consent	x								
Baseline variables	x	x							
Laboratory tests	x	x		x	x	x	x	x	x
Vaccination		x							
Local/systemic reactogenicity		x	x	x					
Serious adverse events		x	x	x	x	x	x	x	x
HBV disease markers		x				x	x	x	x
Immunogenicity assessment		x			x	x	x	x	x

2.6 MEDIA FOR IMMUNOASSAYS

R0 – RPMI (Gibco) with 1% by volume 200mM L-glutamine (Sigma) and 1% by volume 100x penicillin/streptomycin solution (Sigma)

R10 – R0 with 10% by volume heat inactivated foetal bovine serum (FBS) (Hyclone)

RH10 – R0 with 10% by volume human serum (Sigma)

Thawing media – RH10 with DNAse I type IV, final concentration 60µg/mL (Roche)

Freezing media –By volume 50% FBS (Hyclone), 40% RPMI (Gibco), 10% DMSO (Sigma)

2.7 BLOOD PROCESSING

2.7.1 Peripheral blood mononuclear cell isolation and freezing

Whole blood was collected in sodium heparin vacutainers (BD). PBMC isolation for HBV001 cohorts 1 and 2 (healthy volunteers) took place at the Good Clinical Practice (GCP) Laboratory, University of Liverpool, UK. Samples were cryopreserved in liquid nitrogen and transported to the Peter Medawar Building, Oxford. Peripheral blood mononuclear cell (PBMC) isolation for HBV001 cohorts 3 and 4 (participants with chronic HBV) took place at the Peter Medawar Building, Oxford. PBMCs were isolated by layering whole blood diluted in R0 media onto a Lymphoprep density gradient (STEMCELL) and centrifuging for 24 minutes at 2400 rpm with slow acceleration and brake. Cells were counted on a Muse cell analyser (Luminex) and were either used fresh or cryopreserved in freezing media in 1ml cryovials in liquid nitrogen.

2.7.2 Peripheral blood mononuclear cell thawing

Cryovials of frozen PBMC were removed from liquid nitrogen and placed in a water bath at 37 degrees centigrade until partially thawed. Cells we diluted by adding the thawed solution drop by drop into 10ml of R0. After washing, cells were resuspended in 10ml thawing media and rested for 2-3 hours before using in immunoassays.

2.8 HBV001 PEPTIDES

Custom made peptides over 80% pure, 15 amino acids in length (15-mer) and overlapping by 11 amino acids covering the entire genotype C HBV immunogen were supplied by Mimotopes, Australia (Appendix 6a). In addition, peptides covering the amino acid sequence of human invariant chain (not in the vaccine sequence) were obtained. Genotype C peptide numbers 362-365 (within HBV surface) were unable to be synthesised to the desired purity.

Genotype D peptides corresponding to the HBV immunogen were also supplied where the amino acid sequence varied from the vaccine sequence (Appendix 6b). Genotype D peptide number 31 (within the HBV core region) was unable to be synthesised to the desired purity. Individual peptides were dissolved in DMSO at a concentration of 40 mg/mL. Peptides were pooled together in genotype C and genotype D pools and minipools for ELISpot or ICS at a concentration of 500 mg/mL (pooling strategy Table 2.10). All peptides were stored at -80 degrees centigrade.

Table 2.10: Pooling strategy for HBV001 peptides

	Name	**Peptide ID numbers**	**Total peptides**
ELISpot Pool	sIi (Shark invariant chain)	1 - 7	7
	hIi (human invariant chain)	Human 1 - 4	4
	F2A/TPA/Linkers	5 -9, 60 – 64, 272 – 275, 307 – 311, 338 – 343, 312 - 326	40
	HBV Core (geno C)	Geno C 7 - 62	56
	HBV Polymerase 1 (geno C)	Geno C 61 - 116	56
	HBV Polymerase 2 (geno C)	Geno C 117 - 168	52
	HBV Polymerase 3 (geno C)	Geno C 169 - 221	53
	HBV Polymerase 4 (geno C)	Geno C 222 - 275	54
	HBV Pre-S1/pre-S2 (geno C)	Geno C 273 – 310, 324 - 341	56
	HBV Surface (geno C)	Geno C 340 – 396 **Not synthesised (362-365)**	53
	HBV Core (geno D)	Geno C (7-16, 24-29, 42, 46-62) Geno D (17-23, 30-41, 43-45) **Not synthesised (31)**	55
	HBV Polymerase 1 (geno D)	Geno C (61-63, 67-76, 101, 106-108) Geno D (64-66, 77-100, 102-105, 109-116)	56

Table 2.10: Pooling strategy for HBV001 peptides (continued)

	Name	Peptide ID numbers	Total peptides
ELISpot Pool	HBV Polymerase 2 (geno D)	Geno C (155-156, 165-168) Geno D (117-154, 157-164)	52
	HBV Polymerase 3 (geno D)	Geno C (169, 174, 184, 189-202, 207-209, 219-221) Geno D (170-173, 175-183, 185-188, 203-206, 210-218)	53
	HBV Polymerase 4 (geno D)	Geno C (222-233, 245-246, 251-257, 269-272) Geno D (234-244, 247-250, 258-268, 273-275)	54
	HBV Pre-S1/pre-S2 (geno D)	Geno C (290, 301-302, 324-326) Geno D (273-289, 291-300, 303-310, 327-341)	56
	HBV Surface (geno D)	Geno C (345-350, 356, 361-366, 377-379, 386-391, 395-396) **Not synthesised (362-365)** Geno D (340-344, 351-355, 357-360, 367-376, 380-385, 392-394)	53
ELISpot minipool	G1 (HBV geno C surface)	Geno C 340-348	9
	G2 (HBV geno C surface)	Geno C 349-356	8
	G3 (HBV geno C surface)	Geno C 357-364 **Not synthesised (362-365)**	4
	G4 (HBV geno C surface)	Geno C 365-372	8
	G5 (HBV geno C surface)	Geno C 373-380	8
	G6 (HBV geno C surface)	Geno C 381-388	8
	G7 (HBV geno C surface)	Geno C 389-396	8
ICS Pool	sIi	1-7	47
	HBV Core	Geno C 7 - 62	56
	Pol1+2 (HBV Polymerase 1+2)	Geno C 61 – 116, 117 - 168	108
	Pol3+4 (HBV Polymerase 3+4)	Geno C 169 – 221, 222 - 275	107
	HBV Pre-S1/pre-S2/surface	Geno C 273 – 310, 324 – 341, 340 – 396 **Not synthesised (362-365)**	109

2.9 HBV001 IMMUNOASSAYS

2.9.1 Enzyme linked immunospot assay (ELISpot)

Enzyme linked immunospot (ELISpot) assays were conducted as described previously (443). ELISpot plates (Millipore, MSIP54510) were coated with human anti-IFNγ monoclonal antibody (MabTech, 1-D1K) and incubated overnight at 4 degrees centigrade. After washing 5 times with PBS, plates were blocked with R10 media (when using freshly isolated PBMCs) or RH10 media (when using previously frozen PBMCs) for 2 hours at 37 degrees centigrade. HBV peptide pools, minipools or peptides (section 2.8) diluted in R10 (final concentration in well 3μg/mL) were incubated with $2x10^5$ splenocytes plated in triplicate at 37 degrees centigrade overnight (16-20 hours). Cells were diluted in R10 media. DMSO diluted in R10 was used as a negative control. Concanavalin A (Sigma, final concentration10μg/mL), cytomegalovirus (CMV)-lysate (Virusys, 0.5mg/mL diluted 1:200) and FEC (BEI resources, HLA class I peptides for Influenza, Epstein-Barr virus and CMV, final concentration 3μg/mL) were used as positive controls.

After washing 7 times with 0.05% PBS-Tween, plates were incubated with biotinylated human anti-IFNγ (MabTech, 7-B6-1) for 2 hours at room temperature. After washing a further 4 times with PBS, plates were incubated with alkaline phosphatase conjugated goat anti-biotin (Vector laboratories) for 2 hours at room temperature. After washing a further 4 times with PBS, plates were developed with NBT/BCIP substrate (Thermo Fisher Scientific) for 5 minutes. Plates were washed with distilled water and air dried. Plates were counted using an AID ELISpot plate reader and final results were recorded as background subtracted values (calculated by positive spots in the DMSO negative control well).

A positive ELISpot assay was defined as a response equal to or exceeding 50 IFNγ spot forming units per million PBMC, over 2-fold greater than the background well and at least 2 times higher than at baseline, in line with previous studies of therapeutic HBV vaccination candidates (325). In addition, the number of spots in the negative control well (R10 with DMSO) was required to be under 100 SFU per million PBMCs with a positive response in the Concanavalin A well.

2.9.2 Intracellular cytokine staining (ICS)

Intracellular cytokine staining assays were conducted as described previously (443). HBV peptide pools (section 2.8) diluted in stimulation cocktail (Table 2.11, final peptide concentration in well 1μg/mL) were incubated with $1x10^6$ PBMCs at 37 degrees centigrade for 6 hours in 96 well U-bottom plates. As a positive control, cells were cultured with phorbol myristate acetate (PMA, Sigma, 500μg/mL) and ionomycin (Sigma, 500μg/mL diluted) made as a mixed PMA/ionomycin solution (8μL PMA and 20μL ionomycin diluted in 450 μL of R10, working solution diluted 1:10 in R10). As a negative control, cells were cultured with DMSO diluted in R10 media. In addition, CMV-lysate (Virusys, 0.5mg/mL diluted 1:200) was used as an internal positive control.

Table 2.11 Stimulation cocktail for ICS

Reagent	Clone	Fluorochrome	Dilution	Company
Anti-human CD107	H4A3	BV421	1:625	BD Biosciences
Brefeldin A	-	-	1:500	Sigma
Monensin	-	-	1:625	BioLegend

After washing, cells were stained with live/dead aqua (Invitrogen) diluted in PBS at room temperature for 20 minutes. Cells were then fixed in fixation/permeabilization solution for 20 minutes at 4 degrees centigrade (BD Biosciences, Cytofix/Cytoperm). After further washing, cells were stained with antibody cocktail and were diluted in perm/wash buffer (BD Biosciences, Cytofix/Cytoperm) for 20 minutes at room temperature (Table 2.12). After washing and resuspending in PBS, cells were acquired on a BD LSRII. FlowJo (TreeStar), Pestle and SPICEv6 (441) were used for flow cytometry analysis. Final results were recorded as background subtracted values (calculated by the percentage of positive cytokine responses in the DMSO negative control well).

Table 2.12: Antibody cocktail for HBV001 ICS assay

Antibody target	Clone	Fluorochrome	Company
Anti-human CD8a	RPA T8	PerCp-Cy5.5	BioLegend
Anti-human CD4	RPA T4	PE Dazzle 594	BioLegend
Anti-human CD3	SK7	APC Fire 750	BioLegend
Anti-human CD154	24-31	PE-Cy7	BioLegend
Anti-human IFNγ	B27	APC	BD Biosciences
Anti-human TNFα	Mab11	FITC	BD Biosciences
Anti-human IL-2	MQ1-17HI2	PE	BD Biosciences

2.10 STATISTICAL ANALYSIS

Statistical analysis was conducted using GraphPad Prism (version 8.0). All statistical tests used were non-parametric unless otherwise stated and median with interquartile range are displayed as the summary statistics for graphical representation of results. The Mann-Witney-U test was used to compared result between two groups. The Wilcoxon test was used to

compare paired results from the same individual. Spearman rank was used for correlations between two variables. The Kruskal-Wallis test with Dunn's correction for multiple comparisons was used when comparing more than two groups. Statistical significance was defined as a p value < 0.05.

CHAPTER 3: Vaccination with ChAdOx1-HBV and MVA-HBV induces T cell and humoral responses in mice

3.1 PRIMARY HYPOTHESIS

Vaccination with ChAdOx1-HBV and/or MVA-HBV can induce Hepatitis B virus (HBV) specific T cell and humoral responses in healthy mice.

3.2 INTRODUCTION

An estimated 257 million people are infected with HBV chronically worldwide, leading to many developing liver cirrhosis and hepatocellular carcinoma (1). Current therapy suppresses viral replication but is not able to eliminate the virus, which persists as covalently closed circular DNA (cccDNA) within the hepatocyte nucleus. Most individuals with chronic HBV require lifelong treatment as when treatment is stopped, viral load rebounds (8). New therapeutic strategies are needed to control the virus without the need for prolonged treatment courses.

Although the vast majority of individuals with chronic HBV have lifelong infection, a small proportion clear hepatitis B surface antigen (HBsAg) either spontaneously or during treatment (16,103). This phenomenon, known as ‘functional cure’, is associated with a reduced risk of developing the liver related complications of HBV infection such as HCC (9). Current guidelines support functional cure as the goal of therapy for chronic HBV (8).

Therapeutic vaccination is a promising method to induce immune control of chronic HBV (444). Although the successful control of acute HBV is associated with strong CD8 T cell responses (178), HBV specific T cells in chronic infection are dysfunctional (140).

Therapeutic vaccination aims to induce functional cure by restoring HBV specific T cell responses and inducing neutralising anti-HBs Ab (13,253).

Viral vaccine vectors ChAdOx1 (a modified chimpanzee adenovirus (ChAd) (401)) and Modified Vaccinia Ankara (MVA) were chosen to develop novel HBV therapeutic vaccines ChAdOx1-HBV and MVA-HBV (346). These vectors have been shown to generate high magnitude CD8 T cell responses in multiple disease settings and have a good safety record in human phase I studies (405,406,409,413,423).

In this chapter, I assessed ChAdOx1-HBV and MVA-HBV in mice, to investigate their ability to induce high magnitude HBV specific T cell responses and antibody responses in the murine immune system, to provide evidence to support their development for use in humans. CD8 T cell responses were assessed in depth to investigate the magnitude, breath and polyfunctionality of HBV specific T cell responses induced by vaccination. Epitopes induced in H-2D^{b}/H-2K^{b} restricted C67BL/6J mice were identified in order to develop tools to study vaccine induced responses *ex-vivo* in greater phenotypic depth. Finally, ChAdOx1-HBV and MVA-HBV were assessed in humanised HLA-A*02 restricted mice to investigate the breath of vaccine induced responses that might be induced in humans with the HLA-A*02 haplotype. Overall, this data provides evidence that ChAdOx1-HBV and MVA-HBV are immunogenic in healthy mammals, justifying their further investigation in human trials.

3.3 KEY QUESTIONS

1. Does ChAdOx1-HBV and MVA-HBV vaccination induce high magnitude, polyfunctional T cells specific to all major HBV antigens in the vaccine immunogen (HBV core, polymerase and surface) in mice?
2. Does MVA-HBV boost the magnitude and breadth of ChAdOx1-HBV primed T cell responses?
3. Do ChAdOx1-HBV and MVA-HBV vaccination induce anti-HBs Ab production?
4. Can the fine specificity of HBV specific T cells induced by ChAdOx1-HBV and MVA-HBV vaccination be identified, to develop immunological tools to study vaccine induced responses *ex-vivo*?
5. Does ChAdOx1-HBV and MVA-HBV vaccination induce HLA-A*02 restricted T cell responses in humanised HHD mice?
6. Are any previously described HLA-A*02 restricted HBV epitopes in HHD mice vaccinated with ChAdOx1-HBV and MVA-HBV?

Much of this work is published in the following manuscript:

Chinnakannan SK*, Cargill TN*, Donnison TA, Ansari MA, Sebastian S, Lee LN, et al. The Design and Development of a Multi-HBV Antigen Encoded in Chimpanzee Adenoviral and Modified Vaccinia Ankara Viral Vectors, A Novel Therapeutic Vaccine Strategy against HBV. Vaccines. 2020 Apr 14;8(2).

***** equal contribution

3.4 CHAPTER SPECIFIC MATERIALS AND METHODS

3.4.1 Animal experiments

Animal experiments are discussed in detail in Chapter 2. Adult C57BL/6J, BALBc, CD1 or HHD mice (437), aged over 8 weeks were vaccinated intramuscularly into the hind leg with

ChAdOx1, MVA or DNA plasmid vaccines in a total volume of 50μl of PBS. Mice were sacrificed 7 to 14 days after the last vaccination and splenocytes were isolated by mechanical disruption for use in immunoassays. Sera was obtained and stored at -20 degrees centigrade. In all experiments, unvaccinated animals were used as negative controls (n=1-3 per experiment). In results presented in Figures 3.1-3.17, individual mice were not randomly assigned to vaccine regime and investigators were aware of the intervention. In Figures 3.18-3.24, individual mice were randomly assigned to vaccine regime and investigators were blinded to the intervention until after the analysis of results had been completed.

3.4.2 Immunoassays

Immunoassays are discussed in detail in Chapter 2. Splenocytes were stimulated with peptide pools, minipools or individual peptides in duplicate in overnight interferon gamma (IFNγ) ELISpot assays or for 5 hours in intracellular staining assays (ICS) to detect cytokine production by flow cytometry. Anti-HBs Ab was detected in sera of vaccinated mice by ELISA.

3.4.3 Epitope prediction and identification

For epitope prediction and identification, the freely available epitope prediction algorithms hosted through the Immune Epitope Database and Analysis Resource (IEDB) (445), SYFPEITHI (446) and Net MHC (447,448) were used to predict vaccine induced epitopes using vaccine immunogen sequences. The freely available databases of published HBV epitopes, IEDB (445) and Hepitopes, (449) were interrogated to identify known epitopes induced by vaccination.

3.5 RESULTS

3.5.1 ChAdOx1-HBV and MVA-HBV vaccination induce high magnitude T cell responses in several mouse strains

In order to assess the ability of ChAdOx1-HBV alone or in combination with MVA-HBV to induce vaccine specific T cell responses in mammals, C57BL/6J, BALBc or CD1 mice were vaccinated intramuscularly with ChAdOx1-HBV (prime) either alone or followed 4-8 weeks later by MVA-HBV (boost) (Figure 3.1). Splenocytes were isolated 14 days after the last vaccination (peak response) and responses to HBV peptides were tested in IFNγ ELISpot assays.

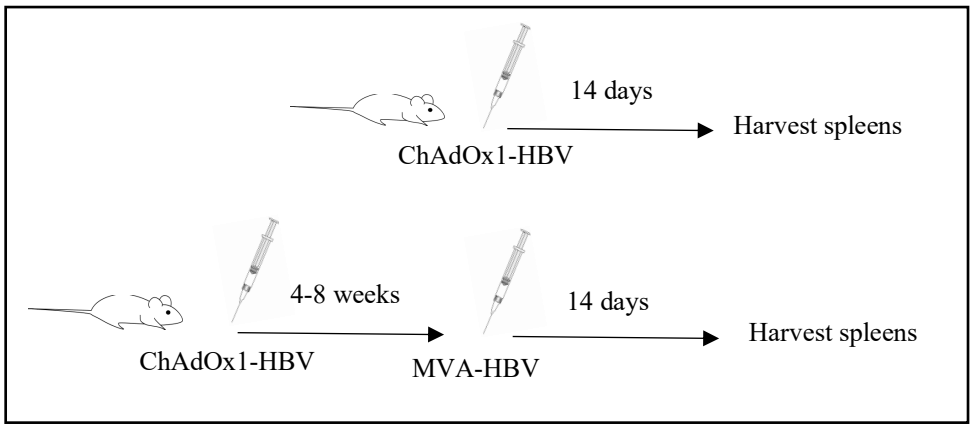


Figure 3.1. Experimental design of ChAdOx1-HBV and MVA-HBV vaccination in mice.

C57BL/6J, BALBc or CD1 mice were vaccinated intramuscularly with ChAdOx1-HBV (prime) either alone or followed 4-8 weeks later by MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination and spleens were harvested for immunological analysis.

C57BL/6J mice

First ChAdOx1-HBV and MVA-HBV were tested in heterologous-prime boost regimes in inbred C57BL/6J mice. C57BL/6J mice are the background strain used in the AAV and adenovirus models of chronic HBV infection (450,451). Understanding the immune response in healthy C57BL/6J mice will be important for future comparison in these mouse models of HBV. The magnitude and breadth of HBV specific T cell responses were compared between mice receiving a single vaccination of ChAdOx1-HBV and mice receiving ChAdOx1-HBV

(prime) followed 4 or 7-8 weeks later by MVA-HBV (boost) (Figure 3.2). The total magnitude of HBV specific T cells induced by vaccination was significantly increased in mice that received MVA-HBV boost at 7-8 weeks after ChAdOx1-HBV prime compared to ChAdOx1-HBV prime alone (median 5325, IQR 2653-6448 vs median 438, IQR 248-616, spot forming units (SFU) per 10^6 splenocytes, p=0.0002) and when MVA-HBV was administered at 4 weeks after ChAdOx1-HBV prime (median 1231, IQR 811-2008 vs median 438, IQR 248-616, p=0.0335) (Figure 3.2.A). In unvaccinated mice, responses were not detectable (data not shown). The breadth of response, defined by the number of positive HBV peptide pools induced after vaccination, was also increased in mice that received MVA-HBV boost at 7-8 weeks after ChAdOx1-HBV prime compared to ChAdOx1-HBV prime alone (median 6 vs 2 positive pools, p=0.0017) although not when MVA-HBV was administered at 4 weeks after ChAdOx1-HBV prime (Figure 3.2.B).

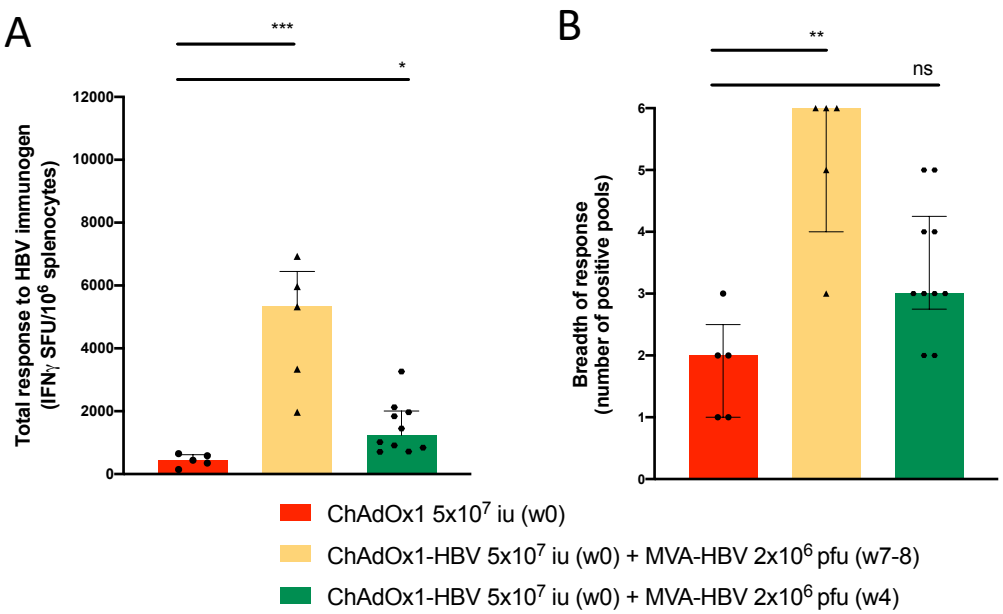


Figure 3.2. T cell responses in C57BL/6J mice after ChAdOx1-HBV and MVA-HBV vaccination.

C57BL/6J were vaccinated intramuscularly with $5x10^7$ infectious units (iu) ChAdOx1-HBV (prime) either alone (week 0, w0) or followed 4-8 weeks later (w4 or w7-8) by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination and spleens were harvested for immunological analysis. Splenocytes were isolated and plated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays.

A: The total magnitude of vaccine induced responses in IFNγ spot forming units per million splenocytes (SFU per 10^6 splenocytes) was calculated by the addition of responses to all peptide pools and shown for each vaccine regimen.
B: The breadth of vaccine induced responses was defined as the number of positive peptide pools.

Results are a summation of 1-2 experiments with 5-10 mice per group. Each colored bar represents a different vaccine regime. Median and interquartile range shown. Total magnitude and breath of responses compared using Kruskal-Wallis test with Dunn's multiple comparison, where each group is compared to the response in C57BL/6J mice after ChAdOx1-HBV prime alone. ***$p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

Next, low dose ($5x10^5$ infectious units (iu)) and high dose ($5x10^7$ infectious units (iu)) ChAdOx1-HBV prime vaccine followed 7-8 weeks later by MVA-HBV were compared to assess whether dosing of ChAdOx1-HBV affected the response magnitude (Figure 3.3). The high dose was chosen as it lies within the most common dosing range for adenoviral vector vaccines in mice at between 10^7-10^{10} viral particles (vp) (452). The low dose was chosen as

few studies have used lower doses of under 10^6 vp in mice, and we hypothesized it might be less immunogenic (452). We used infectious units (iu) to dose ChAdOx1-HBV as this results in a more consistent delivery of live virus between vaccine batches, which might have different ratios of viral particles (viable and nonviable virus) to infectious units (viable virus). Unexpectedly there was no significant difference between the magnitude (Figure 3.3.A) or breadth (Figure 3.3.B) of T cell responses induced after low dose or high dose ChAdOx1-HBV, followed by MVA-HBV boost.

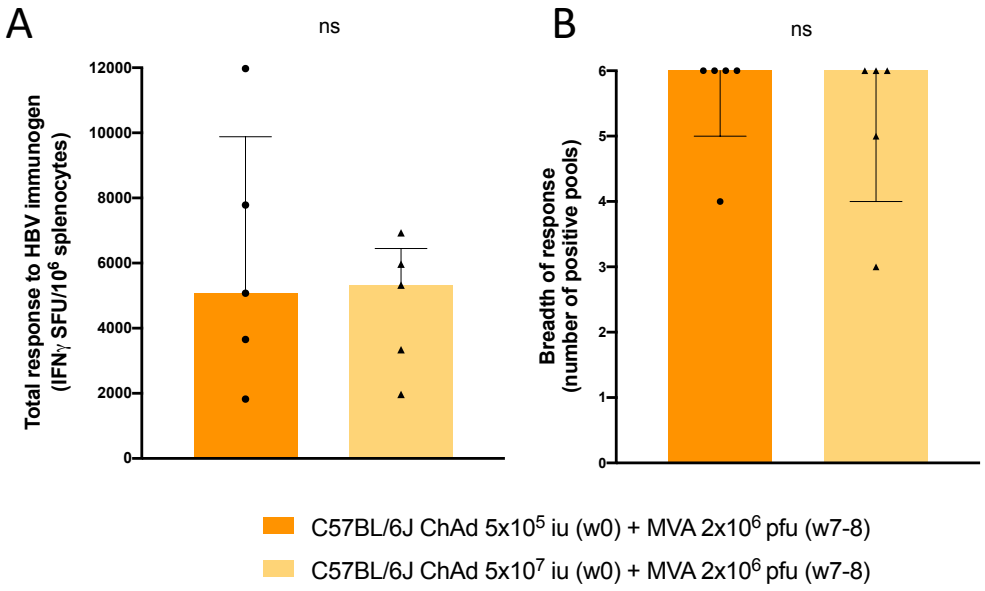


Figure 3.3. T cell responses in C57BL/6J mice after low compared to high dose ChAdOx1-HBV followed by MVA-HBV boost vaccination.

C57BL/6J were vaccinated intramuscularly with low dose $5x10^5$ infectious units (iu) (orange bars) or high dose $5x10^7$ iu (yellow bars) ChAdOx1-HBV (prime, week 0, w0) followed 7-8 weeks later (w7-8) by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination and spleens were harvested for immunological analysis. Splenocytes were isolated and plated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays.

A: The total magnitude of vaccine induced responses in IFNγ spot forming units per million splenocytes (SFU per 10^6 splenocytes) was calculated by the addition of responses to all peptide pools.
B: The breadth of vaccine induced responses was defined as the number of positive peptide pools.

Results are a summation of 1-2 experiments with 5-10 mice per group Median and interquartile range shown. Total magnitude and breath of responses compared between groups using Mann-Whitney-U test. ns = not significant.

BALBc mice

Next, ChAdOx1-HBV and MVA-HBV were tested in heterologous-prime boost regimes in inbred BALBc mice. This strain was chosen to assess the ability of the vaccines to induce immune responses in mice with a different HLA background to C57BL/6J mice. The magnitude and breadth of T cell responses induced after MVA-HBV alone compared to low dose ChAdOx1-HBV followed 7-8 weeks later with MVA-HBV were compared (Figure 3.4). The magnitude of T cell responses induced by ChAdOx1-HBV followed by MVA-HBV was significantly higher than after MVA-HBV alone (median 4906, IQR 2981-6295 vs median 584, IQR 502-1049, p=0.0027 (Figure 3.4.A)). However, there was no difference in the breadth of response between groups (Figure 3.4.B).

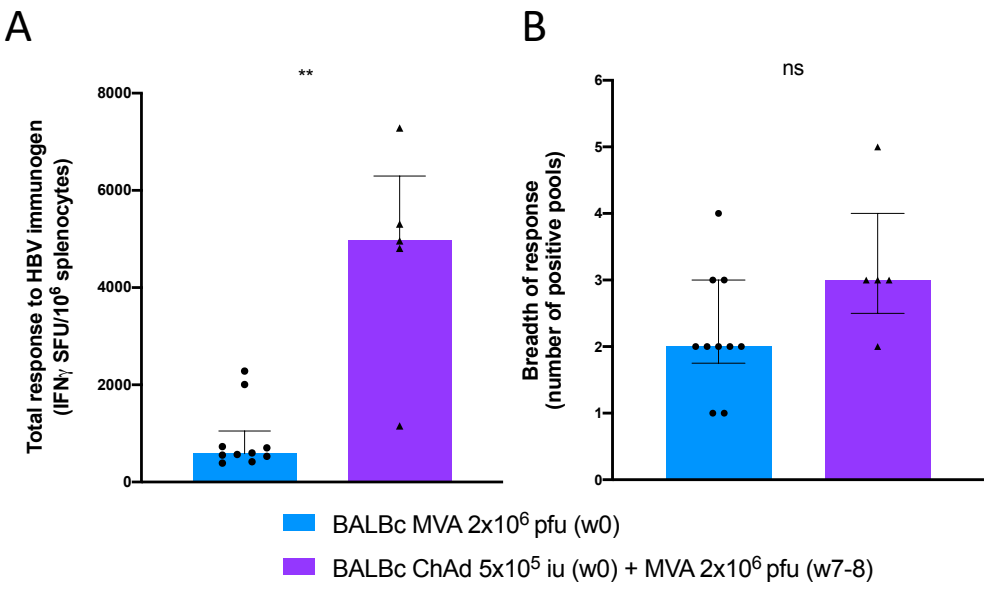


Figure 3.4. T cell responses in BALBc mice after ChAdOx1-HBV and MVA-HBV vaccination.

BALBc were vaccinated intramuscularly with $2x10^6$ plaque forming units (pfu) of MVA-HBV alone or $5x10^5$ infectious units (iu) ChAdOx1-HBV (prime) (week 0, w0) followed 7-8 weeks later (w7-8) by $2x10^6$ pfu of MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination and spleens were harvested for immunological analysis. Splenocytes were isolated and plated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays.

A: The total magnitude of vaccine induced responses in IFNγ spot forming units per million splenocytes (SFU per 10^6 splenocytes) was calculated by the addition of responses to all peptide pools and shown for each vaccine regimen.
B: The breadth of vaccine induced responses was defined as the number of positive peptide pools.

Results are a summation of 1-2 experiments with 5-10 mice per group Each colored bar represents a different vaccine regime. Median and interquartile range shown. Total magnitude and breath of responses compared between groups using Mann-Whitney-U test. ** $p<0.01$, ns = not significant.

CD1 mice

Next ChAdOx1-HBV and MVA-HBV were tested in heterologous-prime boost regimes in outbred CD1 mice. These mice have a higher genetic diversity than inbred strains and therefore are more likely to have heterogenous responses to vaccination, more analogous to vaccine responses in humans. The magnitude and breath of ChAdOx1-HBV prime followed 4 weeks later by MVA-HBV boost was compared between C57BL6/J (inbred) mice and CD1 (outbred mice). There was no significant difference in the magnitude of T cell responses induced by vaccination (Figure 3.5.A), however the breadth of responses was significantly lower in CD1 mice, with wider range of the breath of responses (median 2, IQR 1-3 vs median 3, IQR 3-4 positive peptide pools, p=0.0042, Figure 3.5.B).

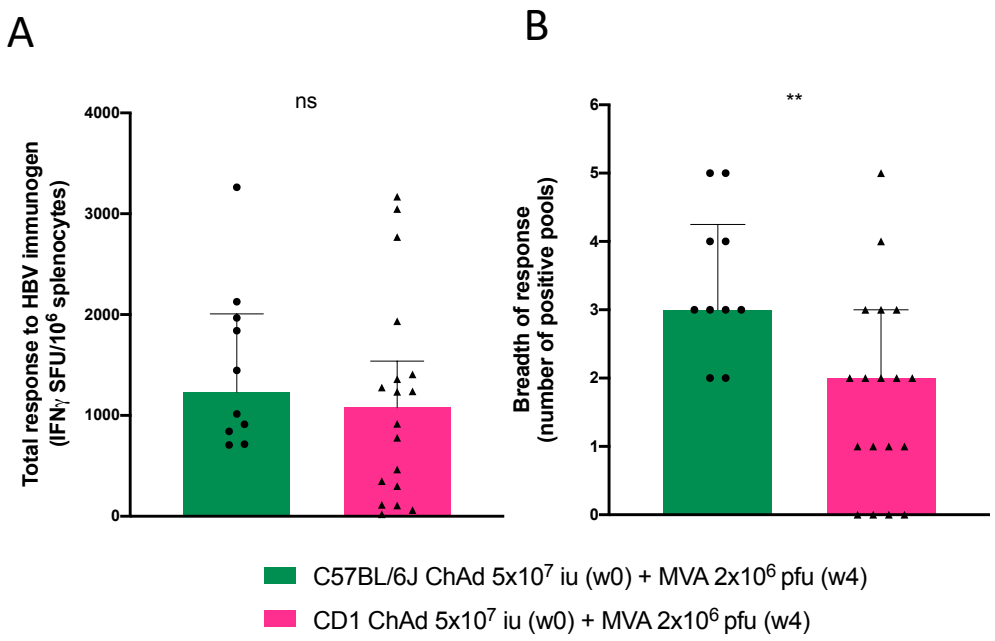


Figure 3.5. T cell responses in C57BL/6J or CD1 mice after ChAdOx1-HBV and MVA-HBV vaccination

C57BL/6J or CD1 mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) ChAdOx1-HBV (prime, week 0, w0) followed 4 weeks later (w4) by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination and spleens were harvested for immunological analysis. Splenocytes were isolated and plated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays.

A: The total magnitude of vaccine induced responses in IFNγ spot forming units per million splenocytes (SFU per 10^6 splenocytes) was calculated by the addition of responses to all peptide pools and shown for each vaccine regimen.
B: The breadth of vaccine induced responses was defined as the number of positive peptide pools.

Results are a summation of 1-2 experiments with 5-10 mice per group Each colored bar represents a different vaccine regime. Median and interquartile range shown. Total magnitude and breath of responses compared between groups using Mann-Whitney-U test. ** $p<0.01$, ns = not significant.

3.5.2 T cells responses induced by ChAdOx1-HBV and MVA-HBV vaccination in mice are specific to the HBV polymerase and HBV surface regions

The regions of the ChAdOx1/MVA-HBV immunogen to which vaccine induced T cells were specific to, was assessed in inbred (C57BL/6J and BALBc) and outbred (CD1) mice (Figure 3.6). T cell responses were detectable to peptide pools corresponding to the HBV polymerase (Pol 1-4) and surface (pre-S1, pre-S2 and S) regions. In C57BL/6J mice T cell responses were detectable to the HBV polymerase region (pol2 peptide pool) and the HBV pre-S1, Pre-S2 and surface regions (Figure 3.6.A). In BALBc mice (Figure 3.6.B), responses were directed to the first part of the HBV polymerase region (pol1 peptide pool) and the HBV surface region (surface peptide pool). In CD-1 mice (Figure 3.6.C), responses were directed towards HBV polymerase (pol1 and pol4 peptide pools) and the HBV surface region (surface peptide pool).

Reassuringly there were no detectable T cell responses to non-HBV vaccine components including shark invariant chain (sIi), furin-2A cleavage region (F2A) and linker regions (Linker). However, responses to HBV core region were largely absent across all mouse strains and regimens tested (Figure 3.6).

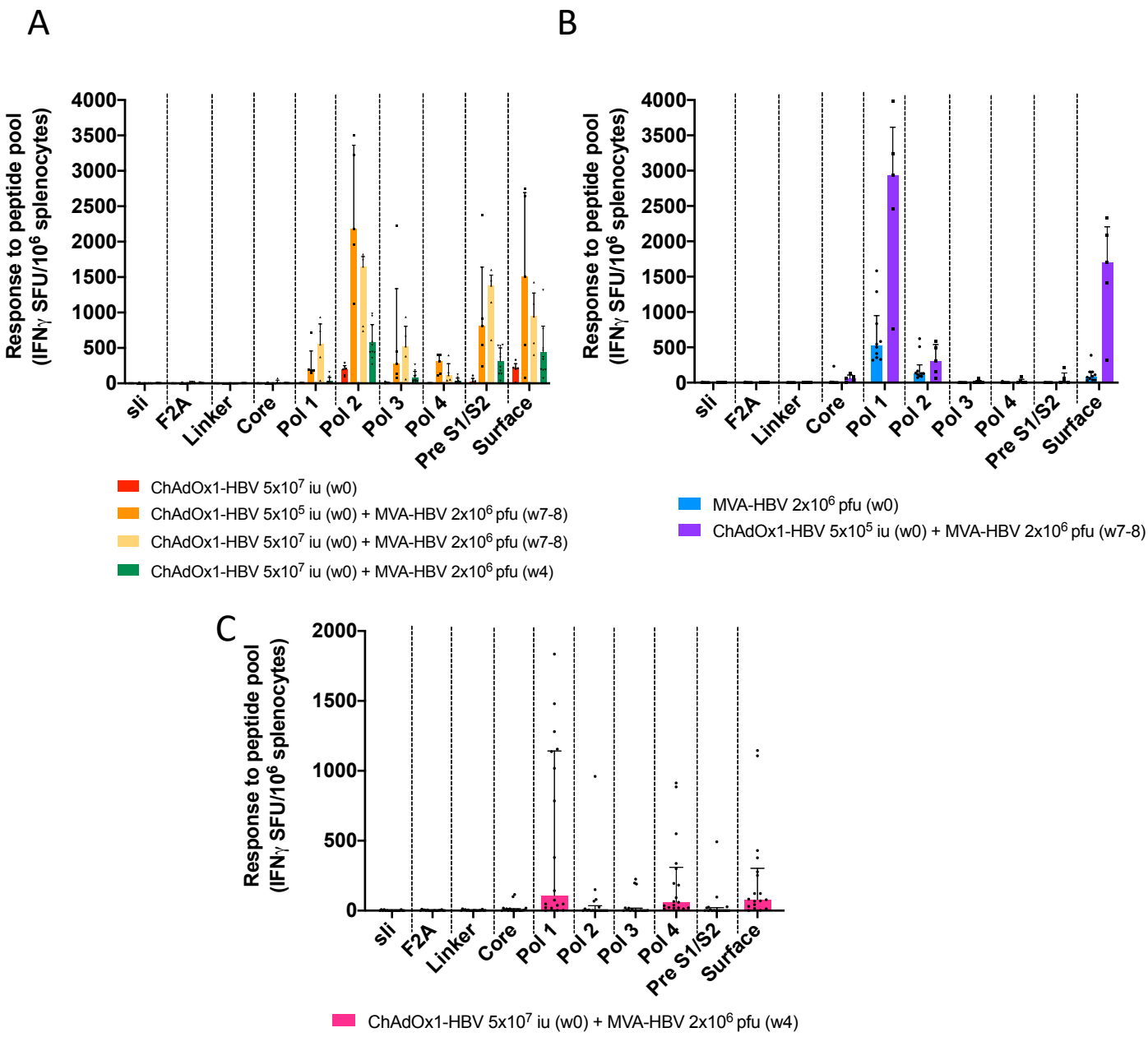


Figure 3.6 The regions of HBV to which T cell responses are induced by ChAdOx1-HBV and MVA-HBV vaccination in mice.

C57BL/6J **(A)**, BALBc **(B)**, or CD1 **(C)** mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) ChAdOx1-HBV or $2x10^6$ plaque forming units (pfu) MVA-HBV alone (week 0, w0) or $5x10^5$ or $5x10^7$ iu ChAdOx1-HBV (prime, w0) followed 4-8 weeks later (w4 or w7-8) by $2x10^6$ pfu MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination and spleens were harvested for immunological analysis. Splenocytes were isolated and plated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays.

The magnitude of the T cell response to each peptide pool is displayed as spot forming units (SFU) per million (10^6) splenocytes. Peptide pools from left to right correspond to truncated shark invariant chain (sIi), furin-2A (F2A), linkers (linker), HBV core (core), HBV polymerase (pol1-pol4), HBV pre-S1 and pre-S2 (pre S1/S2) and HBV surface (surface).

Results are a summation of 1-2 experiments with 5-10 mice per group Each colored bar represents a different vaccine regime. Median and interquartile range shown. No statistical comparison is made between groups.

3.5.3 ChAdOx1-HBV and MVA-HBV vaccination induces polyfunctional CD8 and CD4 T cell responses

The ability of splenocytes from vaccinated C57BL/6J, BALBc and CD-1 mice to produce cytokines upon re-stimulation with pools of HBV peptides was assessed in intracellular staining (ICS) assays by flow cytometry (gating strategy shown in Figure 3.7).

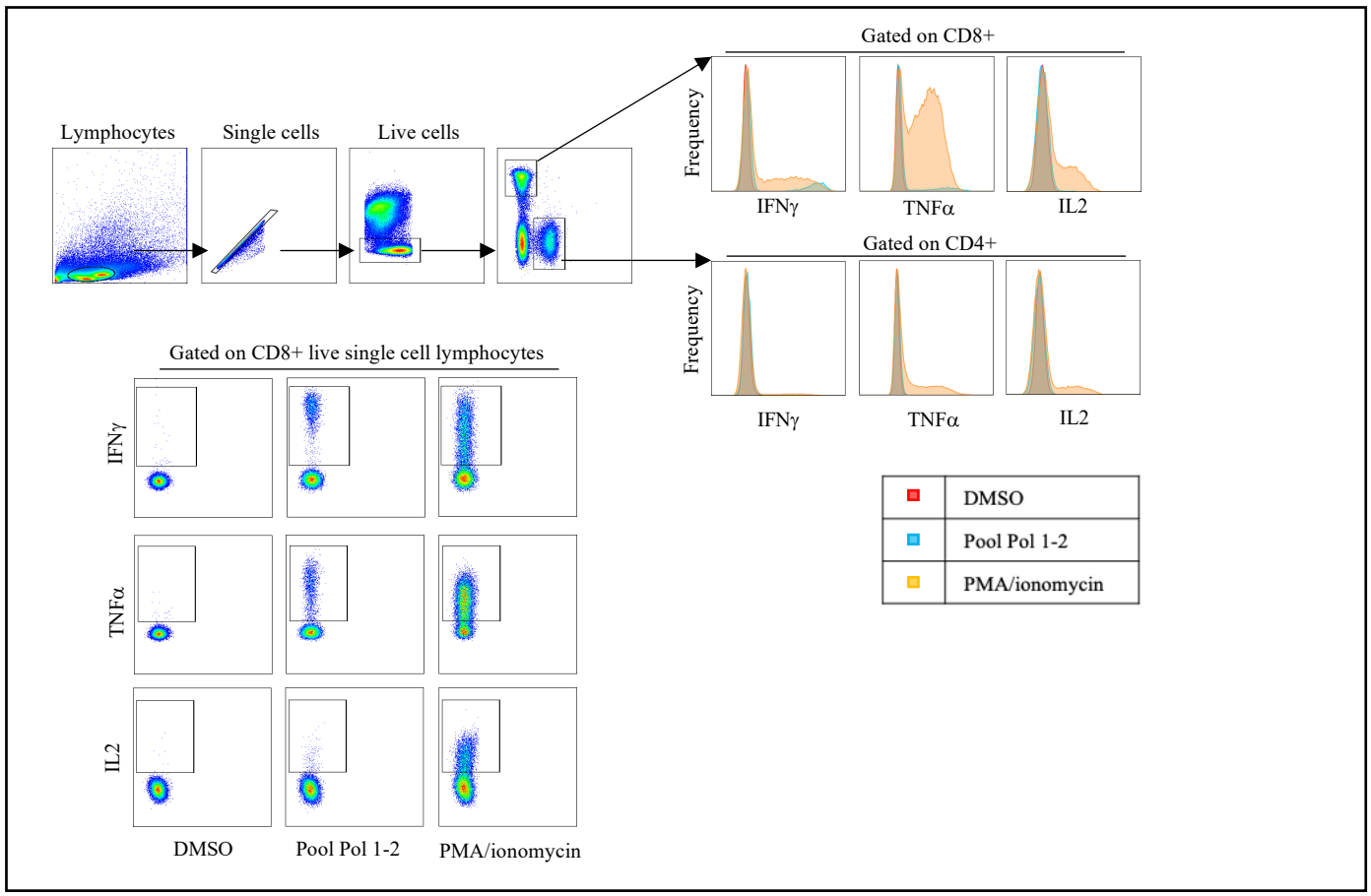


Figure 3.7 The detection of cytokine production by vaccine induced T cells by flow cytometry

Gating strategy to detect live, singlet CD8 or CD4 lymphocytes is shown top left. Histograms show modal expression of IFNγ, TNFα or IL2 on CD8 or CD4 cells after stimulation with dimethyl suphoxide (DMSO) as a negative control (red), pooled peptide Pol 1-2 (polymerase 1 and 2) as test condition (blue) or phorbol myristate acetate and ionomycin (PMA/ionomycin) as a positive control (orange) shown top right. Gating strategy to detect the percentage of CD8 live singlet lymphocytes producing IFNγ, TNFα or IL2 after stimulation with DMSO, peptide pool Pol 1-2 or PMA/ionomycin shown bottom right.

C57BL/6J mice

The cytokine production of vaccine induced T cells was compared between C57BL/6J mice vaccinated with either a single vaccination of ChAdOx1-HBV alone or followed 4-8 weeks later by MVA-HBV (boost) (Figure 3.8). CD8 T cells produced IFNγ when cultured with peptide pools corresponding to the first half of HBV polymerase (pol 1-2), the second half of HBV polymerase (pol 3-4) and the HBV pre-S1, pre-S2 and surface regions (preS1/S2/S) (Figure 3.8.A). The highest percentage of CD8 T cells produced IFNγ in response to the pol 1-2 peptide pool in all vaccine regimes. Compared to ChAdOx1-HBV prime alone, all combinations of ChAdOx1-HBV prime followed by MVA-HBV boost induced a significantly higher percentage of CD8 T cells producing IFNγ in response to the pol 1-2 peptide pool. The largest difference in the percentage of CD8 T cells producing IFNγ to peptide pool pol 1-2 was observed between high dose ChAdOx1-HBV prime followed 7-8 weeks later by MVA-HBV compared with ChAdOx1-HBV prime alone (median 7.10% vs 1.96%, p=0.0006, Figure 3.8A).

CD8 T cells produced tumour necrosis factor alpha (TNFα) when cultured with peptide pools corresponding to the first half of HBV polymerase (pol 1-2), the second half of HBV polymerase (pol 3-4) and the HBV pre-S1, pre-S2 and surface regions (preS1/S2/S), (Figure 3.8.B). The highest percentage of CD8 T cells produced TNFα in response to the pol 1-2 peptide pool in all vaccine regimes. The percentage of CD8 T cells producing TNFα in response to the pol 1-2 peptide pool increased the most after low dose ChAdOx1-HBV prime followed 7-8 weeks later by MVA-HBV boost compared to ChAdOx1-HBV prime alone (median 5.87% vs 0.72%, p<0.0001, Figure 3.8B).

CD8 T cells produced interleukin 2 (IL-2) when cultured with peptide pools corresponding to the first half of HBV polymerase (pol 1-2), (Figure 3.8.C). Vaccination with low dose ChAdOx1-HBV prime followed 7-8 weeks later by MVA-HBV boost led to the highest median percentage of CD8 T cells producing IL-2 to the pol 1-2 peptide pool (median 0.37%, Figure 3.8.C). There were no significant differences in IL-2 production between different vaccine regimes (Figure 3.8.C).

A lower proportion of CD4 cells produced cytokines than CD8 T cells. CD4 T cells produced IFNγ when cultured with peptide pools corresponding to the first half of HBV polymerase (pol 1-2), the second half of HBV polymerase (pol 3-4) and the HBV pre-S1, pre-S2 and surface regions (preS1/S2/S), (Figure 3.8.D). In contrast to CD8 T cells, the highest percentage of CD4 T cells producing IFNγ was towards the pol 3-4 peptide pool. Vaccination with low dose ChAdOx1-HBV prime followed 7-8 weeks later by MVA-HBV boost led to the highest median percentage of CD4 T cells producing IFNγ to the pol 3-4 peptide pool (median 0.37%, Figure 3.8.C). There was no significant difference in IFNγ production between different vaccine regimes (Figure 3.8.D).

CD4 T cells produced TNFα when cultured with peptide pools corresponding to the first half of HBV polymerase (pol 1-2), the second half of HBV polymerase (pol 3-4) and the HBV pre-S1, pre-S2 and surface regions (preS1/S2/S), (Figure 3.8.E). The highest percentage of CD4 T cells producing TNFα was towards the pol 3-4 peptide pool and was increased after low dose ChAdOx1-HBV prime followed 7-8 weeks later by MVA-HBV boost compared to ChAdOx1-HBV prime alone (median 0.35% vs 0.08%, $p<0.0001$, Figure 3.8E).

CD4 T cells produced IL-2 when cultured with peptide pools corresponding to the second half of HBV polymerase (pol 3-4) (Figure 3.8.F). There was no significant difference in IL-2 production between different vaccine regimes (Figure 3.8.F). There was no cytokine production by CD8 or CD4 T cells in response to the non-HBV vaccine components shark invariant chain (sIi), furin-2A and linker regions (AIL peptide pool) nor to the HBV core regions (core peptide pool).

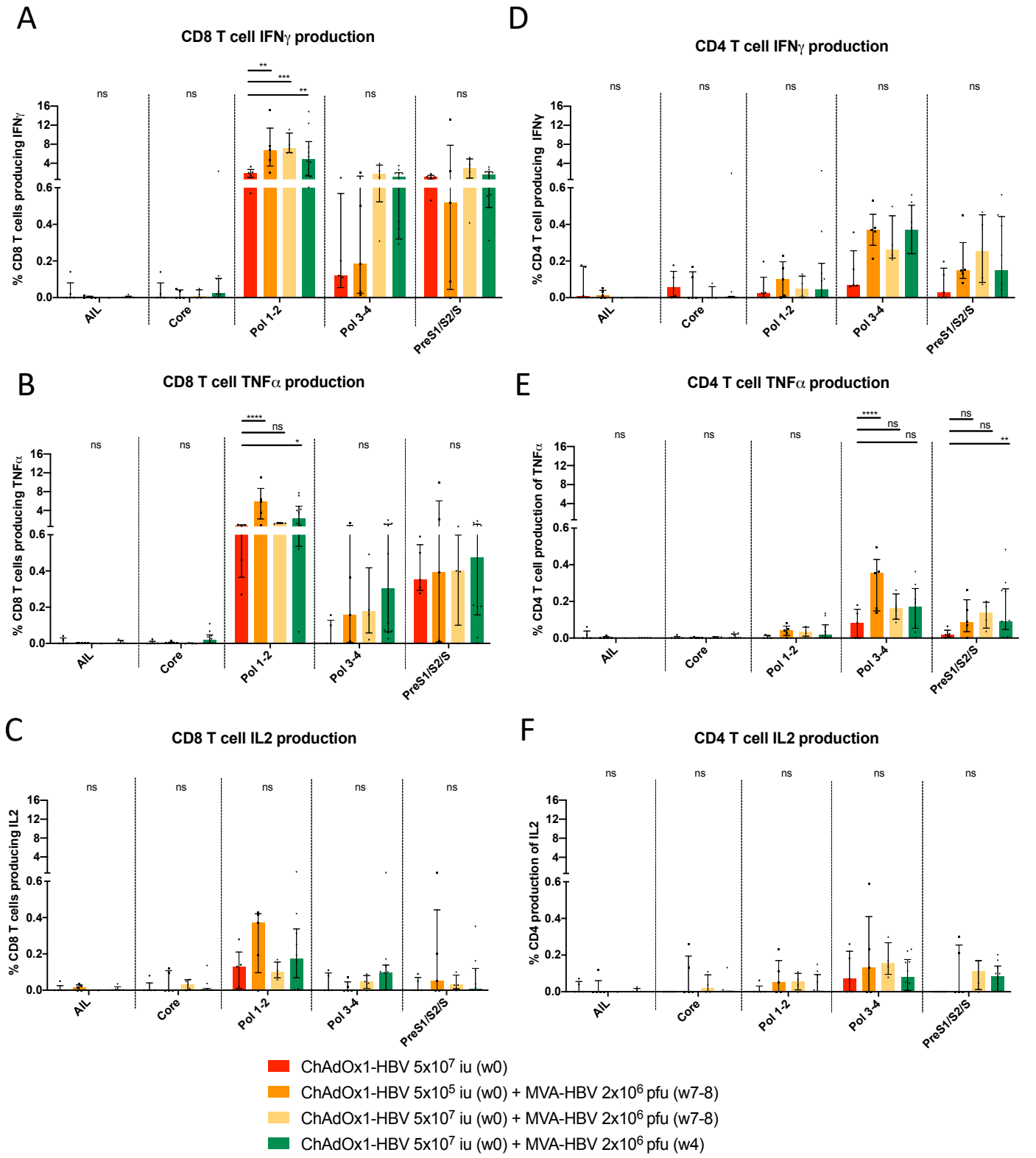


Figure 3.8 Cytokine production by vaccine induced T cells in C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly with $5x10^5$ or $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) either alone (week 0, w0) or followed 4-8 weeks later (w4 or w7-8) by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination to harvest spleens. Splenocytes were isolated and stimulated with peptide pools corresponding to entire vaccine immunogen; shark invariant chain, furin-2A and linker regions (AIL), HBV core (Core), HBV polymerase (Pol1-2 and Pol3-4), HBV surface (PreS1/S2/S). Cells were stained with fluorochrome labelled antibodies to detect intracellular interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα) and interleukin-2 (IL2) production by flow cytometry.

The percentage of CD8 T cells producing IFNγ (**A**), TNFα (**B**) or IL2 (**C**) and the percentage of CD4 T cells producing IFNγ (**D**), TNFα (**E**) or IL2 (**F**) in response to stimulation with peptide pools is shown. Results are a summation of 1-2 experiments with 5-10 mice per group. Each colored bar represents a different vaccine regime. Median and interquartile range shown. Total magnitude of responses compared using 2-way ANOVA test with Dunnett's multiple comparisons, where each vaccine regime is compared to the response after ChAdOx1-HBV prime alone. ****p<0.0001, ***p<0.001, ** p<0.01, * p<0.05, ns = not significant.

Next the polyfunctionality of vaccine induced T cell responses were investigated. The proportion of cytokine producing CD8 or CD4 T cells that produced single (blue), double (pink) or triple (purple) cytokines on re-stimulation were investigated using Boolean gating and SPICE analysis (Figure 3.9). Vaccine induced CD8 T cells were mostly single or double cytokine producers, whereas a greater proportion of CD4 T cells were double or triple cytokine producers.

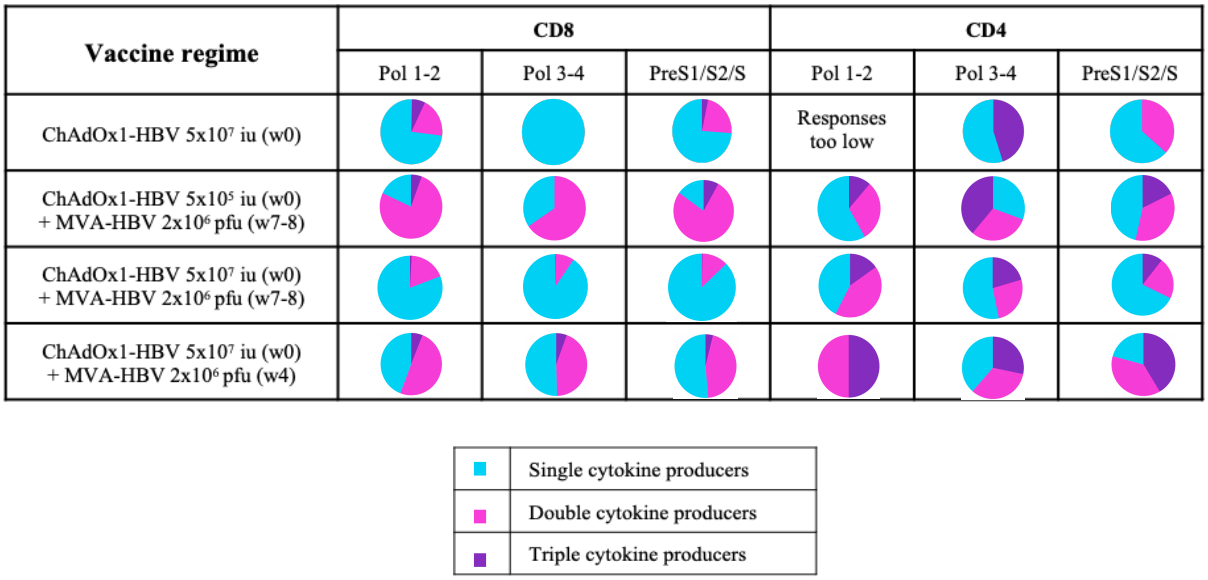


Figure 3.9 Polyfunctionality of T cell responses induced by ChAdOx1-HBV and MVA-HBV vaccination in C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly with $5x10^5$ or $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime, week 0, w0) either alone or followed 4-8 weeks later (w4 or w7-8) by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination to harvest spleens. Splenocytes were isolated and stimulated with peptide pools corresponding to the vaccine immunogen; HBV polymerase (pol 1-2 and pol 3-4) and HBV pre-S1, pre-S2 and surface (PreS1/S2/S). Cells were stained with fluorochrome labelled antibodies to detect intracellular interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα) and interleukin-2 (IL2) production by flow cytometry.

Pie charts show the proportion of cytokine producing CD8 or CD4 T cells that produce single (blue), double (pink) or triple (purple) cytokines in response to stimulation with each peptide pool after vaccination with different regimes. Base of pie chart = median.

BALBc mice

Next, the cytokine production of vaccine induced T cells were compared between BALBc mice vaccinated after a single vaccination of MVA-HBV alone or low dose ChAdOx1-HBV (prime) followed 7-8 weeks later by MVA-HBV (boost) (Figure 3.10).

CD8 T cells produced IFNγ to peptide pools corresponding to the first half of HBV polymerase (pol 1-2) and the HBV pre-S1, pre-S2 and surface regions (preS1/S2/S), (Figure 3.10.A). The highest percentage of CD8 T cells produced IFNγ in response to the pol 1-2 peptide pool. CD8 IFNγ responses to pol 1-2 were significantly increased after ChAdOx1-HBV followed by MVA-HBV boost compared to MVA-HBV alone (median 11.61% vs 0.80%, $p<0.0001$, Figure 3.10A).

T cells produced TNFα to peptide pools corresponding to the first half of HBV polymerase (pol 1-2) and the HBV pre-S1, pre-S2 and surface regions (preS1/S2/S), (Figure 3.10.B). The highest percentage of CD8 T cells produced TNFα in response to the pol 1-2 peptide pool. CD8 TNFα responses to pol 1-2 were significantly increased after ChAdOx1-HBV followed by MVA-HBV boost compared to MVA-HBV alone (median 7.94% vs 0.75%, $p<0.0001$, Figure 3.10.B).

CD8 T cells produced IL-2 to peptide pools corresponding to the first half of HBV polymerase (pol 1-2) and the HBV pre-S1, pre-S2 and surface regions (preS1/S2/S), (Figure 3.10.C). The highest percentage of CD8 T cell produced IL-2 in response to the pol 1-2 peptide pool. CD8 IL-2 responses to pol 1-2 were significantly increased after ChAdOx1-HBV followed by MVA-HBV boost compared to MVA-HBV alone (median 0.65% vs 0.29%, $p<0.0001$, Figure 3.10.B).

Less CD4 than CD8 T cell produced cytokines in response to peptide pool stimulation. CD4 T cells produced IFNγ or TNFα or IL-2 to peptide pools corresponding to the first half of HBV polymerase (pol 1-2) (Figure 3.10. D, E, F). The percentage of CD4 T cells producing TNFα was significantly higher after ChAdOx1-HBV followed by MVA-HBV boost compared to MVA-HBV alone (median 0.19% vs 0.10%, $p<0.0035$, Figure 3.10.E) but there were no differences in IFNγ or IL-2 production.

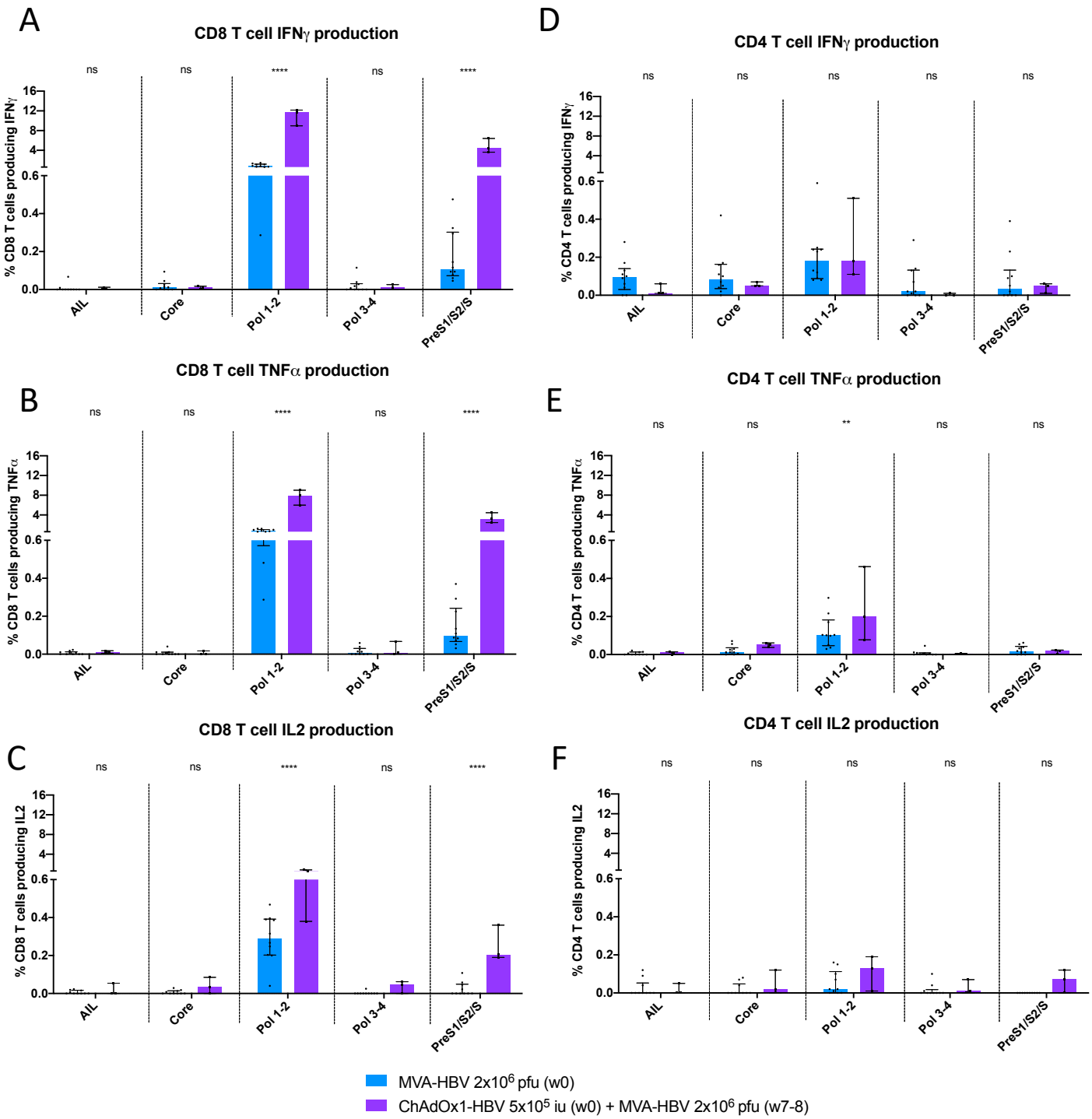


Figure 3.10 Cytokine production by vaccine induced T cells in BALBc mice

BALBc mice were vaccinated intramuscularly with $2x10^6$ plaque forming units (pfu) of MVA-HBV alone or $5x10^5$ infectious units (iu) of ChAdOx1-HBV (prime, week 0, w0) followed 7-8 weeks later (w7-8) by $2x10^6$ pfu of MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination to harvest spleens. Splenocytes were isolated and stimulated with peptide pools corresponding to entire vaccine immunogen; shark invariant chain, furin-2A and linker regions (AIL), HBV core (Core), HBV polymerase (pol 1-2 and pol 3-4), HBV pre-s1, pre-S2 and surface (PreS1/S2/S). Cells were stained with fluorochrome labelled antibodies to detect intracellular interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα) and interleukin-2 (IL2) production by flow cytometry.

The percentage of CD8 T cells producing IFNγ (**A**), TNFα (**B**) or IL2 (**C**) and the percentage of CD4 T cells producing IFNγ (**D**), TNFα (**E**) or IL2 (**F**) in response to stimulation with peptide pools is shown. Results are a summation of 1-2 experiments with 3-10 mice per group. Each colored bar represents a different vaccine regime. Median and interquartile range shown. Total magnitude of responses compared using 2-way ANOVA test with Sidak's multiple comparisons. ****p<0.0001, ** p<0.01, ns = not significant.

Next the polyfunctionality of vaccine induce T cell responses were investigated. The proportion of cytokine producing CD8 or CD4 T cells that produced single (blue), double (pink) or triple (purple) cytokines on re-stimulation were investigated using Boolean gating and SPICE analysis (Figure 3.11). Vaccine indued CD8 T cells specific for pol 1-2 were mostly double or triple cytokine producers, whereas a greater proportion of CD4 T cells were single cytokine producers.

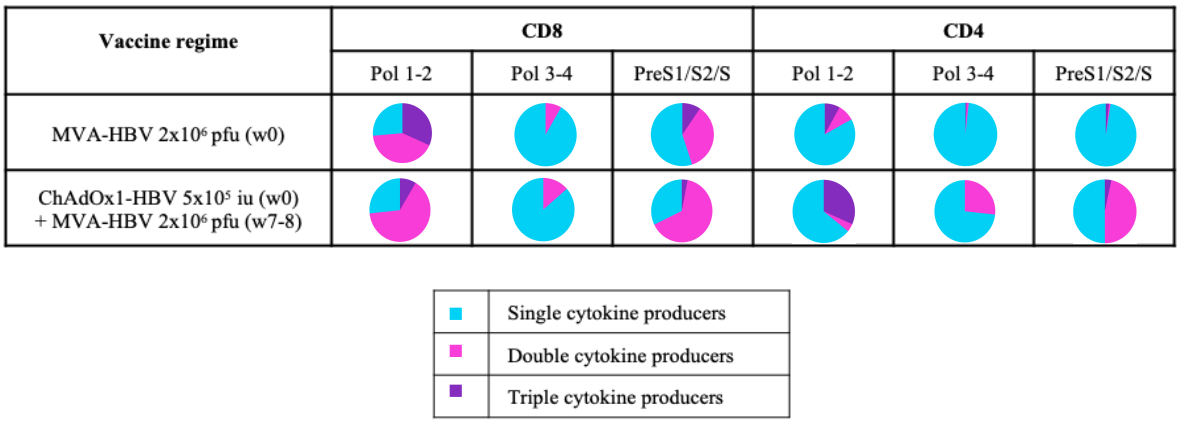


Figure 3.11 Polyfunctionality of T cell responses induced by ChAdOx1-HBV and MVA-HBV vaccination in BALBc mice

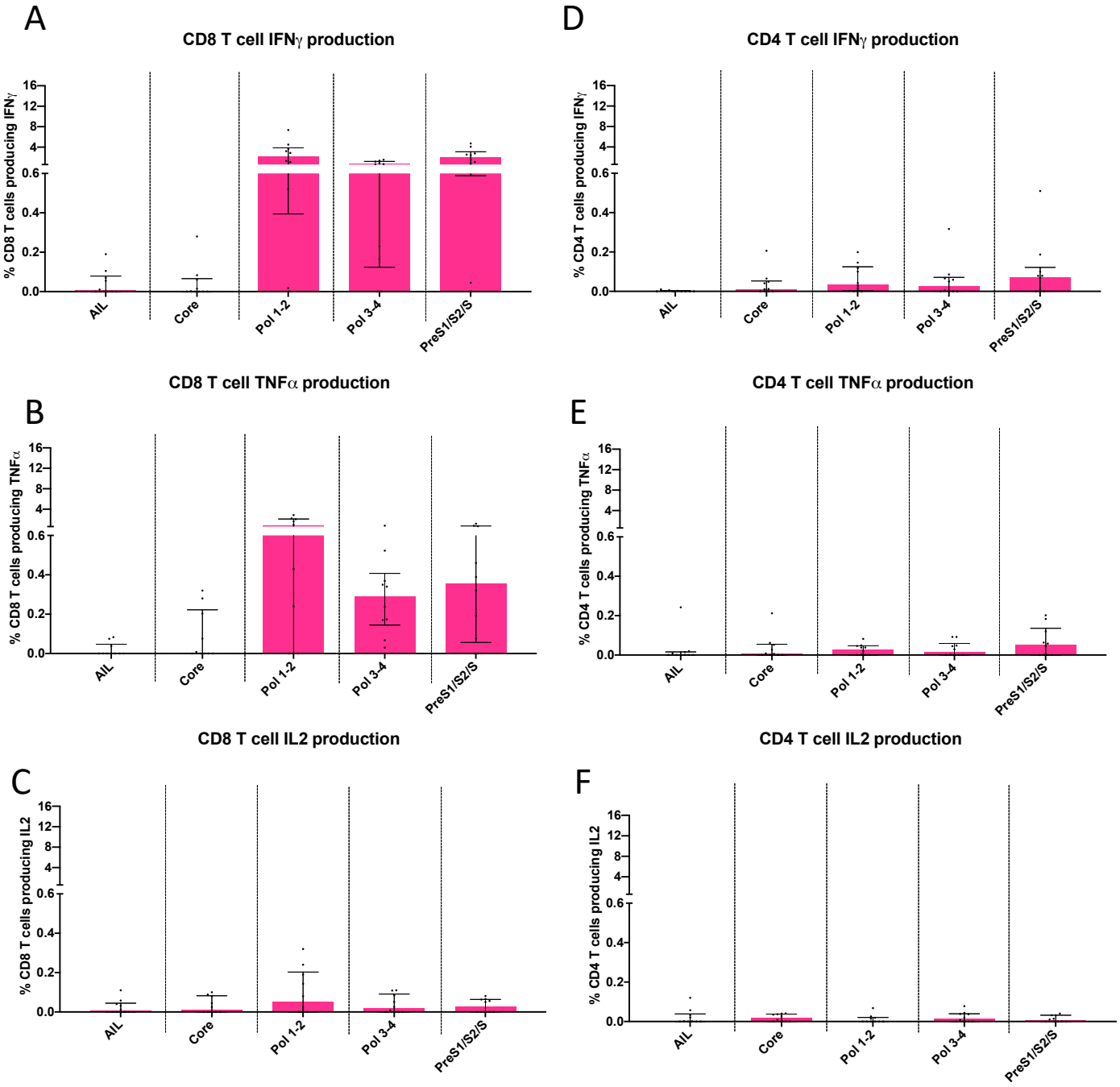
BALBc mice were vaccinated intramuscularly with $2x10^6$ plaque forming units (pfu) of MVA-HBV alone or $5x10^5$ infectious units (iu) of ChAdOx1-HBV (prime, week 0, w0) followed 7-8 weeks later (w7-8) by $2x10^6$ pfu of MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination to harvest spleens. Splenocytes were isolated and stimulated with peptide pools corresponding to the vaccine immunogen; HBV polymerase (pol 1-2 and pol 3-4) and HBV pre-S1, pre-S2 and surface (PreS1/S2/S). Cells were stained with fluorochrome labelled antibodies to detect intracellular interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα) and interleukin-2 (IL2) production by flow cytometry.

Pie charts show the proportion of cytokine producing CD8 or CD4 T cells that produce single (blue), double (pink) or triple (purple) cytokines in response to stimulation with each peptide pool after vaccination with different regimes. Base of pie chart = median.

CD1 mice

Finally, the cytokine production of vaccine induced T cells was investigated in CD-1 mice after vaccination with ChAdOx1-HBV prime followed 4 weeks later by MVA-HBV boost (Figure 3.12).

CD8 T cells produced IFNγ and TNFα to peptide pools corresponding to the first half of HBV polymerase (pol 1-2), the second half of polymerase (pol 3-4) and the HBV pre-S1, pre-S2 and surface regions (preS1/S2/S), (Figure 3.12.A and B). Only minimal CD8 T cells produced IL-2 (Figure 3.12.C) and CD4 cells produced cytokines only occasionally (Figure 3.12.D, E and F). The majority of cytokine producing T cells were single cytokine producers (Figure 3.12.G).



G

Vaccine regime	CD8			CD4		
	Pol 1-2	Pol 3-4	PreS1/S2/S	Pol 1-2	Pol 3-4	PreS1/S2/S
ChAdOx1-HBV 5x10⁷ iu (w0) + MVA-HBV 2x10⁶ pfu (w4)						

	Single cytokine producers
	Double cytokine producers
	Triple cytokine producers

Figure 3.12 Cytokine production by vaccine induced T cells in CD-1 mice.

CD-1 mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime, week 0, w0) followed 4 weeks later (w4) by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were sacrificed 14 days after their last vaccination to harvest spleens. Splenocytes were isolated and stimulated with peptide pools corresponding to entire vaccine immunogen; shark invariant chain, furin-2A and linker regions (AIL), HBV core (Core), HBV polymerase (pol 1-2 and pol 3-4), HBV pre-S1, pre-S2 and surface (PreS1/S2/S). Cells were stained with fluorochrome labelled antibodies to detect intracellular interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα) and interleukin-2 (IL2) production by flow cytometry.

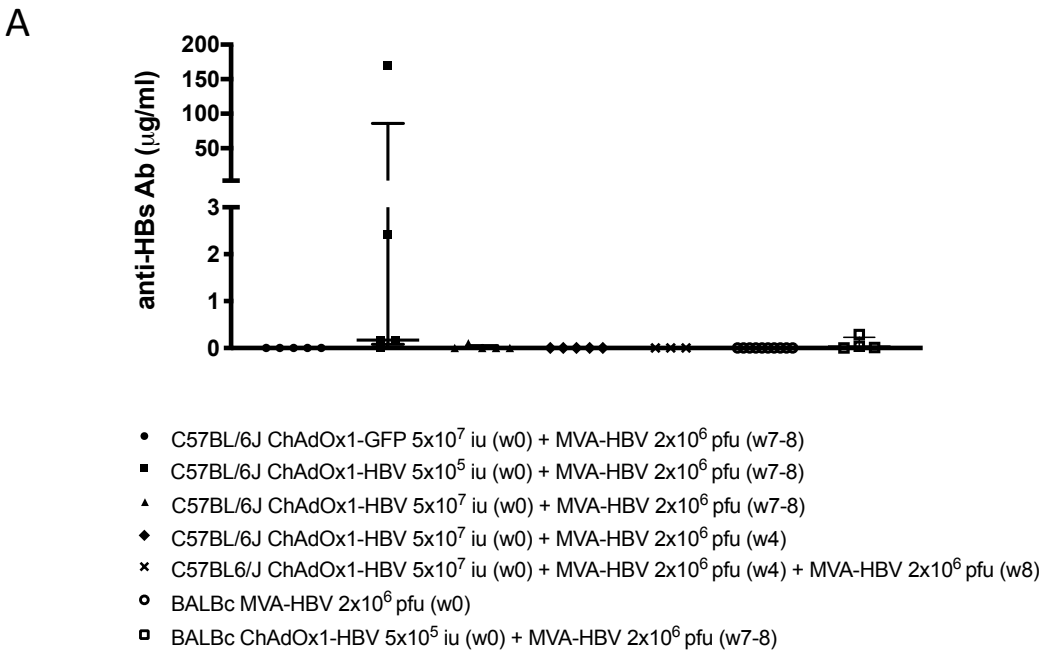
The percentage of CD8 T cells producing IFNγ (**A**), TNFα (**B**) or IL2 (**C**) and the percentage of CD4 T cells producing IFNγ (**D**), TNFα (**E**) or IL2 (**F**) in response to stimulation with peptide pools is shown. Results are a summation of 2 experiments with total 18 mice. Median and interquartile range shown. Pie charts (**G**) show the proportion of cytokine producing CD8 or CD4 T cells that produce single (blue), double (pink) or triple (purple) cytokines in response to stimulation with each peptide pool after vaccination with different regimes. Base of pie chart = median.

3.5.4 ChAdOx1-HBV and MVA-HBV vaccination does not reproducibly induce anti-HBV surface antibody

In order to assess the ability of vaccination to induce humoral responses, sera from C57BL/6J and BALBc vaccinated mice were tested for the presence of anti-Hepatitis B surface antibody (anti-HBs Ab) in enzyme-linked immunosorbent assays (ELISA). C57BL/6J mice receiving ChAdOx1-HBV followed 7-8 weeks later by MVA-HBV had detectable anti-HBs Ab titers (median 0.17μg/mL, IQR 0.08–86.05, figure 3.13.A). However, anti-HBs Ab was not induced by any other vaccine regime. Sera were also tested at later time points after MVA-HBV boost (week 5 and week 8 after vaccination) and again anti-HBs Ab was not detectable (data not shown).

In the C57BL/6J mice that had detectable anti-HBs Ab responses to vaccination the relationship between CD4 and CD8 cytokine responses and anti-HBs Ab titers were investigated. There was no correlation between anti-HBs Ab and the percentage of CD4 or CD8 cells producing cytokine in response to stimulation with the peptide pool corresponding

to HBV pre-S1, pre-S2 and surface (preS1/S2/S) (Figure 3.13.B and C), nor CD8 responses to the peptide pool corresponding to HBV polymerase (pol 1-2) (Figure 3.13.D).



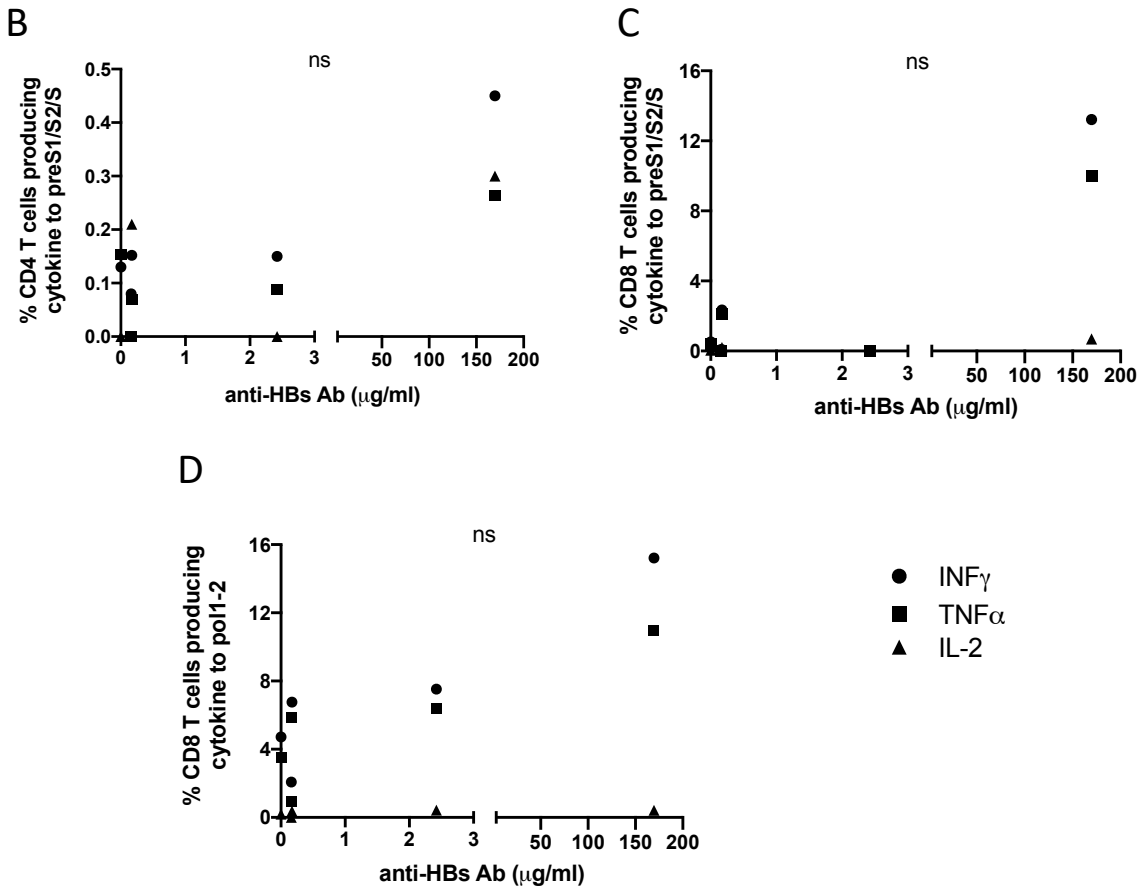


Figure 3.13 Anti-HBV surface antibody (anti-HBs Ab) induction by ChAdOx1-HBV and MVA-HBV vaccination in mice

C57BL6/J or BALBc mice were vaccinated intramuscularly with $5x10^5$ or $5x10^7$ infectious units (iu) of ChAdOx1-HBV or ChAdOx1-GFP (prime, week 0, w0) followed 4-8 weeks later (w4 or w7-8) by $2x10^6$ plaque forming units of MVA-HBV (boost) either once or twice. Mice were sacrificed 2-3 weeks after their last vaccination and sera tested for the presence of anti-HBs Ab in enzyme-linked immunosorbent assays (ELISA). **A:** anti-HBs Ab titer (μg/mL) detection after different vaccination regimes.

Anti-HBs Ab titers of C57BL6/J mice vaccinated with $5x10^5$ infectious units of ChAdOx1-HBV (prime) followed 7-8 week later by $2x10^9$ plaque forming units of MVA-HBV (boost) were correlated with percentage of T cells producing IFNγ (circles), TNFα (squares) or IL2 (triangles) in response to peptide pools corresponding to HBV pre-S1, pre-S2 and surface (S1/S2/S) or HBV polymerase (pol 1-2) in intracellular staining assays. **B**: CD4 T cell responses to S1/S2/S. **C:** CD8 cell responses to S1/S2/S. **D:** CD8 cell responses to pol 1-2.

Results are a summation of single experiment with 3-5 mice per group. Median and interquartile range shown. Correlations assessed by Spearman's rank test, ns = not significant.

3.5.5 Immunogenic regions of ChAdOx1-HBV and MVA-HBV vaccines are identified in HBV polymerase and surface

To further characterise the regions of the HBV vaccine immunogen that induced murine T cell responses, splenocytes from vaccinated C57BL/6J, BALBc and CD1 mice were stimulated in IFNγ ELISpot assays with minipools consisting of ten peptides, 15 amino acids in length, covering the entire vaccine immunogen. In each minipool with the highest responses, the individual 15-amino acid peptides were then tested separately.

C57BL6

Splenocytes from C57BL/6J mice 2 weeks after vaccination with $5x10^5$ or $5x10^7$ iu ChAdOx1-HBV followed 4 weeks later by MVA-HBV responded most strongly to minipools P8 (peptide numbers 134-143, median 583, IQR 313-955, SFU per 10^6 splenocytes), P9 (peptide numbers 144-153, median 599, IQR 431-875 SFU per 10^6 splenocytes) and P10 (peptide numbers 154-163, median 470, IQR 280-687 SFU per 10^6 splenocytes) within the HBV polymerase region of the immunogen (Figure 3.14.A). Within HBV surface, the highest

magnitude responses were seen towards minipools S1 (peptide numbers 282-291, median 294, IQR 137-443 SFU per 10^6 splenocytes), S2 (peptide numbers 292-301, median 279, IQR 153-508 SFU per 10^6 splenocytes), S8 (peptide numbers 352-361, median 228, IQR 64-417 SFU per 10^6 splenocytes) and S9 (peptide numbers 362-371, median 300, IQR 216-592 SFU per 10^6 splenocytes, Figure 3.14.A).

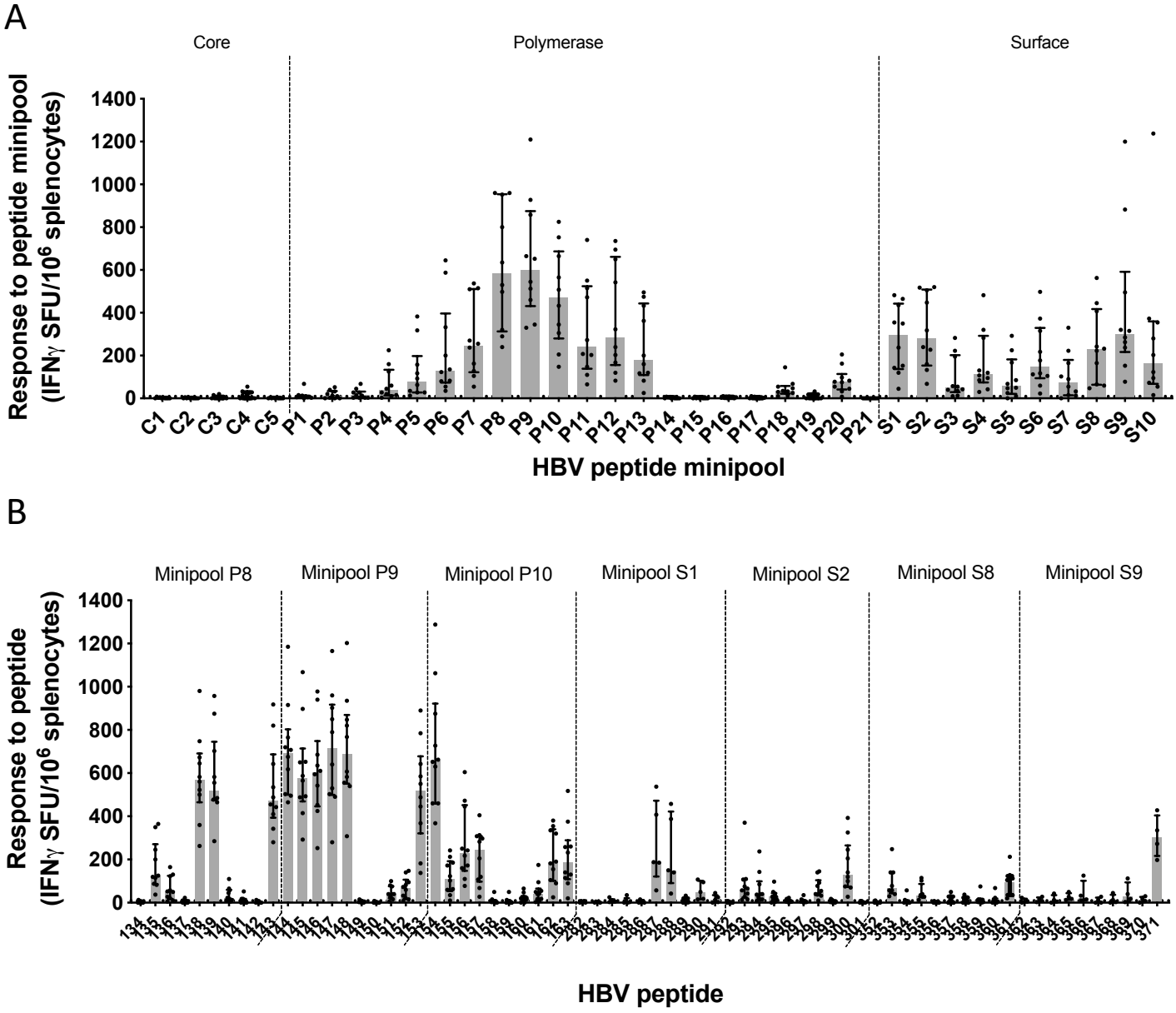


Figure 3.14 Identification of regions of the HBV immunogen that induce T cell responses after vaccination with ChAdOx1-HBV and MVA-HBV in C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly with $5x10^5$ or $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime, week 0, w0) followed 4 weeks later (w4) by $2x10^6$ plaque forming units (pfu) of MVA-HBV. Mice were sacrificed 14 days after their last vaccination. Splenocytes were isolated and plated in interferon-gamma (IFNγ) ELISpot assays with minipools corresponding to the entire vaccine immunogen or to individual peptides in responsive minipools. The magnitude of vaccine responses was recorded as IFNγ spot forming units per million splenocytes (IFNγ SFU/10^6 splenocytes). **A** Minipool and **B** peptide responses.

Results are a summation of 1-2 experiments with total 9 mice per group. Median and interquartile range shown.

The highest responding peptides within each minipool are shown in Figure 3.14.B, with corresponding amino acid sequences in Table 3.1. Within HBV polymerase the highest responses were recorded towards peptide numbers 138-139 in minipool P8 (peptide 138 median 571, IQR 465- 691 SFU per 10^6 splenocytes), peptides 143-148 in minipools P8-P9 (peptide 147 median 715, IQR 499-917 SFU per 10^6 splenocytes) and peptides 153-154 in minipools P9-P10 (peptide 154 median 658, IQR 460-922 SFU per 10^6 splenocytes). Within HBV surface the highest responses were recorded towards peptide numbers 287-288 in minipool S1 (peptide 287 median 188, IQR 121-473 SFU per 10^6 splenocytes), peptide number 300 in minipool S2 (median 130, IQR 74-265 SFU per 10^6 splenocytes) and peptide number 371 in minipool S9 (median 304, IQR 216-404 SFU per 10^6 splenocytes).

Table 3.1: HBV peptide regions that induce HBV specific T cells after vaccination with ChAdOx1-HBV and MVA-HBV in mice

Strain	MHC Class I	Minipool	Peptide numbers	Amino acid sequence
BALBc	$H2K^d$ $H2D^d$ $H2L^d$	P4	98-99	HAVNHYFKTRHYLHTLWKA
		P10	159	PHNTTESRLVVDFSQ
		S6	332	TIPQSLDSWWTSLNF
		S10	374-375	IWMMWYWGPSLYNILSPFL
C57BL/6J	$H2K^b$ $H2D^b$ H2Lnull	P8	138-139	SSSGHAVELHNIPPSSARS
		P8- P9	143-148	SEGPIFSAWWLQFRNSKPASDYALTHIVNLLEDWG
		P9-P10	153- 154	GEHNIRIPRTPARVTGGVF
		S1	287-288	PLGFFPDHQLDPAFGANSN
		S2	300	GLLGWSPQAQGILTT
		S9	371	FVGLSPTVWLSVIWM
CD1	Outbred	P4	98-99	HAVNHYFKTRHYLHTLWKA
		P21	269	PSVPSHLPDRVHFAS
		S9	362	CTCIPIPSSWAFARF

BALBc

Splenocytes from BALBc mice, harvested 2 weeks after vaccination with $5x10^7$ iu ChAdOx1-HBV responded most strongly to minipools P4 (peptide numbers 94-103, median 715, IQR 276-1110 SFU per 10^6 splenocytes) and P10 (peptide numbers 154-163, median 65, IQR 11-80 SFU per 10^6 splenocytes) within HBV polymerase region and S6 (peptide numbers 332-341, median 183, IQR 54-391 SFU per 10^6 splenocytes) and S10 (peptide

numbers 372-379, median 93, IQR 35-286 SFU per 10^6 splenocytes) within the HBV surface region (Figure 3.15.A).

The highest responding peptides within each minipool are shown in Figure 3.15.B, with corresponding amino acid sequences in Table 3.1. Within HBV polymerase, specific responses were recorded towards peptide numbers 98-99 in minipool P4 (peptide 99 median 440, IQR 191-671 SFU per 10^6 splenocytes) and peptide number 159 in minipool P10 (median 80, IQR 51-107 SFU per 10^6 splenocytes). Within HBV surface, responses were recorded specific for peptide number 332 in minipool S6 (median 270, IQR 35-590 SFU per 10^6 splenocytes) and numbers 374-375 in minipool S10 (peptide 375 median 165, IQR 34-273 SFU per 10^6 splenocytes).

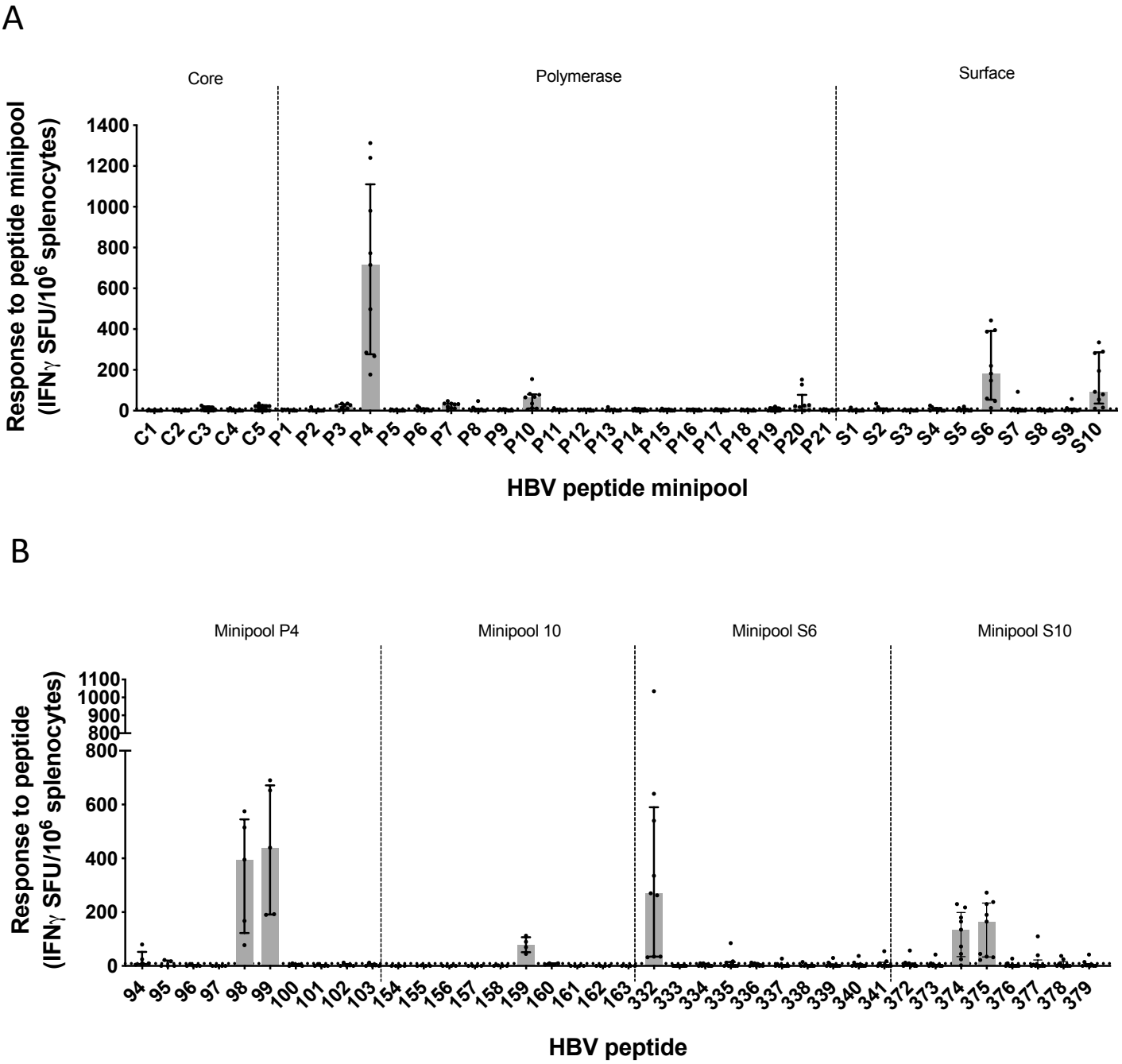


Figure 3.15 Identification of regions of the HBV immunogen that induce T cell responses after vaccination with ChAdOx1-HBV and MVA-HBV in BALBc mice

BALBc mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-HBV alone. Mice were sacrificed 14 days later. Splenocytes were isolated and plated in interferon-gamma (IFNγ) ELISpot assays with minipools corresponding to the entire vaccine immunogen or to individual peptides in responsive minipools. The magnitude of vaccine responses was recorded as IFNγ spot forming units per million splenocytes (IFNγ SFU/10^6 splenocytes). **A** Minipool and **B** peptide responses. Results are a summation of 1-2 experiments with total 9 mice per group. Median and interquartile range shown.

CD1

Splenocytes from CD1 mice 2 weeks after vaccination with $5x10^7$ iu ChAdOx1-HBV 4 weeks later by MVA-HBV responded most strongly to minipools P4 (peptide numbers 94-103, median 5, IQR 0-1113 SFU per 10^6 splenocytes) and P21 (peptide numbers 264-273, median 138, IQR 45-253 SFU per 10^6 splenocytes) within HBV polymerase and S9 (peptide numbers 362-371, median 123, IQR 18-365 SFU per 10^6 splenocytes) within the HBV surface region of the immunogen (Figure 3.16.A).

The highest responding peptides within each minipool are shown in Figure 3.16.B, with corresponding amino acid sequences in Table 3.1. Within HBV polymerase responses were recorded towards peptide numbers 98-99 in minipool P4 (peptide number 98, median 0, IQR 0-375 SFU per 10^6 splenocytes) and peptide number 269 in minipool P21 (median 440, IQR 86-601 SFU per 10^6 splenocytes). Within HBV surface minipool S9, responses were recorded towards peptide 362 (median 645, IQR 255-924 SFU per 10^6 splenocytes).

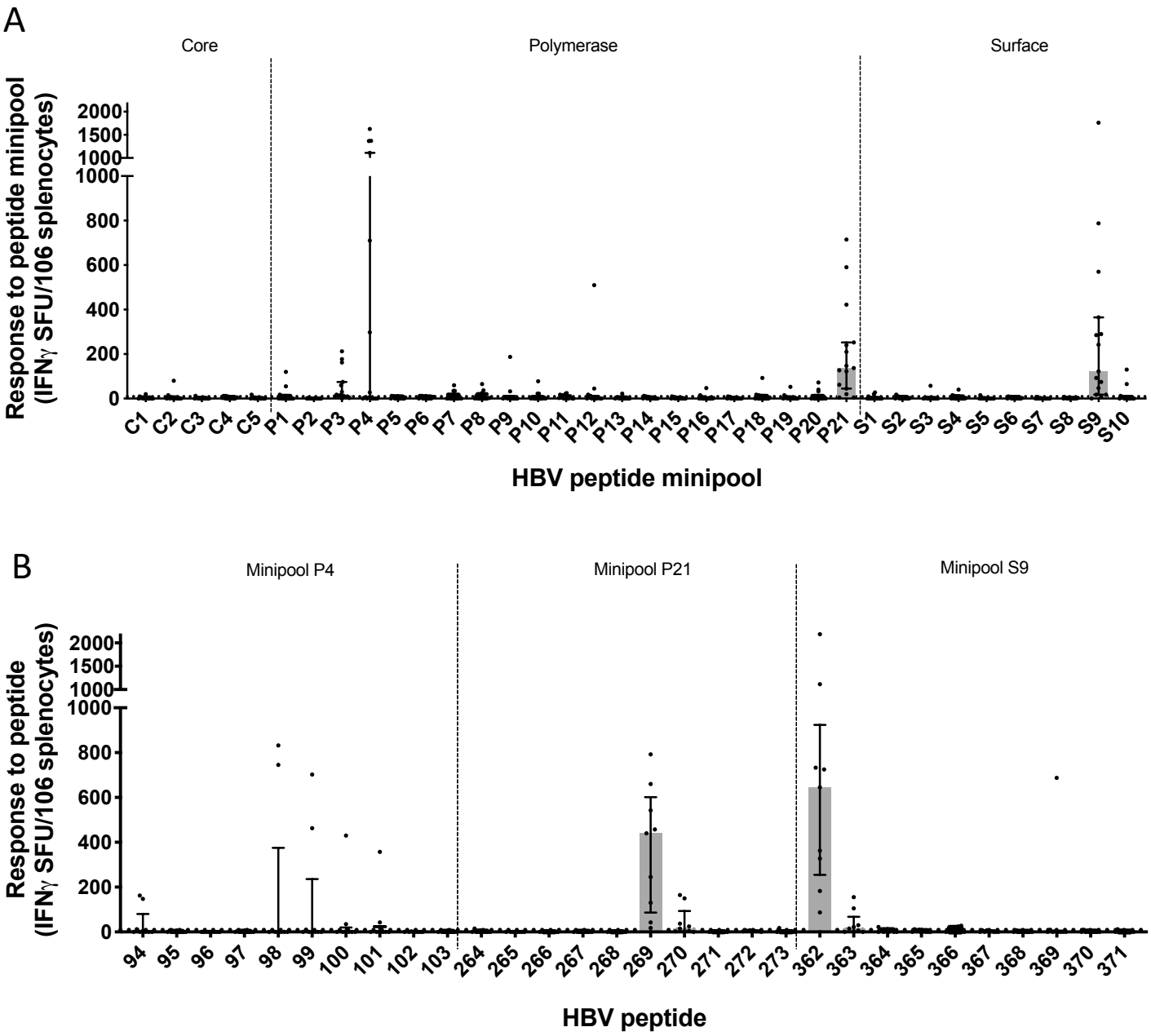


Figure 3.16 Identification of regions of the HBV immunogen that induce T cell responses after vaccination with ChAdOx1-HBV and MVA-HBV in CD-1 mice

CD-1 mice were vaccinated intramuscularly with $5x10^5$ or $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime, week 0, w0) followed 4 weeks later (w4) by $2x10^6$ plaque forming units (pfu) of MVA-HBV. Mice were sacrificed 14 days later. Splenocytes were isolated and plated in interferon-gamma (IFNγ) ELISpot assays with minipools corresponding to the entire vaccine immunogen or to individual peptides in responsive minipools. The magnitude of vaccine responses was recorded as IFNγ spot forming units per million splenocytes (IFNγ SFU/10^6 splenocytes). **A** Minipool and **B** peptide responses. Results are a summation of 1-2 experiments with total 9 mice per group. Median and interquartile range shown.

3.5.6 $H2D^b$ and $H2K^b$ restricted epitopes induced by vaccination can be identified in C57BL/6J mice

C57BL/6J mice were chosen to further define epitope regions, with the aim of developing or purchasing a fluorochrome labelled multimer so as to identify vaccine induced cells *ex-vivo* without peptide stimulation. Within HBV polymerase, the amino acid sequences corresponding to peptide numbers 138-139 (SSSGHAVELHNIPPSSARS), 143-148 (SEGPIFSAWWLQFRNSKPASDYALTHIVNLLEDWG) and 153-153 (GEHNIRIPRTPARVTGGVF) were identified by ELISpot as being immunogenic (Figure 3.14.B, Table 3.1). These regions were investigated for known or putative epitopes using three online prediction algorithms; Immune Epitope Database and Analysis Resource (IEDB) (445), SYFPEITHI (446) and Net MHC (447,448). No $H2K^b$ or $H2D^b$ restricted epitopes had previously been described within these regions. Five peptides, 9 amino acids in length (SSSGHAVEL, YALTHIVNL, LQFRNSKPA, ASDYALTHI and THIVNLLED) were predicted as being intermediate or strong binders to either $H2K^b$ or $H2D^b$ by one or more of the algorithms (Table 3.2).

Table 3.2: Prediction of epitopes within the HBV polymerase region in C57BL/6J mice

Predicted epitope	Number of amino acids	IEDB Artificial Neural Network(445) ic50 (nM, <50 high affinity and <500 intermediate affinity binder)		SYFPETHI binding score(446) (>25 high binder, >18 intermediate binder)		NET MHC rank (447,448) (<0.5 strong binder, <2 intermediate binder)	
		$H2K^b$	$H2D^b$	$H2K^b$	$H2D^b$	$H2K^b$	$H2D^b$
SSSGHAVEL	9	2256.86	23269.18	Not predicted	Intermediate 18	3.5	8.5
YALTHIVNL	9	Intermediate 157.25	Intermediate 115.53	Not predicted	Intermediate 17	Strong 0.4	Strong 0.07
LQFRNSKPA	9	4988.41	7788.72	Not predicted	12	10	Intermediate 1.3
ASDYALTHI	9	4949.6	Not predicted	Not predicted	Intermediate 19	7	6
THIVNLLED	9	22188.79	28222.88	Not predicted	Intermediate 19	31	12

Splenocytes from C57BL/6J mice vaccinated with $5x10^7$ iu ChAdOx1-HBV followed 4 weeks later by MVA-HBV were plated with peptides (SSSGHAVEL, YALTHIVNL, LQFRNSKPA, ASDYALTHI and THIVNLLED) in IFNγ ELISpot assays (Figure 3.17). Splenocytes from vaccinated mice were responsive to all peptides tested. The two highest responses were towards peptides YALTHIVNL (median 1219, IQR 900-1483 SFU per 10^6 splenocytes) and THIVNLLED (median 1083, IQR 788-1447 SFU per 10^6 splenocytes). Peptide SSSGHAVEL had the lowest responses (median 36.65, IQR 0-169). There were no commercially available fluorochrome multimers available with these specificities and none had previously been described as epitopes on the IEDB database (445).

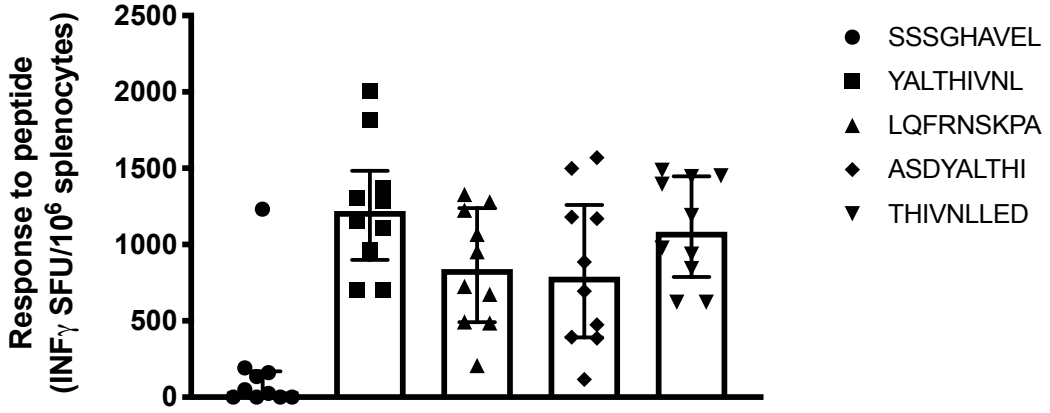


Figure 3.17 Identification of epitopes within the HBV polymerase region that induce T cell responses after vaccination with ChAdOx1-HBV and MVA-HBV in C57BL/6J mice

C57BL6/J mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-HBV followed 4 weeks later by $2x10^6$ plaque forming units of MVA-HBV. Mice were sacrificed 14 days after their last vaccination. Splenocytes were isolated and plated in interferon-gamma (IFNγ) ELISpot assays with peptides of putative polymerase epitopes. The magnitude of vaccine responses were recorded as IFNγ spot forming units per million splenocytes (IFNγ SFU /10^6 splenocytes). Results are a summation of single experiment with 10 mice per group. Median and interquartile range shown.

Within HBV surface, the amino acid sequences corresponding to peptide numbers 287-288 (PLGFFPDHQLDPAFGANSN), 300 (GLLGWSPQAQGILTT) and 371 (FVGLSPTVWLSVIWM) were identified by ELISpot as being immunogenic (Figure 3.14.B, Table 3.1). These sequences were assessed for known or putative epitopes. No peptide was predicted as being intermediate or strong binders to either $H2K^b$ or $H2D^b$ by one or more of the algorithms (Table 3.3, best scoring peptides shown). However, within the peptide 371 sequence (FVGLSPTVWLSVIWM) an epitope in C57BL/6J mice had previously been described *in-vivo* (338,453–464) despite having low prediction binding scores (Table 3.3).

Table 3.3: In-silico prediction of epitopes within the HBV surface region in C57BL/6J mice

Predicted epitope	Number of amino acids	IEDB Artificial Neural Networkic50 (nM, <50 high affinity and <500 intermediate affinity binder)		SYFPETHI binding score (>25 high binder, >18 intermediate binder)		NET MHC rank (<0.5 strong binder, <2 intermediate binder)	
		$H2K^b$	$H2D^b$	$H2K^b$	$H2D^b$	$H2K^b$	$H2D^b$
WSPQAQGIL	9	10259.48	13770.86	Not predicted	15	13	4
VGLSPTVWL	9	2616.87	8881.44	Not predicted	15	4	2.5
VWLSVIWM (338,453–464)	8	3720.7	23086.36	7	Not predicted	5.5	8.5

3.5.7 HBV core specific T cells are induced in C57BL/6J mice when the immunogen is restricted to the HBV core region

Thus far vaccine induced responses towards the HBV core region of the immunogen had not been observed in mice. It was hypothesised that the immunodominant T cell responses induced by vaccination to the HBV polymerase and HBV surface regions might mean subdominant responses in HBV core were not detectable. To investigate whether it was possible to generate H-$2K^b$ or H-$2D^b$ restricted T cell responses towards the HBV core region when the HBV polymerase and HBV surface regions were not present, C57BL/6J mice were vaccinated with DNA-plasmid vaccines encoding the HBV core region alone. Mice received 3 intramuscular doses of DNA-plasmid vaccine HBV-core genotype C (DNA-HBVc) or HBV-core genotype D (DNA-HBVd) to assess cross-reactivity of responses, or RHV immunogen (DNA-RHV) as a negative control. Mice were vaccinated in homologous prime-

boost strategies at 4-week intervals. Splenocytes were harvested at 10 days after final vaccination for immunological assessment (Figure 3.18).

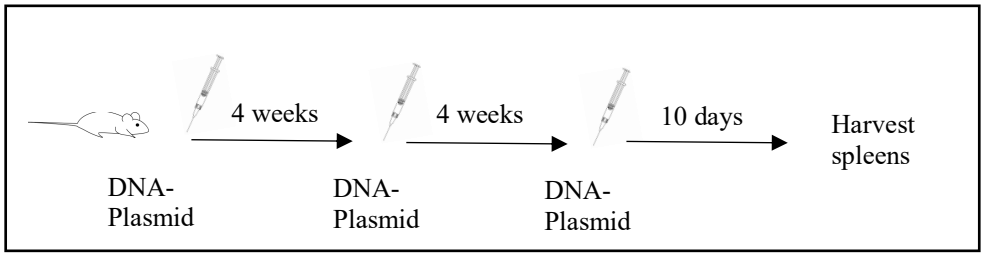


Figure 3.18 Vaccination of C57BL/6J mice with DNA-plasmid vaccines

C57BL/6J mice were vaccinated intramuscularly with 100µg DNA-plasmid vaccine 3 times at 4 weekly intervals. Mice received DNA-plasmid HBV-core genotype C (DNA-HBVc) or HBV-core genotype D (DNA-HBVd) or RHV immunogen (DNA-RHV) as a negative control.

After vaccination pooled peptides corresponding to the entire HBV core region, were incubated with splenocytes in IFNγ ELISpot assays (Figure 3.19.A). Frozen peptides can lose their ability to stimulate T cells over time. For this reason, a new peptide set corresponding to the genotype C vaccine immunogen was manufactured (new core geno C, Appendix 3a) for comparison with the original peptide set (original core geno C, Appendix 2). In order to assess genotype D specific responses, pooled peptides specific to HBV core regions where genotype C and genotype D differed in amino acid sequence were also assessed (core geno D Appendix 3b). Finally pooled peptides corresponding to the HBV polymerase region 2 were used as a negative control (pol 2).

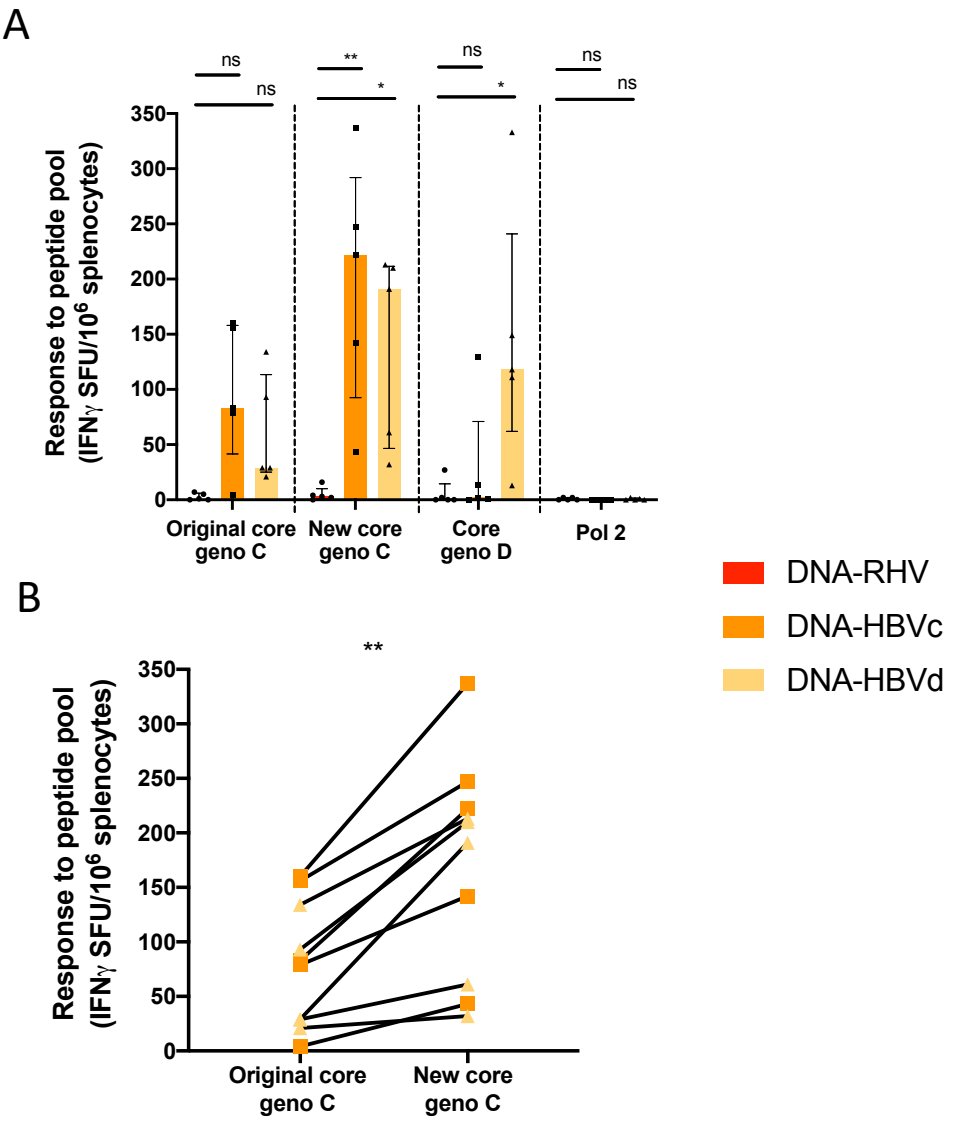


Figure 3.19 T cell responses induced by DNA-plasmid vaccines in C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly with 100µg DNA-plasmid vaccine 3 times at 4 weekly intervals. Mice received DNA-plasmid HBV-core genotype C (DNA-HBVc) or HBV-core genotype D (DNA-HBVd) or RHV immunogen (DNA-RHV) as a negative control. Splenocytes were isolated 10 days after the third vaccination.

Splenocytes were incubated with pools of HBV peptides in interferon-gamma (IFNγ) ELISpot assays. Peptide pools corresponded to the HBV core region and were either the original peptides corresponding to the vaccine genotype C sequence (original core geno C), newly synthesised to the genotype C vaccine sequence (new core geno C) or specific to the HBV core genotype D sequence, where it varied from genotype C at the amino acid level (core geno D). Peptide pool pol 2 corresponding to a region within HBV polymerase was used as a negative control.

A: The magnitude of responses to each peptide pool after each vaccine regime. B: Pairwise comparison between responses to the original peptides corresponding to the vaccine genotype C sequence (original core geno C), newly synthesised to the genotype C vaccine sequence (new core geno C) after HBV-DNAc or HBV-DNAd vaccines.

As expected, after vaccination with DNA-RHV, splenocyte responses towards the HBV peptide pools were minimal (all under 20 SFU per 10^6 splenocytes, red bar, Figure 3.19.A). T cell responses were detected towards the original HBV core genotype C peptide pool (original core geno C) after vaccination with either DNA-HBVc (orange bar, median 83, IQR 42-158 SFU per 10^6 splenocytes) or DNA-HBVd (yellow bar, median 29, IQR 25-114 SFU per 10^6 splenocytes) but responses were not significantly higher than splenocytes from the DNA-RHV vaccine group (red bar, median 1, IQR 0-6 SFU per 10^6 splenocytes, Figure 3.19.A). However, T cell responses towards the new HBV core genotype C peptide pool (new core geno C) were significantly increased after DNA-HBVc (median 222, IQR 93-292 SFU per 10^6 splenocytes, p=0.0047) or DNA-HBVd vaccination (median 191, IQR 47-212 SFU per 10^6 splenocytes, p=0.0473) compared to DNA-RHV vaccination (median 3, IQR 1-10, Figure 3.19.A). Responses to HBV core genotype D peptides (core geno D) were significantly increased after DNA-HBVd (median 118, IQR 62-241 SFU per 10^6 splenocytes, p=0.0182) but not DNA-HBVc vaccination (median 2, IQR 5-71 SFU per 10^6 splenocytes) compared with DNA-RHV vaccination (median 0, IQR 0-15 SFU per 10^6 splenocytes, Figure 3.19.A). As expected, no vaccine regime induced detectable T cell responses to the Pol 2 peptide pool Figure 3.19.A.

To assess whether the original peptide set (original core geno C) had a reduced ability to simulate T cells after nearly 3 years frozen storage, pairwise T cell responses to the original core geno C and new core geno C peptide pools were assessed (Figure 3.19.B). Responses to the new peptides (median 201, IQR 57-229 SFU per 10^6 splenocytes) were significantly higher than the original peptides (median 81, IQR 27-140 SFU per 10^6 splenocytes, p=0.0020 Figure 3.19.B).

The ability of vaccine induced T cells to produce cytokines IFNγ, TNFα or IL-2 in response to HBV genotype C core peptides (new core geno C) or HBV polymerase peptides (Pol 2) were assessed in intracellular staining assays (Figure 3.20). Vaccination with DNA-HBVc induced CD8 T cells that produced IFNγ (median 0.67, IQR 0-0.99% of CD8 T cells), TNFα (median 0.60, IQR 0.17-0.78% of CD8 T cells) or IL-2 (median 0.25%, IQR 0.01-0.34% of CD8 T cells) upon re-stimulation with the new core geno C peptide pool, but these responses were not significantly increased compared to the DNA-RHV group. Vaccination with DNA-HBVc induced a significantly higher percentage of CD4 cells producing IFNγ (median 0.72, IQR 0.28-1.38% of CD4 T cells, p=0.0159) compared with DNA-RHV vaccination. No significant differences in CD4 TNFα (median 0.74, IQR 0.37-0.93% of CD4 T cells), or IL-2 (median 0.94, IQR 0.07-1.36% of CD4 T cells) were observed (Figure 3.20). As expected, T cell responses to the Pol 2 peptide pool were minimal (Figure 3.20).

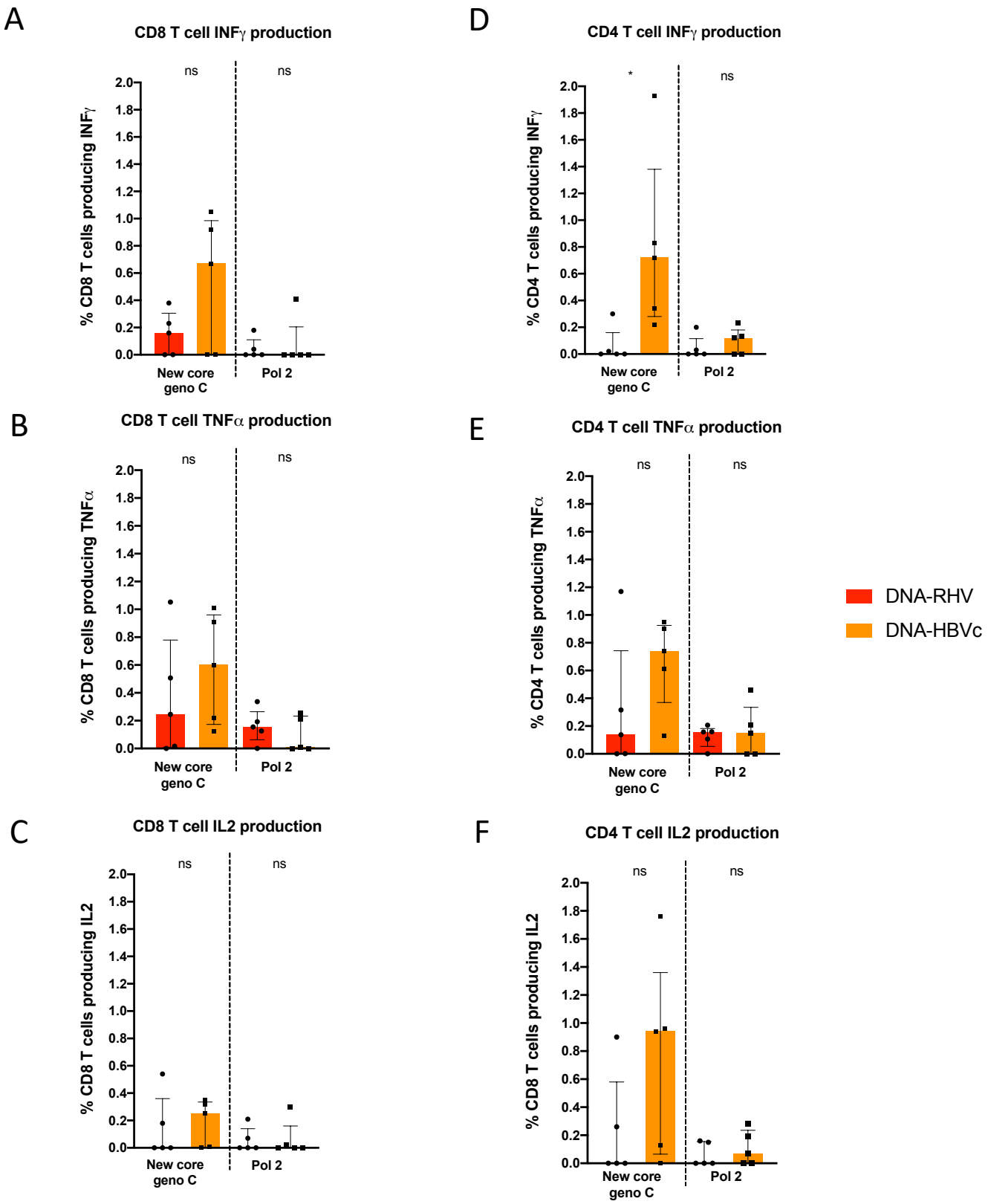


Figure 3.20 T cell cytokine production after vaccination with DNA-plasmid vaccines

C57BL/6J mice were vaccinated intramuscularly with 100µg DNA-plasmid vaccine 3 times at 4 weekly intervals. Mice received DNA-plasmid HBV-core genotype C (DNA-HBVc) or RHV immunogen (DNA-RHV) as a negative control. Splenocytes were isolated 10 days after the third vaccination and plated with peptide pools corresponding to HBV core (new core geno C) or HBV polymerase (Pol 2) in intracellular staining assays. Cells were stained with fluorochrome labelled antibodies to detect intracellular interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα) and interleukin-2 (IL2) production by flow cytometry.
The percentage of CD8 T cells producing IFNγ (**A**), TNFα (**B**) or IL2 (**C**) and the percentage of CD4 T cells producing IFNγ (**D**), TNFα (**E**) or IL2 (**F**) in response to stimulation with peptide pools is shown. Results are a summation of a single experiment with 5 mice per group. Median and interquartile range shown. Total magnitude of responses compared using Mann Whitney-U test. * $p<0.05$, ns = not significant.

3.5.8 ChAdOx1-HBV and MVA-HBV vaccination induces HBV core specific T cell responses in humanised HHD mice

In order to investigate whether responses towards the HBV core region could be induced in T cells with human MHC molecules, HHD mice (MHC class I restricted to human HLA-A*0201) (437) were vaccinated with ChAdOx1-HBV or ChAdOx1-GFP as a negative control, followed 4 weeks later by MVA-HBV (Figure 3.21).

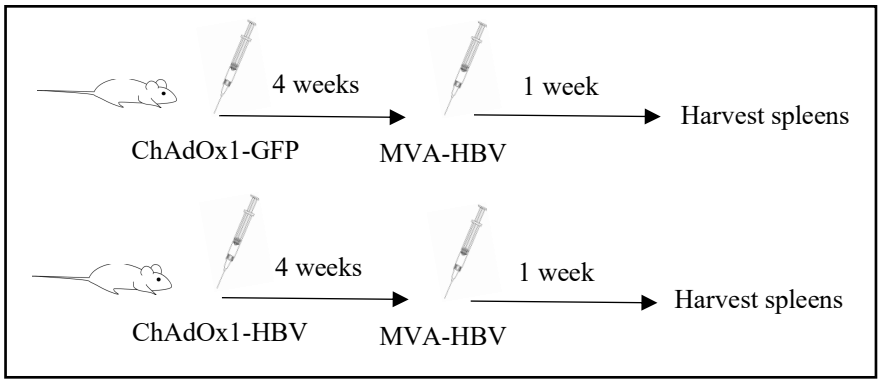


Figure 3.21 Vaccination of HHD mice with ChAdOx1-HBV and MVA-HBV
HHD mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-GFP or ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were sacrificed 1 week after their last vaccination to harvest spleens for immunological assessment.

Splenocytes were harvested 1 week after final vaccination and were incubated with peptide pools corresponding to the entire vaccine immunogen in IFNγ ELISpot assays (Figure 3.22). ChAdOx1-HBV followed by MVA-HBV induced significantly higher magnitude (median 518 vs 58 SFU per 10^6 splenocytes, p=0.0007, Figure 3.22.A) and breadth (median 5 vs 1 positive peptide pool(s), p=0.0005, Figure 3.22.B) of total T cell responses as compared to vaccination with ChAdOx1-GFP followed 4 weeks later by MVA-HBV.

The magnitude of response to each peptide pool was compared between the two vaccination regimes (Figure 3.22.C). Responses towards the peptide pool corresponding to HBV core

(core) were significantly higher in mice receiving ChAdOx1-HBV followed by MVA-HBV compared to ChAdOx1-GFP followed by MVA-HBV (median 99 vs. 0 SFU per 10^6 splenocytes, p=0.0046). Surprising, the highest responses were detected towards the peptide pool corresponding to truncated shark invariant chain (sIi), the non-HBV component included in the vaccine immunogen as a molecular adjuvant. Responses to the sIi peptide pool were significantly increased in mice receiving ChAdOx1-HBV followed by MVA-HBV compared to ChAdOx1-GFP followed by MVA-HBV (median 639 vs. 5 SFU per 10^6 splenocytes, p=0.0009). Responses were also detectable towards HBV polymerase (pol 1, pol 3 and pol 4 peptides pools) and HBV surface (preS1/S2) but these were not significantly different between vaccination regimes (Figure 3.22.C).

The ability of vaccine induced CD8 and CD4 T cells to produce cytokines IFNγ, TNFα or IL-2 on stimulation with HBV peptide pools was assessed in intracellular staining assays and compared between the two vaccination regimes (Figure 3.23). The percentage of CD8 T cells producing IFNγ (median 7.27% vs. 0.13% of CD8 T cells, p=0.0063) and TNFα (median 1.34% vs. 0.03% of CD8 T cells, p=0.0438) towards the AIL pool (shark invariant chain, furin-2-A and linker regions) were significantly increased in mice receiving ChAdOx1-HBV followed by MVA-HBV compared to ChAdOx1-GFP followed by MVA-HBV (Figure 3.23.A and B). The percentage of CD8 T cells producing TNFα (median 0.38% vs. 0.01% of CD8 T cells, p=0.0442) towards the HBV core pool (core) were also significantly increased in mice receiving ChAdOx1-HBV followed by MVA-HBV compared to ChAdOx1-GFP followed by MVA-HBV (Figure 3.23.B). CD4 T cells producing IFNγ or TNFα were detectable in response to HBV polymerase 3 and 4 (pol 3-4) peptide pool. However, there were no significant differences between vaccination groups (Figure 3.23.C).

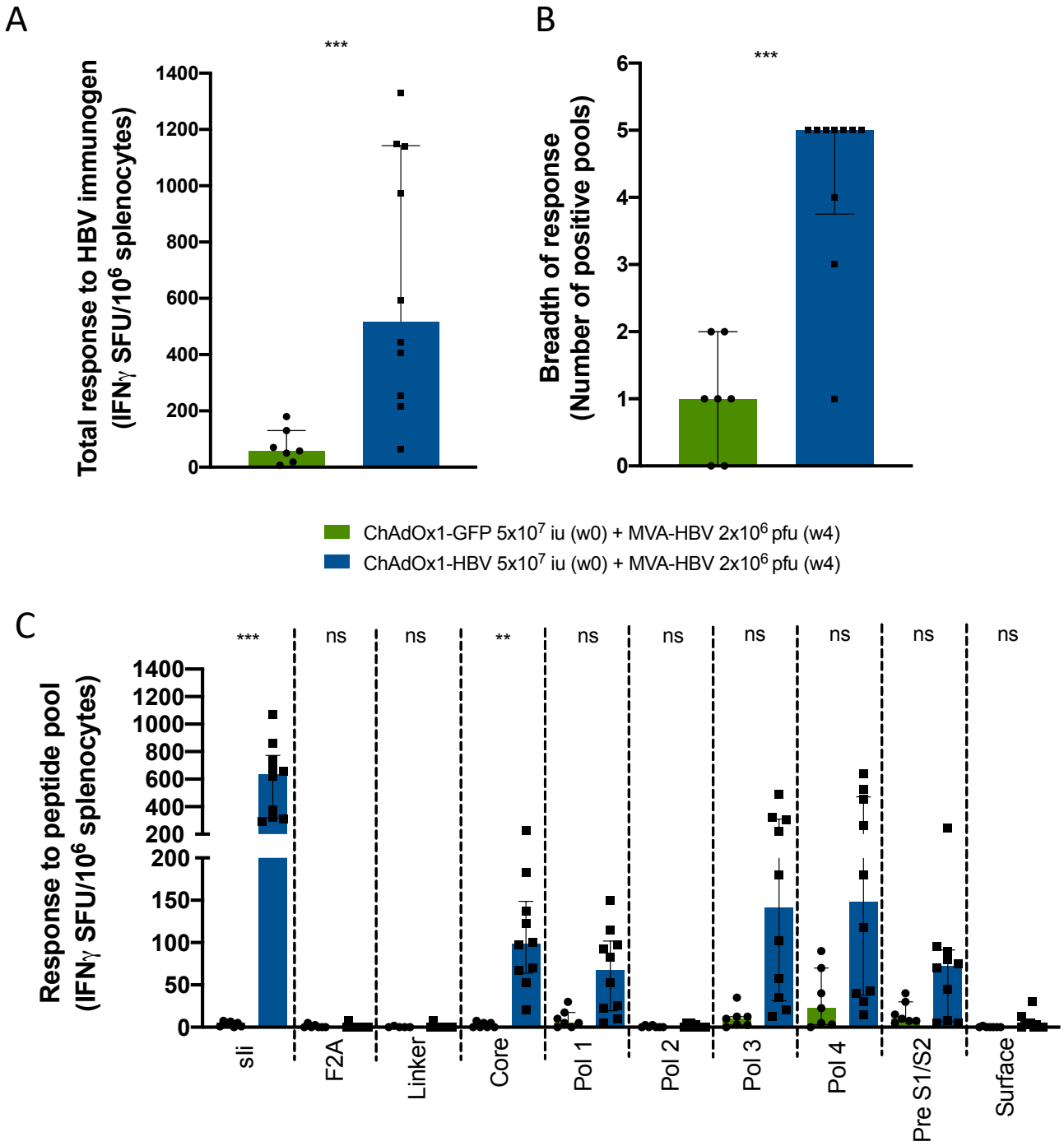


Figure 3.22 T cell responses in HHD mice after ChAdOx1-HBV and MVA-HBV vaccination

HHD mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-GFP or ChAdOx1-HBV (prime, week 0, w0) followed 4 weeks later (w4) by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were sacrificed 1 week after their last vaccination to harvest spleens for immunological assessment.

Splenocytes were isolated and incubated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. **A:** The total magnitude of vaccine induced responses to the HBV immunogen in IFNγ spot forming units per million splenocytes (SFU /10^6 splenocytes). **B:** The breadth of vaccine induced responses was defined as the number of positive peptide pools. **C:** The magnitude of the T cell response to each peptide pool from left to right correspond to truncated shark invariant chain (sIi), furin-2A (F2A), linkers (linker), HBV core (core), HBV polymerase (pol1-pol4), HBV pre-S1 and pre-S2 (pre S1/S2) and HBV surface (surface).

Results are a summation of 2 experiments with 7-10 mice per group. Median and interquartile range shown. Mann-Whitney test used for comparisons between two groups. Multiple responses compared using Kruskal-Wallis test with Dunn's multiple comparison, where each group is compared to the response in HHD mice after ChAdOx1-GFP followed by MVA-HBV. ***$p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

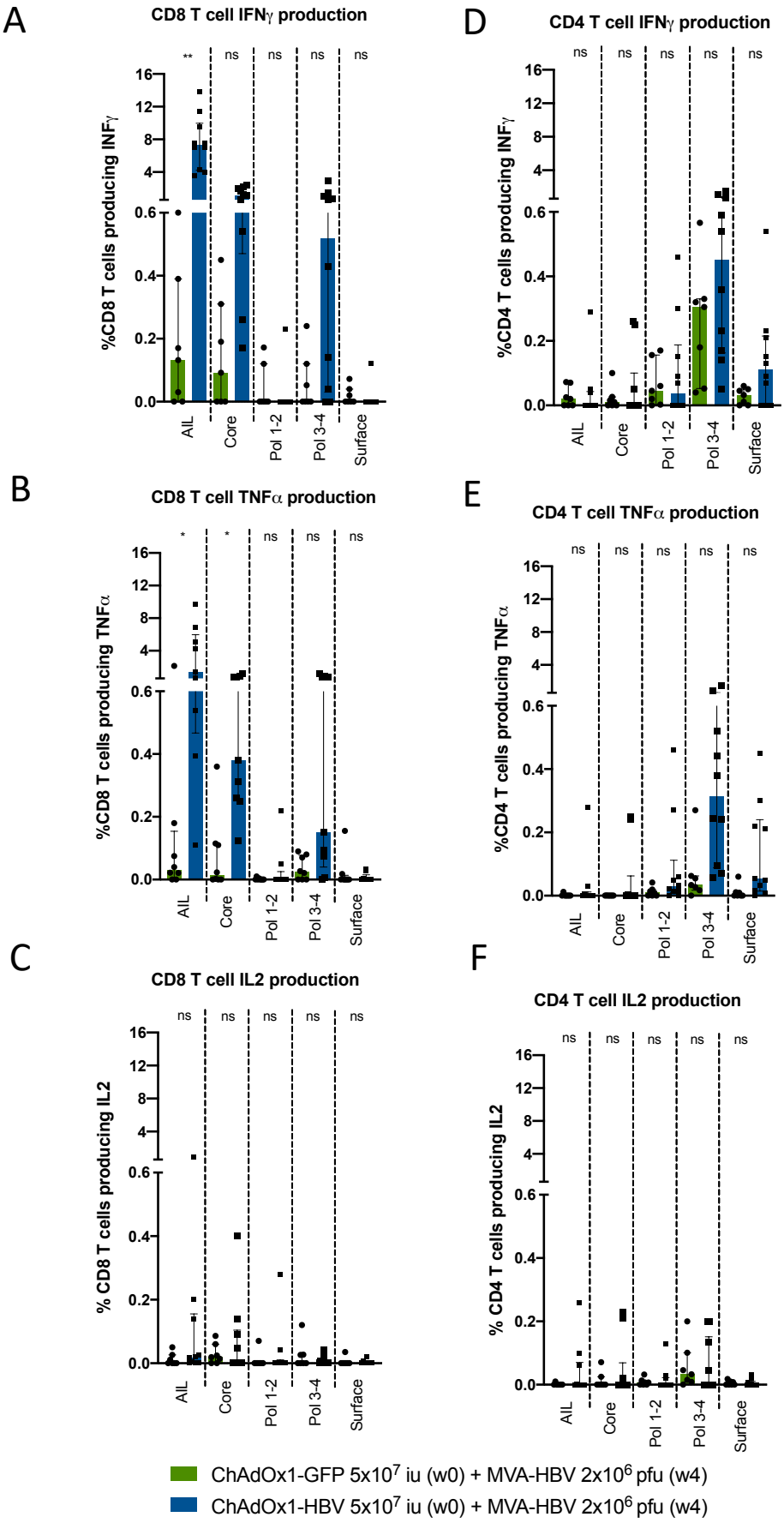

A
CD8 T cell IFNγ production
%CD8 T cells producing INFγ
B
CD8 T cell TNFα production
%CD8 T cells producing TNFα
C
CD8 T cell IL2 production
% CD8 T cells producing IL2
D
CD4 T cell IFNγ production
%CD4 T cells producing INFγ
E
CD4 T cell TNFα production
%CD4 T cells producing TNFα
F
CD4 T cell IL2 production
% CD4 T cells producing IL2
AIL
Core
Pol 1-2
Pol 3-4
Surface
ns
ChAdOx1-GFP 5x10^7 iu (w0) + MVA-HBV 2x10^6 pfu (w4)
ChAdOx1-HBV 5x10^7 iu (w0) + MVA-HBV 2x10^6 pfu (w4)

Figure 3.23 Cytokine production by vaccine induced T cells in HHD mice after ChAdOx1-HBV and MVA-HBV vaccination

HHD mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-GFP or ChAdOx1-HBV (prime, week 0, w0) followed 4 weeks later (w4) by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were sacrificed 1 week after their last vaccination to harvest spleens for immunological assessment. Splenocytes were isolated and incubated with pools of peptides corresponding to the entire vaccine immunogen in intracellular staining assays. Cells were stained with fluorochrome labelled antibodies to detect intracellular interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα) and interleukin-2 (IL2) production by flow cytometry.

The percentage of CD8 T cells producing IFNγ (**A**), TNFα (**B**) or IL2 (**C**) and the percentage of CD4 T cells producing IFNγ (**D**), TNFα (**E**) or IL2 (**F**) in response to stimulation with peptide pools is shown. Results are a summation of 2 experiments with 5-10 mice per group. Median and interquartile range shown. Multiple responses compared using Kruskal-Wallis test with Dunn's multiple comparisons ** $p<0.01$, * $p<0.05$, ns = not significant.

3.5.9 Immunogenic regions of ChAdOx1-HBV and MVA-HBV vaccines are identified in HBV core, polymerase, and surface in HHD mice

To investigate the immunogenic regions of the HBV vaccines in HLA-A*0201 restricted HHD mice in more detail, mice were vaccinated with ChAdOx1-HBV followed 4 weeks later by MVA-HBV. Splenocytes 7 days after the last vaccination were incubated with minipools of ten peptides covering HBV core, polymerase and surface regions of the vaccine immunogen in IFNγ ELISpot assays (Figure 3.24.A). The individual peptides making up the minipools with the highest responses were then tested separately (Figure 3.24.B).

The highest responding minipools were C3 (median 25, IQR 15-56 SFU per 10^6 splenocytes) within HBV core, P15 (median 53, IQR 14-213 SFU per 10^6 splenocytes), P18 (median 103, IQR 51-110 SFU per 10^6 splenocytes) and P20 (median 43, IQR 29-200 SFU per 10^6 splenocytes) within HBV polymerase and S2 (median 50, IQR 25-150 SFU per 10^6 splenocytes) within HBV surface (Figure 3.24.A).

Peptides with the highest responses within each minipool are shown in Figure 3.24.B, with corresponding amino acid sequences in Table 3.4. Within HBV core, responses were

recorded towards peptide number 31 in minipool C3 (peptide 31 median 105, IQR 54-234 SFU per 10^6 splenocytes). Within HBV polymerase, responses were recorded towards peptide number 207 in minipool P15 (peptide 207 median 25, IQR 20-190 SFU per 10^6 splenocytes), peptide number 243 in minipool P18 (peptide 243 median 258, IQR 161-351 SFU per 10^6 splenocytes) and peptides 257-258 in minipool P20 (peptide 258 median 324, IQR 230-465 SFU per 10^6 splenocytes). Within HBV surface responses were recorded towards peptides 293-294 in minipool S2 (peptide 293 median 155, IQR 115-233 SFU per 10^6 splenocytes).

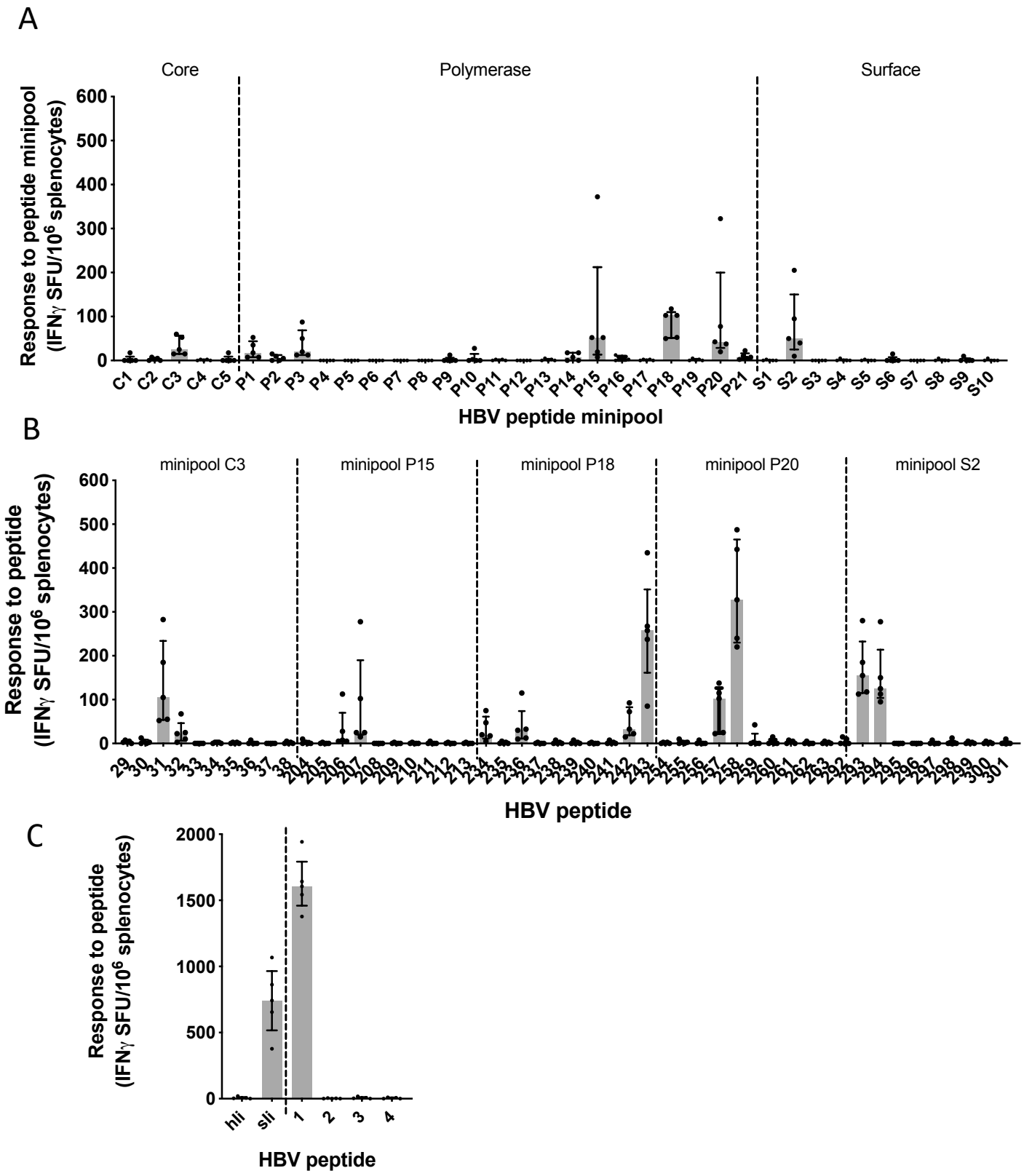


Figure 3.24 Identification of regions of the HBV immunogen that induce T cell responses after vaccination with ChAdOx1-HBV and MVA-HBV in HHD mice

Mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV. Mice were sacrificed 14 days after their last vaccination. Splenocytes were isolated incubated with with peptide minipools corresponding to the entire vaccine immunogen or to individual peptides in responsive minipools in interferon-gamma (IFNγ) ELISpot assays. The magnitude of vaccine responses was recorded as IFNγ spot forming units per million splenocytes (IFNγ SFU/10^6 splenocytes). **A** Minipool and **B** individual peptide responses in HHD mice. **C** Responses towards pool of human invariant chain peptides (hIi), shark invariant chain peptides (sIi) and the individual peptides that comprise the shark invariant chain pool (1-4). Results are a summation of a single experiment with 5 mice per group. Median and interquartile range shown.

In order to identify known HLA-A*0201 restricted epitopes, the databases Hepitopes (449) (database of human HLA class I HBV epitopes) and IEDB (445) were searched with the immunogenic peptide sequences identified by IFNγ ELISpot (peptide 31 AILCWGELMNLATWV, peptide 207 NFLLSLGIHLNPNKT, peptide 243 RGTFVAPLPIHTAEL, peptides 257-258 LRGTSFVYVPSALNPAADP and peptides 293-294 FNPNKDHWPEANQVGAGAF, Table 3.4).

Table 3.4: Identification of T cell epitopes induced by HBV vaccination in HHD mice

Strain	**MHC Class I**	**Minipool**	**Peptide numbers**	**Amino acid sequence**
HHD	HLA-A2.1	C3	31	AILCWGELMNLATWV
		P15	207	NFLLSLGIHLNPNKT
		P18	243	RGTFVAPLPIHTAEL
		P20	257-258	LRGTSFVYVPSALNPAADP
		S2	293-294	FNPNKDHWPEANQVGAGAF

Six epitopes had previously been described (Table 3.5) in patients with acute or chronic HBV or through vaccination in HLA-A2 restricted mice. Core specific epitopes LCWGELMNL (465), ELMNLATWV(466) and LATWVGNNLE(467) have been described in the peptide 31 region (AILCWGELMNLATWV). Polymerase specific epitope FLLSLGIHL(236,468,469) has been described in the peptide 207 region (NFLLSLGIHLNPNKT), PLPIHTAEL(470) in the peptide 243 region (RGTFVAPLPIHTAEL) and ILRGTSFVYV(471,472) in the peptide 257-258 region (LRGTSFVYVPSALNPAADP). No epitopes have been described in the peptide 293-294 region of HBV surface (FNPNKDHWPEANQVGAGAF).

Table 3.5: Known HLA-A0201 restricted T cell epitopes within immunogenic regions of the HBV immunogen in HHD mice. Underlined regions within the amino acid sequence of the reactive HBV peptide indicate the sequence of a described epitope

Peptide number	Amino acid sequence	HBV region	Described Epitope	Number of amino acids	Database	Description
31	AILCWGELMNLATWV	Core	LCWGELMNL	9	Hepitopes IEDB	Chronic HBV infection (465)
31	AILCWGELMNLATWV	Core	ELMNLATWV	9	Hepitopes	Mice and healthy humans (466)
31	AILCWGELMNLATWV	Core	LATWVGNNLE	10	Hepitopes	Chronic HBV infection (467)
207	NFLLSLGIHLNPNKT	Polymerase	FLLSLGIHL	9	Hepitopes IEDB	Acute and chronic HBV infection (236,468,469)
243	RGTFVAPLPIHTAEL	Polymerase	PLPIHTAEL	9	Hepitopes	Vaccination HLA-A2 restricted transgenic mice (470)
257-258	LRGTSFVYVPSALNPAADP	Polymerase	ILRGTSFVYV	10	Hepitopes	Acute and chronic HBV infection (471,472)
293-294	FNPNKDHWPEANQVGAGAF	Surface	None described	-	-	-

The highest magnitude responses in HHD mice were directed towards the truncated shark invariant chain region of the immunogen (Figure 3.24.C). In order to investigate this further, splenocytes were plated in IFNγ assays with peptides coving human invariant chain (hIi, to assess cross-reactivity), truncated shark invariant chain (sIi) and the 4 individual peptides (peptide numbers 1-4) that correspond to the entire sIi sequence (Figure 3.24.C). The highest responses were detected towards peptide number 1 (amino acid sequence SLLWGGVTVLAAMLI, median 1605, IQR 1460-1793 SFU per 10^6 splenocytes). Responses to hIi were not detected.

3.5.10 In-silico analysis predicts HLA-A*02 restricted epitopes within truncated shark invariant chain (sIi)

Unexpectedly in HLA-A*02 restricted HHD mice high magnitude T cell responses specific for the sIi region were observed. If this response is also present in humans, it might divert the immune responses to vaccination towards sIi at the expense of responses to the HBV regions of the vaccine immunogen or generate auto-reactive immune responses towards human invariant chain. Therefore, I identified putative epitopes within sIi and investigated whether the amino acid sequence could be altered *in-silico* to disrupt predicted epitopes using the using IEDB (445), SYFPEITHI (446) and Net MHC (447) databases.

The truncated sIi sequence in the vaccine is 26 amino acids in length (SLLWGGVTVLAAMLIAGQVASVVFLV). The immunogenic region in HHD mice was at the beginning of the of the sIi sequence (SLLWGGVTVLAAMLI), **Table 3.6**. This region was investigated for known human epitopes on the IEDB database (445) but there were none recorded.

Table 3.6: Amino acid sequence of truncated shark invariant chain and the immunogenic region in HHD mice

Amino acid position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
sIi sequence	S	L	L	W	G	G	V	T	V	L	A	A	M	L	I	A	G	Q	V	A	S	V	V	F	L	V
Immunogenic region in HHD mice	S	L	L	W	G	G	V	T	V	L	A	A	M	L	I											

Next, putative epitopes within the region SLLWGGVTVLAAMLI were investigated using SYFPEITHI (446) and Net MHC (447). Three epitopes were predicted to be strong binders to several HLA-A (Figure 3.25.A) and HLA-B haplotypes (Figure 3.25.B, Table 3.7). These may represent a single epitope as their amino acid sequences nearly entirely overlap.

Table 3.7: Predicted epitopes (red) for the truncated shark invariant chain region SLLWGGVTVLAAMLI on SYFPEITHI and Net MHC

Epitope sequence (underlined)	Number of amino acids	HLA allele
SLLWGGVTVLAAMLI	9	HLA-A *0201, *0206, *0211, *0212, *0216, *0219, *0250, *6901
SLLWGGVTVLAAMLI	8	HLA-A *0207, *0211, *0212, *0216, *0219
SLLWGGVTVLAAMLI	9	HLA-A *0201, *0207, *0211, *0212, *0216, *0217, *0219, *0250, *3201. HLA-B*1502, *0413, *4801

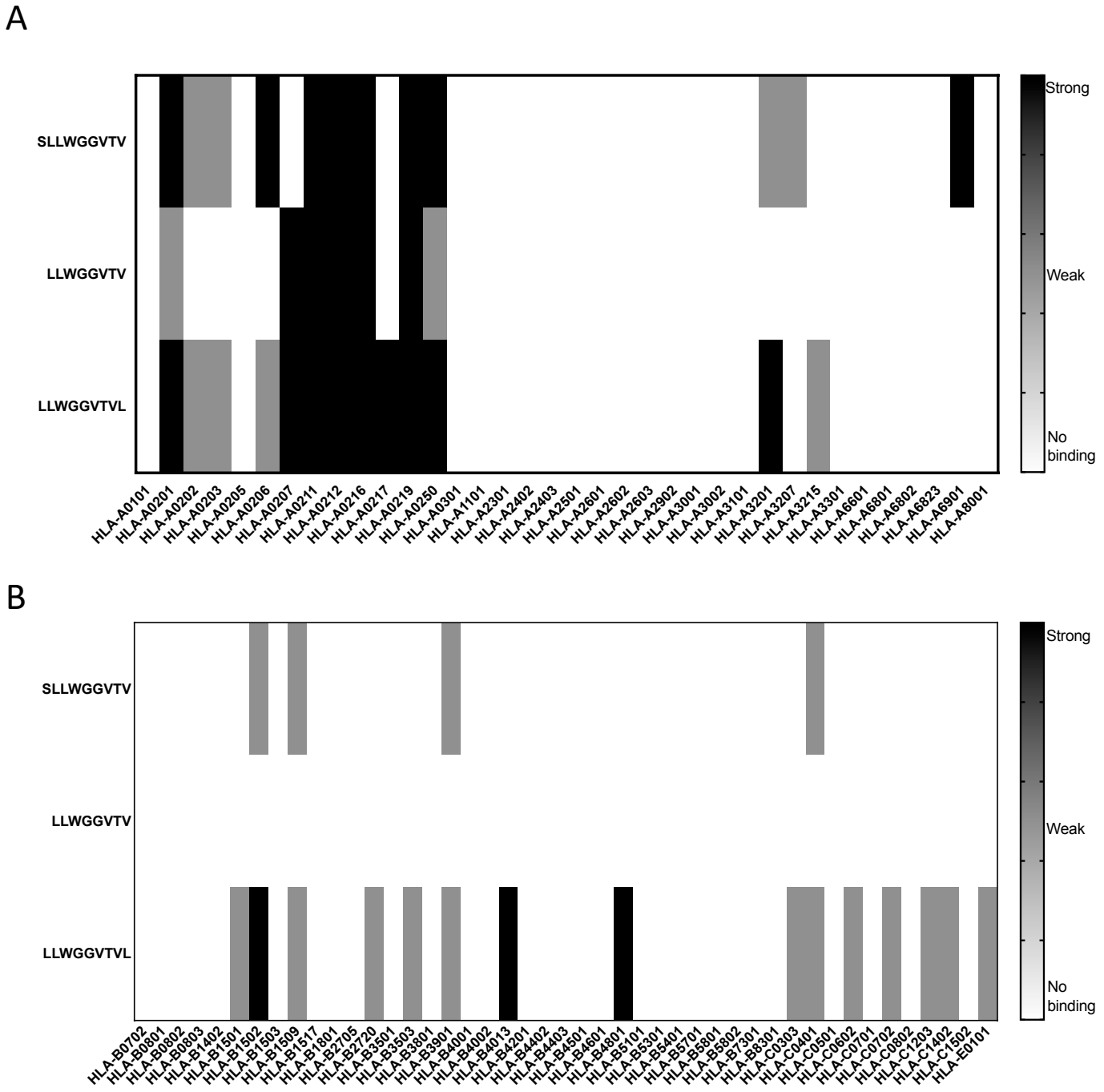


Figure 3.25 Predicted epitopes for sIi region SLLWGGVTVLAAMLI

The region of truncated shark invariant chain (sIi) with amino acid sequence SLLWGGVTVLAAMLI was investigated for putative MHC class I restricted epitopes using the online SYFPEITHI and Net MHC resources. Amino acid sequences of putative epitopes (listed on the left side of the figure) were predicted as being strong (black bars), weak (grey bars) or non-binders (white) to **A:** HLA-A molecules or **B:** HLA-B, -C or –E molecules. Strong binding peptides were defined as having a SYFPEITHI binding score of over 18 or a Net MHC rank threshold of 0.5% or under. Weak binding peptides were defined as having a Net MHC rank threshold of 0.5-2%.

Next, I sought to disrupt the predicted epitopes in sIi (either SLLWGGVTV, predicted epitope (i) or LLWGGVTV, predicted epitope (ii)) such that they were no longer predicted to bind strongly to HLA-A*02. I used a trial-and-error approach, replacing amino acids with different biochemical properties at the anchor residues 2 and 9, which are integral for peptide binding to HLA-A*02 molecules (473–475). Replacing the position 2 and 9 hydrophobic amino acids leucine or valine (L or V) with aromatic amino acid tryptophan (W), was successful in disrupting the peptide sequences such that they were no longer predicted to be strong binders of HLA-A or HLA-B molecules using SYFPEITHI (Table 3.8).

Table 3.8: Amino acid sequence of predicted epitopes in truncated shark invariant chain and their disruption by amino acid replacement at positions 2 and 9 (original amino acid = blue epitope (i) or green epitope (ii), replacement amino acid = red)

Amino acid position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**HLA-A and HLA-B binding prediction**
Predicted epitope (i)	S	**L**	L	W	G	G	V	T	**V**	Strong
Disrupted epitope (i)	S	**W**	L	W	G	G	V	T	**W**	No strong binders
Predicted epitope (ii)	L	**L**	W	G	G	V	T	V	**L**	Strong
Disrupted epitope (ii)	L	**W**	W	G	G	V	T	V	**W**	No strong binders

Next, I examined whether any new epitopes were predicted in a longer sIi sequence (15 amino acids in length), after these amino acid replacements (**Table 3.9**). I chose to include the amino acid methionine (M) at the start of this sequence, as in the ChAdOx1-HBV vaccine immunogen this is the start amino acid followed directly by the sIi sequence.

Table 3.9: Amino acid sequence of the first part of truncated shark invariant chain in the HBV vaccine immunogen and the altered sequence by amino acid replacement (original amino acid = blue epitope (i) or green epitope (ii), replacement amino acid = red)

Amino acid position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sIi sequence	M	S	L	L	W	G	G	V	T	V	L	A	A	M	L
Altered sequence (i)	M	S	W	L	W	G	G	V	T	W	L	A	A	M	L
Altered sequence (ii)	M	S	L	W	W	G	G	V	T	V	W	A	A	M	L

Unfortunately, there were now some putative epitopes with high binding score predictions to HLA-A and HLA-B alleles within the longer altered sIi sequences using SYFPEITHI (446). There were two predicted epitopes WLWGGVTW (8 amino acids) and GVTWLAAML (9 amino acids) within the altered sIi sequence (i) MSWLWGGVTWLAAML (**Figure 3.26.A**). The peptide WLWGGVTW had a high SYFPETHI score of 29, predicting strong binding to HLA-A*0201. There were also two predicted epitopes, SLWWGGVTV (9 amino acids) and GVTVWAAML (9 amino acids), within altered sIi sequence (ii) MSLWWGGVTVWAAML (**Figure 3.26.B**). The peptide SLWWGGVTV had a high SYFPETHI score of 29, predicting strong binding to HLA-A*0201. Further alterations of the sequence using different amino acids, introducing more than two replacement amino acids or sequentially altering the amino

acid sequence in triplicate, did not easily remove predicted epitopes with high binding scores on either SYFPEITHI (446) or Net MHC (447) (data not shown).

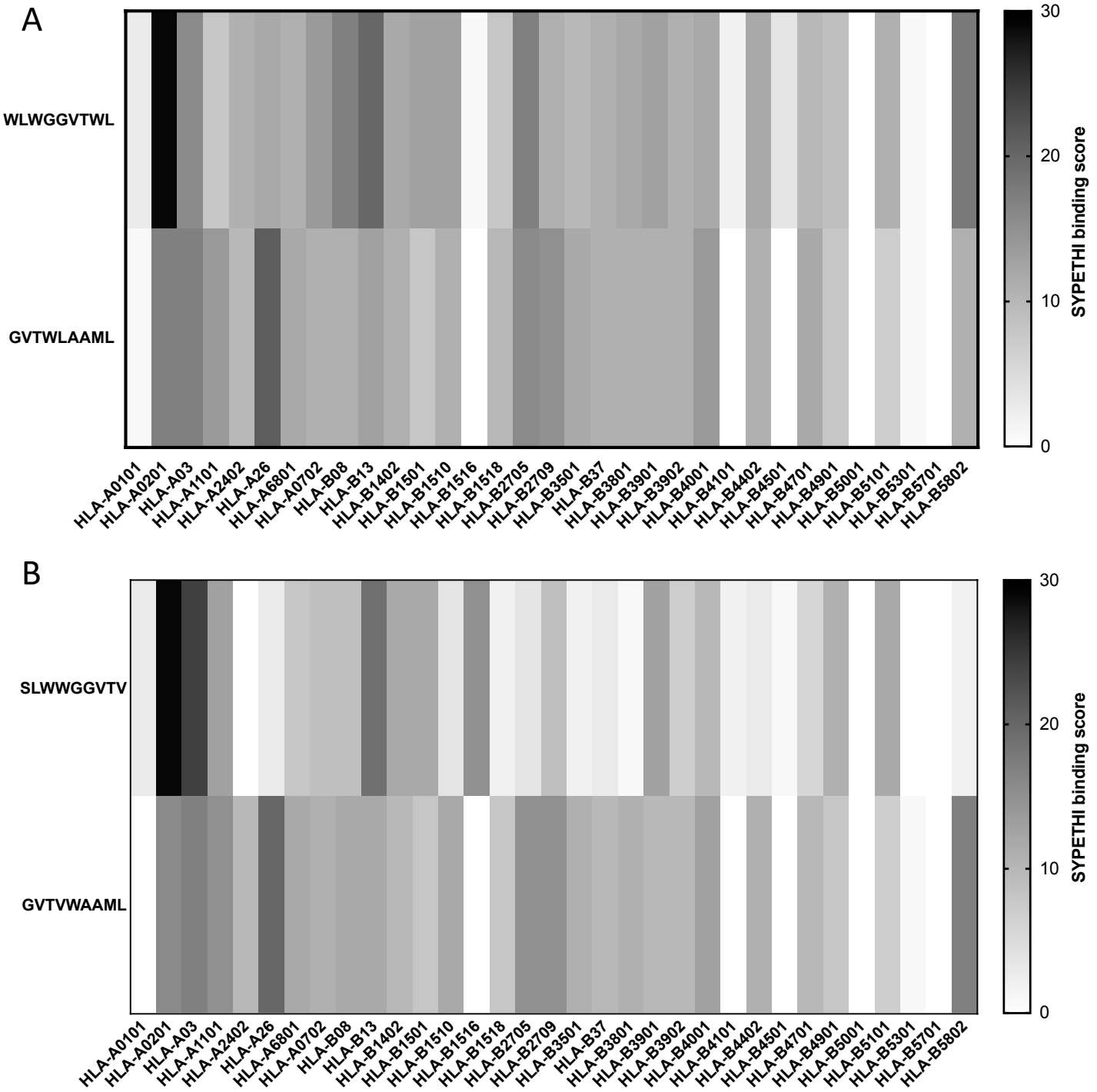


Figure 3.26 Predicted epitopes for altered sIi regions MSWLWGGVTWLAAML and MSLWWGGVTVWAAML

A: The altered region of truncated shark invariant chain (sIi) with amino acid sequence MSWLWGGVTWLAAML was investigated for putative MHC class I restricted epitopes using the online SYFPEITHI resource.
B: The altered region of truncated shark invariant chain (sIi) with amino acid sequence MSLWWGGVTVWAAML was investigated for putative MHC class I restricted epitopes using the online SYFPEITHI and Net MHC resource.
Amino acid sequences of putative epitopes (listed on the left side of the figure) were predicted as being strong binders (SYFPEITHI score 30) to non-binders (SYFPEITHI score 0).

Next, to ensure there were no other HLA-A restricted epitopes predicted within sIi, the entire sIi sequence (26 amino acids) was investigated using SYFPEITHI (446) and Net MHC (447). Three epitopes with strong binding were predicted (Table 3.10, Figure 3.27**,** orange, blue and green boxes).

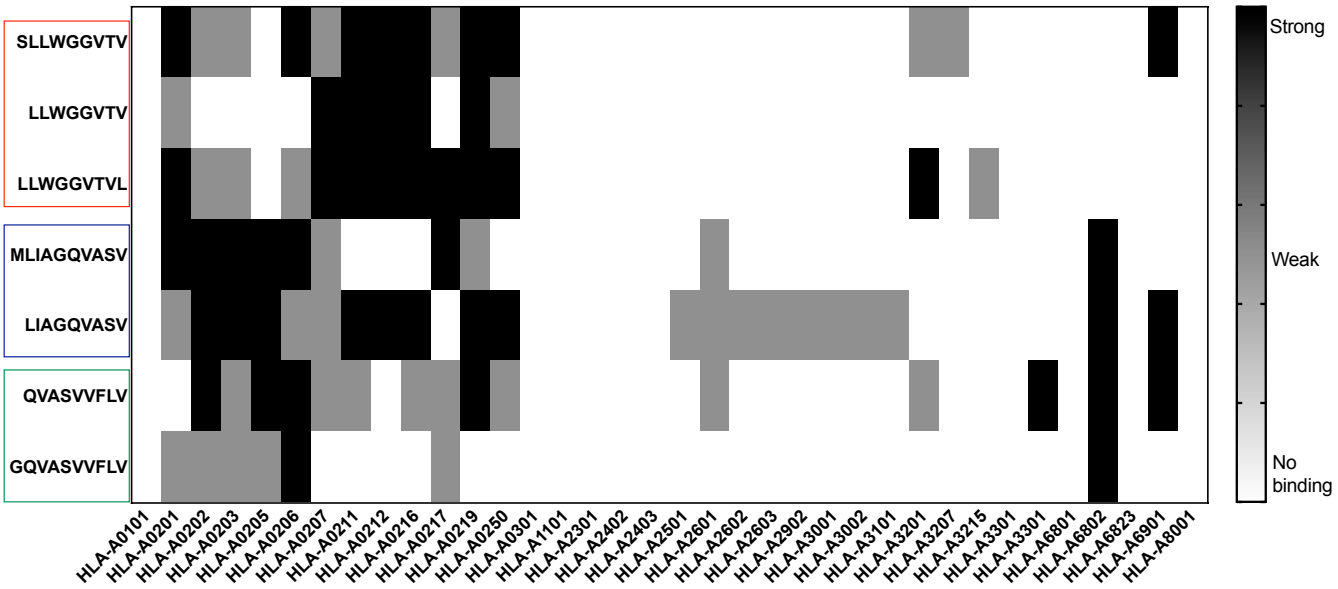


Figure 3.27 Predicted epitopes for 26 amino acids sIi sequence SLLWGGVTVLAAMLIAGQVASVVFLV

The truncated shark invariant chain (sIi) amino acid sequence SLLWGGVTVLAAMLIAGQVASVVFLV was investigated for putative MHC class I restricted epitopes using the online SYFPEITHI and Net MHC resources. Amino acid sequences of putative epitopes (listed on the left side of the figure) were predicted as being strong (black bars), weak (grey bars) or non-binders (white) to HLA-A molecules. Strong binding peptides were defined as having a SYFPEITHI binding score of over 18 or a Net MHC rank threshold of 0.5% or under. Weak binding peptides were defined as having a Net MHC rank threshold of 0.5-2%. Predicted epitopes with largely overlapping sequences are regions SLLWGGVTVL (orange box), MLIAGQVASV (blue box) and GQVASVVFLV (green box).

The first epitope (a) SLLWGGVTV is described above (sIi amino acid positions 1-9, orange box Figure 3.27, Table 3.10). The other predicted epitopes were towards the end of the sIi sequence (b) LIAGQVASV (sIi amino acid positions 14-22, blue box Figure 3.27, Table 3.10) and (c) QVASVVFLY (sIi amino acid positions 18-26, green box Figure 3.27, Table 3.10).

Table 3.10: Amino acid sequence of truncated shark invariant chain and putative epitopes (red) predicted by SYFPETHI and Net MHC

Amino acid position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
sIi sequence	S	L	L	W	G	G	V	T	V	L	A	A	M	L	I	A	G	Q	V	A	S	V	V	F	L	V
Predicted epitope (a)	**S**	**L**	**L**	**W**	**G**	**G**	**V**	**T**	**V**																	
Predicted epitope (b)														**L**	**I**	**A**	**G**	**Q**	**V**	**A**	**S**	**V**				
Predicted epitope (c)																		**Q**	**V**	**A**	**S**	**V**	**V**	**F**	**L**	**V**

Disruption of all 3 epitopes was attempted by replacing amino acids at positions 2 and 9 of each predicted epitope as described above. However, any replacement that disrupted the epitope in question led to new predicted epitopes using both SYFPEITHI (446) and Net MHC (447) (data not shown). Furthermore, epitopes (b) and (c) lie within a relatively conserved region of truncated invariant chain sequences between different species (431). Disruption in these positions leads to loss of the adjuvant function of the invariant chain (431). Therefore, further disruption of the sIi sequence was not pursued.

Invariant chains from other species have been shown to have a similar adjuvant effect on CD8 T cell responses induced by ChAd vectored vaccines (431). These include full length trout, truncated chicken and truncated human invariant chains (431), which could be used as alternative to sIi as a molecular adjuvant in ChAdOx1-HBV (amino acid sequences in Table 3.11).

Table 3.11: Aligned amino acid sequences of truncated shark, truncated trout, truncated chicken, and truncated human invariant chain

shark	S	L	L	-	W	G	G	V	T	V	L	A	A	M	L	I	A	G	Q	V	A	S	V	V	F	L	V
Trout	R	A	F	K	I	A	G	F	T	L	L	A	C	L	L	I	A	G	Q	A	L	T	A	Y	F	V	L
Chicken	R	T	A	-	L	S	A	L	S	I	L	V	A	L	L	I	A	G	Q	A	V	T	I	Y	Y	V	Y
Human	G	A	L	-	Y	T	G	F	S	I	L	V	T	L	L	L	A	G	Q	A	T	T	A	Y	F	L	Y

I investigated whether there were any predicted MHC class I epitopes within truncated trout, chicken and human invariant chains, to assess their suitability to replace sIi as a molecular adjuvant using SYFPEITHI (446) and Net MHC (447).

In truncated trout invariant chain there was a single region in which SYFPEITHI (446) predicted epitopes would bind moderately to HLA*A0201, with binding scores of between 21 and 23 (yellow square, Figure 3.28.A). There were 5 regions in which Net MHC predicted strong binders to HLA-A molecules (red, orange, yellow, green and blue squares, Figure

3.28.B). The putative epitope CLLIAGQAL was predicted to strongly bind to HLA-A alleles by both algorithms.

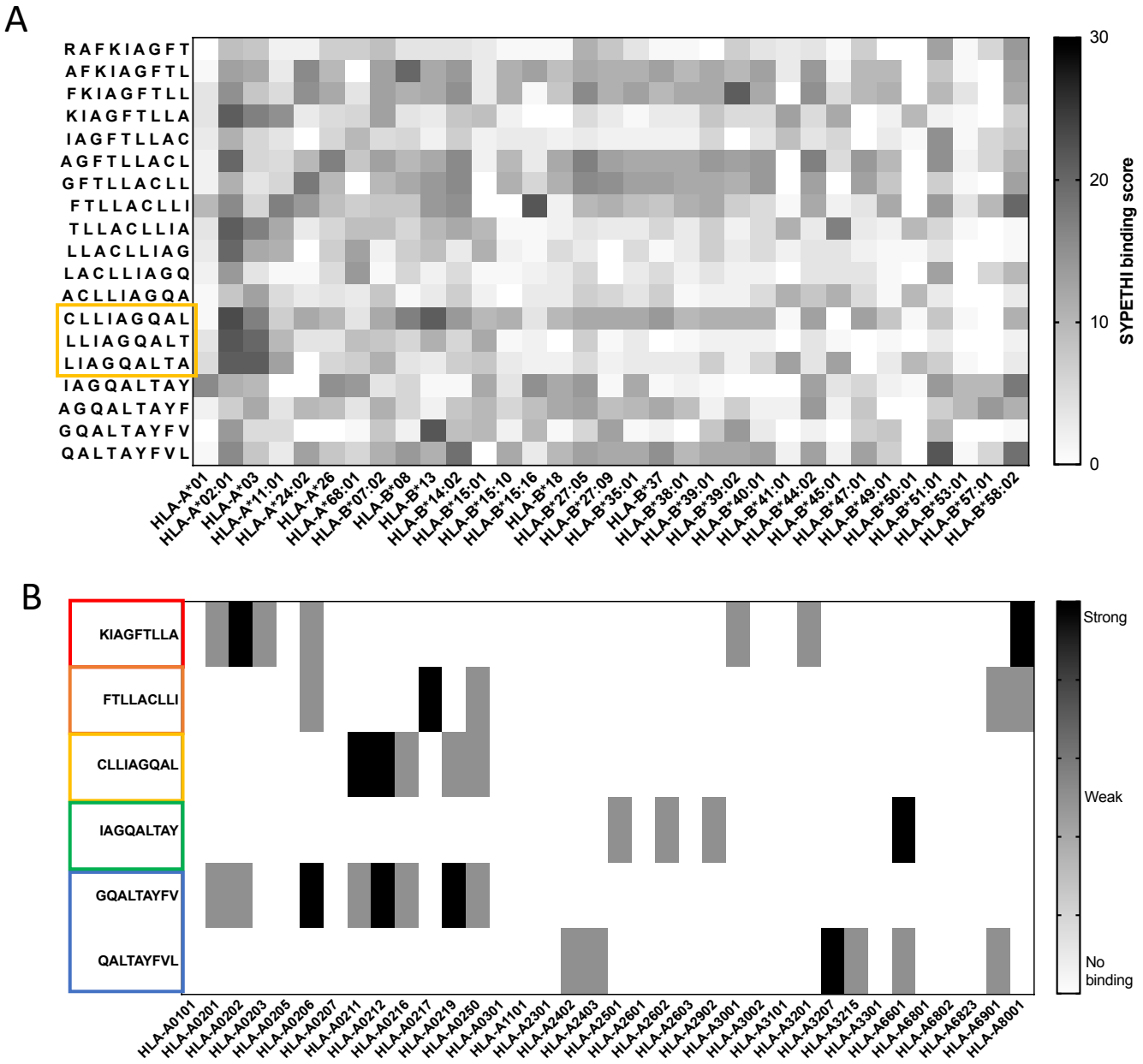


Figure 3.28 Predicted epitopes for truncated trout invariant chain amino acid sequence RAFKIAGFTLLACLLIAGQALTAYFVL

The truncated trout invariant chain amino acid sequence RAFKIAGFTLLACLLIAGQALTAYFVL was investigated for putative MHC class I restricted epitopes. Amino acid sequences of putative epitopes are listed on the left side of the figure and sequences with high overlap are boxed in red, orange, yellow, green, or blue.
A: Putative epitopes identified using SYFPEITHI were predicted as being strong binders (black bars, SYFPEITHI binding score of 30) or non-binders (white bars, SYFPEITHI binding score of 0).
B: Putative epitopes identified using Net MHC were predicted as being strong binders (black bars, Net MHC rank threshold of 0.5% or under), weak binders (grey bars, Net MHC rank threshold of 0.5-2%) or non-binders (Net MHC rank threshold of over 2%).

In truncated chicken invariant chain there were 2 regions in which SYFPEITHI (446) predicted epitopes would bind moderately or highly to HLA*A0201, with binding scores of between 22 and 29 (orange and yellow boxes, Figure 3.29.A). There were 4 regions in which Net MHC predicted strong binders to HLA-A molecules (red, yellow, and green boxes, Figure 3.29.B). The putative epitope ALLIAGQAV was predicted to strongly bind to HLA-A alleles by both algorithms.

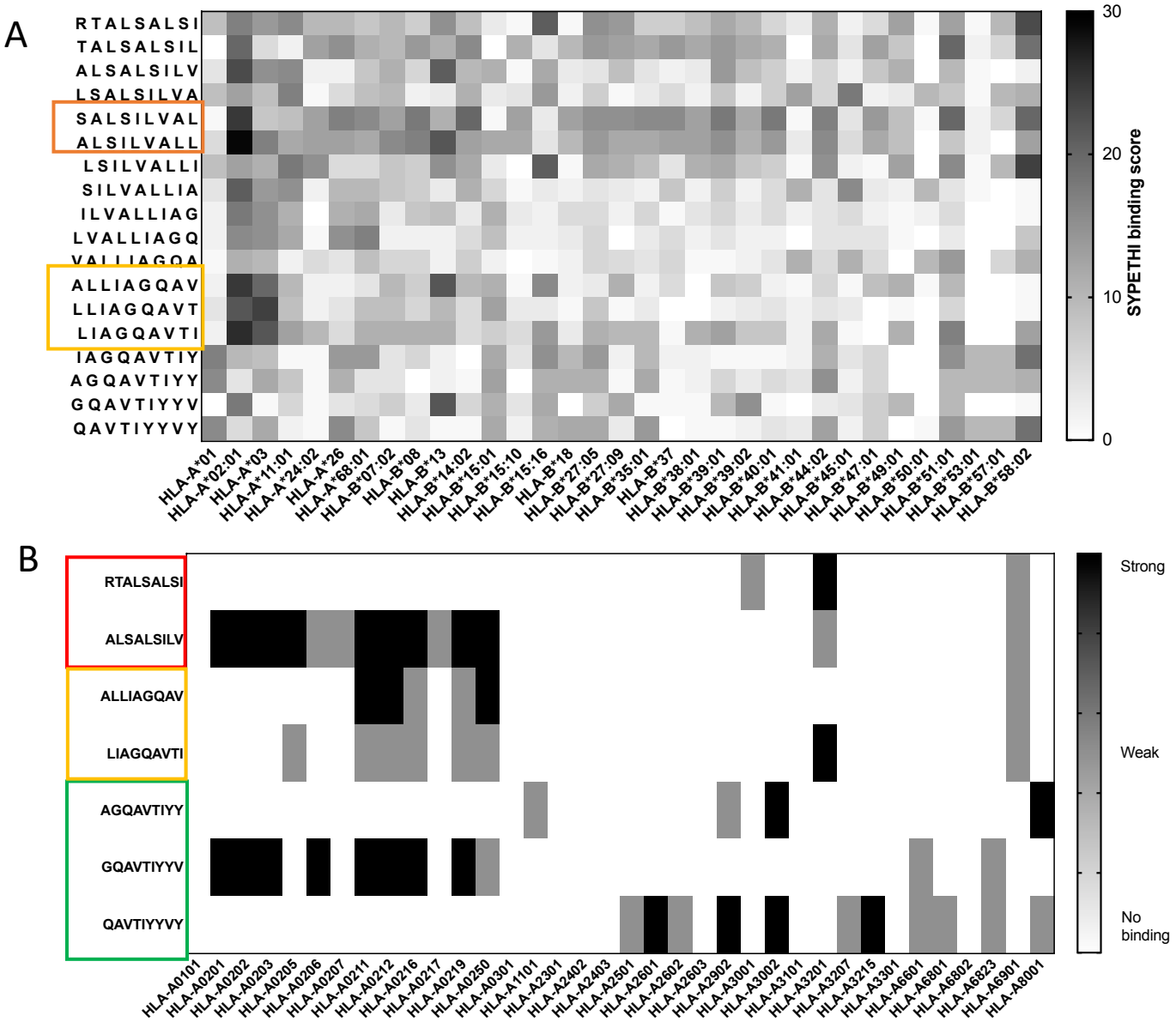


Figure 3.29 Predicted epitopes for truncated chicken invariant chain amino acid sequence RTALSALSILVALLIAGQAVTIYYVY

The truncated chicken invariant chain amino acid sequence RTALSALSILVALLIAGQAVTIYYVY was investigated for putative MHC class I restricted epitopes. Amino acid sequences of putative epitopes are listed on the left side of the figure and sequences with high overlap are boxed in red, orange, yellow or green. **A:** Putative epitopes identified using SYFPEITHI were predicted as being strong binders (black bars, SYFPEITHI binding score of 30) or non-binders (white bars, SYFPEITHI binding score of 0). **B:** Putative epitopes identified using Net MHC were predicted as being strong binders (black bars, Net MHC rank threshold of 0.5% or under), weak binders (grey bars, Net MHC rank threshold of 0.5-2%) or non-binders (Net MHC rank threshold of over 2%).

In truncated human invariant chain there were 2 regions in which SYFPEITHI (446) predicted epitopes would bind moderately or highly to HLA*A0201, with binding scores of between 22 and 25 (Figure 3.30.A). There were 4 regions in which Net MHC predicted strong binders to HLA-A molecules (Figure 3.30.B). The putative epitope ALYTGFSIL was predicted to moderately or strongly bind HLA-A alleles by both algorithms. Therefore, all truncated invariant chain sequences investigated had some putative HLA-A restricted epitopes that could potentially induce T cell responses in humans.

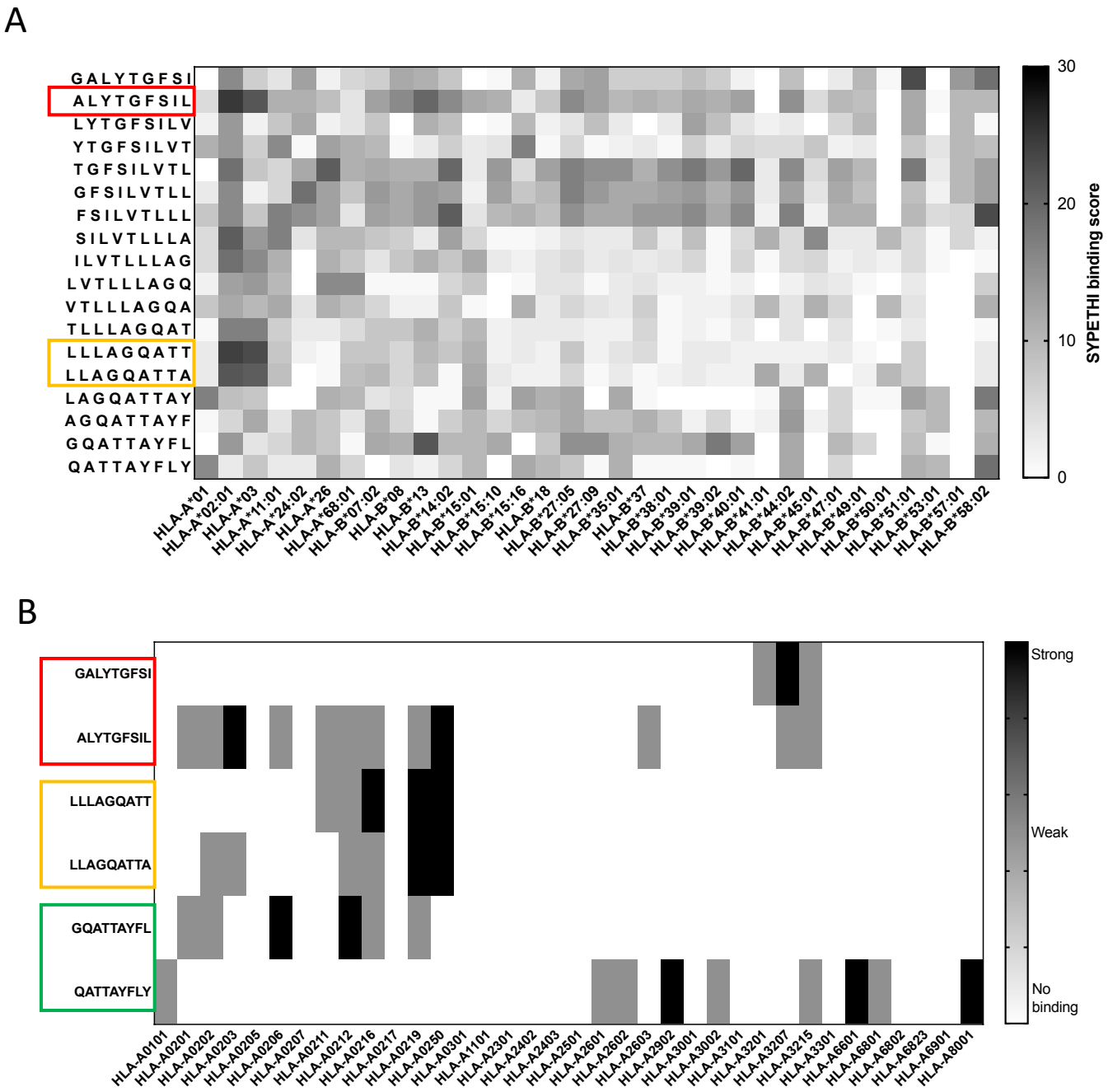


Figure 3.30 Predicted epitopes for truncated human invariant chain amino acids sequence GALYTGFSILVTLLLAGQATTAYFLY

The truncated human invariant chain amino acid sequence GALYTGFSILVTLLLAGQATTAYFLY was investigated for putative MHC class I restricted epitopes. Amino acid sequences of putative epitopes are listed on the left side of the figure and sequences with high overlap are boxed in red, orange, yellow or green.

A: Putative epitopes identified using SYFPEITHI were predicted as being strong binders (black bars, SYFPEITHI binding score of 30) or non-binders (white bars, SYFPEITHI binding score of 0).
B: Putative epitopes identified using Net MHC were predicted as being strong binders (black bars, Net MHC rank threshold of 0.5% or under), weak binders (grey bars, Net MHC rank threshold of 0.5-2%) or non-binders (Net MHC rank threshold of over 2%).

3.5.11 Truncated human invariant chain (hIi) is alternative molecular adjuvant in ChAdOx1-HBV

It was not possible to abrogate the sIi epitope by sequential amino acid replacement. Although putative MHC class I epitopes were predicted in the truncated human invariant chain sequence in-silico (Figure 3.30), full length human invariant chain in a ChAd vectored HCV vaccine did not induce any auto-reactive T cell or antibody responses in a recent human trial (443). Therefore, it was hypothesised that using truncated human invariant chain instead of sIi would be unlikely to induce cellular immune responses towards the human invariant chain, whilst disrupting the functional HLA-A2 restricted sIi epitope and maintaining the adjuvant effect of invariant chain on CD8 T cell responses.

ChAdOx1-sIi-HBV (with truncated shark invariant chain), ChAdOx1-hIi-HBV (with truncated human invariant chain) or ChAdOx1-GFP (as a negative control) were administered by intramuscular injection into the hind leg of HHD mice at a dose of $5x10^7$ infectious units. Mice were sacrificed 2 weeks after vaccination for assessment of immune responses (Figure 3.31).

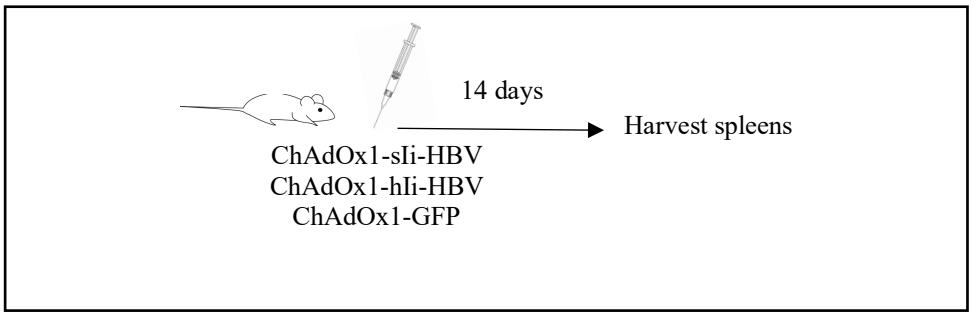


Figure 3.31. Experimental design of ChAdOx1 vaccination in HHD mice.

HHD mice were vaccinated intramuscularly with ChAdOx1-sIi-HBV, ChAdOx1-hIi-HBV or ChAdOx1-GFP. Mice were sacrificed 14 days after vaccination and spleens were harvested for immunological analysis.

Splenocytes were incubated with peptide pools corresponding to the entire HBV vaccine immunogen (HBV core, polymerase, pre-S1, pre-S2 and surface), the putative sIi epitope SLLWGGVTV and a peptide pool corresponding to the truncated human invariant chain. Vaccination with ChAdOx1-sIi-HBV induced high magnitude T cell responses towards the peptide SLLWGGVTV, confirming the region as the T ell epitope within sIi (Figure 3.32). No T cell responses to this peptide were detected in mice that had received the ChAdOx1-hIi-HBV vaccine (Figure 3.32). There was no significant difference between the total magnitude of T cell responses towards the HBV vaccine immunogen between mice that had received ChAdOx1-sIi-HBV and mice that had received ChAdOx1-hIi-HBV (median total response 243 vs 280 IFNγ SFU per million splenocytes, Figure 3.32).

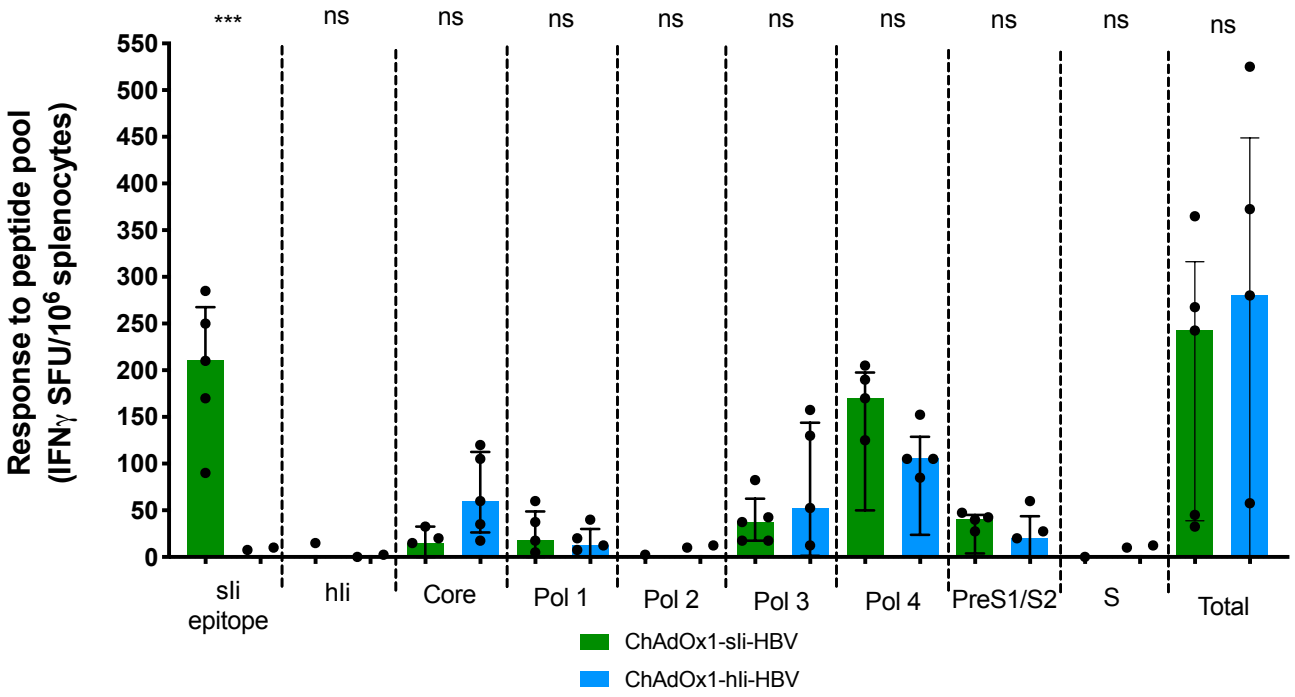


Figure 3.32 T cell responses in HHD mice after ChAdOx1-sIi-HBV or ChAdOx1-hIi-HBV vaccination

HHD mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) ChAdOx1-sIi-HBV or ChAdOx1-hIi-HBV. Mice were sacrificed 14 days after vaccination and spleens were harvested for immunological analysis. Splenocytes were isolated and plated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. The magnitude of the T cell response to each peptide pool is displayed as spot forming units (SFU) per million (10^6) splenocytes. Peptide pools from left to right correspond to truncated shark invariant chain (sIi) epitope SLLWGGVTV, truncated human invariant chain (hIi), HBV core (core), HBV polymerase (pol1-pol4), HBV pre-S1 and pre-S2 (pre S1/S2) and HBV surface (surface). Results are from a single experiment with 5 mice per group. Each colored bar represents a different vaccine regime. Median and interquartile range shown. Total magnitude of responses compared using 2-way ANOVA test with Sidak's multiple comparisons. ***$p<0.001$, ns = not significant.

3.6 DISCUSSION

The findings in this chapter support the primary hypothesis that vaccination with ChAdOx1-HBV and MVA-HBV can induce HBV specific T cell responses in healthy mice targeting multiple epitopes.

Vaccination with ChAdOx1-HBV and/or MVA-HBV induced high magnitude, polyfunctional CD8 and CD4 T cells specific to HBV polymerase and surface antigens in inbred and outbred mice. Strong CD8 T cell responses are important for clearance of acute HBV infection (168,176,178,476–478). Therefore, the ability of these vaccines to induce HBV specific T cells supports their ongoing investigation as therapeutic vaccine candidates in humans.

Heterologous vaccination with ChAdOx1-HBV (prime) and MVA-HBV (boost) significantly increased the magnitude and breadth of T cell responses in C57BL/6J mice. These findings are in agreement with other studies employing heterologous ChAdOx1/ MVA vaccine regimes, in which the MVA vaccination increases the magnitude of the ChAdOx1 primed T cell response in both mice (410,425) and humans (405,406,409).

The data in this chapter suggests that a longer interval between ChAdOx1-HBV and MVA-HBV vaccination (7-8 weeks rather than 4), is required to significantly boost HBV specific T cell responses. However, other studies using heterologous ChAdOx1/ MVA vaccine regimes have not found a particular interval between prime and boost vaccination to be consistently superior in increasing the T cell response magnitude. In a phase I human study of influenza vaccines ChAdOx1-NP+M1 and MVA-NP+M1, there was no significant difference in peripheral blood mononuclear cell (PBMC) T cell response magnitude as measured by area under the curve, between regimes given 8 compared to 52 weeks apart (406). In another study

employing the same vaccines, the greatest fold change of T cell responses compared to baseline was in the group given vaccinations 14 weeks as opposed to 7 weeks apart, although the two regimes were not directly compared statistically (405). Conversely in a study of mycobacterium tuberculosis (MTB) vaccines in BALBc mice delivered intranasally, ChAdOx1-85A and MVA-85A vaccination regimes given 2 weeks apart had significantly increased T cell responses in the lung compared to 4 or 8 week regimes, whereas responses in the spleen were highest in the 4 week regime (407). It is likely therefore, that the magnitude of ChAdOx1/ MVA vaccine induced T cell responses are dependent on several factors including the vaccine immunogen, route of vaccination, test species and organ in which the response is measured. Therefore, dose regime intervals for the ChAdOx1-HBV and MVA-HBV vaccines in mice are unlikely to reliably infer the best vaccine intervals in humans.

Chronic HBV infection is characterised by the persistence of HBsAg in the blood for over 6 months. Waning titers and clearance of HBsAg occurs rarely in individuals with chronic HBV infection (16,103) but the immune mechanisms underlying this are not fully understood. Sustained HBsAg loss (functional cure) is associated with a lower risk of developing cirrhosis and hepatocellular carcinoma (9). To emulate this phenomenon, it is desirable for therapeutic vaccination to induce anti-HBs Ab, with the hypothesis that it will reduce HBsAg titers through neutralisation. However, mice vaccinated with ChAdOx1-HBV and MVA-HBV did not reproducibly induce detectable titers of anti-HBs Ab. In mice where anti-HBs Ab was detectable, there was no association between CD4 T cell IFNγ responses specific to HBV surface antigen, implying T cell help might not be the limiting step in antibody production. The ELISA assay used is able to detect antibodies towards HBV surface but not HBV preS1 or pre-S2. Further work is necessary to determine whether antibodies are

induced in mice after vaccination, but towards different HBV antigens. Ultimately clinical trials will be required to assess whether the vaccines induce anti-HBs Ab in humans.

In vaccinated mice, T cell responses with the greatest magnitude were generated towards the HBV polymerase and HBV surface regions of the immunogen. Immunogenic regions in C57BL/6J mice were studied in greater detail in order to identify candidates for fluorochrome labeled multimers that could be used to study vaccine induced T cell phenotype in greater depth. Within the HBV polymerase antigen five H-2D^b/H-2K^b restricted epitopes were predicted, of which four had high responses in IFNγ ELISpot. Within the HBV surface antigen, no epitopes were predicted but the H-2K^b restricted epitope VWLSVIWM has previously been described (453–456). These epitopes are all potential candidates for fluorochrome labeled multimers that could be developed to assess the therapeutic potential of vaccine responses in the AAV HBV mouse model of chronic HBV infection, first described in HLA-A2/DR1 mice (450) but more recently in C57BL/6 mice (340,479).

Responses to the HBV core antigen were low or undetectable in C57BL6/J, BALBc and CD1 mice after ChAdOx1-HBV / MVA-HBV vaccination. In order to investigate whether HBV core epitopes could be presented on H-2D^b/H-2K^b molecules, plasmid vaccines containing only the HBV core region were tested. These vaccinations were able to induce detectable HBV core specific T cell responses in C57BL/6J mice. Further, ChAdOx1-HBV/ MVA-HBV vaccinations were able to induce HLA-A*02 restricted HBV core specific responses in HHD mice. Together these data suggest ChAdOx1-HBV / MVA-HBV vaccines are able to induce HBV core specific T cells in H-2D^b/H-2K^b restricted C57BL/6J mice, but they are not detectable due to HBV polymerase and surface epitope immunodominance.

It is desirable for an HBV therapeutic vaccine to be able to induce strong T cell responses towards all major HBV antigens in humans, to emulate acute infection where T cells specific for HBV core (170,171,173,476), polymerase (174) and surface (471) regions are present. In humanised HHD mice, T cell responses directed towards HBV core, polymerase and surface antigens were detectable after vaccination, demonstrating that in HLA-A*02 positive humans, ChAdOx1-HBV / MVAHBV vaccines have the potential to induce T cells towards all major HBV antigens. Within the most immunogenic regions of the vaccines in HHD mice, six HLA-A*02 restricted epitopes have been previously described in individuals with acute or chronic HBV infection (465–472,480). However, it is not known if these epitopes, nor other previously describe epitopes with particular HBV protein specificities will be efficacious in inducing HBV immune control in those with chronic HBV infection. Studies of these vaccines in individuals with chronic HBV will be necessary to determine this.

The responses of greatest magnitude in HLA-A*02 restricted HHD mice were directed towards the truncated shark invariant chain (sIi) region of the insert. This region was included as a molecular adjuvant, shown to increase the magnitude of vaccine induced T cell responses (346,431). Responses were directed towards an sIi region in which two epitopes with strong HLA-A*02 binding are predicted but have not previously been reported. This is consistent with results from ChAd/MVA vaccines trialed in humans recently, in which high magnitude responses were recorded towards the same region of sIi in HLA-A*02 restricted individuals (Amy Flaxman and Adrian Hill, personal correspondence). I confirmed SLLWGGVTV as the amino acid sequence of the sIi epitope in further experiments in HHD mice. Substitution of truncated sIi with truncated human invariant chain (hIi) prevented this T cell response and did not alter the magnitude of HBV specific T cell responses induced by vaccination. Reassuringly there was no cross reactivity with human invariant chain peptides in HHD mice

after vaccination with either ChAdOx1-sIi-HBV or ChAdOx1-hIi-HBV, suggesting they are unlikely to induce autoreactive T cell responses in humans. Trials of ChAdOx1-HBV and MVA-HBV in humans will be required to investigate this further.

3.7 CONCLUSION

ChAdOx1-HBV / MVA-HBV vaccination induced high magnitude T cell responses in mice. Epitopes have been identified that might enable *ex-vivo* analysis of vaccine induced T cells in mice using different regimes or additional therapies to improve vaccine immunogenicity going forward.

Studies in healthy human volunteers will be necessary to determine the immunogenic regions of the vaccine in humans with various HLA types in order to develop tools to study vaccine induced T cell responses in man. The vaccines will also need to be studied in individuals with chronic HBV to determine whether they are able to induce effective T cell responses with the ability to control the virus in the liver.

3.8 SUMMARY OF RESULTS

- ChAdOx1-HBV / MVA-HBV vaccination induces high magnitude polyfunctional CD8 and CD4 T cell responses in several mouse strains
- MVA-HBV vaccination boosts the magnitude and breadth of ChAdOx1-HBV primed T cell responses in mice
- ChAdOx1-HBV/ MVA-HBV vaccination does not reproducibly induce HBV surface antibody production in mice
- $H2D^b$ and $H2K^b$ restricted epitopes specific to HBV polymerase and surface regions are induced by vaccination can be identified in C57BL/6J mice

- HBV core specific T cells are induced C57BL/6J mice when the immunogen is restricted to the HBV core region
- ChAdOx1-HBV and MVA-HBV vaccination induces high magnitude T cell responses in HLA-A*02 restricted HHD mice towards HBV core, polymerase and surface regions but also towards the sIi region of the vaccine
- Substitution of truncated sIi with truncated hIi prevented the T cell response to sIi in HHD mice and did not alter the magnitude of HBV specific T cell responses induced by vaccination

CHAPTER 4: Investigating the effect of ChAdOx1-HBV and MVA-HBV vaccination route on the magnitude and phenotype of HBV-specific CD8 T cells in the blood and liver

4.1 PRIMARY HYPOTHESIS

The magnitude of vaccine induced HBV specific T cells in the liver can be augmented by intravenous as opposed to intramuscular administration of MVA-HBV (boost) vaccination.

4.2 INTRODUCTION

The primary immunological goal of an HBV therapeutic vaccine candidate is its ability to induce robust HBV specific CD8 T cell responses. This is based on evidence that CD8 responses are necessary for viral clearance of acute HBV infection (178) and that individuals that go on to develop chronic HBV have dysfunctional HBV specific CD8 cells (231). An in depth understanding of the CD8 response to vaccination with ChAdOx1-HBV and MVA-HBV is therefore a key read-out of potential vaccine efficacy.

Vaccine induced T cell immunogenicity is traditionally assessed in spleen derived lymphocytes in mice, due to the large numbers of lymphocytes that can be processed at any one time or blood derived lymphocytes in mice and humans, favored as a relatively non-invasive method of tracking longitudinal responses. When considering the immunogenicity of therapeutic vaccination for HBV, evaluating T cell responses in the liver is more relevant than responses in the spleen or blood, as the virus exclusively infects hepatocytes (481). Functional responses of mice vaccinated with ChAdOx1-HBV and MVA-HBV in the spleen have been assessed (seen Chapter 1, (346)) but responses in the liver have yet to be evaluated.

Surface expression of receptors on lymphocytes can be evaluated by flow cytometry to differentiate CD8 T cell populations with distinct phenotypes with associated functional properties, including effector T cells, effector memory and central memory subsets (482–487). Cell phenotyping in this way can complement functional assays where cells are re-stimulated with antigen or can be used to identify vaccine induced cells directly *ex-vivo*, using fluorochrome labelled multimers (488). The latter approach has the advantage of evaluating T cell phenotypes as they are *in-vivo* rather than assessing changes that happen as a result of artificial *ex-vivo* stimulation. More recently, populations of memory T cells with distinct surface expression patterns have been described that remain resident in liver without recirculating (489,490). Liver resident memory T cells (T_{RM}) are enriched in the liver of individuals with chronic HBV and negatively correlate with circulating HBV DNA titres (491). T_{RM} cells are therefore likely to be important in controlling HBV infection. As yet, the memory phenotype of CD8 cells induced by ChAdOx1-HBV and MVA-HBV vaccination in the liver has not been investigated.

Commonly vaccines are administered intramuscularly. However, recent studies investigating vaccine candidates for tuberculosis, cancer and malaria have shown increased efficacy when administered intravenously, as opposed to intramuscularly (492–494). In mice, intravenous vaccination with malaria or Hepatitis C virus (HCV) vaccines increased the magnitude of vaccine induced CD8 T cells in the liver, which displayed a T_{RM} phenotype (492,495). It is not yet known whether vaccination with ChAdOx1-HBV and MVA-HBV can induce HBV-specific liver T_{RM} and whether intravenous boost vaccination is associated with improved CD8 targeting to the liver.

In this chapter, the phenotype of CD8 T cells and HBV specific CD8 T cells induced by vaccination with ChAdOx1-HBV and MVA-HBV are evaluated by flow cytometry. Cell phenotypes are compared between organ of origin at between 14 and 60 days after vaccination. The presence of vaccine induced resident memory cells in the liver is assessed. Finally, intramuscular and intravenous routes of MVA-HBV boost vaccination are investigated for their ability to enhance the magnitude and phenotype of CD8 responses in the liver.

4.3 KEY QUESTIONS

1. Can vaccine induced HBV specific T cells be identified in the blood by flow cytometry using a fluorochrome labelled pentamer?
2. Can pentamer positive HBV specific T cells be used as a surrogate for the total splenocyte response to vaccination as measured in interferon-gamma ELISpot assays?
3. Can the phenotype of pentamer positive HBV specific T cells be examined *ex-vivo* in the blood by flow cytometry?
4. Does intravenous as opposed to intramuscular administration of boost vaccination with MVA-HBV increase the magnitude of HBV specific T cells in the blood, liver, spleen or lung?
5. Does vaccination with ChAdOx1-HBV and MVA-HBV change the phenotype of CD8 T cells compared to T cells in unvaccinated mice?
6. Does the phenotype of CD8 T cells alter by vaccination regime, timing of response or organ of origin?
7. What is the phenotype of pentamer positive HBV specific T cells induced by vaccination?

8. Does the phenotype of pentamer positive HBV specific T cells alter by vaccination regime, timing of response or organ of origin?
9. Is the expression of liver tissue residency marker CD69 expanded on liver as compared to blood derived CD8 or HBV specific T cells?
10. Do CD69 positive CD8 or HBV specific T cells co-express other liver tissue residency markers compared to CD69 negative cells.

4.4 CHAPTER SPECIFIC MATERIALS AND METHODS

4.4.1 Animal experiments

Animal experiments are discussed in detail in Chapter 2. Adult C57BL/6J mice (aged over 8 weeks) were vaccinated intramuscularly into the hind leg with ChAdOx1-HBV or MVA-HBV in a total volume of 50µl of PBS or intravenously into the tail vein in a total volume of 200µl of PBS. Mice were sacrificed at 14 or 60 days after their last vaccination. Liver, lung and spleen derived lymphocytes were isolated by mechanical disruption for use in immunoassays. In all experiments, unvaccinated animals were used as negative controls (n=1-5 per experiment). Individual mice were not randomly assigned to vaccine regime and investigators were aware of the intervention received.

4.4.2 Immunoassays

Immunoassays are discussed in detail in Chapter 2. Splenocytes were stimulated with peptide pools, minipools or individual peptides in duplicate in overnight interferon gamma (IFNγ) ELISpot assays.

4.4.3 Identification of HBV specific cells by flow cytometry

Flow cytometry is discussed in detail in Chapter 2. Lymphocytes were incubated with fluorochrome labelled pentamer specific to VWLSVIWM (ProImmune, UK) and monoclonal antibodies specific for CD8, CD44, KLRG1, CD127, CD62L, CX3CR1, CD69, CD11a and CXCR6. Cells were acquired on an LSRII flow cytometer (BD) and analysed on FlowJo version 10.0 (BD).

4.5 RESULTS

4.5.1 ChAdOx1-HBV and MVA-HBV vaccination induces HBV specific T cells in C57BL/6J mice that can be identified by flow cytometry using fluorochrome labelled multimers

In order to study vaccine induced T cells directly, fluorochrome labelled multimers can be used to identify T cells specific for a particular epitope. This enables the study of vaccine induced T cells directly *ex-vivo*, without the need to re-activate them. In chapter 3, data showed that the peptide number 371 (amino acid sequence FVGLSPTVWLSVIWM) was an immunogenic region in C57BL/6J mice within the HBV surface region of the vaccine. Within this sequence, the H-2K^{b} restricted epitope VWLSVIWM had previously been described (338,453–464).

To assess whether a fluorochrome labelled pentamer specific to VWLSVIWM (ProImmune, UK) could identify HBV specific vaccine induced T cells in C57BL/6J mice, blood derived lymphocytes from vaccinated and unvaccinated mice were incubated with fluorochrome labelled monoclonal antibodies and VWLSVIWM-pentamer. HBV specific T cells were

identified by flow cytometry by gating live, single cell CD8 T cells (representative gating show in Figure 4.1).

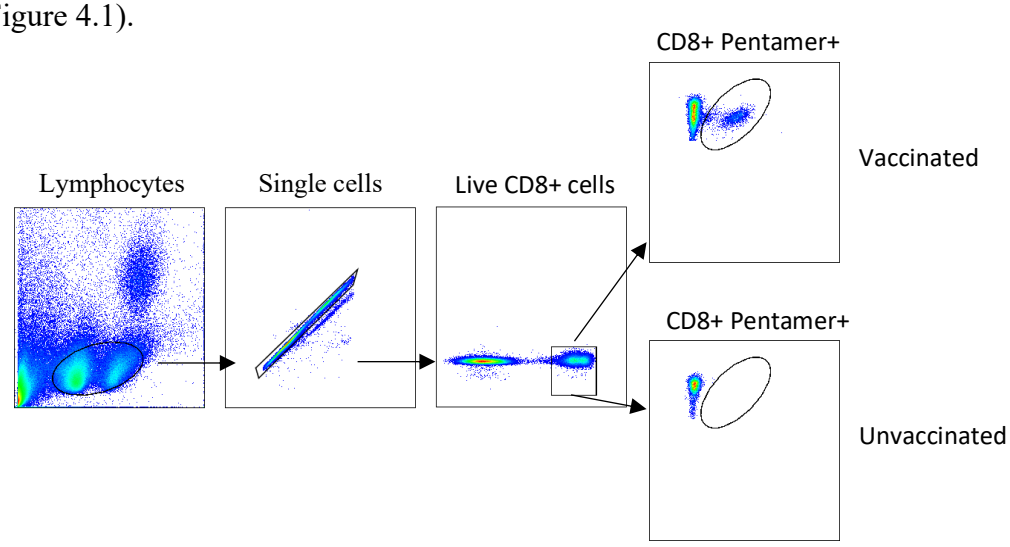


Figure 4.1 Identification of HBV surface specific CD8 T cells using a pentamer in C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4-8 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Unvaccinated animals were used as controls. Mice were bled at 2 weeks after final vaccination and lymphocytes were stained with fluorochrome labelled monoclonal antibodies and a pentamer specific to HBV surface (epitope sequence VWLSVIWM) to identify vaccine induced T cells by flow cytometry. Representative gating of VWLSVIWM-specific T cells induced by vaccination in the blood is shown. A cluster of pentamer positive cells is clearly seen in vaccinated animals but is absent in unvaccinated animals.

The dynamics of HBV specific T cell responses in blood derived lymphocytes over time were compared between unvaccinated mice (grey line) and mice receiving regimens of ChAdOx1-HBV (prime) alone (green line) or with MVA-HBV (boost) at either 4 weeks (blue line) or 8 weeks (orange line) after prime (Figure 4.2). Pentamer positive HBV specific T cells were identified in vaccinated but not unvaccinated mice (grey line). After ChAdOx1-HBV (prime) all groups of vaccinated mice had detectable pentamer positive HBV specific T cells, which peaked at 2 weeks after prime (pentamer positive T cells median 14.30%, IQR 5.4 – 21.3% of all CD8 T cells). The response was highly variable across all groups (green, blue and orange lines) and there was no significant difference in the percentage of pentamer positive T cells across the groups at week 2 after prime.

The percentage of pentamer positive T cells increased between pre MVA-HBV and 7 days post MVA-HBV boost in mice vaccinated 4 weeks (blue line, pre-boost median 1.1% vs post-boost median 7.9% pentamer positive T cells, p=0.0079) or 8 weeks (orange line, pre-boost median 1.3% vs post-boost median 7.1% pentamer positive T cells, p=0.0159) after ChAdOx1-HBV prime (Figure 4.2).

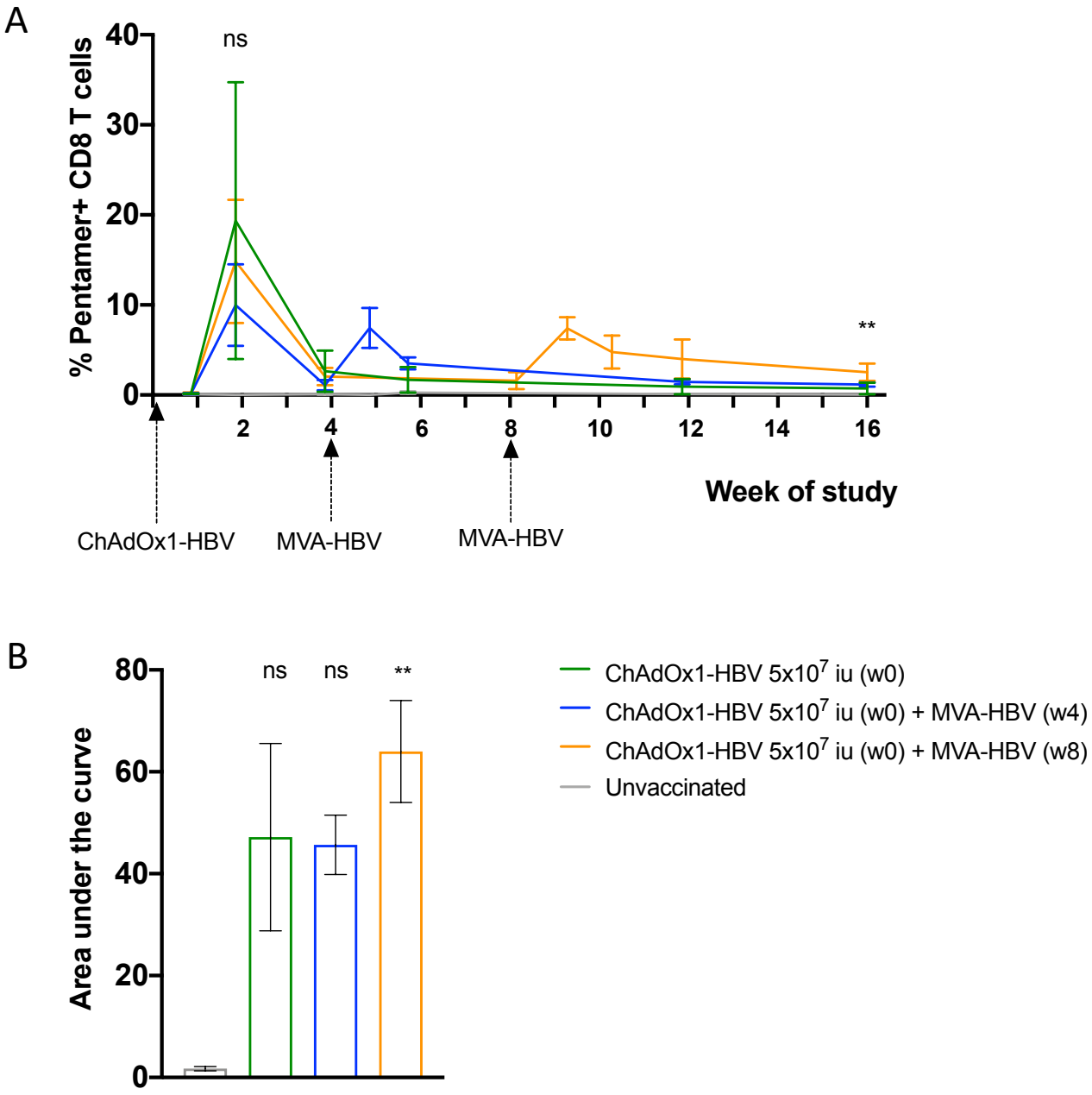


Figure 4.2 Longitudinal magnitude of HBV surface specific CD8 T cells induced by ChAdOx1-HBV and MVA-HBV vaccination

C57BL/6J mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) either alone (green line) or followed 4 weeks (blue line) or 8 weeks (orange line) later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Unvaccinated animals were used as controls (grey line). Mice were bled at intervals up to 16 weeks after prime vaccination and lymphocytes were stained with fluorochrome labelled monoclonal antibodies and a pentamer specific to HBV surface (epitope sequence VWLSVIWM) to identify vaccine induced T cells by flow cytometry. **A:** Pentamer positive cells as a percentage of CD8 T cells in the blood from 0 to 16 weeks after ChAdOx1-HBV vaccination. **B**: Area under the curve of pentamer positive cells as a percentage of CD8 T cells between 0 and 16 weeks after ChAdOx1-HBV vaccination. Results are a summation of single experiment with 5 mice per group (vaccinated animals) or 3 mice per group (unvaccinated animals). Each colored line represents a different vaccine group (green, blue, orange, grey).

Median and interquartile range shown. Percentage of VWLSVIWM- specific T cells compared between groups using Kruskal-Wallis test with Dunn's multiple comparisons, where each group is compared to the response in unvaccinated mice. Area under the curve between groups compared using one-way ANOVA test with multiple comparisons, where each group is compared to the response in unvaccinated mice. **$p<0.01$, ns = not significant

At week 16 after ChAdOx1-HBV prime the median percentage of pentamer positive T cells was significantly different between groups of mice (p=0.0019), being significantly higher in mice that had received ChAdOx1-HBV and MVA-HBV vaccines 8 weeks apart (orange line, median 2.45, IQR 1.71-3.36%) as compared to unvaccinated mice (grey line, median 0.04, IQR 0.04-0.04%, p=0.0135, Figure 4.2). The area under the curve (AUC) was also significantly greater in mice that had received ChAdOx1-HBV and MVA-HBV vaccines 8 weeks apart (orange line, AUC 63.77) as compared to unvaccinated mice (grey line, AUC 1.734, p=0.0076, Figure 4.2).

To determine whether the percentage of blood derived pentamer positive T cells was associated with the total HBV response to vaccination in the spleen, pentamer positive T cell responses were compared to total responses of splenocytes in IFNγ ELISpot assays. There was a significant positive correlation between total response to vaccination in the spleen (IFNγ spot forming units/million splenocytes) and pentamer positive T cell responses as a percentage of CD8 T cells in blood at week 16 post prime vaccination (r=0.51, p=0.0443, Figure 4.3).

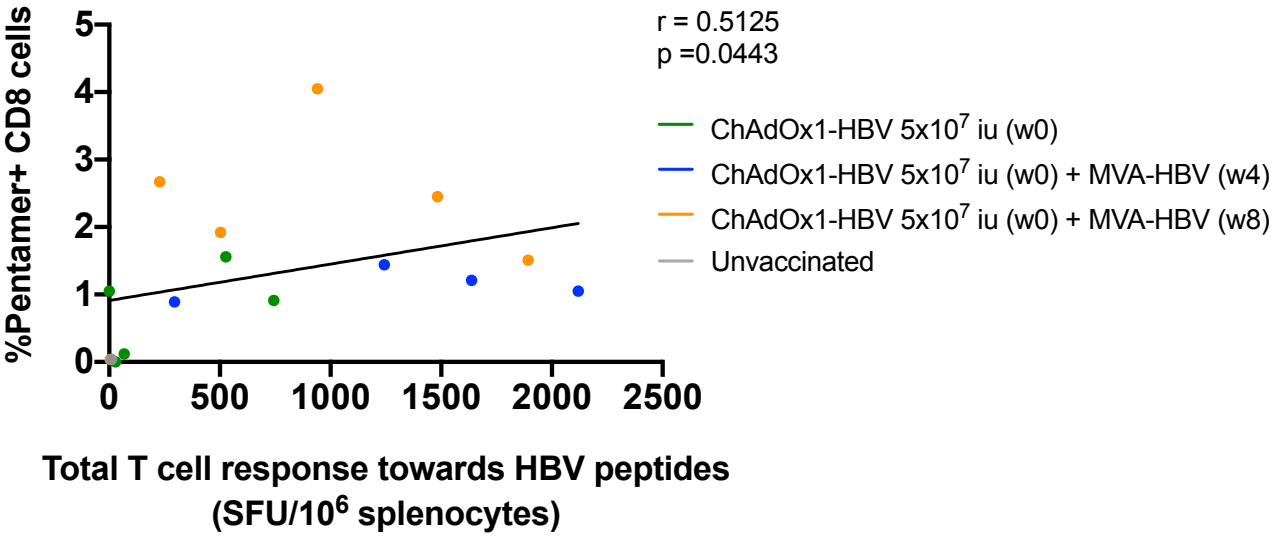
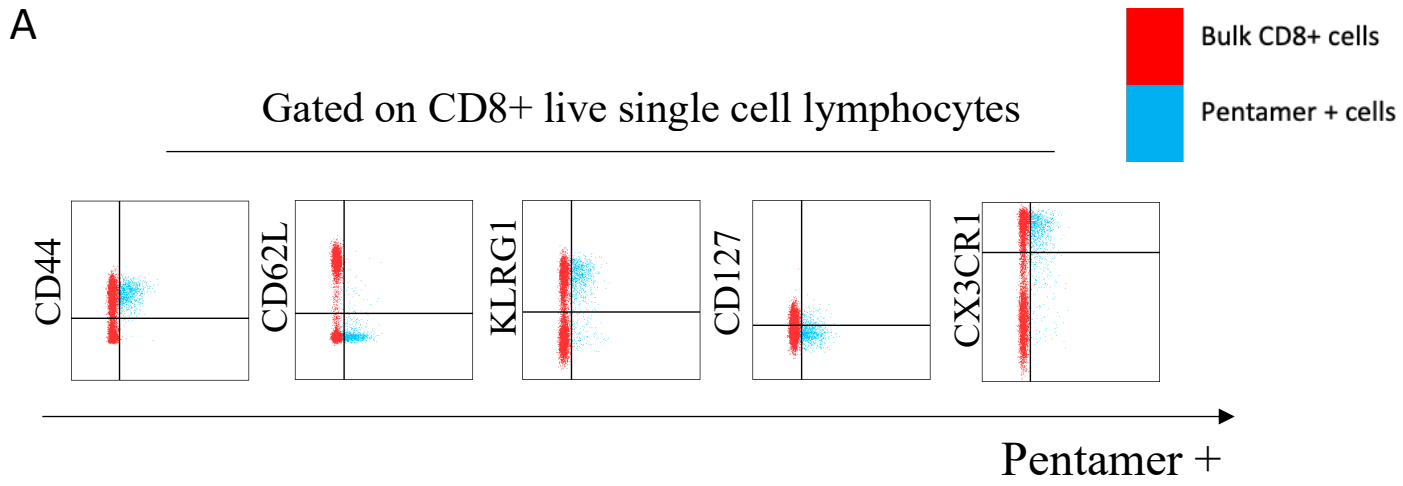


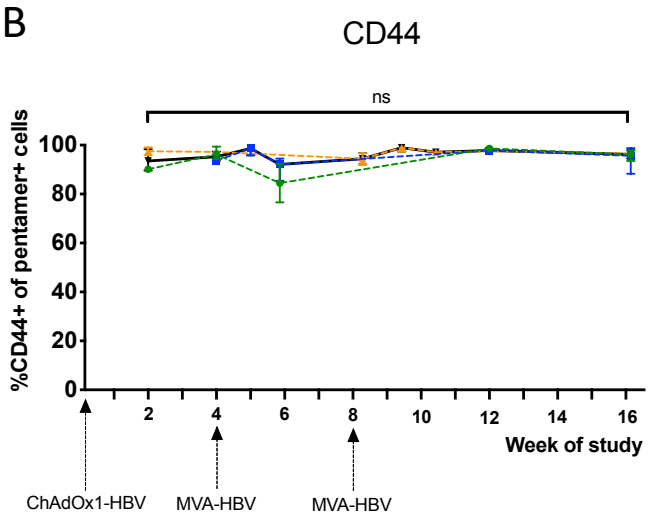
Figure 4.3 The relationship between total T cell response on ELISpot and the percentage of pentamer positive CD8 T cells after ChAdOx1-HBV vaccination

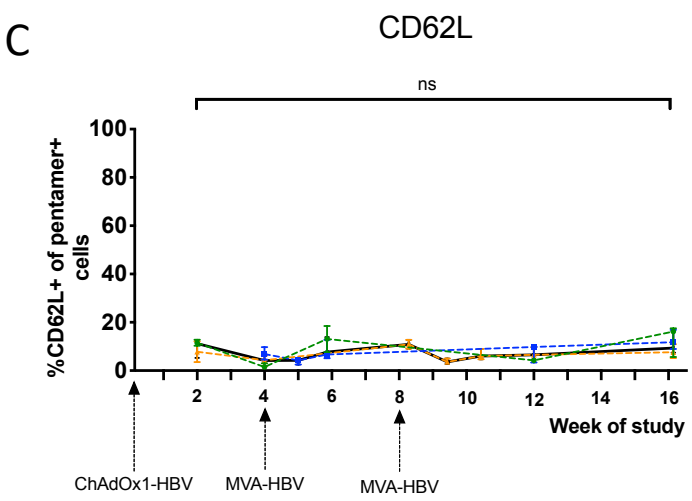
C57BL/6J mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) either alone (green) or followed 4 weeks (blue) or 8 weeks (orange) later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Unvaccinated animals were used as controls (grey). Mice were sacrificed 16 weeks after ChAdOx1-HBV vaccination for spleen and blood lymphocyte isolation. Blood derived lymphocytes were stained with fluorochrome labelled monoclonal antibodies and a pentamer specific to HBV surface (epitope sequence VWLSVIWM) to identify vaccine induced T cells by flow cytometry. Splenocytes were incubated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays (HBV core, polymerase (pol1-4), pre-S1/Pre-S1 and surface peptide pools). The percentage of blood derived CD8 T cells that were VWLSVIWM-pentamer positive at week 16 after prime vaccination were compared to the total response to vaccination as measured by INFγ spot forming units per million splenocytes at the same time point. Correlation calculated using Spearman rank.

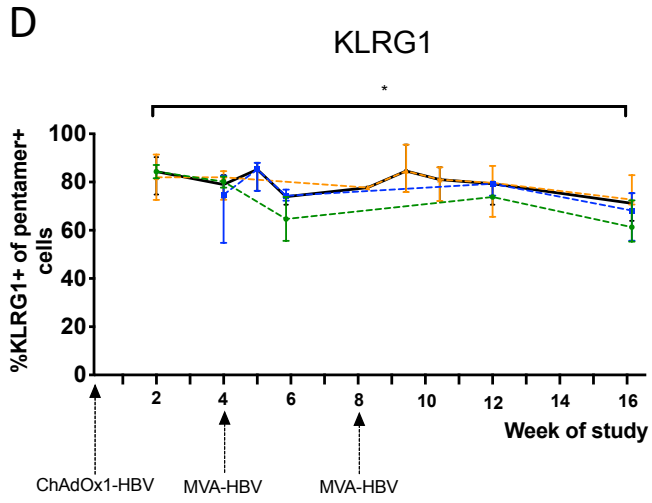
Finally, the phenotype of pentamer positive T cells was investigated by flow cytometry to determine the expression of CD44, CD62L, KLRG1, CD127 and CX3CR1 on vaccine induced cells (representative gating show in Figure 4.4). The median percentage of pentamer positive CD8 T cells expressing each marker was similar between the different vaccine regimes (green, blue and orange lines, with all regimes displayed as black line, Figure 4.4). CD44 was highly expressed (median 96.20, range 83.70 - 99.40% CD44 positive at week 16). CD62L by contrast had low expression (median 9.33, range 3.26 – 17.50% CD62L positive at week 16). Pentamer positive T cells also highly expressed KLRG1 (median 71.20, range 44.90 - 86.50% KLRG1 positive at week 16) and CX3CR1 (median 75.40, range 56.90 -

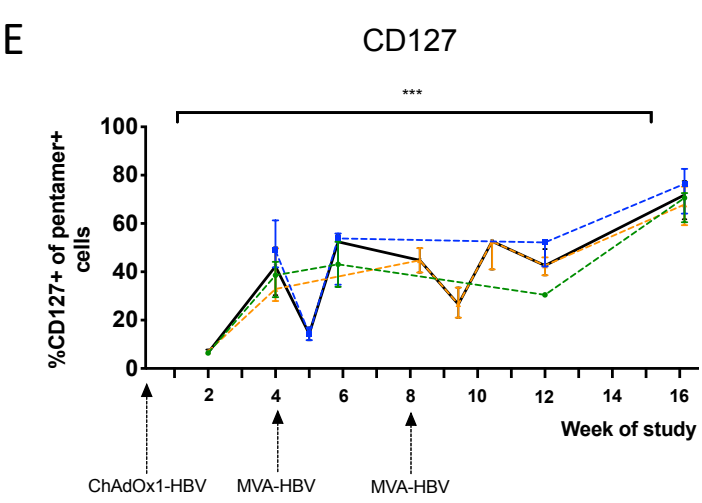
82.00% CX3CR1 positive at week 16). CD127 expression significantly increased between week 2 (median 6.5, range 5.83 – 8.12%) and week 16 post prime (median 71.80, range 55.60 - 83.90%, p=0.0008). CX3CR1 expression significantly decreased between week 2 (median 98.95, range 91.40 – 99.60 %) and week 16 post prime (median 75.40, range 56.90 - 82.00 %, p=0.0008).

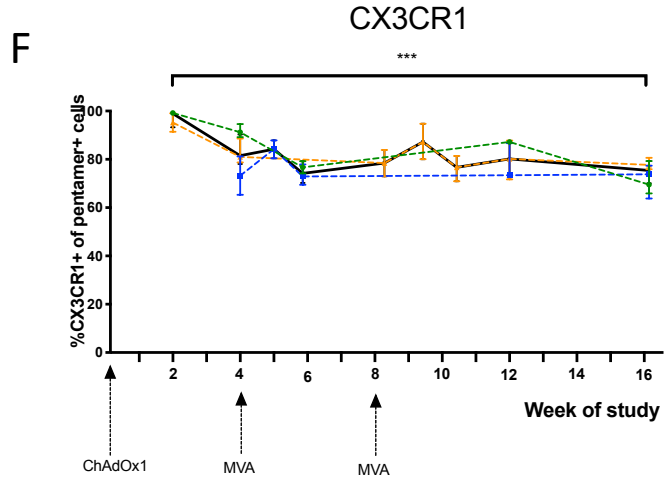

A
Gated on CD8+ live single cell lymphocytes
Bulk CD8+ cells
Pentamer + cells
CD44
CD62L
KLRG1
CD127
CX3CR1
Pentamer +


B
CD44
ns
%CD44+ of pentamer+ cells
Week of study
ChAdOx1-HBV
MVA-HBV
MVA-HBV


C
CD62L
ns
%CD62L+ of pentamer+ cells
Week of study
ChAdOx1-HBV
MVA-HBV
MVA-HBV


D
KLRG1
*
%KLRG1+ of pentamer+ cells
Week of study
ChAdOx1-HBV
MVA-HBV
MVA-HBV


E
CD127
***
%CD127+ of pentamer+ cells
Week of study
ChAdOx1-HBV
MVA-HBV
MVA-HBV


F
CX3CR1
***
%CX3CR1+ of pentamer+ cells
Week of study
ChAdOx1
MVA
MVA

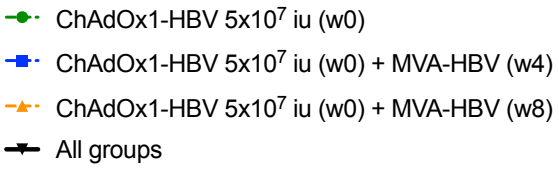

ChAdOx1-HBV 5x10^7 iu (w0)
ChAdOx1-HBV 5x10^7 iu (w0) + MVA-HBV (w4)
ChAdOx1-HBV 5x10^7 iu (w0) + MVA-HBV (w8)
All groups

Figure 4.4 The phenotype of blood derived HBV specific vaccine induced T cells

C57BL/6J mice were vaccinated intramuscularly with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) either alone (green line) or followed 4 weeks (blue line) or 8 weeks (orange line) later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost). Mice were bled at intervals up to 16 weeks after prime vaccination and lymphocytes were stained with fluorochrome labelled monoclonal antibodies and a pentamer specific to HBV surface (epitope sequence VWLSVIWM) to identify vaccine induced T cells by flow cytometry.
A: Representative gating and expression of CD44, CD62L, KLRG1, CD127 and CX3CR1 on bulk CD8 T cells (red) and VWLSVIWM pentamer positive cells (blue).
Percentage of VWLSVIWM-specific T cells expressing **B:** CD44, **C:** CD62L, **D:** KLRG1, **E:** CD127 and **F:** CX3CR1 over time. Results are a summation of single experiment with 5 mice per group. Each colored line represents a different vaccine group (green, blue, orange). The summation of results from all groups is shown with a black line. The percentage of HBV pentamer specific T cells expressing each marker at week 2 was compared to week 16 (all groups combined) using Mann Whitney U test. Median and interquartile range shown. *** $p<0.001$, *$p<0.05$, ns = not significant

4.5.2 The magnitude of HBV specific CD8 T cells is increased in the liver after intravenous as compared with intramuscular MVA-HBV boost vaccination in C57BL/6J mice after ChAdOx1-HBV prime

Previous studies suggest vaccine induced T cell responses can be enhance by administering vaccine intravenously (i.v.) as opposed to intramuscularly (i.m.) (492,494). To investigate whether the magnitude of HBV vaccine induced T cell responses could be enhanced by alternative routes of vaccination in mice, T cell responses induced by ChAdOx1-HBV (prime) followed 4 weeks later MVA-HBV (boost) were compared between groups receiving i.m. prime followed by i.m. boost (i.m.i.m.) or i.m. prime followed by i.v. boost (i.m.i.v.). The magnitude of HBV-specific T cells was assessed at 14 days (peak response) or over 60 days (long-lived response) after the last vaccination in the blood, liver, spleen and lungs of vaccinated animals (experimental set up shown in Figure 4.5.A). Vaccine induced pentamer positive HBV specific T cells could be detected in all organs assessed in vaccinated mice (gating strategy shown in Figure 4.5.B).

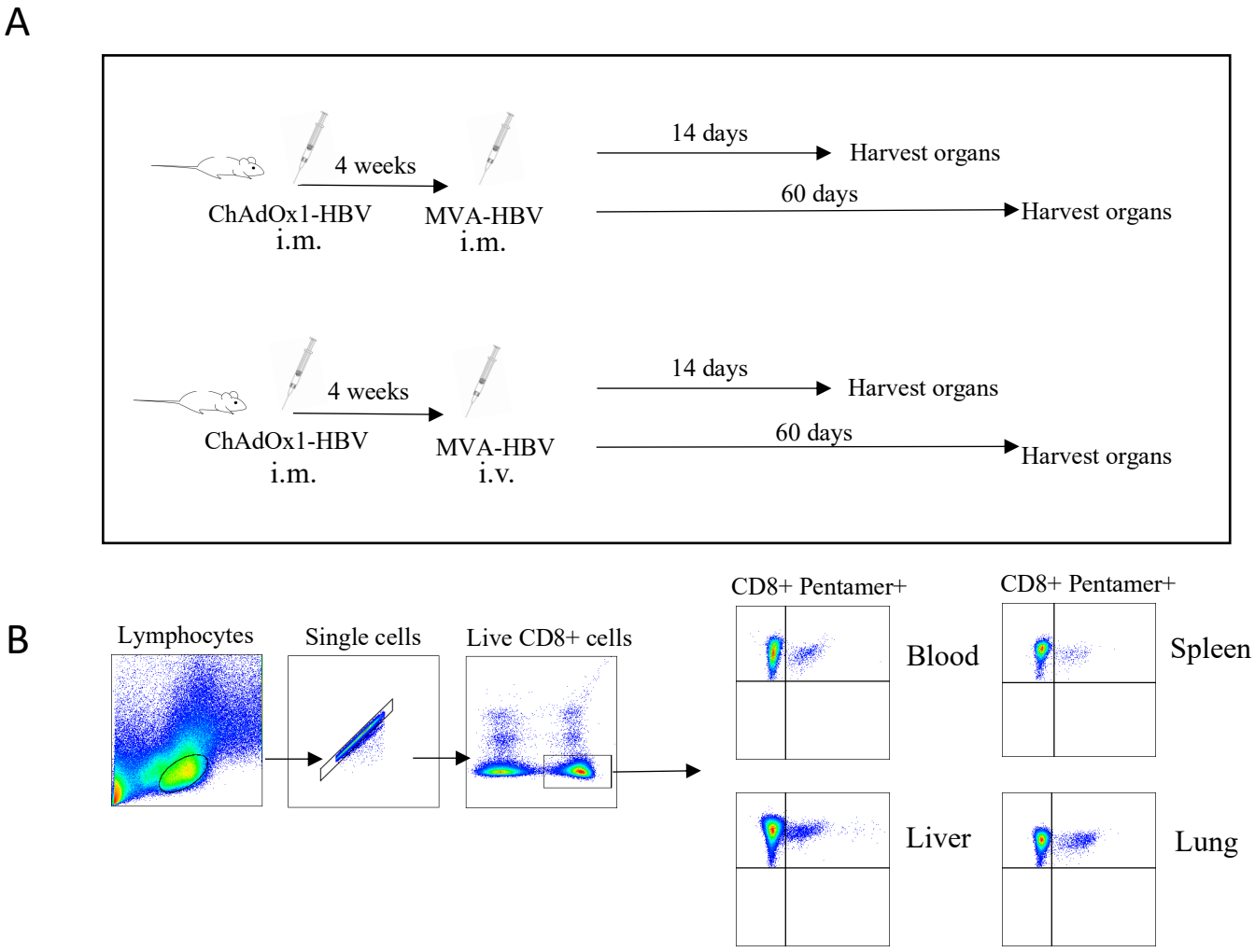


Figure 4.5 Experimental set-up to compare intramuscular ChAdOx1-HBV (prime) and intravenous as compared with intramuscular MVA-HBV (boost) vaccination in C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice were sacrificed 14 days (peak response) 60 or more days (long lived response) after final vaccination to assess vaccine induced CD8 T cell responses in the blood, liver, spleen, and lungs, identified by HBV pentamer (epitope sequence VWLSVIWM).
A: Experimental set up.
B: Representative gating of VWLSVIWM-specific T cells induced by vaccination in lymphocytes derived from the blood, liver, spleen, and lung.

Mice that had received the i.m.i.m. regimen had a significantly higher percentage of pentamer positive T cells compared to unvaccinated animals, where pentamer positive T cells were low or undetectable (blood median 3.61 vs. 0.30% $p < 0.0001$, liver median 3.45 vs 0.10% $p<0.0001$, spleen median 1.74 vs 0.10% $p<0.0001$ and lung median 3.52 vs 0.12% $p=0.0004$, Figure 4.6.A).

To investigate whether there were differences between the magnitude of response in the different organ compartments, pentamer positive T cells as a percentage of CD8 T cells were compared between blood, liver, spleen and lung at day 14 after the i.m.i.m. vaccine regimen (Figure 4.6.B). The percentage of pentamer positive T cells was significantly higher in the blood than in the spleen (median blood 3.61% vs spleen 1.74%, $p=0.0150$). The percentage of pentamer positive T cells was similar in the blood (median 3.61, IQR 2.97-5.59%), liver (median 3.45, IQR 1.36-5.52%) and lung (median 3.52, IQR 1.62-6.80%).

To investigate whether there were differences between the magnitude of response within organ compartments, pentamer positive T cells as a percentage of CD8 T cells were compared between different vaccine regimes (i.m.i.m. and i.m.i.v.) and at different timepoints after vaccination (day 14 and day 60, Figure 4.6.C). At day 14 after vaccination, responses in the liver were significantly increased in mice that had received the i.m.i.v. as compared to i.m.i.m. vaccine regimen (i.m.i.v. 5.49% vs im.im 3.45%, $p=0.0331$) but not in the blood, spleen or lung. At day 60 after vaccination, responses in the liver were not significantly different between vaccine regimes. There were no significant differences between the magnitude of T cell responses at day 14 compared to day 60 after vaccination in any organ.

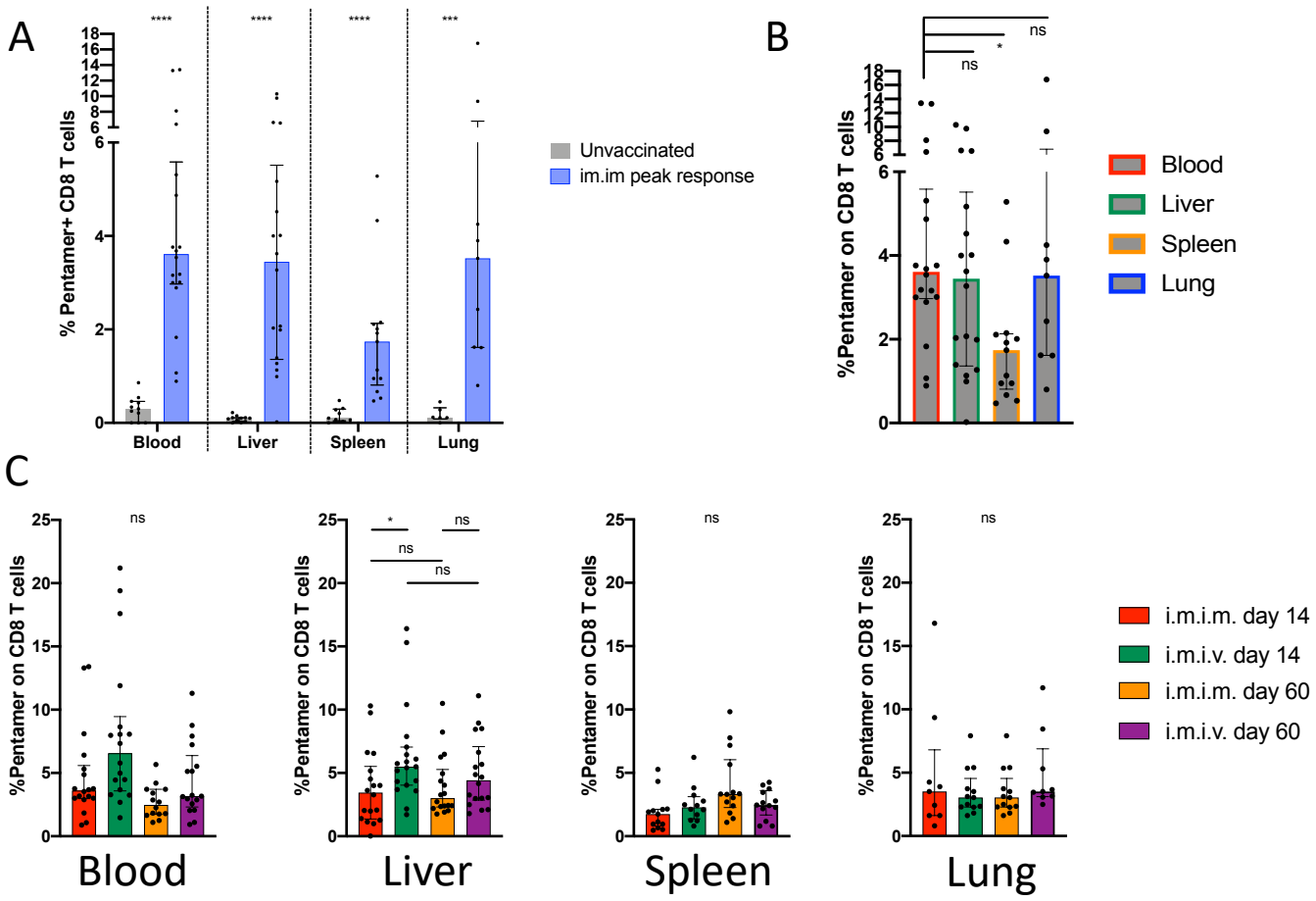


Figure 4.6 The magnitude of HBV specific CD8 T cells after intramuscular ChAdOx1-HBV (prime) and intravenous as compared with intramuscular HBV (boost) vaccination in C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days (peak response) 60 or more days (long lived response) after final vaccination to assess vaccine induced CD8 T cell responses in the blood, liver, spleen, and lungs, identified by HBV pentamer (epitope sequence VWLSVIWM).

A: Percentage of pentamer positive CD8 T cells in unvaccinated mice compared to mice vaccinated with the i.m.i.m. regimen in the blood, liver, spleen, and lung at day 14 after vaccination.
B: Percentage of pentamer positive CD8 T cells in the blood, liver, spleen, and lung at day14 after mice were vaccinated with the i.m.i.m. regimen.
C: Percentage of pentamer positive CD8 T cells after i.m.i.m. or i.m.i.v. vaccination regimens at day 14 or day 60 after vaccination. Results shown from left to right for blood, liver, spleen, and lung derived T cells.

Results are a summation of 5 experiments with 3-5 mice per group. Median and interquartile range shown. Comparisons between two groups were performed using Mann-Whitney U test or between more than two groups using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, * $p<0.05$, ns = not significant.

4.5.3 The memory phenotype of CD8 T cells is not changed after intravenous as compared with intramuscular MVA-HBV boost vaccination in C57BL/6J mice after ChAdOx1-HBV prime

To investigate the phenotype of T cells after vaccination, surface expression of markers associated with T cell differentiation were assessed by flow cytometry on CD8 cells derived from the blood, liver, spleen and lung. Expression of markers on bulk (total) CD8 T cells were compared between unvaccinated mice and mice vaccinated with im.im or i.m.i.v. regimes of ChAdOx1-HBV and MVA-HBV (experimental set up as per Figure 4.5.A). Surface marker expression identified antigen experienced cells (CD44+), effector T cells (KLRG1+), memory T cells (CD127-), central memory T cells (T_{CM} CD8+CD127+KLRG1-CD62L+), effector memory T cells (T_{EM} CD8+CD127+KLRG1-CD62L-) and cytotoxic CX3CR1 positive T_{EM} cells (representative gating strategy shown in Figure 4.7)(483,496).

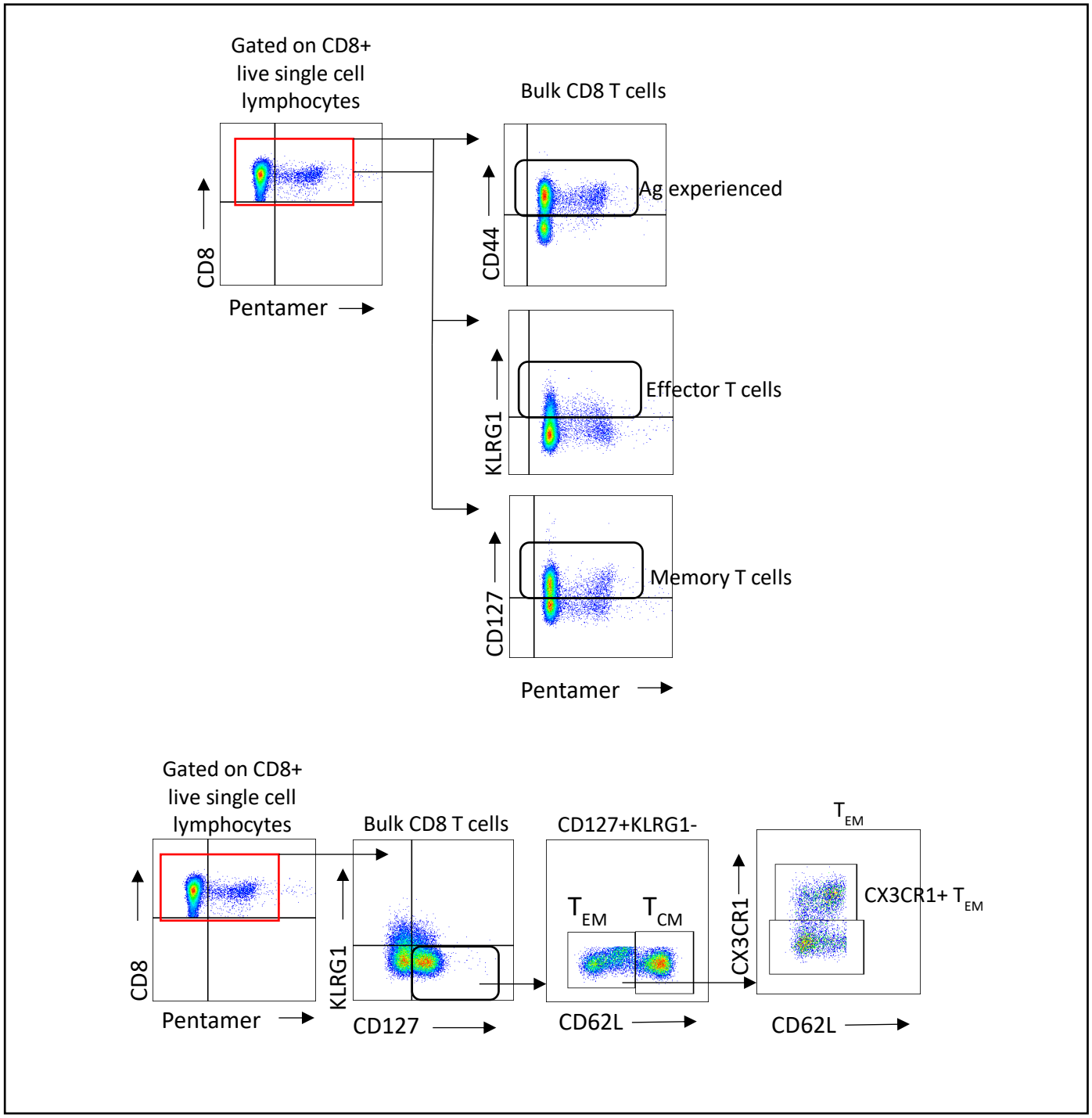


Figure 4.7 Gating strategy to determine the phenotype of CD8 T cells in C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lungs were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on bulk CD8 T cells by flow cytometry. Gating strategy to identify antigen experienced (Ag experienced), effector T cells, memory T cells, T central memory (T_{CM}) and T effector memory (T_{EM}) T cells shown.

There were significant differences in surface expression of markers between CD8 T cells from unvaccinated compared to vaccinated mice. In all organs in unvaccinated mice, a low percentage of CD8 T cells expressed CD44 (antigen experienced cells, range of medians 16.3% - 30.2%, Appendix 7, Table 1). Expression was higher in vaccinated compared with unvaccinated animals (Figure 4.8.A). In blood derived CD8 T cells, CD44 expression was significantly higher compared with spleen derived CD8 T cells in unvaccinated mice (median 30.10% vs 16.20%, p=0.0465, figure 4.8.B). After vaccination CD44 expression on CD8 T cells was significantly higher the blood (day 14 after i.m.i.m. and i.m.i.v. regimen), liver (all regimens) and spleen (day 14 after i.m.i.v. and day 60 after i.m.i.m. and i.m.i.v. regimens) compared to unvaccinated mice (Appendix 7, Table 1, figure 4.8.C). There were no significant differences in CD44 expression between the i.m.i.m. and i.m.i.v. regimens at either timepoint in any organ (Appendix 7, Table 1, figure 4.8.C).

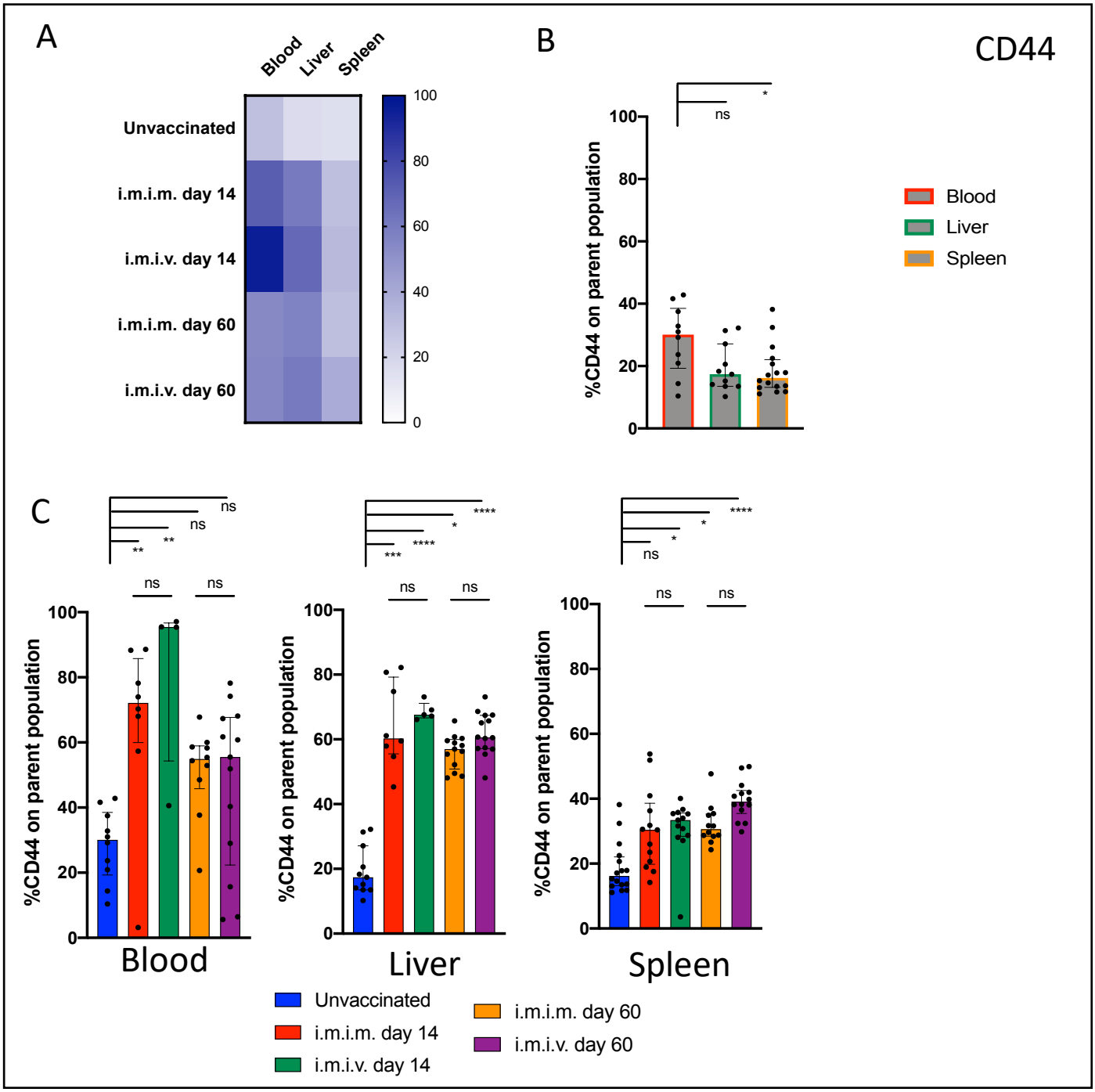


Figure 4.8 CD44 expression on bulk CD8 T cells in unvaccinated and vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver and spleen were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on bulk CD8 T cells by flow cytometry.

A: Heatmap showing median percentage of bulk CD8 T cells expressing CD44 in the blood, liver, and spleen in in unvaccinated and vaccinated mice. **B:** Percentage of CD8 T cells expressing CD44 in the blood, liver, and spleen in unvaccinated mice at day 14 of the experiment. **C:** Percentage of CD8 T cells that express CD44 in unvaccinated mice compared to mice vaccinated with i.m.i.m. or i.m.i.v. regimen at day 14 or day 60 after vaccination in the blood, liver, or spleen. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

Surface expression of KLRG1 (effector T cells) was low on CD8 T cells in all organs of unvaccinated mice (range of medians 1.09% - 4.68%, Appendix 7, Table 2) but was higher on CD8 T cells from vaccinated mice (Figure 4.9.A). In unvaccinated mice, blood derived CD8 cells had a higher expression of KLRG1 than spleen derived CD8 cells (median 4.68% vs 1.09%, $p<0.0001$, Figure 4.9.B). After vaccination KLRG1 expression on CD8 T cells was significantly higher in the blood (day 14 after i.m.i.m. and i.m.i.v. regimen), liver (day 14 after i.m.i.m. and i.m.i.v. regimen and day 60 after i.m.i.m. regimen), spleen (all regimens) and lung (all regimens) compared to unvaccinated mice (Appendix 7, Table 2, Figure 4.9.C). KLRG1 was significantly decreased on liver derived CD8 T cells in mice vaccinated with the i.m.i.v. compared to i.m.i.m. regimen at day 60 (Appendix 7, Table 2, Figure 4.9.C).

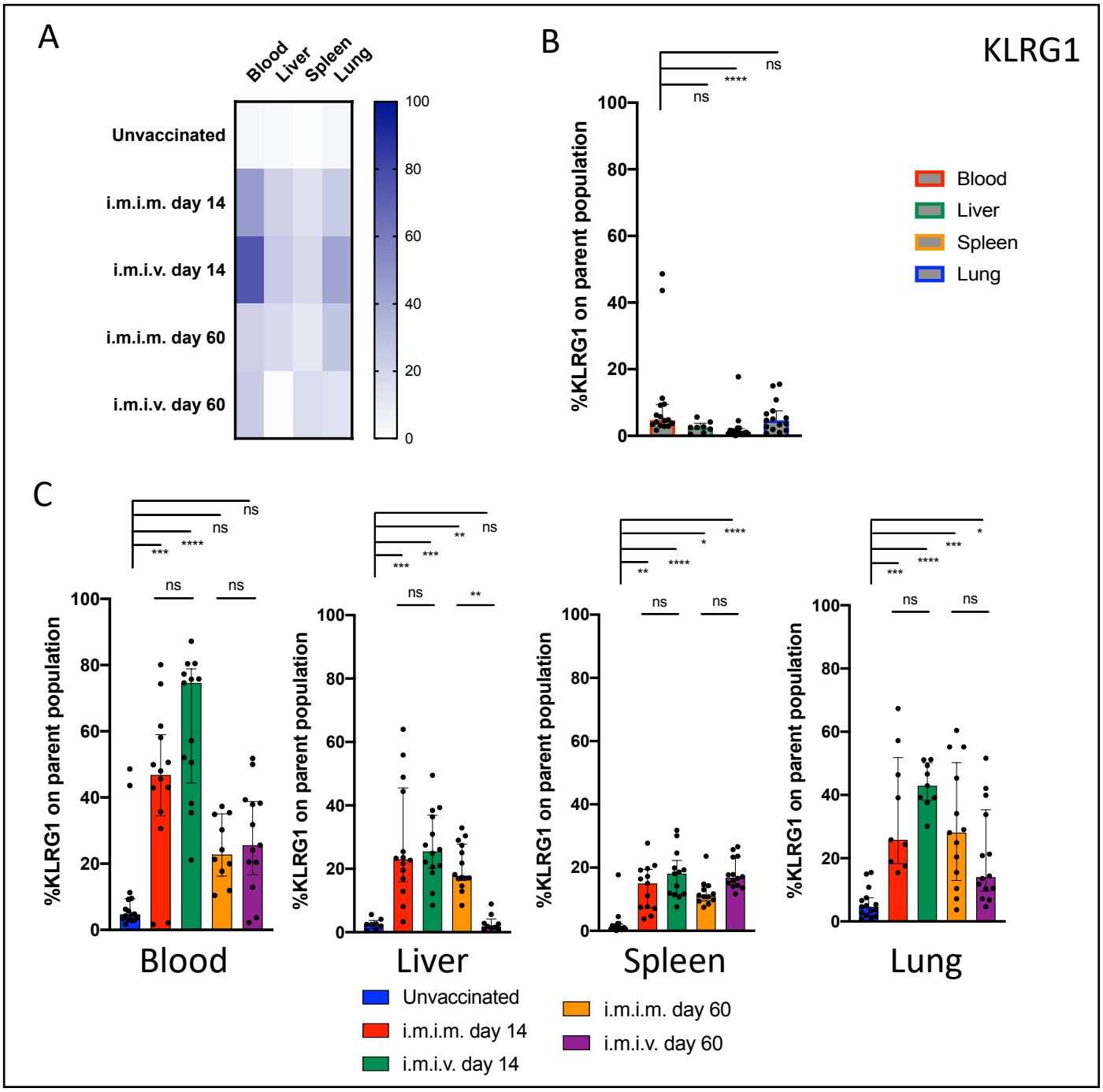


Figure 4.9 KLRG1 expression on bulk CD8 T cells in unvaccinated and vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on bulk CD8 T cells by flow cytometry.

A: Heatmap showing median percentage of bulk CD8 T cells expressing KLRG1 in the blood, liver, spleen, and lung in unvaccinated and vaccinated mice. **B:** Percentage of CD8 T cells expressing KLRG1 in the blood, liver, spleen, and lung in unvaccinated mice at day 14 of the experiment. **C:** Percentage of CD8 T cells that express KLRG1 in unvaccinated mice compared to mice vaccinated with i.m.i.m. or i.m.i.v. regimen at day 14 or day 60 after vaccination in the blood, liver, or spleen. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

CD127 expression (memory T cells) on CD8 cells was moderate in unvaccinated mice (range of medians 52.9% - 75.2%, Appendix 7, Table 3, Figure 4.10.A) and there were no significant differences in expression between organs (Figure 4.10.B). On liver derived CD8 cells, CD127 expression significantly decreased at day 14 but not at day 60 after vaccination. There were no significant differences in CD127 expression in the in the blood, spleen or lung (Appendix 7, Table 3, Figure 4.10.C). There were no significant differences in CD127 expression between the i.m.i.m. and i.m.i.v. regimens at either timepoint in any organ (Appendix 7, Table 3, Figure 4.10.C).

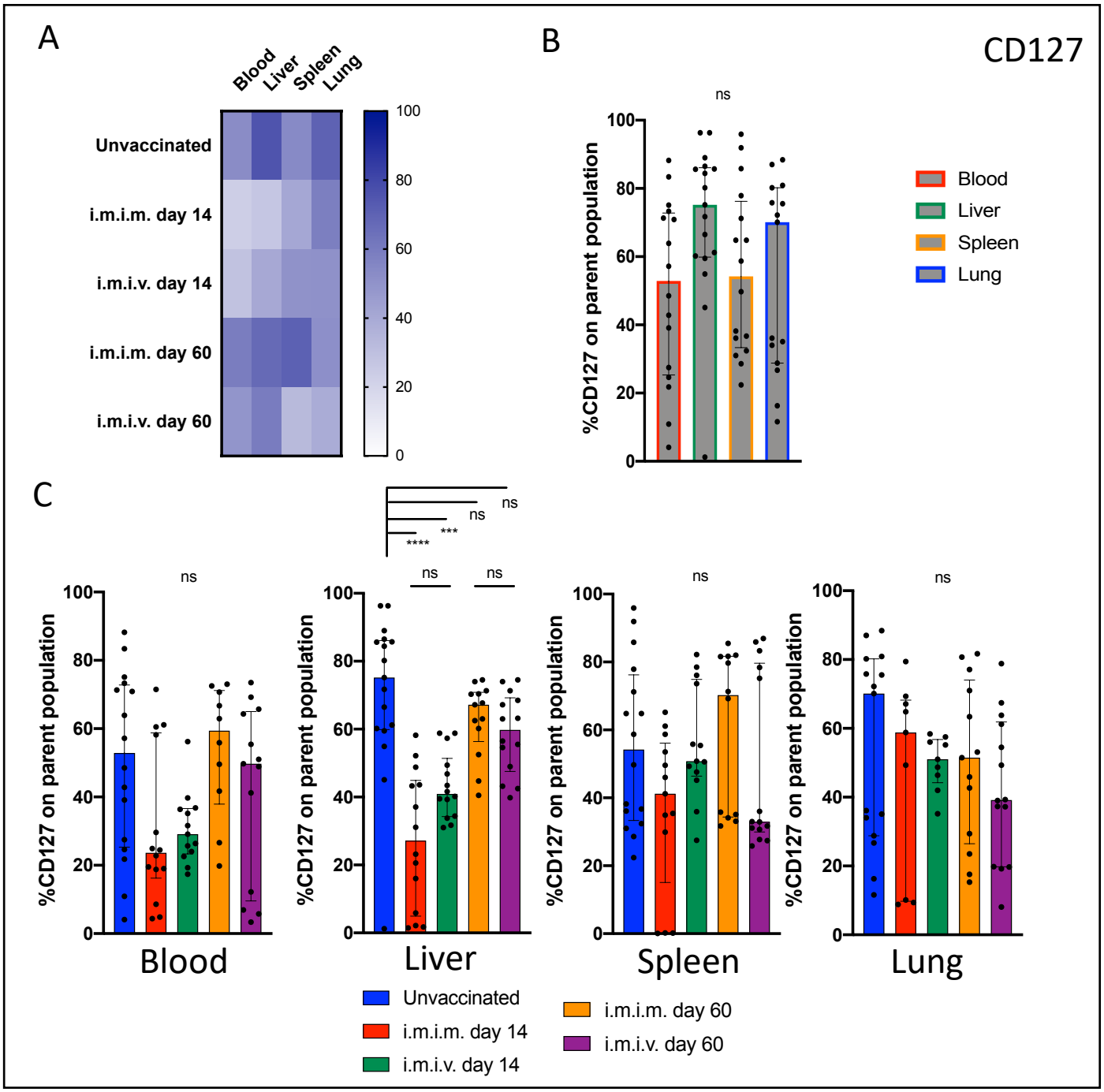


Figure 4.10 CD127 expression on bulk CD8 T cells in unvaccinated and vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on bulk CD8 T cells by flow cytometry.

A: Heatmap showing median percentage of bulk CD8 T cells expressing CD127 in the blood, liver, spleen, and lung in unvaccinated and vaccinated mice. **B:** Percentage of CD8 T cells expressing CD127 in the blood, liver, spleen, and lung in unvaccinated mice at day 14 of the experiment. **C:** Percentage of CD8 T cells that express CD127 in unvaccinated mice compared to mice vaccinated with i.m.i.m. or i.m.i.v. regimen at day 14 or day 60 after vaccination in the blood, liver, or spleen. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

The majority of CD127+KLRG1- memory T cells had a central memory phenotype in the liver, spleen and lung (T_{CM} CD8+CD127+KLRG1-CD62L+, Appendix 7, Table 4, Figure 4.11.A). A significantly higher proportion of CD8 T cells with a T_{CM} phenotype were present in the liver, spleen and lung compared to the blood (Figure 4.11.B). After vaccination by at least one regimen T_{CM} cells decreased as a proportion of memory T cells in all organs (Appendix 7, Table 4, Figure 4.11.C). There were no significant differences in T_{CM} expression between the i.m.i.m. and i.m.i.v. regimens at either timepoint in any organ (Appendix 7, Table 4, Figure 4.11.C).

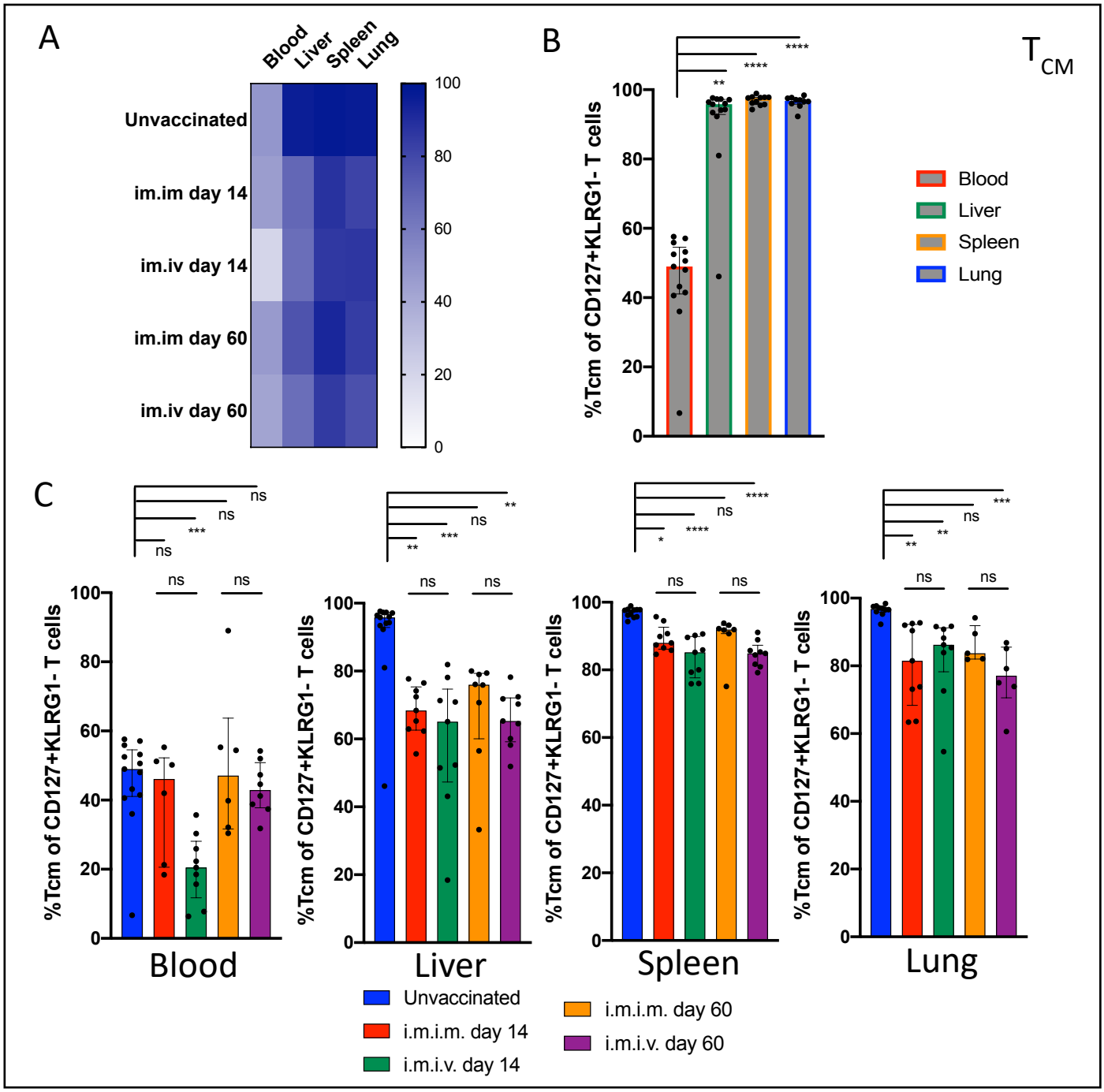


Figure 4.11 CD8 central memory T cells in unvaccinated and vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on bulk CD8 T cells by flow cytometry.
A: Heatmap showing median percentage of bulk CD8 T cells with a T central memory phenotype (T_{CM}, CD8+CD127+KLRG1-CD62L+) in the blood, liver, spleen, and lung in unvaccinated and vaccinated mice. **B:** Percentage of CD8 T cells with a T_{CM} phenotype in the blood, liver, spleen, and lung in unvaccinated mice at day 14 of the experiment. **C:** Percentage of CD8 T cells with a T_{CM} phenotype in unvaccinated mice compared to mice vaccinated with i.m.i.m. or i.m.i.v. regimen at day 14 or day 60 after vaccination in the blood, liver, or spleen. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

Effector memory T cells (T_{EM} CD8+CD127+KLRG1-CD62L-) were significantly higher in blood compared to liver, spleen and lung derived CD8 cells in unvaccinated mice (Appendix 7, Table 5, Figure 4.12.A and B). After vaccination by at least one regimen T_{EM} cells increased in the blood and liver, but vaccination did not affect expression in the spleen and lungs (Appendix 7, Table 5, Figure 4.12.C). There were no significant differences in T_{EM} expression between the i.m.i.m. and i.m.i.v. regimens at either timepoint in any organ (Appendix 7, Table 5, Figure 4.12.C).

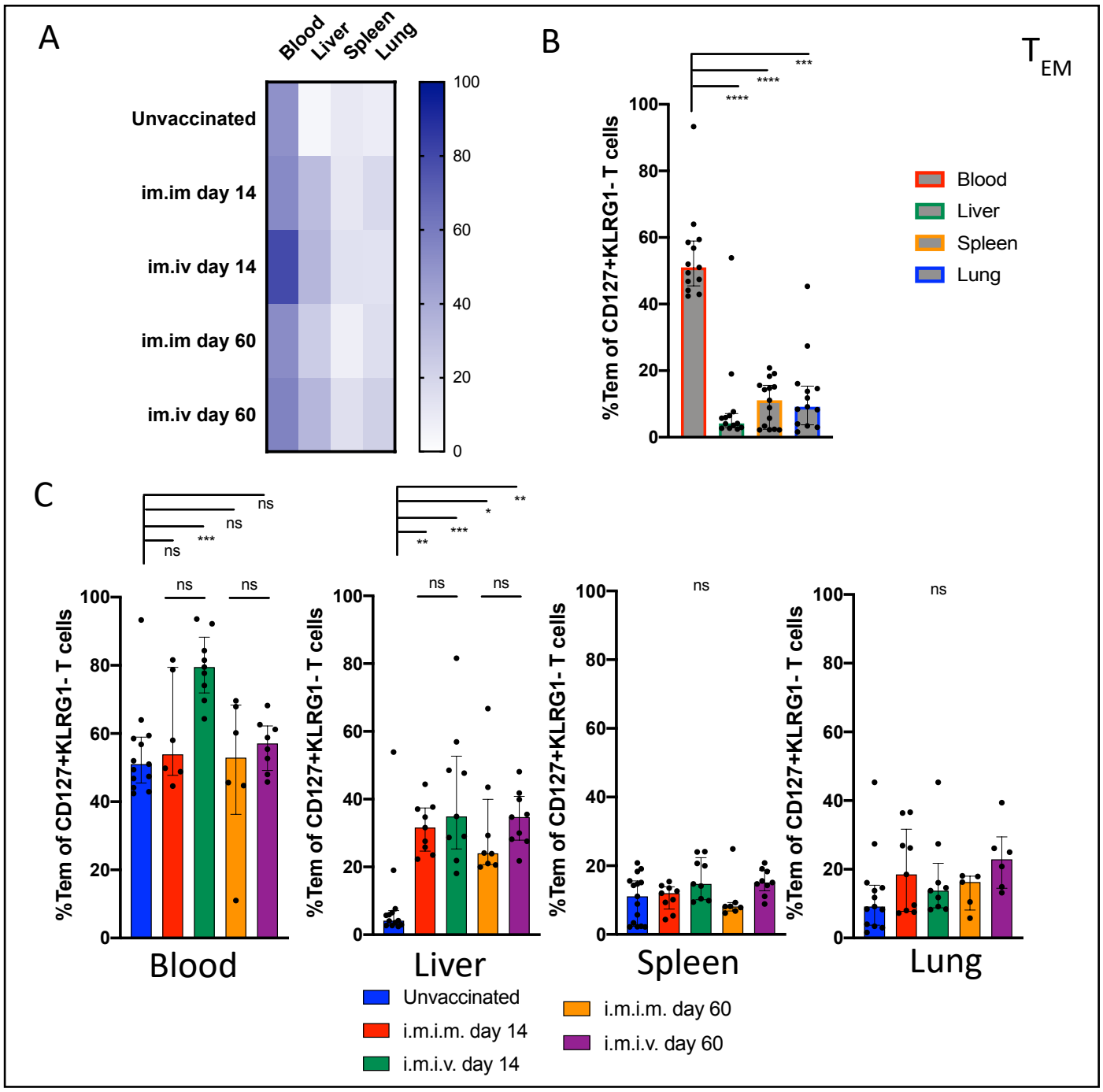


Figure 4.12 CD8 effector memory T cells in unvaccinated and vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on bulk CD8 T cells by flow cytometry.

A: Heatmap showing median percentage of bulk CD8 T cells with a T effector memory phenotype (T_{EM}, CD8+CD127+KLRG1-CD62L-) in the blood, liver, spleen, and lung in unvaccinated and vaccinated mice. **B:** Percentage of CD8 T cells with a T_{EM} phenotype in the blood, liver, spleen, and lung in unvaccinated mice at day 14 of the experiment. **C:** Percentage of CD8 T cells with a T_{EM} phenotype in unvaccinated mice compared to mice vaccinated with i.m.i.m. or i.m.i.v. regimen at day 14 or day 60 after vaccination in the blood, liver, or spleen. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

CX3CR1 expression on T_{EM} cells was low in unvaccinated mice and there were no significant differences between organs (range of medians 4.24% - 12.9%, Appendix 7, Table 6, Figure 4.13.A and B). After vaccination by at least one regimen CX3CR1 expression on T_{EM} cells increased in all organs but there were no differences between the i.m.i.m. and i.m.i.v. regimens (Appendix 7, Table 6, figure 4.13.B).

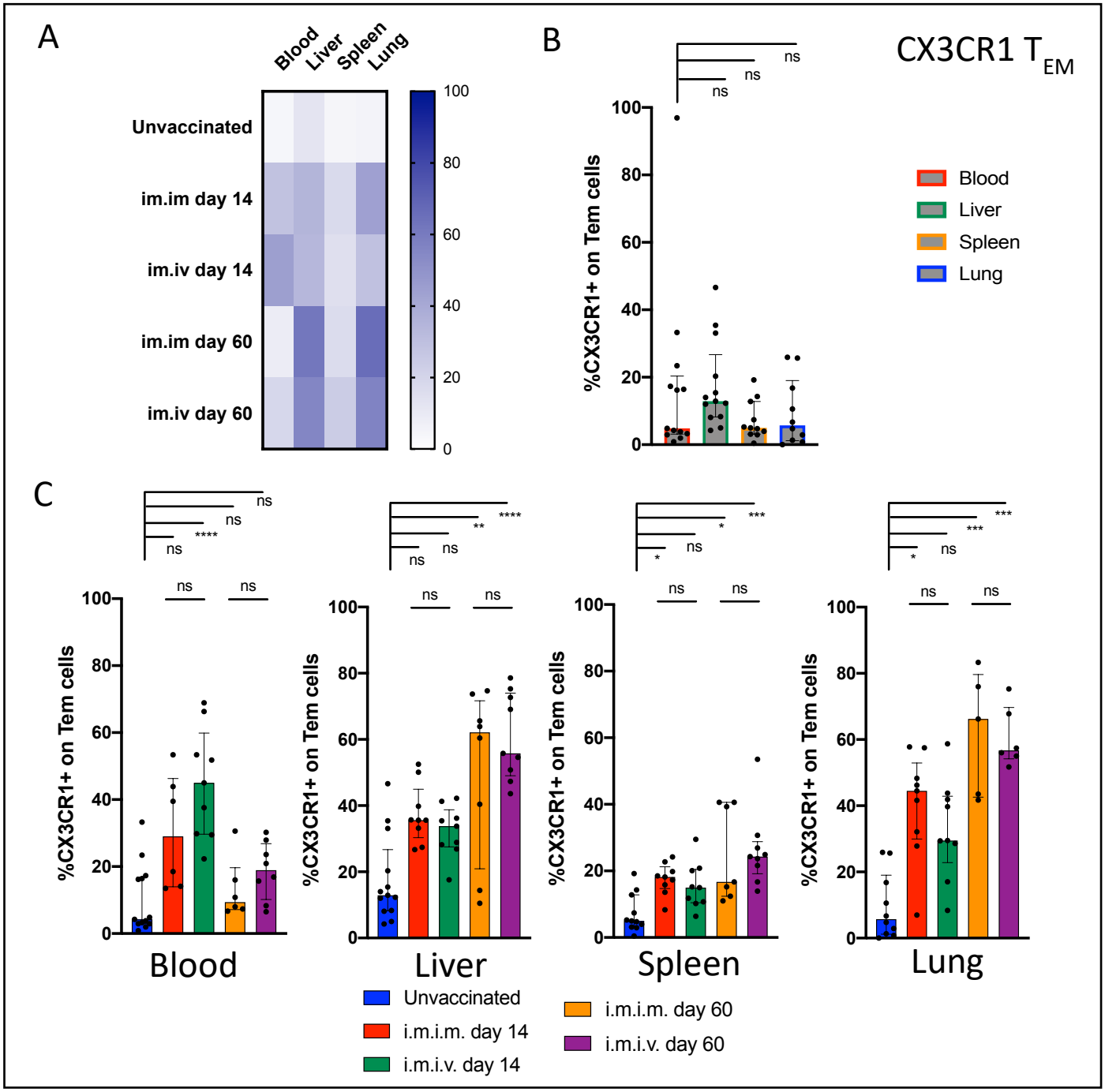


Figure 4.13 CX3CR1 expression on CD8 effector memory T cells in unvaccinated and vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on bulk CD8 T cells by flow cytometry.

A: Heatmap showing median percentage expression of CX3CR1 on CD8 T effector memory cells (CX3CR1+ T_{EM}, CD8+CD127+KLRG1-CD62L-CX3CR1+) in the blood, liver, spleen, and lung in unvaccinated and vaccinated mice. **B:** CX3CR1 expression on CD8 T_{EM} cells in the blood, liver, spleen, and lung in unvaccinated mice at day 14 of the experiment. **C:** CX3CR1 expression on CD8 T_{EM} cells in unvaccinated mice compared to mice vaccinated with i.m.i.m. or i.m.i.v. regimen at day 14 or day 60 after vaccination in the blood, liver, or spleen. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

4.5.4 The memory phenotype of pentamer positive HBV specific CD8 T cells does not differ after intravenous as compared with intramuscular MVA-HBV boost vaccination in C57BL/6J mice

In order to investigate the phenotype of vaccine induced pentamer positive HBV specific T cells, the surface expression of markers associated with T cell differentiation (CD44, CD62L, KLRG1, CD127 and CX3CR1 (483,496)) were compared between vaccination regimen and between different organs (experimental set up as per Figure 4.5.A, representative gating strategy shown in Figure 4.14). Pentamer positive HBV specific T cells showed consistent expression of markers across organs, vaccine regimes and time points.

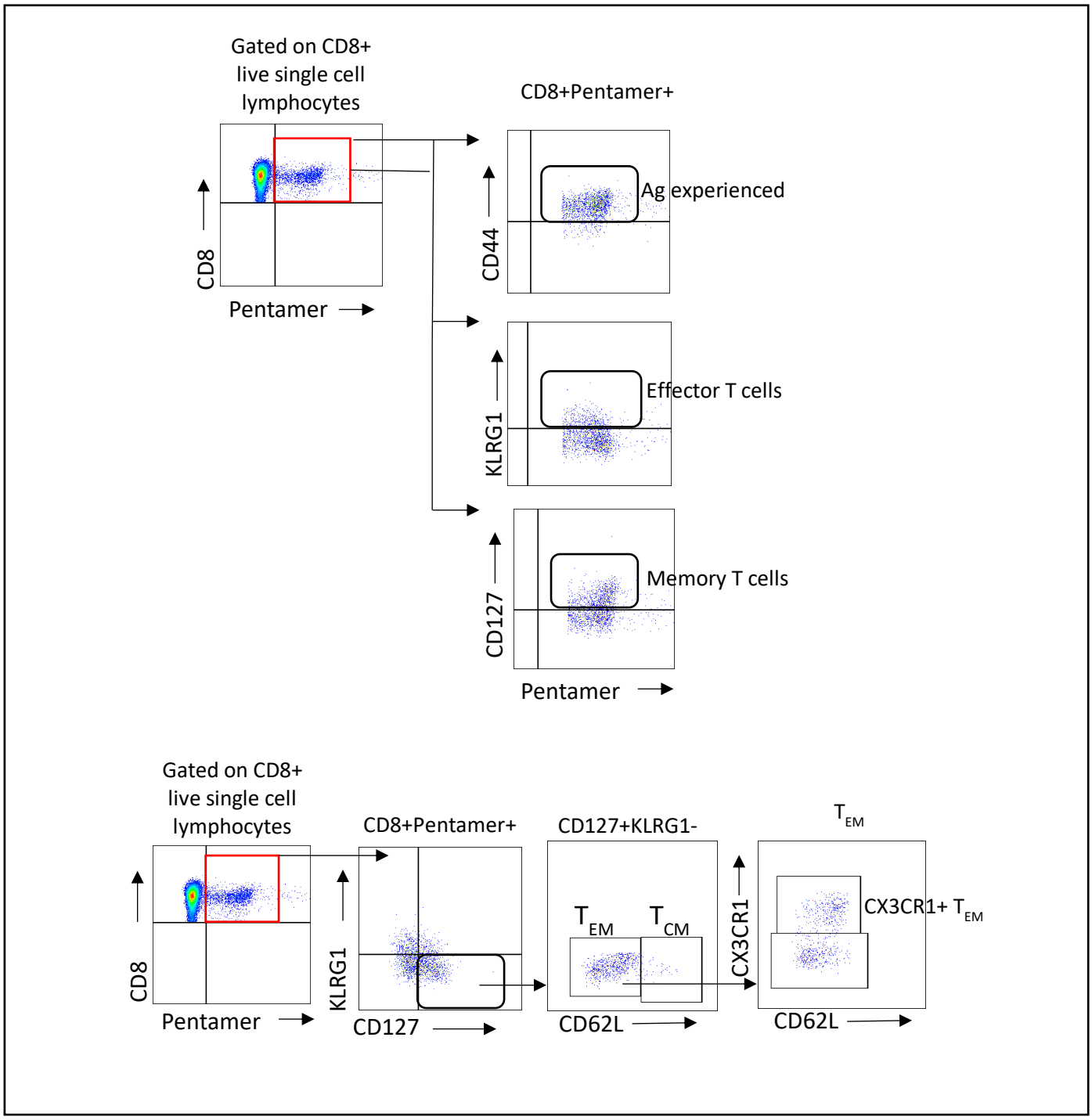


Figure 4.14 Gating strategy to determine the phenotype of pentamer positive HBV specific CD8 T cells in C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lungs were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on pentamer positive HBV specific CD8 T cells by flow cytometry. Gating strategy to identify antigen experienced (Ag experienced), effector T cells, memory T cells, T central memory (T_{CM}) and T effector memory (T_{EM}) T cells shown.

CD44 expression was high on pentamer positive cells (range of medians 83.0% - 98.9%, Appendix 7, Table 7, Figure 4.15.A) and did not significantly vary between organs (i.m.i.m. regimen, day 14 after vaccination) (Figure 4.15.B). CD44 expression was significantly lower on blood derived and higher on spleen derived pentamer positive cells at day 60 compared with day 14 after the i.m.i.v. vaccination regimen (Appendix 7, Table 7, Figure 4.15.C). In the liver, CD44 expression was significantly lower on pentamer positive cells at day 60 compared with day 14 after the i.m.i.v. vaccination regimen (Figure 4.4.C). There were no differences in CD44 expression on pentamer positive cells between the i.m.i.m. and i.m.i.v. regimens in any organ at any timepoint (Appendix 7, Table 7, Figure 4.15.C).

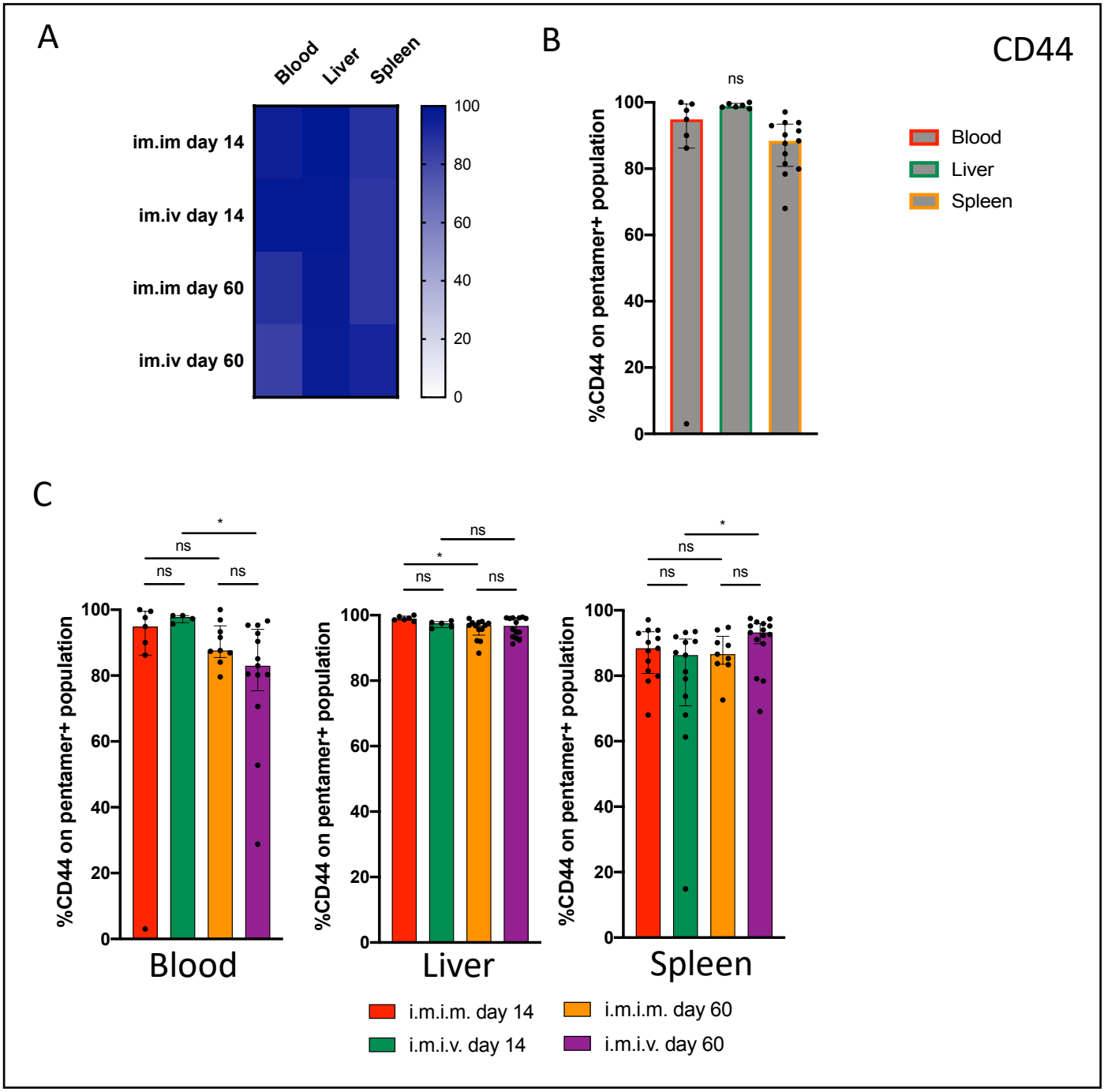


Figure 4.15 CD44 expression on pentamer positive HBV T cells in vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver and spleen were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on pentamer positive HBV specific CD8 T cells by flow cytometry.

A: Heatmap showing median percentage of pentamer positive CD8 T cells expressing CD44 in the blood, liver, and spleen in vaccinated mice. **B:** Percentage of pentamer positive CD8 T cells expressing CD44 in the blood, liver, and spleen 14 days after vaccination with the i.m.i.m. regimen. **C:** Percentage of pentamer positive CD8 T cells that express CD44 at day 14 or day 60 after vaccination with i.m.i.m. or i.m.i.v. regimens in the blood, liver, or spleen. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

KLRG1 expression was moderate on pentamer positive cells in all organs (range of medians 33.4% - 78.7%, Appendix 7, Table 8, Figure 4.16.A). There were no differences in expression on cells derived from different organs at day 14 after the i.m.i.m. regimen (Figure 4.16.B). KLRG1 expression was significantly lower on pentamer positive cells on day 60 compared to day 14 after vaccination with the i.m.i.v. regimen in the lung but there were no differences in expression between the i.m.i.m. and i.m.i.v. regimens (Appendix 7, Table 8, Figure 4.16.C).

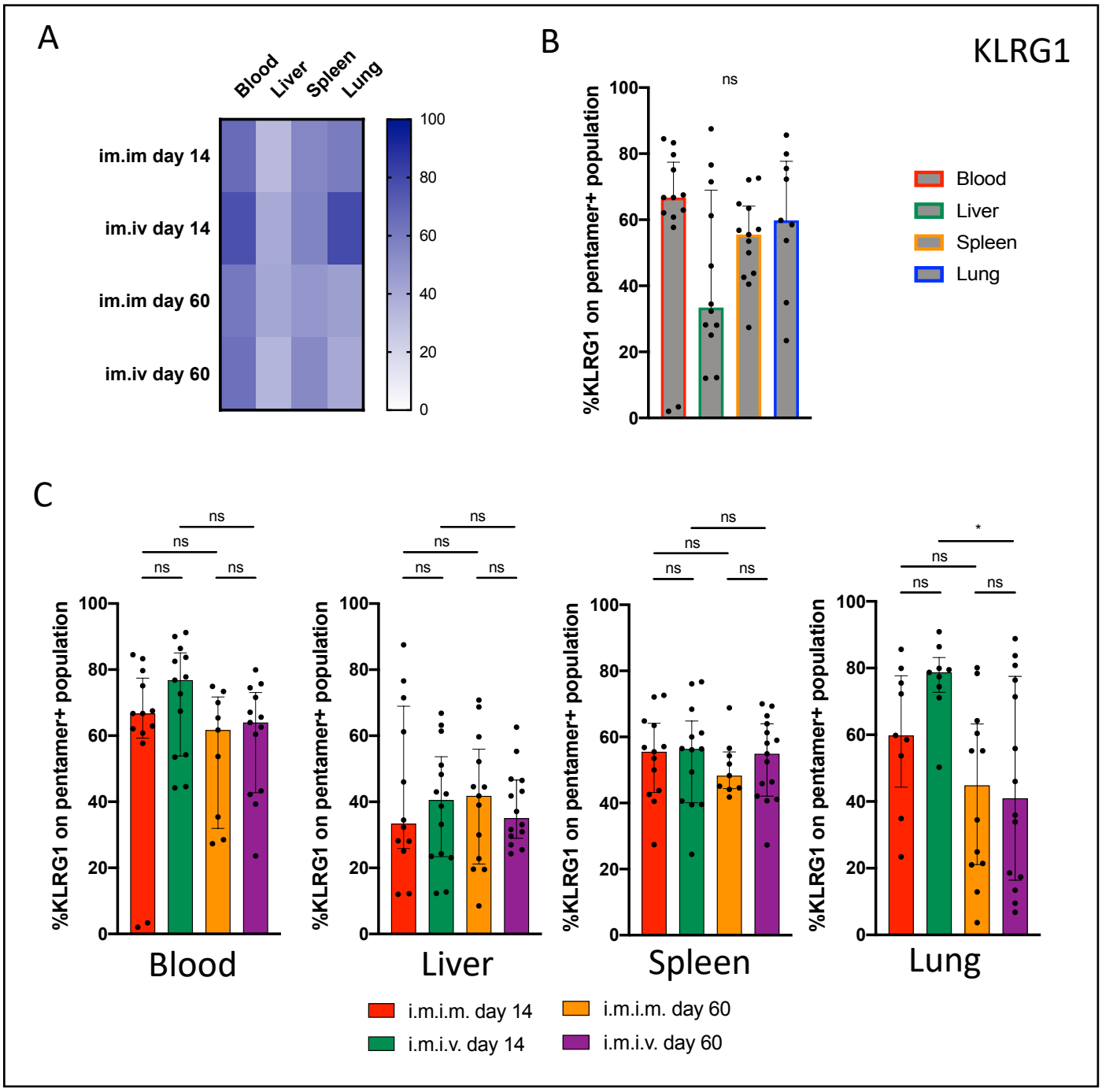


Figure 4.16 KLRG1 expression on pentamer positive HBV T cells in vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on pentamer positive HBV specific CD8 T cells by flow cytometry.

A: Heatmap showing median percentage of pentamer positive CD8 T cells expressing KLRG1 in the blood, liver, spleen, and lung in vaccinated mice. **B:** Percentage of pentamer positive CD8 T cells expressing KLRG1 in the blood, liver, spleen, and lung 14 days after vaccination with the i.m.i.m. regimen. **C:** Percentage of pentamer positive CD8 T cells that express KLRG1 at day 14 or day 60 after vaccination with i.m.i.m. or i.m.i.v. regimens in the blood, liver, spleen, and lung. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

CD127 expression was also moderate on pentamer positive cells in all organs (range of medians 21.9% - 70.7%, Appendix 7, Table 9, Figure 4.17.A). There were no differences in expression on cells derived from the blood, liver, spleen or lung at day 14 after the i.m.i.m. regimen Figure 4.17.B). CD127 expression was significantly higher on pentamer positive cells at day 60 compared to day 14 after the i.m.i.m. regimen in blood, the i.m.i.m. and i.m.i.v. regimens in the liver and the i.m.i.m. and i.m.i.v. regimens in the spleen. There were no differences in expression between the i.m.i.m. and i.m.i.v. regimens compared at the same timepoint (Figure 4.17.C).

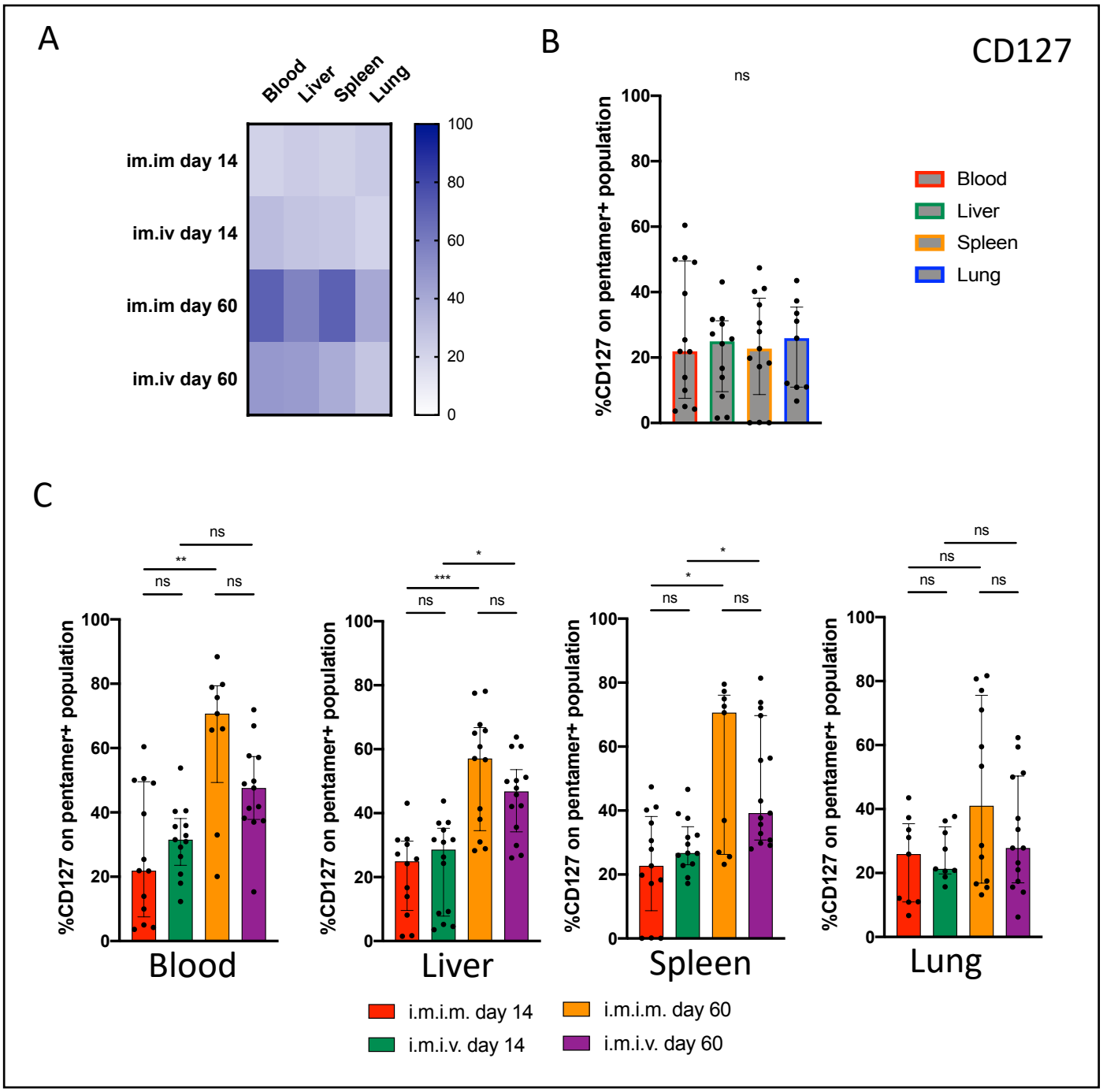


Figure 4.17 CD127 expression on pentamer positive HBV T cells in vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on pentamer positive HBV specific CD8 T cells by flow cytometry.

A: Heatmap showing median percentage of pentamer positive CD8 T cells expressing CD127 in the blood, liver, spleen and lung in vaccinated mice. **B:** Percentage of pentamer positive CD8 T cells expressing CD127 in the blood, liver, spleen, and lung 14 days after vaccination with the i.m.i.m. regimen. **C:** Percentage of pentamer positive CD8 T cells that express CD127 at day 14 or day 60 after vaccination with i.m.i.m. or i.m.i.v. regimens in the blood, liver, spleen, and lung. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

A low proportion of CD127+KLRG1- memory pentamer positive T cells were of a T_{CM} phenotype (range of medians 5.91% - 22.6%, Appendix 7, Table 10, Figure 4.18.A). There were no significant differences in expression on pentamer positive cells between organs at day 14 after the i.m.i.m. regimen (Figure 4.18.B). T_{CM} cells increased at day 60 compared to day 14 in the blood after the i.m.i.v. regimen (Figure 4.18.C). There were no differences in expression between the i.m.i.m. and i.m.i.v. regimens compared at the same timepoint (Figure 4.18.C).

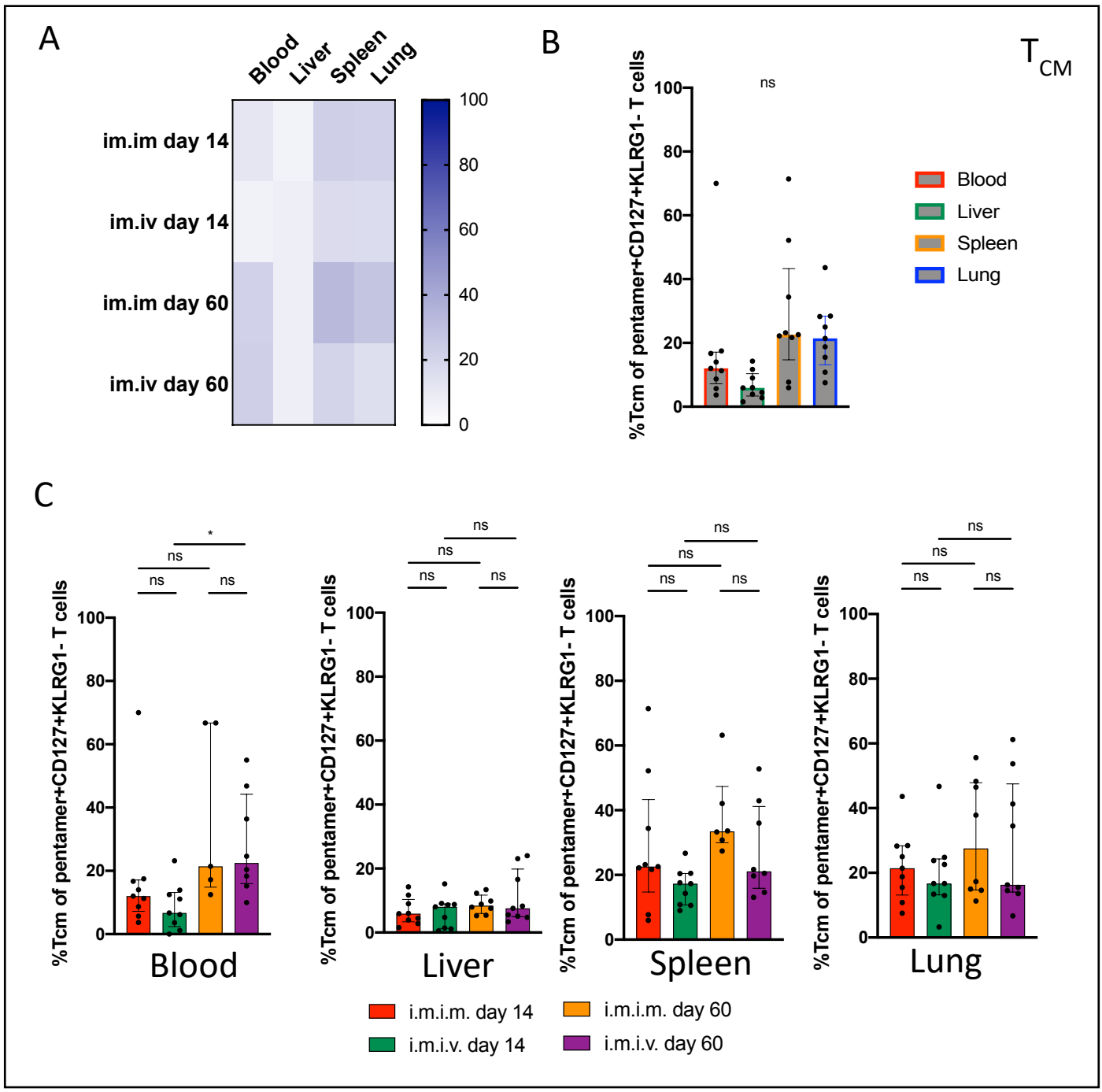


Figure 4.18 Pentamer positive central memory T cells in vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Unvaccinated mice were used as negative controls. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on pentamer positive CD8 T cells by flow cytometry. **A:** Heatmap showing median percentage of pentamer positive T cells with a T central memory phenotype (T_{CM}, CD8+CD127+KLRG1-CD62L+) in the blood, liver, spleen, and lung in vaccinated mice. **B:** Percentage of pentamer positive T cells with a T_{CM} phenotype in the blood, liver, spleen, and lung 14 days after vaccination with the i.m.i.m. regimen. **C:** Percentage of pentamer positive T cells with a T_{CM} phenotype at day 14 or day 60 after vaccination with i.m.i.m. or i.m.i.v. regimens in the blood, liver, spleen, and lung. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

Most CD127+KLRG1- memory pentamer positive T cells had a T_{EM} phenotype (range of medians 75.0% - 94.1%, Appendix 7, Table 11, Figure 4.19.A). There were no significant differences in expression on pentamer positive cells between organs (Figure 4.19.B). T_{EM} cells decreased at day 60 compared to day 14 in the blood after the i.m.i.v. regimen (Figure 4.19.C). There were no differences in expression between the i.m.i.m. and i.m.i.v. regimens compared at the same timepoint (Figure 4.19.C).

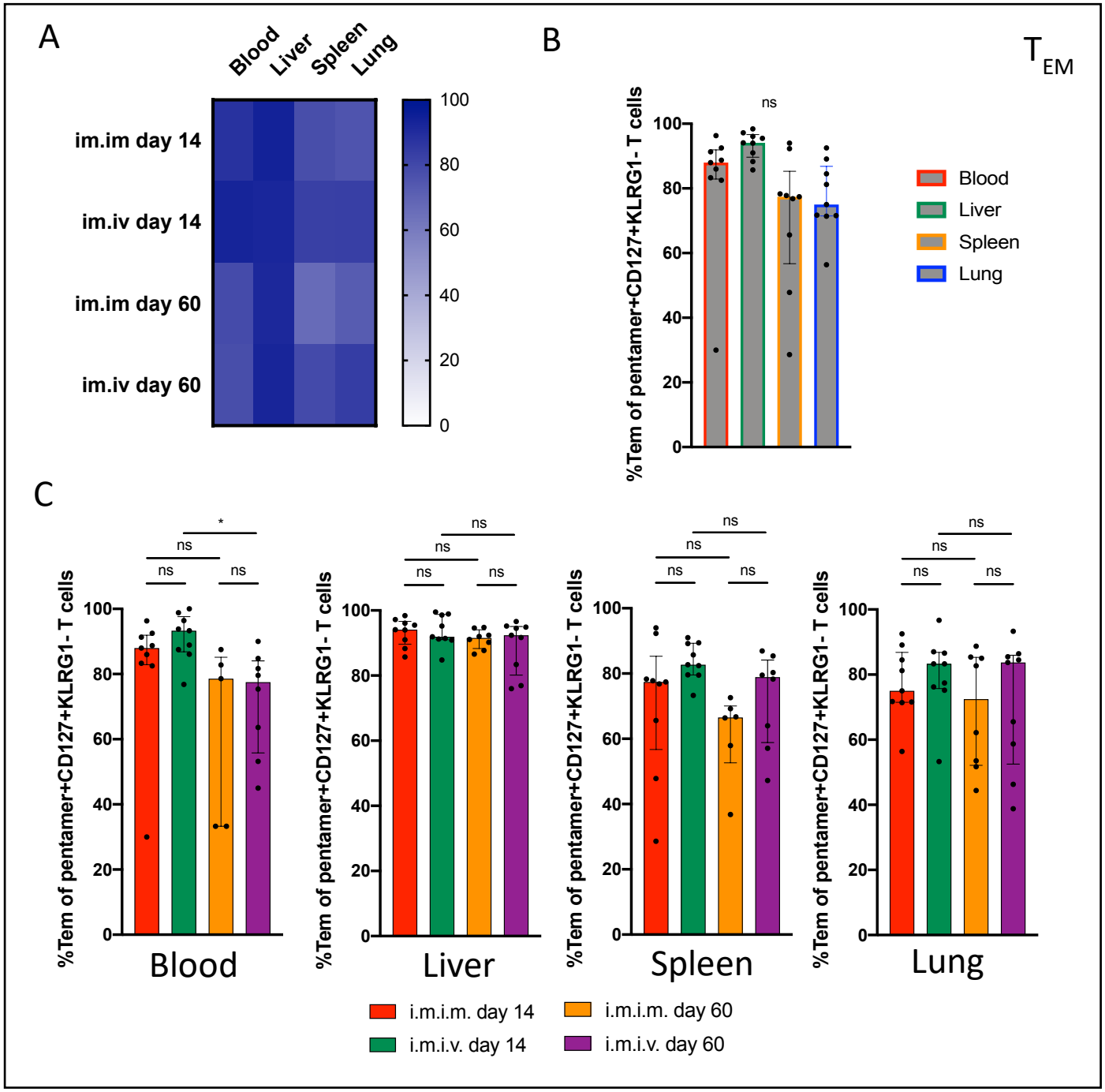


Figure 4.19 Pentamer positive effector memory T cells in vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on pentamer positive CD8 T cells by flow cytometry.

A: Heatmap showing median percentage of pentamer positive T cells with a T effector memory phenotype (T_{EM}, CD8+CD127+KLRG1-CD62L-) in the blood, liver, spleen, and lung in vaccinated mice. **B:** Percentage of pentamer positive T cells with a T_{EM} phenotype in the blood, liver, spleen, and lung 14 days after vaccination with the i.m.i.m. regimen. **C:** Percentage of pentamer positive T cells with a T_{EM} phenotype at day 14 or day 60 after vaccination with i.m.i.m. or i.m.i.v. regimens in the blood, liver, spleen, and lung. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

CX3CR1 expression on T_{EM} cells was moderate (range of medians 23.3% – 57.9%, Appendix 7, Table 12, Figure 4.20.A). CX3CR1 expression was significantly lower on spleen as compared to blood derived pentamer positive T_{EM} cells (23.3% vs. 55.7%, p=0.0323, Figure 4.4.20.B). There were no differences in expression between the i.m.i.m. and i.m.i.v. regimens compared at the same timepoint. However, in the liver CX3CR1 expression was higher on pentamer positive T_{EM} cells at day 60 compared to day 14 after the i.m.i.v. regimen (Figure 4.4.20.C).

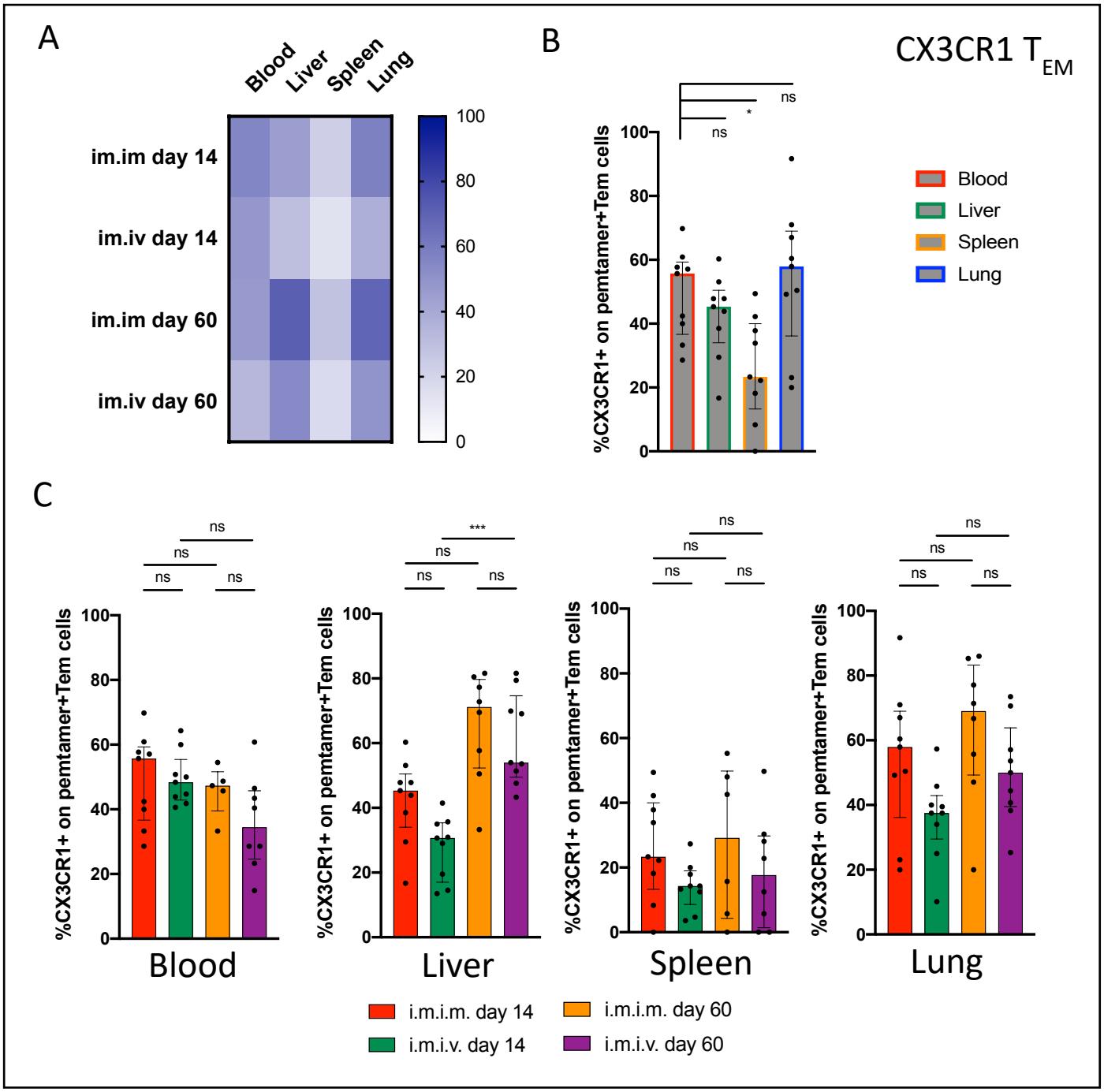


Figure 4.20 CX3CR1 expression on pentamer positive effector memory T cells in vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of differentiation markers on pentamer positive CD8 T cells by flow cytometry.

A: Heatmap showing median percentage expression of CX3CR1 on pentamer positive T cells with a T effector memory phenotype (T_{EM}, CD8+CD127+KLRG1-CD62L-) in the blood, liver, spleen, and lung in vaccinated mice. **B:** Percentage expression of CX3CR1 on pentamer positive T cells with a T_{EM} phenotype in the blood, liver, spleen, and lung 14 days after vaccination with the i.m.i.m. regimen. **C:** Percentage expression of CX3CR1 on pentamer positive T cells with a T_{EM} phenotype at day 14 or day 60 after vaccination with i.m.i.m. or i.m.i.v. regimens in the blood, liver, spleen, and lung. Results are a summation of 4 experiments with 3-5 mice per group. Comparisons between more than two groups performed using Kruskal-Wallis test with Dunn's multiple comparisons. Median and interquartile range shown.
**** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

4.5.5 Liver derived CD8 T cells express higher tissue residency marker CD69 than blood derived T cells in unvaccinated C57BL/6J mice

Liver resident CD8 T cells are a specialised population of cells that patrol the liver sinusoids without recirculating, providing a local immune response important for the control of hepatotropic infections including HBV and malaria (489–491). In order to investigate whether liver tissue resident cells were enriched in the liver compared with the blood, expression of surface markers associated with liver tissue residency and homing ($CD69^{hi}$, $CD11a^{hi}$, $CXCR6^{hi}$, $KLRG1^{lo}$, $CX3CR1^{lo}$ (489,490)) were investigated on lymphocytes derived from the blood and liver of unvaccinated mice (representative gating strategy shown in Figure 4.21.A).

CD69 expression was significantly increased on liver compared to blood derived bulk CD8 T cells (median 0.90% vs 12.25%, p=0.0039 figure 4.21.B). CD69 positive cells derived from the blood had significantly higher expression of surface markers CD11a (median 75.0% vs. 10.9%, p<0.0001) and CXCR6 (median 43.1% vs. 0.52%, p<0.0001, figure 4.21.C) compared to CD69 negative cells. In the liver CD69 positive cells also had higher expression of CD11a (median 83.0% vs. 13.6%, p<0.0001) and CXCR6 (median 54.5% vs. 3.43%, p<0.0001, figure 4.21.D) than CD69 negative cells. In both the blood and liver, KLRG1 and CX3CR1 expression was low on CD69 positive and negative CD8 T cells. In liver derived CD69 positive cells, CX3CR1 expression was higher (13.1% vs 22.4%, p=0.0212 figure 4.21.D) than on CD69 negative cells.

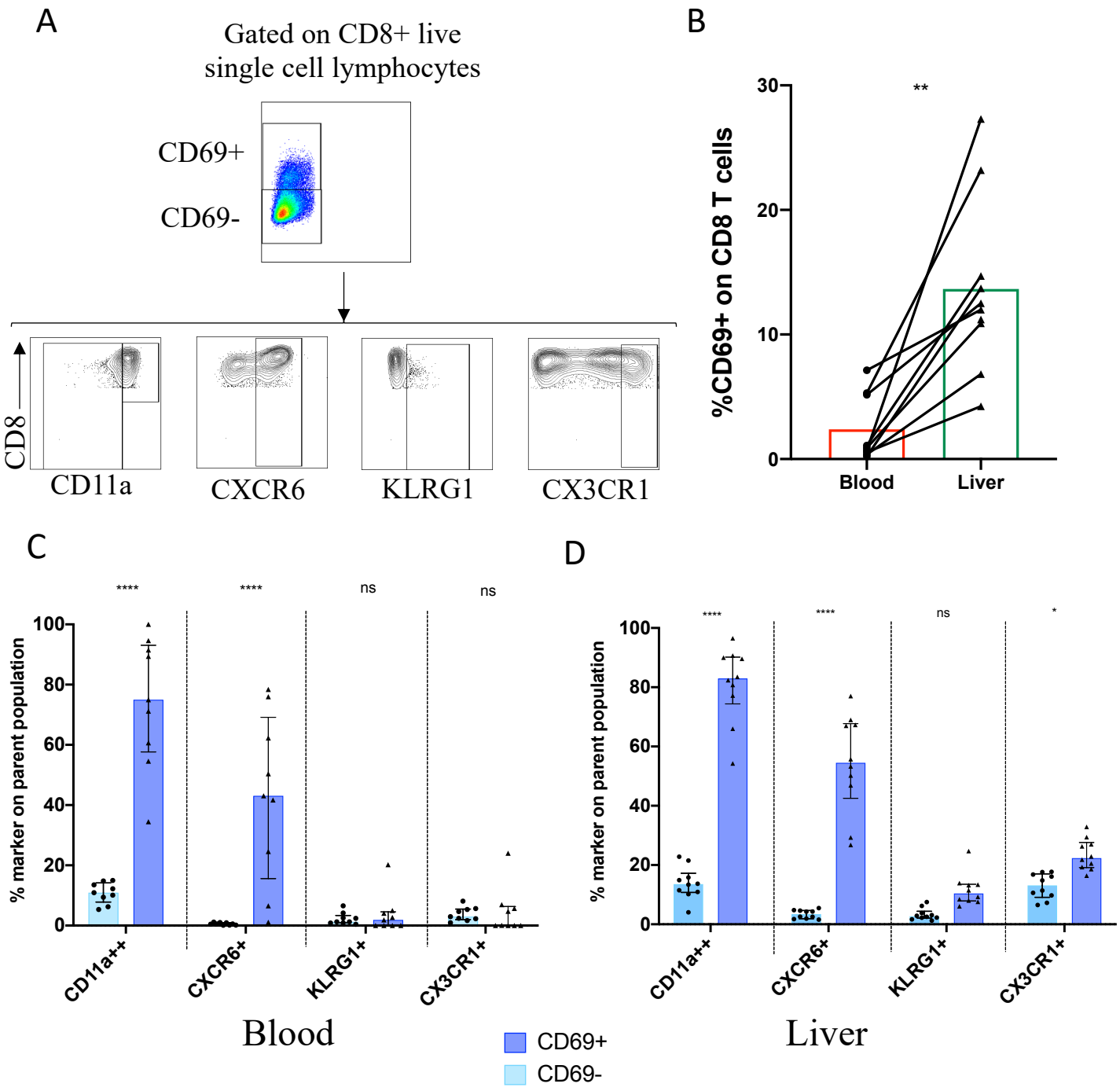


Figure 4.21 Expression of CD69 on blood and liver derived CD8 T cells in unvaccinated C57BL/6J mice

C57BL/6J mice were sacrificed and lymphocytes derived from blood and liver were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of CD69, CD11a, CXCR6, KLRG1 and CX3CR1 on bulk CD8 T cells.
A: Representative gating strategy of CD69 and CD11a, CXCR6, KLRG1 and CX3CR1 on CD8 T cells.
B: Percentage of bulk CD8 T cells that express CD69 in the blood as compared to the liver
C: Percentage of CD11a, CXCR6, KLRG1 and CX3CR1 on CD69 positive (CD69+) compared to CD69 negative (CD69-) bulk CD8 T cells from the blood (left panels) or the liver (right panels).
Results are a summation of single experiments with 10 mice. Median and interquartile range shown.
Percentage of CD8 T cells expressing surface marker compared between two groups using Wicoxon test or between more than two groups using ANOVA test with Sidak's multiple comparisons.
**** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

4.5.6 Liver derived bulk and pentamer positive HBV specific CD8 T cells express higher tissue residency marker CD69 than blood derived T cells after vaccination with ChAdOx1-HBV and MVA-HBV in C57BL/6J mice

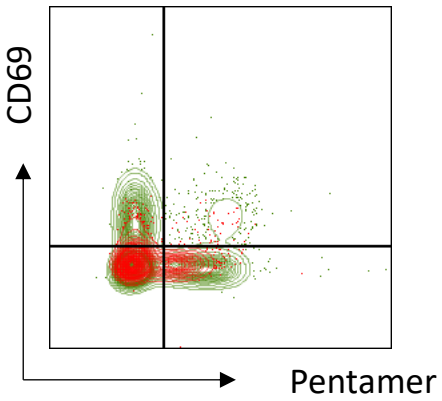
Liver resident CD8 T cells expressing CD69 are associated with controlling HBV infection and remain in the liver after HBV resolution (491). Surface expression of markers associated with tissue residency and liver homing ($CD69^{hi}$, $CD11a^{hi}$, $CXCR6^{hi}$, $KLRG1^{lo}$, $CX3CR1^{lo}$ (489,490)) were investigated on liver and blood derived HBV specific VWLSVIWM-T cells induced by vaccination with i.m.i.m. or i.m.i.v. regimes of ChAdOx1-HBV and MVA-HBV and unvaccinated mice as controls (experimental set up as per Figure 4.5.A, representative gating for CD69 on blood and liver CD8 and pentamer (VWLSVIWM) T cells shown in Figure 4.22.A).

In all paired liver compared to blood derived bulk CD8 T cells, the percentage of CD69 positive cells was increased (median blood CD69 2.72% vs. liver CD69 5.94%, p=0.0012, Figure 4.22.B). CD69 was significantly increased on liver compared to blood CD8 T cells at day 14 after i.m.i.v. vaccination (median blood 2.79% vs. liver 23.10%, p<0.0001) but not by other routes or time points after vaccination (Figure 4.22.C).

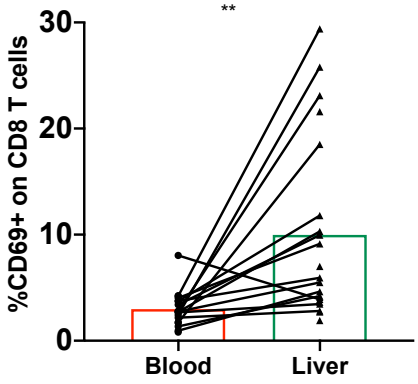
In liver compared to blood derived pentamer positive T cells, the percentage of CD69 positive cells was also increased (median blood 5.58% vs. liver 9.56%, p=0.0098, Figure 4.22.D). There were no significant differences between vaccination regime and timepoint of response (Figure 4.22.E).

A

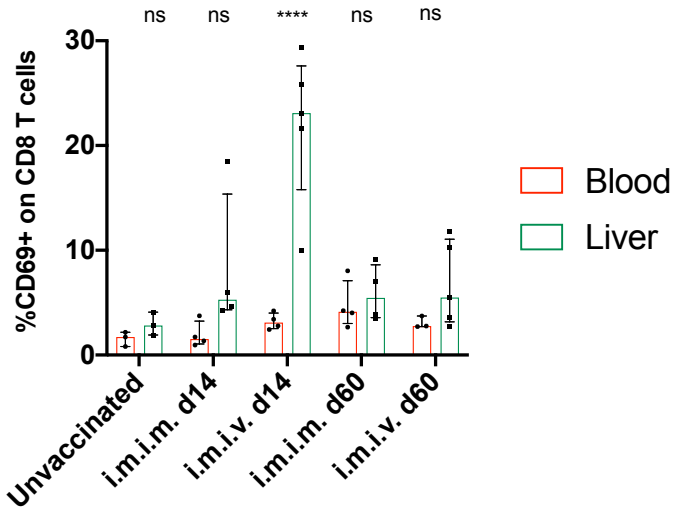
Gated on CD8+ live
single cell lymphocytes



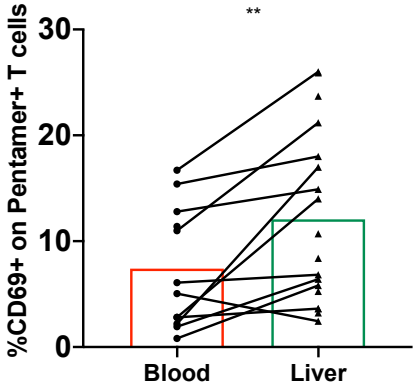
B



C



D



E

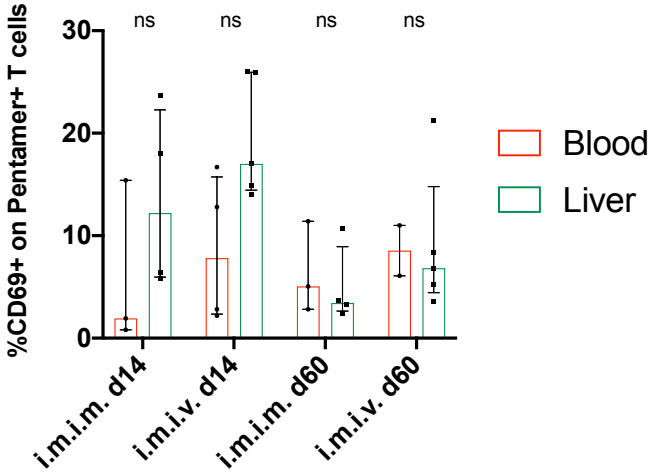


Figure 4.22 CD69 expression on blood and liver derived bulk and pentamer positive CD8 T cells in vaccinated C57BL/6J mice

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v.. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of CD69, CD11a, CXCR6, KLRG1 and CX3CR1 on bulk CD8 T cells and pentamer positive VWLSVIWM-specific T cells, by flow cytometry.
A: Representative gating of CD69 and HBV pentamer (VWLSVIWM) on CD8 T cells induced by vaccination in the blood (red) and liver (green).
B: Percentage of bulk CD8 T cells that express CD69 in the blood as compared to the liver in all samples or **C:** by vaccination regiment
D: Percentage of VWLSVIWM-specific pentamer positive T cells that express CD69 in the blood as compared to the liver in all samples and E: by vaccination regimen
Results are a summation of a single experiment with 3-5 mice per group. Median and interquartile range shown. Percentage of CD8 T cells expressing surface marker compared between two groups using Wilcoxon test or between more than two groups using ANOVA test with Sidak's multiple comparisons. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.

Next, the expression of CD11a, CXCR6, KLRG1 and CX3CR1 were compared between CD69 positive and negative HBV specific pentamer positive CD8 T cells after vaccination in the blood and the liver to assess whether they co-expressed other markers associated with liver residency (representative gating shown in Figure 4.23).

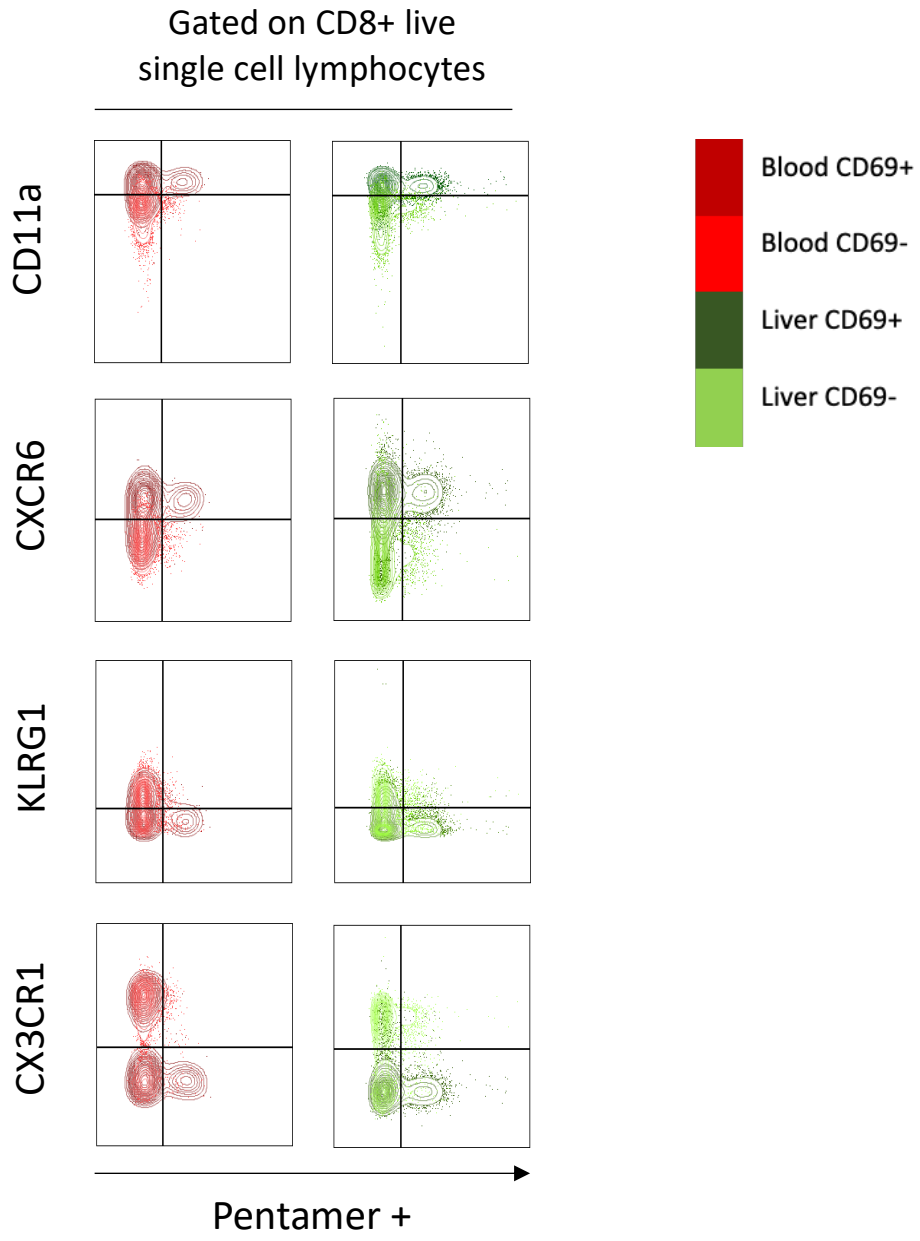


Figure 4.23 Surface expression of liver tissue residence markers on blood and liver derived bulk and pentamer positive CD8 T cells

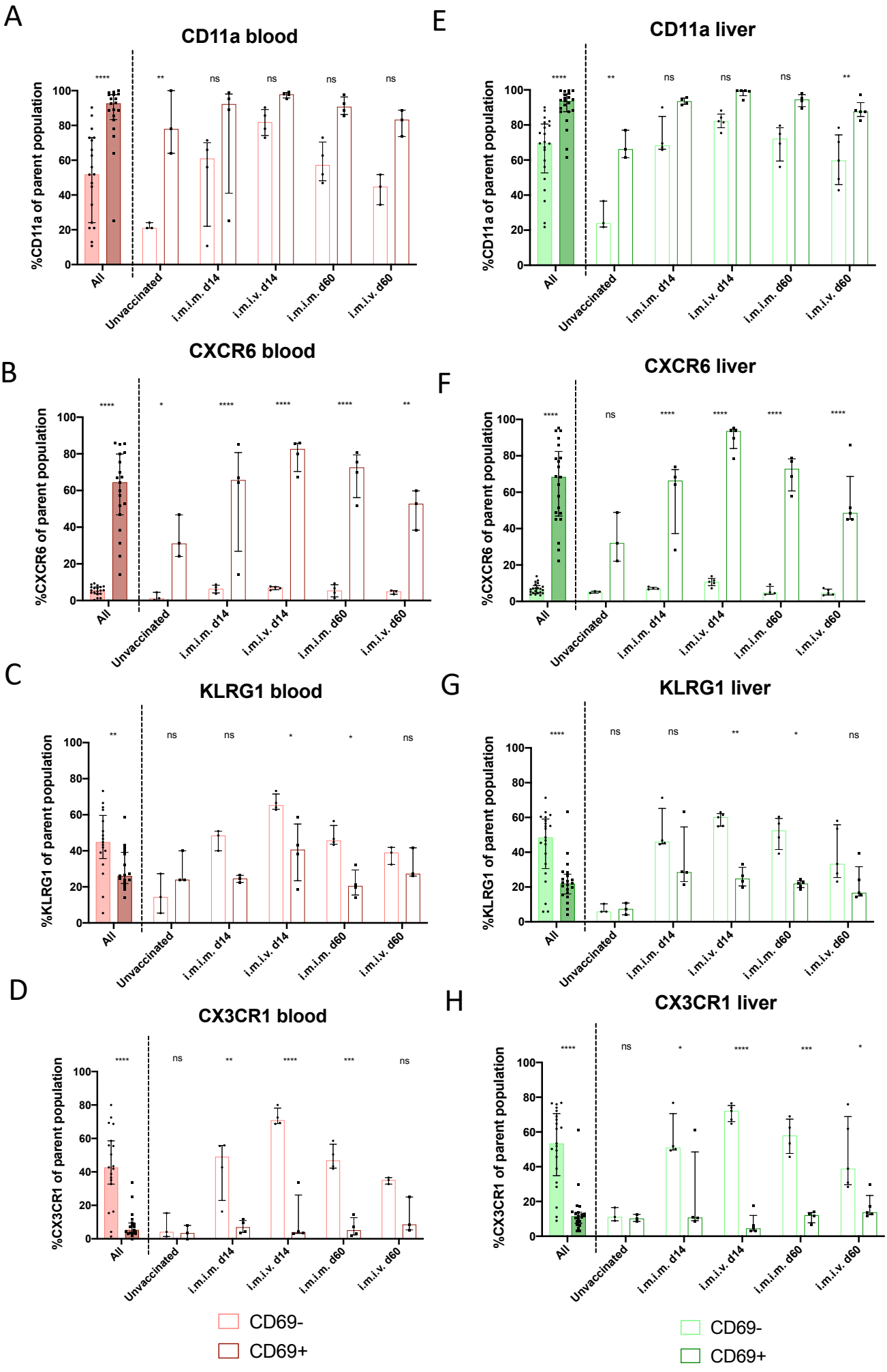
C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v.. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of CD69, CD11a, CXCR6, KLRG1 and CX3CR1 on bulk CD8 T cells and pentamer positive VWLSVIWM-specific T cells, by flow cytometry. Representative gating of CD11a, CXCR6, KLRG1 and CX3CR1 on CD69 positive (CD69+) or CD69 negative (CD69-) VWLSVIWM-specific pentamer positive T cells in the blood (red) and liver (green).

On blood derived bulk CD8 T cells after vaccination (Figure 4.24, red bars), CD69 positive cells expressed significantly higher CD11a (median 92.7% vs 51.9%, $p<0.0001$, Figure 4.24.A) and CXCR6 (median 64.5% vs 5.57%, $p<0.0001$, Figure 4.24.B) and lower KLRG1 (median 26.0% vs 44.8%, $p=0.0066$, Figure 4.24.C) and CX3CR1 (median 5.33% vs 42.7%, $p<0.0001$, Figure 4.24.D) compared with CD69 negative cells.

On liver derived bulk CD8 T cells after vaccination (Figure 4.24, green bars), CD69 positive cells expressed significantly higher CD11a (median 93.7% vs 69.5%, $p<0.0001$, Figure 4.24.E) and CXCR6 (median 95.4% vs 6.60%, $p<0.0001$, Figure 4.24.F) and lower KLRG1 (median 22.0% vs 48.5%, $p<0.0001$ Figure 4.24.G) and CX3CR1 (median 11.5% vs 53.4%, $p<0.0001$, Figure 4.24.H) compared with CD69 negative cells.

Figure 4.24 Surface expression of liver tissue residence markers on blood and liver derived CD69 positive and negative bulk CD8 T cells

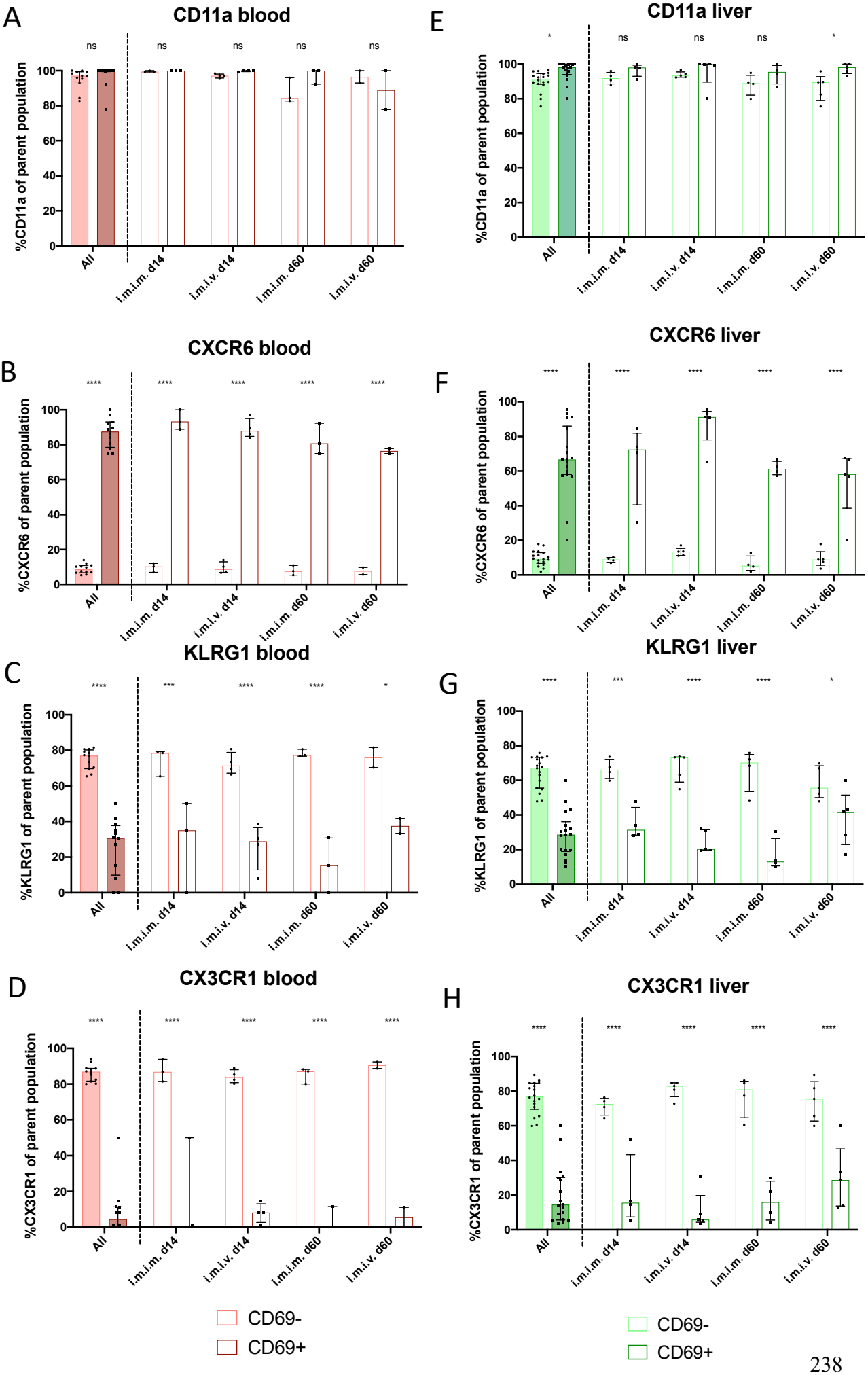
C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of CD69, CD11a, CXCR6, KLRG1 and CX3CR1 on bulk CD8 T cells and pentamer positive VWLSVIWM-specific T cells, by flow cytometry. Percentage of **A:** CD11a, **B:** CXCR6, **C:** KLRG1 and **D:** CX3CR1 on CD69 positive (CD69+) compared to CD69 negative (CD69-) bulk CD8 T cells derived from the blood (red bars). Percentage of **E:** CD11a, **F:** CXCR6, **G:** KLRG1 and **H:** CX3CR1 on CD69 positive (CD69+) compared to CD69 negative (CD69-) bulk CD8 T cells derived from the liver (green bars).
Results are a summation of single experiments with 3-5 mice per group. Median and interquartile range shown. Percentage of CD8 T cells expressing surface marker compared between two groups using Wilcoxon test or between more than two groups using ANOVA test with Sidak's multiple comparisons. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns = not significant.


A
CD11a blood
%CD11a of parent population
B
CXCR6 blood
%CXCR6 of parent population
C
KLRG1 blood
%KLRG1 of parent population
D
CX3CR1 blood
%CX3CR1 of parent population
E
CD11a liver
%CD11a of parent population
F
CXCR6 liver
%CXCR6 of parent population
G
KLRG1 liver
%KLRG1 of parent population
H
CX3CR1 liver
%CX3CR1 of parent population
All
Unvaccinated
i.m.i.m. d14
i.m.i.v. d14
i.m.i.m. d60
i.m.i.v. d60
CD69-
CD69+

On blood derived pentamer positive HBV specific T cells (Figure 4.25, red bars), there was no significant difference in CD11a expression between CD69 positive or negative cells (Figure 4.25.A). CD69 positive pentamer positive T cells expressed significantly higher CXCR6 (median 87.5% vs 8.65%, p<0.0001, Figure 4.25.B) and lower KLRG1 (median 30.7% vs %.77.0, p<0.0001, Figure 4.25.C) and CX3CR1 (median 4.46% vs 85.95%, p<0.0001, figure 4.25.D) compared with CD69 negative cells. Liver derived CD69 positive pentamer positive T cells (Figure 4.25, green bars), expressed significantly higher CD11a (median 98.2% vs 91.5%, p=0.0107, Figure 4.24.E), CXCR6 (median 67.36% vs 9.69%, p<0.0001, Figure 4.24.F) and lower KLRG1 (median 28.65% vs 67.3%, p<0.0001, Figure 4.24.G) and CX3CR1 (median 14.45% vs 76.85%, p<0.0001, Figure 4.24.F) compared with CD69 negative cells (Figure 4.6.E, right panel green bars).

Figure 4.25 Surface expression of liver tissue residence markers on blood and liver derived CD69 positive and negative pentamer positive HBV specific CD8 T cells

C57BL/6J mice were vaccinated intramuscularly (i.m.) with $5x10^7$ infectious units (iu) of ChAdOx1-HBV (prime) followed 4 weeks later by $2x10^6$ plaque forming units (pfu) of MVA-HBV (boost) either intramuscularly (i.m.) or intravenously (i.v.). Mice receiving i.m. prime and i.m. boost are designated as i.m.i.m. Mice receiving i.m. prime and i.v. boost are designated as i.m.i.v. Mice were sacrificed 14 days or over 60 days after final vaccination and lymphocytes derived from blood, liver, spleen, and lung were stained with fluorochrome labeled monoclonal antibodies to detect surface expression of CD69, CD11a, CXCR6, KLRG1 and CX3CR1 on bulk CD8 T cells and pentamer positive VWLSVIWM-specific T cells, by flow cytometry. Percentage of **A:** CD11a, **B:** CXCR6, **C:** KLRG1 and **D:** CX3CR1 on CD69 positive (CD69+) compared to CD69 negative (CD69-) pentamer positive CD8 T cells derived from the blood (red bars). Percentage of **E:** CD11a, **F:** CXCR6, **G:** KLRG1 and **H:** CX3CR1 on CD69 positive (CD69+) compared to CD69 negative (CD69-) pentamer positive CD8 T cells derived from the liver (green bars).
Results are a summation of single experiments with 3-5 mice per group. Median and interquartile range shown. Percentage of CD8 T cells expressing surface marker compared between two groups using Wilcoxon test or between more than two groups using ANOVA test with Sidak's multiple comparisons. **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, ns = not significant.


A
CD11a blood
%CD11a of parent population
E
CD11a liver
%CD11a of parent population
B
CXCR6 blood
%CXCR6 of parent population
F
CXCR6 liver
%CXCR6 of parent population
C
KLRG1 blood
%KLRG1 of parent population
G
KLRG1 liver
%KLRG1 of parent population
D
CX3CR1 blood
%CX3CR1 of parent population
H
CX3CR1 liver
%CX3CR1 of parent population
All
i.m.i.m. d14
i.m.i.v. d14
i.m.i.m. d60
i.m.i.v. d60
CD69-
CD69+

4.6 DISCUSSION

The work outlined in this chapter investigated the magnitude and phenotype of HBV specific T cells induced by ChAdOx1-HBV and MVA-HBV vaccination. The findings support the primary hypothesis that the magnitude of HBV specific CD8 T cells is increased in the liver after intravenous as compared with intramuscular MVA-HBV (boost) vaccination in C57BL/6J mice. This is consistent with recently published work investigating candidate vaccines for malaria and hepatitis C virus, where intravenous vaccination increased vaccine induced T cell responses in the liver (492,495). Other studies have shown that intravenous inoculation with live attenuated or viral vector vaccines, improves protection against TB or malaria challenge and increases vaccine efficacy in murine cancer models (492–494,497,498). In the context of malaria, intravenous administration of the PfSPZ vaccine candidate has been shown to be safe, well tolerated and practicable to administer to populations in malaria endemic areas (499–502). Intravenous administration of malaria vaccine candidate ChAd63.ME-TRAP has recently been given safely to humans in a small phase I study (492). Further studies will be required to determine whether intravenous vaccination in humans can enhance vaccine induced T cell responses in the liver and whether this will confer improvements in vaccine efficacy.

Vaccination with ChAdOx1-HBV and MVA-HBV altered the phenotype of CD8 T cells in the blood, liver, spleen and lung compared to CD8 T cells in unvaccinated mice. CD8 T cells in all compartments had increased CD44 expression after vaccination, in agreement with previous studies of adenoviral vectored vaccines (387). This suggests vaccination with ChAdOx1-HBV/MVA-HBV leads to CD8 T cell activation, as CD44 upregulation occurs after T cell receptor engagement with antigen (503). CD8 expression of KLRG1 also

increased after vaccination with ChAdOx1-HBV/MVA-HBV, suggesting vaccination leads to the expansion of effector T cell populations (504,505). Previous studies of adenoviral vaccination in mice have also shown an increase in KLRG1 expression on CD8 T cells after vaccination (387).

Memory T cells with distinct effector functions and localisations, were originally described by differential CD45RA and CCR7 or CD62L expression (482). CD127 expression has been shown to define a subset of antigen experienced cells that develop into long-lived CD8 memory T cells and can be further delineated by CD62L expression into T cells with central memory or effector memory phenotypes (485–487). I found that in the solid organs (spleen, liver and lung) the majority of CD127 positive CD8 T memory cells had a central memory (T_{CM}) phenotype (KLRG1-CD127+CD62L+) whereas in the blood there were more CD8 T cells with an effector memory phenotype (T_{EM}) (KLRG1-CD127+CD62L-), in keeping with previous observations that CD62L- T_{EM} cells are enriched in the circulation (482). After vaccination, the proportion of T_{EM} cells in the liver, lung and spleen expanded. This is in agreement with a recent study of mice vaccinated with a ChAdOx1 vectored SARS-CoV-2 vaccine, in which spleen derived T cells were CD44+CD62- (506). After ChAdOx1-HBV/MVA-HBV vaccination there was also an increased proportion of CX3CR1 expression on T_{EM} cells, consistent with an expansion of cells with a cytotoxic phenotype able to produce granzyme (496,507). There were no differences in phenotype conferred by intravenous as compared to intramuscular MVA-HBV (boost) vaccination after ChAdOx1-HBV prime in any organ.

HBV specific T cells induced by ChAdOx1-HBV/MVA-HBV were successfully identified by flow cytometry using a fluorochrome labelled multimers in C57BL/6J mice. This will be a

useful tool to evaluate and optimise vaccination strategies using ChAdOx1-HBV and MVA-HBV in mouse models of chronic HBV. Pentamer positive HBV specific cells show a consistent phenotype across all organs. There were no differences in phenotype conferred by intravenous as compared with intramuscular MVA-HBV (boost) vaccination after ChAdOx1-HBV prime. Pentamer positive cells had high CD44 expression, supporting they were antigen experienced (503). Within the pentamer positive HBV specific T cell population, both KLRG1 positive effector T cells and CD127 positive memory cells were identified. CD127 expression increased at long lived compared (day 60) to peak (day 14) time points after vaccination on pentamer positive HBV specific cells derived from the blood, liver and spleen. This is consistent with the increased expression of CD127 on antigen specific cells in mice at long lived time points after infection with Sendai virus (483). The majority of pentamer positive CD127 positive memory T cells had a T_{EM} phenotype (KLRG1-CD127+CD62L-), which contained populations of both CX3CR1 positive and negative cells. This is consistent with studies in humans, in which ChAd and MVA vectored vaccines given in heterologous prime-boost strategies induce cells with a T_{EM} phenotype in the blood (398,508) and in mice vaccinated with human adenoviral vector, in which cytotoxic CX3CR1 positive T_{EM} cells are induced (496).

Markers of tissue residency were investigated on CD8 T cells after ChAdOx1-HBV/MVA-HBV vaccination. A higher percentage of CD8 T cells in the livers of both unvaccinated and vaccinated mice were CD69 positive compared to blood derived counterparts. CD69 was also increased on pentamer positive HBV specific T cells in the liver compared to the blood. These CD69 positive cells have a phenotype consistent with murine liver resident cells (CD11a and CXCR6 high, KLRG1 and CX3CR1 low) (489,490). The ability of an HBV therapeutic vaccine candidate to induce HBV specific T_{RM} cells is likely to be important, as

these cells are associated with viral control in patients with chronic HBV (491,509). These findings are consistent with recent studies in mice vaccinated with malaria or HCV vaccine candidates, which have also shown intrahepatic vaccine induced CD8 cells have a T_{RM} phenotype after intravenous vaccination (492,495). However, in these studies CD69 positive cells predominated in the liver, whereas I found the majority of cells were CD69 negative. It is probable that due to the vascular nature of the liver, some of the isolated cells are actually passing through the liver rather than being truly resident. To better identify true tissue resident lymphocytes, future work should employ intravenous injection of fluorochrome labeled anti-CD8 just prior to mouse sacrifice, which enables the identification of blood derived (CD8 positive) and tissue derived (CD8 negative) cells more accurately (510). T_{RM} cells could also be identified using quantitative histocytometric analysis, which has been shown to more accurately estimate populations of tissue resident lymphocytes (510). Ultimately parabiosis experiments would be required to confirm tissue residency (489).

4.7 CONCLUSION

This data supports that vaccination with ChAdOx1-HBV and MVA-HBV induces HBV specific T cells with T_{EM} and T_{RM} phenotypes. Intravenous boost vaccination increases the magnitude of HBV specific T cells in the liver but does not significantly alter their phenotype. Further studies sampling both blood and liver responses in murine models of chronic HBV and in human clinical trials will be required to determine whether vaccination can induce cells with the same phenotype in the setting of chronic HBV, and whether vaccination will be sufficient to induce and maintain immunological control of the virus.

4.8 SUMMARY OF RESULTS

1. Vaccine induced HBV specific T cells can be identified in the blood by flow cytometry using a fluorochrome labelled pentamer.
2. The percentage of pentamer positive CD8 T cells in the blood positively correlates with total splenocyte responses to vaccination measured in interferon-gamma ELISpot assays.
3. Intravenous as opposed to intramuscular administration of boost vaccination with MVA-HBV increases the magnitude of HBV specific T cells in the liver.
4. Vaccination with ChAdOx1-HBV and MVA-HBV changes the phenotype of CD8 T cells, increasing the percentage that express CD44, KLRG1 and T_{EM} markers. The phenotype is not affected by vaccination regime or timing of response.
5. The majority of pentamer positive HBV specific T cells are CD44 high and show a mixture of effector and T_{EM} cell phenotypes.
6. The expression of liver tissue residency marker CD69 is expanded on liver as compared to blood derived CD8 and HBV specific T cells. These cells co-express other markers of tissue residency.

CHAPTER 5: Vaccination with ChAdOx1-HBV induces T cell responses in healthy volunteers and patients with chronic HBV infection

5.1 PRIMARY HYPOTHESIS

Vaccination with ChAdOx1-HBV is safe and can induce HBV specific T cells in healthy volunteers and patients with chronic HBV infection.

5.2 INTRODUCTION

Chronic infection with Hepatitis B virus remains highly prevalent worldwide contributing to death through chronic liver disease and hepatocellular carcinoma (HCC) (1). Nucleos(t)ide analogues, the mainstay of current therapy, reduce HBV replication but do not eradicate infection and usually require long term treatment (8).

In recent years, efforts to develop novel therapeutic strategies for chronic HBV have amplified (131). Complete eradication of HBV is highly challenging due to its persistence within the nucleus of infected hepatocytes as covalently close circular DNA (cccDNA). Therefore so-called functional cure, the sustained loss of HBsAg, has been agreed on as a more achievable therapeutic goal (511,512). Sustained loss of HBsAg occurs in people that clear acute HBV infection, as well as in a small proportion of patients with chronic infection treated with pegIFNα or long-term nucleos(t)ide analogues (15). In achieving functional cure, immunological control of HBV is realised and maintained without the need for further therapy. Furthermore the long-term risks of liver related complications of HBV infection are reduced, particularly if HBsAg loss occurs before the age of 50 (105,513,514).

Modification of the immune response is one strategy employed by several HBV therapeutics under development (138). The achievement of functional cure in either successful control of acute HBV infection or after long-term treatment in chronic infection, is associated with robust long-lived HBV specific T cell responses (17,18,178). In contrast, chronic HBV infection is characterised by a dysfunctional cellular immune response (515). Therapeutic vaccination is one immunotherapeutic strategy that aims to restore immune function and induce functional cure, by delivering non-infective HBV antigen to boost existing or induce new functional HBV specific T cells.

Several HBV therapeutic vaccine candidates have been trialed, but thus far none have increased rates of HBV functional cure (reviewed in detail in Chapter 1). Strong HBV specific immune responses have never been achieved after therapeutic vaccination in patients with chronic HBV (326,327,319,322,318,321,329,290,296,300,516,306,313,316,323,320,326,362). In several cases, the immunogenicity of therapeutic vaccination candidates has not been tested in healthy volunteers prior to testing in patients with chronic HBV (319–321,329,349,350,352). This means it is difficult to understand whether a vaccine candidate is inherently poorly immunogenic, or if factors relating to chronic HBV infection are responsible for its limited success.

The therapeutic vaccine candidates ChAdOx1-HBV and MVA-HBV have been shown to induce high magnitude HBV specific T cell responses in healthy mice ((346) and Chapter 3). In this chapter I report the results of HBV001, the first in-human clinical trial of ChAdOx1-HBV in both healthy volunteers and patients with chronic HBV, which aimed to assess safety and immunogenicity of ChAdOx1-HBV (347). HBV specific T cell responses to vaccination

are followed longitudinally to assess the magnitude and breath of the responses and the phenotype and functionality of these cells is determined. The results will inform the design of further trials of therapeutic vaccination involving ChAdOx1-HBV.

5.3 KEY QUESTIONS

1. Is vaccination with ChAdOx1-HBV safe and well tolerated in healthy volunteers?
2. Does vaccination of healthy volunteers with ChAdOx1-HBV induce HBV specific T cells towards all major HBV antigens within the vaccine immunogen?
3. What is the phenotype and functionality of T cells induced by ChAdOx1-HBV in healthy volunteers?
4. Is vaccination with ChAdOx1-HBV safe and well tolerated in patients with chronic HBV infection?
5. Does vaccination with ChAdOx1-HBV in patients with chronic HBV infection induce or boost the magnitude of HBV specific T cells?
6. Do HBV specific T cells induced by ChAdOx1-HBV cross react to genotype C and genotype D HBV peptides?

5.4 CHAPTER SPECIFIC MATERIALS AND METHODS

5.4.1 HBV001 trial design

The HBV001 trial is discussed in detail in Chapter 2. Briefly for cohorts 1 and 2, 10 healthy volunteers were enrolled to receive intramuscular injection of ChAdOx1-HBV either at low dose (cohort 1, n=5, 2.5 x10^9 viral particles (vp)) or high dose (cohort 2, n=5, 2.5 x10^{10} vp). All participants were HBsAg and HBcAb negative, prior to vaccination.

At time of writing, 7 volunteers with chronic HBV infection were enrolled to receive intramuscular injection of ChAdOx1-HBV at either low dose (cohort 3, 2.5 x10^9 vp, n=6) or high dose (cohort 4, 2.5 x10^{10} vp, n=1). Blood was drawn at day 0 prior to vaccination and on days 14, 28, 56, 84 and 168 after vaccination for peripheral blood mononuclear cell (PBMC) isolation. All participants in cohorts 3 and 4 of HBV001 had chronic hepatitis B infection evidenced by HBsAg positivity for over 6 months prior to inclusion. All participants were taking nucleos(t)ide analogue therapy (entecavir or tenofovir) for at least 12 months prior to enrolment and had an HBV DNA of under 40 IU/mL for at least 6 months. Quantitative HBsAg levels were required to be equal to or under 10 000 IU/mL at screening. Recruitment and follow up is ongoing.

The HBV001 study design and operation were led by the study sponsor; Vaccitech Limited, Oxford, UK. I was involved in the trial design including the initial advisory board meeting and protocol development. I attended mothy programme meetings at which I regularly presented immunological data from ChAdOx1-HBV and MVA-HBV experiments in mice (chapter 3) as well as the immunological data from the HBV001 trial itself (this chapter). Together with my colleague Anthony Brown (Barnes group research assistant), I wrote the trial standard operating procedures for the immunological assays.

Recruitment and trial procedures for healthy volunteers was conducted in the Medicine Evaluation Unit Manchester and blood was processed at the Good Clinical Practice laboratory, Liverpool and stored in liquid nitrogen before transport to Oxford. I performed the majority of the immunological assays (ELISpot and intracellular staining) and my colleague Anthony Brown (Barnes group research assistant), assisted me or performed assays where necessary.

Recruitment and trial procedures for patients with chronic HBV was conducted by Louise Holland and Paola Cicconi at Oxford University Hospitals NHS Foundation Trust, Oxford. Blood was processed and immunological assays were performed at the Peter Medawar Building Oxford by either myself or Anthony Brown.

5.4.2. The use of additional PBMCs for assay optimisation

Additional PBMCs from healthy unvaccinated controls for use in assay optimisation experiments or as negative controls were recruited to the Oxford BRC GI biobank (REC: Gastro01 16/YH/0247). PBMCs were either used in immunoassays fresh directly from isolation, or frozen in liquid nitrogen for storage.

5.4.3 Immunoassays

Immunoassays are discussed in detail in Chapter 2. Briefly PBMCs were plated with peptide pools corresponding to the ChAdOx1-HBV vaccine immunogen in overnight interferon gamma (IFNγ) ELISpot assays or for 6 hours in intracellular staining assays (ICS) to detect cytokine production by flow cytometry. A positive ELISpot assay was defined as a response equal to or exceeding 50 IFNγ spot forming units per million PBMC, over 2-fold greater than the background well and at least 2 times higher than baseline, in line with previous studies of therapeutic HBV vaccination candidates (325).

5.5 RESULTS

5.5.1 Baseline characteristics of healthy volunteers in HBV001

The baseline characteristics of the 10 healthy volunteers enrolled in HBV001 are shown in Table 5.1. Cohort 1 received low dose (2.5 x10^9 vp) and cohort 2 received high dose (2.5

x10^{10} vp) ChAdOx1-HBV. The median age of participants was 38 (range 30-48), 70% (n=7) were male and 90% (n=9) were of Caucasian ethnicity.

Table 5.1 Baseline characteristics of healthy volunteers enrolled in HBV001

Volunteer ID	Cohort	Age	Gender	Ethnicity
401	1	30	M	Caucasian
402	1	40	F	Caucasian
403	1	31	M	Caucasian
404	1	44	M	Asian
406	1	48	M	Caucasian
405	2	36	F	Caucasian
407	2	41	F	Caucasian
408	2	46	M	Caucasian
411	2	35	M	Caucasian
412	2	33	M	Caucasian

5.5.2 Vaccination with ChAdOx1-HBV in healthy volunteers is well tolerated

Vaccination with ChAdOx1-HBV was well tolerated in all healthy volunteers receiving either low dose or high dose vaccine (cohorts 1 and 2). The most common local adverse reaction within 7 days of vaccination was pain at the injection site in 40% (n=2/5) receiving low dose and 100% (n=5/5) receiving high dose vaccine (Figure 5.1.A). All of these reactions were graded as mild in severity. In the low dose group, a single participant experienced bilateral foot pain, which was graded as moderate in severity and had resolved by 48 hours (Figure 5.1.B left panel). Systemic reactions were more frequent in the high dose group with

participants reporting abdominal pain (n=1/5), fever (n=1/5), headache (n=1/5), vomiting (n=1/5), or feeling generally unwell (n=1/5), which were all mild in severity (Figure 5.1.B right panel).

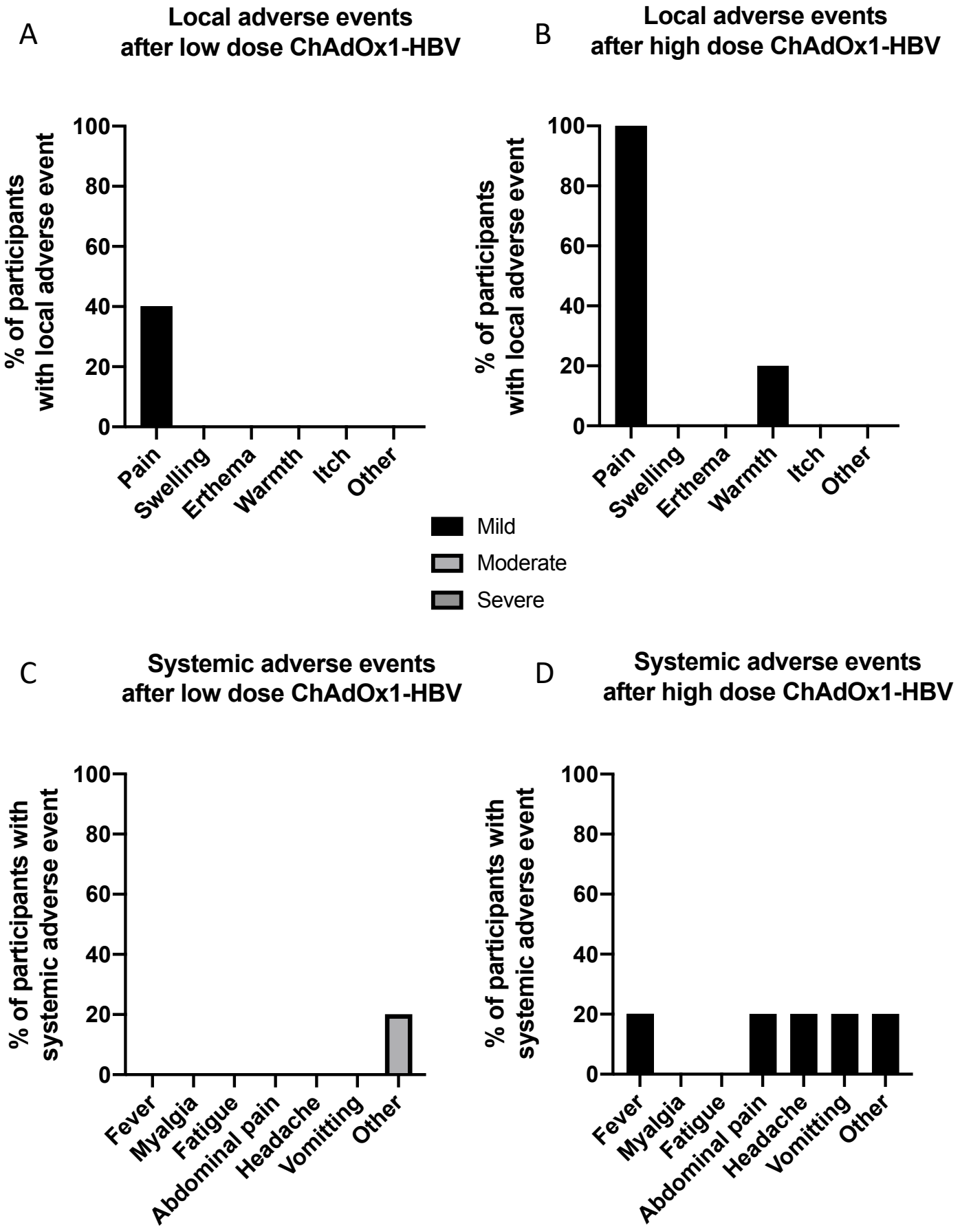


Figure 5.1 Adverse events after vaccination with ChAdOx1-HBV in healthy volunteers

Local adverse events within 7 days after vaccination with **A:** low dose 2.5 x10^{9} viral particles (vp) ChAdOx1-HBV or **B:** high dose 2.5 x10^{10} vp ChAdOx1-HBV. Systemic adverse events within 7 days after vaccination with **C:** low dose (2.5 x10^{9} viral particles (vp) ChAdOx1-HBV or **D:** high dose 2.5 x10^{10} vp ChAdOx1-HBV. Mild (black), moderate (light grey) or severe (dark grey) grading shown. Other = bilateral foot pain or feeling generally unwell.

5.5.3 Vaccination with ChAdOx1-HBV induces high magnitude HBV specific T cell responses in healthy volunteers

PBMCs from healthy volunteers enrolled into HBV001 were tested for pre-existing HBV specific T cell responses in IFNγ ELISpot assays. Due to unavailability of sufficient PBMC sample, no assays to detect baseline responses were conducted for participants 408 and 412 and not all peptide pools could be tested for donors 405, 407 and 411.

Unexpectedly, 25% (n=2) healthy volunteers had T cell responses towards the surface peptide pool (median 58, IQR 22-436 SFU per million PBMCs, Figure 5.2). In addition, participant 401 had detectable responses towards other parts of the HBV immunogen, namely HBV core (150 SFU per million PBMCs), pol4 (168 SFU per million PBMCs) and the linker/TPA/F2A regions (358 SFU per million PBMCs, Figure 5.2).

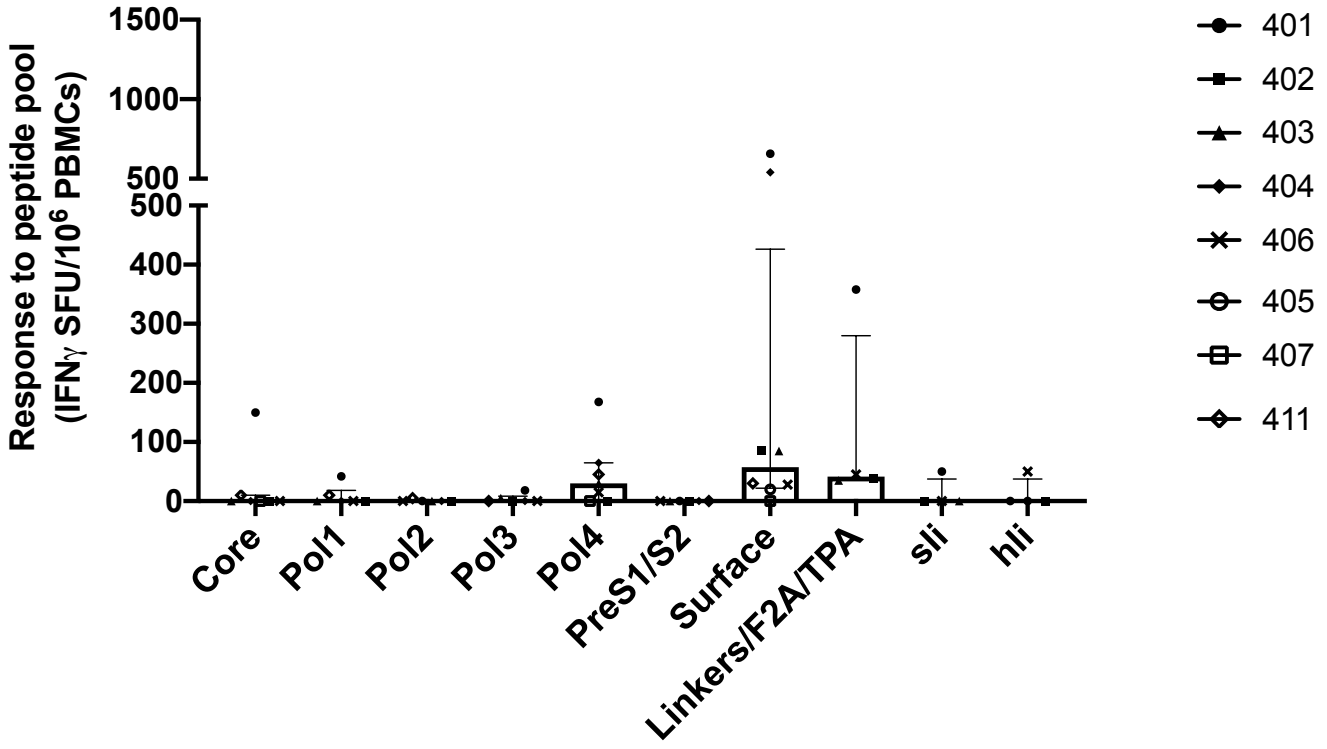


Figure 5.2 Baseline T cell responses towards HBV peptide pools prior to vaccination with ChAdOx1-HBV in healthy participants enrolled in HBV001

Peripheral blood mononuclear cells (PBMC) from healthy volunteers enrolled in HBV001 were isolated prior to vaccination with ChAdOx1-HBV at timepoint day 0. PBMCs from HBV001 participants 401-406 and 405, 407 and 411 were incubated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. From left to right peptide pools corresponded to HBV core (core), HBV polymerase (pol 1–pol 4) HBV preS1 and pre-S2 (preS1/S2), HBV surface (surface) and the non-HBV vaccine components including the linker regions, furin-2A and TPA (linkers/F2A/TPA), truncated shark invariant chain (sIi) and truncated human invariant chain (hIi).The magnitude of response to each peptide pool is recorded as IFNγ spot-forming units per million PBMC (IFNγ SFU/10^6 PBMC). Median and interquartile range shown.

To determine the regions of HBV surface to which baseline responses were directed, PBMCs from participant 401 at baseline were stimulated with HBV surface peptide minipools (G1-G7) containing 8 or 9 peptides making up the HBV surface pool. Positive responses were detected towards minipools G1, G2, G3, G6 and G7, with the highest responses towards the G7 minipool (320 SFU per million PBMCs, Figure 5.3).

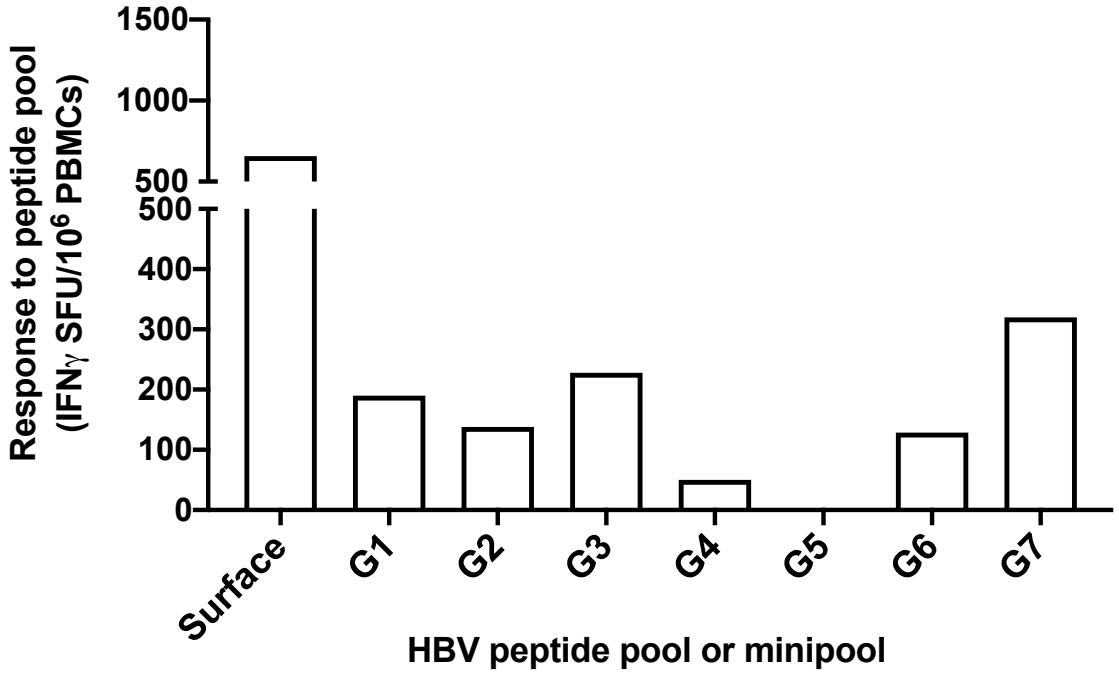


Figure 5.3 Baseline T cell responses towards HBV surface peptide pool and minipools prior to vaccination with ChAdOx1-HBV in healthy participant number 401

Peripheral blood mononuclear cells (PBMC) from healthy volunteer number 401 were isolated prior to vaccination with ChAdOx1-HBV at timepoint day 0. PBMCs were incubated with HBV surface peptide pool and minipools G1-G7 (corresponding to the HBV surface region) in interferon-gamma (IFNγ) ELISpot assays. The magnitude of responses to vaccination were recorded as IFNγ spot-forming units per million PBMC (IFNγ SFU per 10^6 PBMC).

Next, PBMCs from 10 healthy volunteers enrolled in HBV001 collected at day 0, 14, 28, 56, 84 and 168 after vaccination with ChAdOx1-HBV were tested for vaccine induced T cell responses in IFNγ ELISpot assays. The total response towards the HBV components of the vaccine immunogen were calculated by the summation of responses towards the HBV peptide pools core, pol1-pol4, preS1/S2 and surface. Total response to vaccination peaked between day 14 and day 28 after ChAdOx1-HBV (day 14 median 1173, IQR 1173-1553 and day 28 median 1440, IQR 743-1798 SFU per million PBMCs, Figure 5.4.A). Total responses at day 14 and 28 after vaccination were significantly higher than day 0 responses (day 14 median 1173 vs day 0 median 110 SFU per million PBMCs, p=0.0019, day 28 median 1440 vs day 0 median 110 SFU per million PBMCs, p=0.0057, Figure 5.4.A). At day 28 after vaccination a positive response to vaccination was detected in 90% (n=9/10) of participants (Table 5.2). Total responses declined after day 28 and were not significantly increased

compared to baseline. Responses remained detectable at day 168 after vaccination (median 563, IQR 523-732 SFU per million PBMCs, Figure 5.4.A). There was no significant difference in the magnitude of total response between those receiving low dose (2.5 x10^9 viral particles) compared with high dose (2.5 x10^{10} viral particles) ChAdOx1-HBV (Figure 5.4.B).

Table 5.2: Positive IFNγ ELISpot responses in healthy volunteers at day 14 and 28 post vaccination with ChAdOx1-HBV

A positive assay was defined as a response equal to or exceeding 50 IFN-γ spot forming units per million PBMC, over 2-fold greater than the background well and at least 2 times higher than baseline.

Participant	Day 14 total response magnitude SFU/10^6 PBMC	Day 14 Positive/negative	Day 28 total response magnitude SFU/10^6 PBMC	Day 18 Positive/negative
401	1173	Negative	1817	Negative
402	895	Positive	782	Positive
403	1445	Positive	1792	Positive
404	865	Negative	1582	Positive
406	1660	Positive	1661	Positive
405	1357	Positive	1298	Positive
407	2038	Positive	1845	Positive
408	983	Positive	1065	Positive
411	No sample	No sample	625	Positive
412	918	Positive	377	Positive

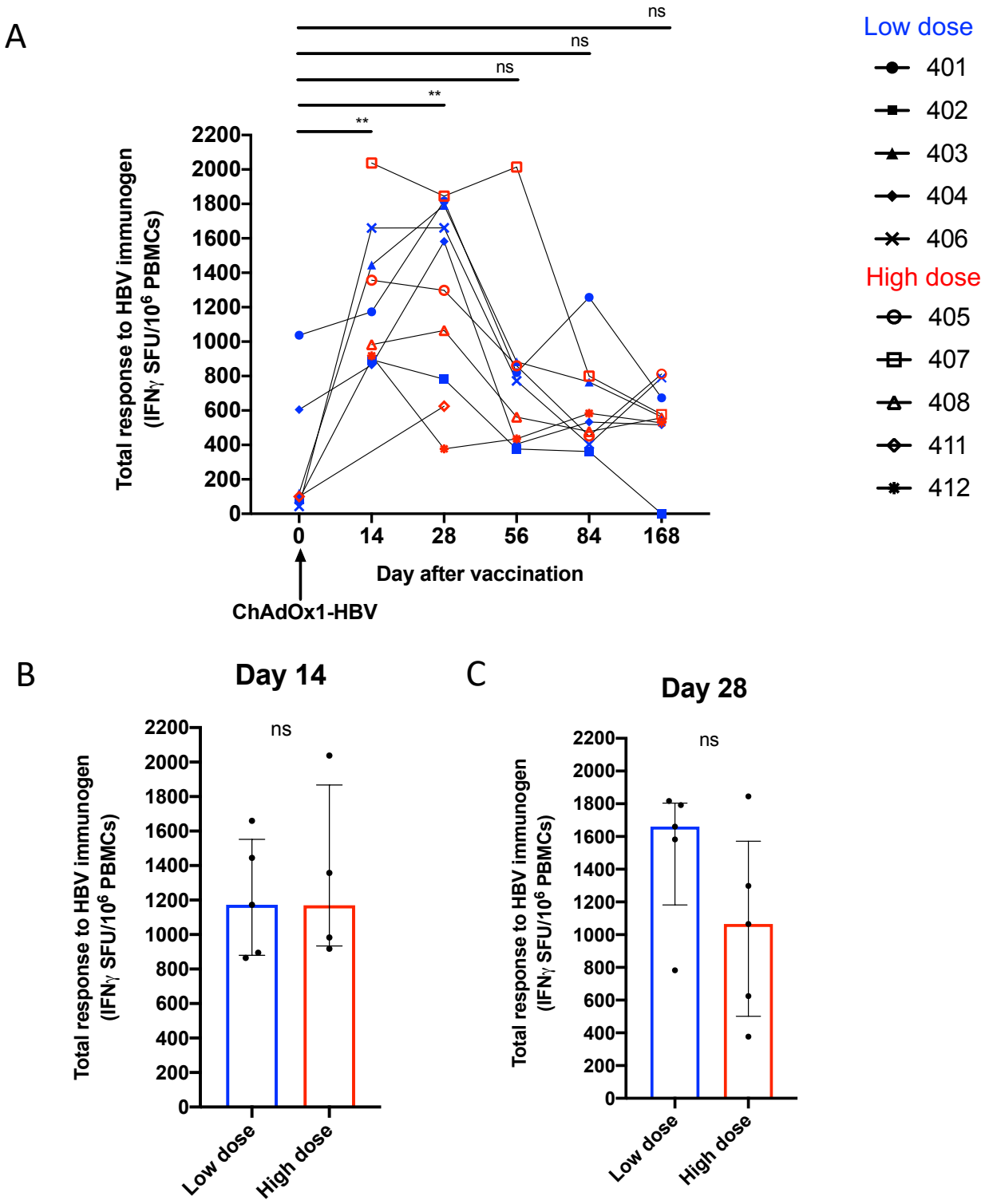


Figure 5.4 Total T cell responses after ChAdOx1-HBV vaccination in healthy volunteers

Peripheral blood mononuclear cells (PBMC) from healthy volunteers enrolled in HBV001 were isolated at timepoint day 0, 14, 28, 56, 84 and 168 after intramuscular vaccination with ChAdOx1-HBV at either low dose (2.5 x10^9 viral particles (vp), participants 401-406, blue symbols) or high dose (2.5 x10^{10} vp, participants 405-412, red symbols). PBMCs were plated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. The magnitude of responses to vaccination were recorded as IFNγ spot-forming units per million PBMC (SFU/10^6 PBMC). **A:** Total vaccine response magnitude at all timepoints (summation of the response to all peptide pools corresponding to HBV components of the vaccine immunogen including core, pol1 - pol4, preS1/S2 and surface). **B:** Total vaccine response at day 14 or **C:** 28 after ChAdOx1-HBV compared between the low dose and high dose groups. Kruskal-Wallis test with Dunn's multiple comparisons used to compare multiple groups. Mann Whitney-U test used for statistical comparison between 2 groups. Median and interquartile range shown. ** $p<0.005$, ns = not significant

T cell responses towards individual HBV antigens within the vaccine immunogen were also investigated. Responses to peptide pool core (corresponding to the HBV core antigen, total 56 overlapping peptides) peaked at day 28 after vaccination (median 163, IQR 80-275 SFU/million PBMCs, Figure 5.5.A) and declined thereafter. Responses at day 14 (median 135 SFU/ million PBMCs) and day 28 (median 163 SFU/ million PBMCs) after vaccination were significantly higher than at baseline (median 0 SFU/ million PBMCs, p=0.0041 and 0.0033 respectively, Figure 5.5.A).

Responses to peptide pools pol1-pol4 (corresponding to the HBV polymerase antigen, total 215 overlapping peptides) were the highest of all HBV regions, peaking between day 14 and day 28 after vaccination (day 14 median 555, IQR 372-644 and day 28 median 572, IQR 261-765 SFU per million PBMCs, Figure 5.5.B) and remained detectable at day 168. Responses at day 14 (median 555 SFU/ million PBMCs) and day 28 (median 571 SFU/ million PBMCs) after vaccination were significantly higher than at baseline (median 48 SFU/ million PBMCs, p=0.0004 and 0.0005 respectively, Figure 5.5.B).

Responses towards peptide pool preS1/S2 (corresponding to the HBV preS1 and pre-S2 regions, total 56 overlapping peptides) were the lowest of all HBV regions in the vaccine immunogen, peaking at day 14 after vaccination (median 100, IQR 57-197 SFU per million PBMCs, Figure 5.5.C). Responses at day 14 (median 100 SFU/ million PBMCs), day 28 (median 36 SFU/ million PBMCs) and day 56 (median 72 SFU/ million PBMCs) after vaccination were significantly higher than at baseline (median 0 SFU/ million PBMCs, p=0.0002, p=0.0068 and p=0.0010 respectively, Figure 5.5.C).

Responses towards peptide pool surface (corresponding to HBV surface antigen, total 57 overlapping peptides) peaked between day 14 and 28 after vaccination (day 14 median 460, IQR 357-611 and day 28 median 643, IQR 514-706 SFU per million PBMCs, Figure 5.5.D). Responses at day 14 (median 460 SFU/ million PBMCs) and day 28 (median 514 SFU/ million PBMCs) after vaccination were significantly higher than at baseline (median 58 SFU/ million PBMCs, $p=0.0171$ and $p=0.0142$ respectively, Figure 5.5.D).

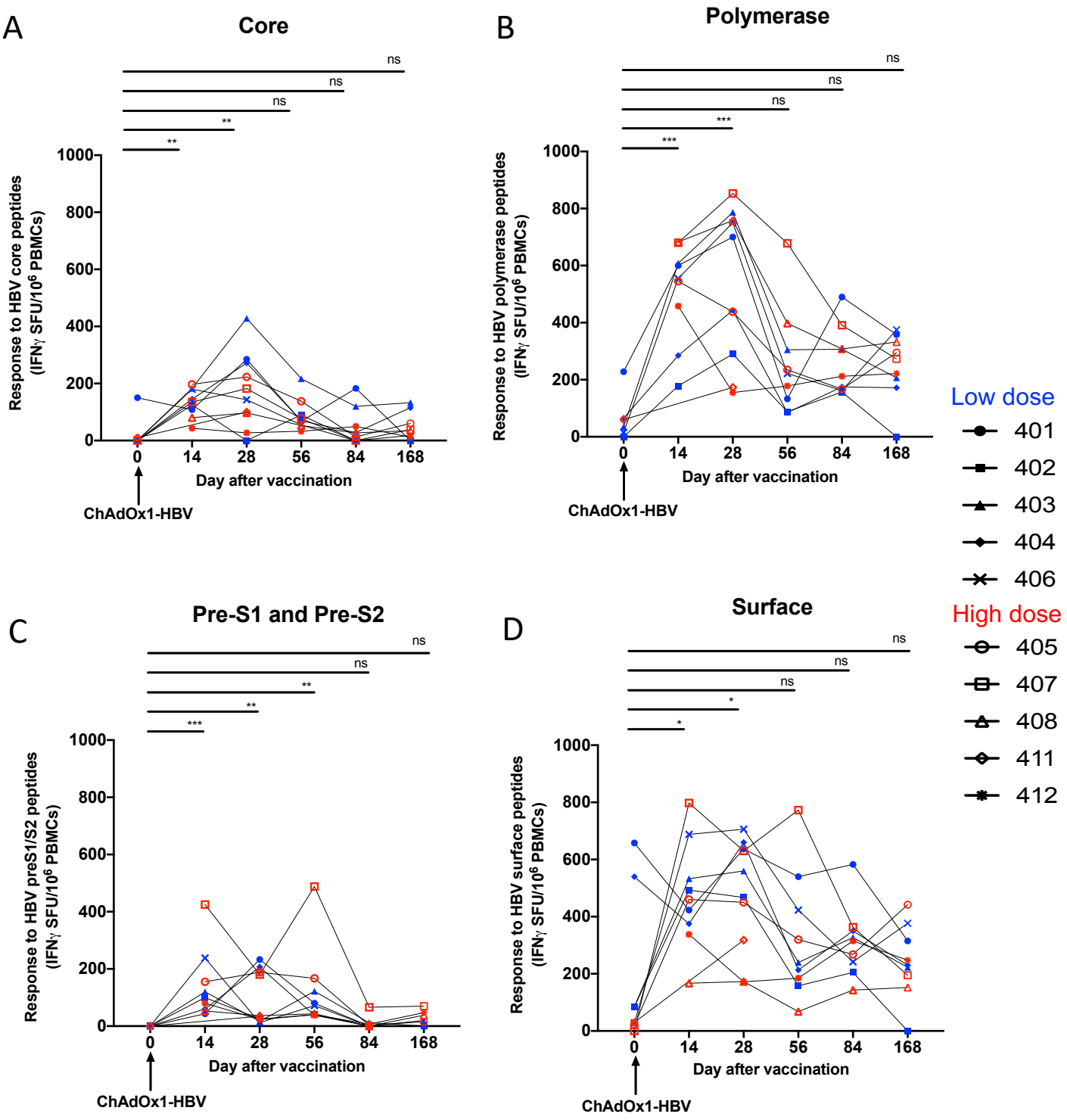


Figure 5.5 HBV specific T cell responses after ChAdOx1-HBV vaccination in healthy volunteers

Peripheral blood mononuclear cells (PBMC) from healthy volunteers enrolled in HBV001 were isolated at timepoint day 0, 14, 28, 56, 84 and 168 after intramuscular vaccination with ChAdOx1-HBV at either low dose (2.5 x10^9 viral particles, participants 401-406, blue symbols) or high dose (2.5 x10^{10} viral particles, participants 405-412, red symbols). PBMCs were plated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. Longitudinal responses to **A:** HBV core (core peptide pool), **B:** HBV polymerase (pol 1- pol 4 peptide pools), **C:** HBV pre-S1 and pre S2 (preS1/S2 peptide pools) and **D:** HBV surface (surface peptide pool) are displayed as IFNγ spot-forming units per million PBMC (SFU/10^6 PBMC). Kruskal-Wallis test with Dunn's multiple comparisons used to compare multiple groups. ***p<0.001, ** p<0.005, *p<0.05, ns = not significant

Finally, T cell responses towards non-HBV components of the vaccine immunogen were examined. Responses to peptide pool linkers/F2A/TPA (corresponding to the vaccine linker sequences, cleavage protein furine-2-A (F2A) and adjuvant tissue plasminogen activator (TPA) with their adjoining HBV immunogen regions, total 40 peptides) were detectable, peaking at 14-28 days after vaccination (day 14 median 208, IQR 129-302 and day 28 median 189, IQR 117-363 SFU per million PBMCs, Figure 5.6.A). Responses were not significantly different between baseline and post vaccination timepoints.

Four participants (numbers 403, 405, 407 and 412) had detectable responses towards the truncated shark invariant chain (sIi) peptide pool (corresponding to the truncated sIi molecular adjuvant, total 7 overlapping peptides). Positive responses peaked at day 28 after vaccination (range of positive responses 95-1072 SFU per million PBMCs, Figure 5.6.B). Responses were not significantly different between baseline and post vaccination timepoints.

There were also detectable responses towards the truncated human invariant chain (hIi) peptide pool (not within the vaccine immunogen, total 4 overlapping peptides) at day 28 or 56 after vaccination (participants 401, 404, 406, 405 and 407), range135-233 SFU per million PBMC (Figure 5.6.C). Responses were not significantly different between baseline and timepoint after vaccination. The hIi peptide pool was included to determine whether there might be any cross reactivity between T cells recognising sIi. Spearman's rank test did not show a positive correlation between responses towards the sIi and hIi pool ($r = 0.27$, $p = 0.44$, Figure 5.6.D).

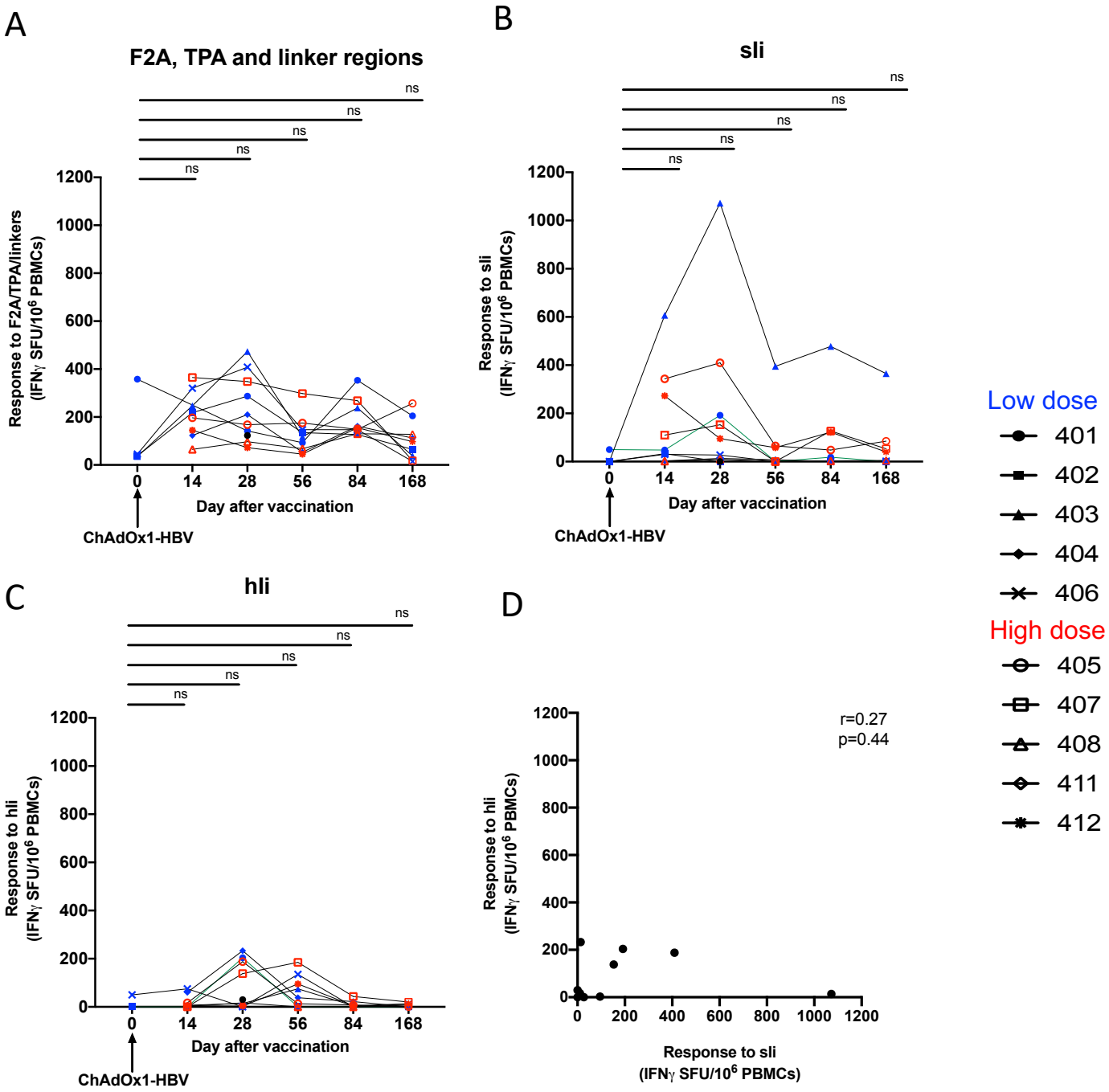


Figure 5.6 T cell responses to non-HBV vaccine components after ChAdOx1-HBV in healthy volunteers

Peripheral blood mononuclear cells (PBMC) from healthy participants enrolled in HBV001 were isolated timepoint day 0 and then days 14, 28, 56, 84 and 168 after intramuscular vaccination with ChAdOx1-HBV at either low dose (2.5 x10^{9} viral particles, participants 401-406, blue symbols) or high dose (2.5 x10^{10} viral particles, participants 405-412, red symbols). PBMCs were plated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. Longitudinal responses to **A:** the furin-2A (F2A), tissue plasminogen activator (TPA) and linker regions, **B:** truncated shark invariant chain (sIi peptide pool) and **C:** truncated human invariant chain (hIi peptide pool) are displayed as IFNγ spot-forming units per million PBMC (SFU/10^{6} PBMC). **D:** Correlations of responses at day 28 after vaccination between sIi and hIi peptide pools. Kruskal-Wallis test with Dunn's multiple comparisons used to compare multiple groups. Correlation calculated using using Spearman's rank test, r value and p value shown. ns = not significant

5.5.4 In healthy volunteers, vaccination with ChAdOx1-HBV induces polyfunctional CD8 and CD4 T cells

T cell responses induced by vaccination with ChAdOx1-HBV were evaluated in intracellular staining assays (ICS) to determine the balance of CD8 and CD4 responses and their cytokine production profiles (gating strategy for flow cytometry Figure 5.7).

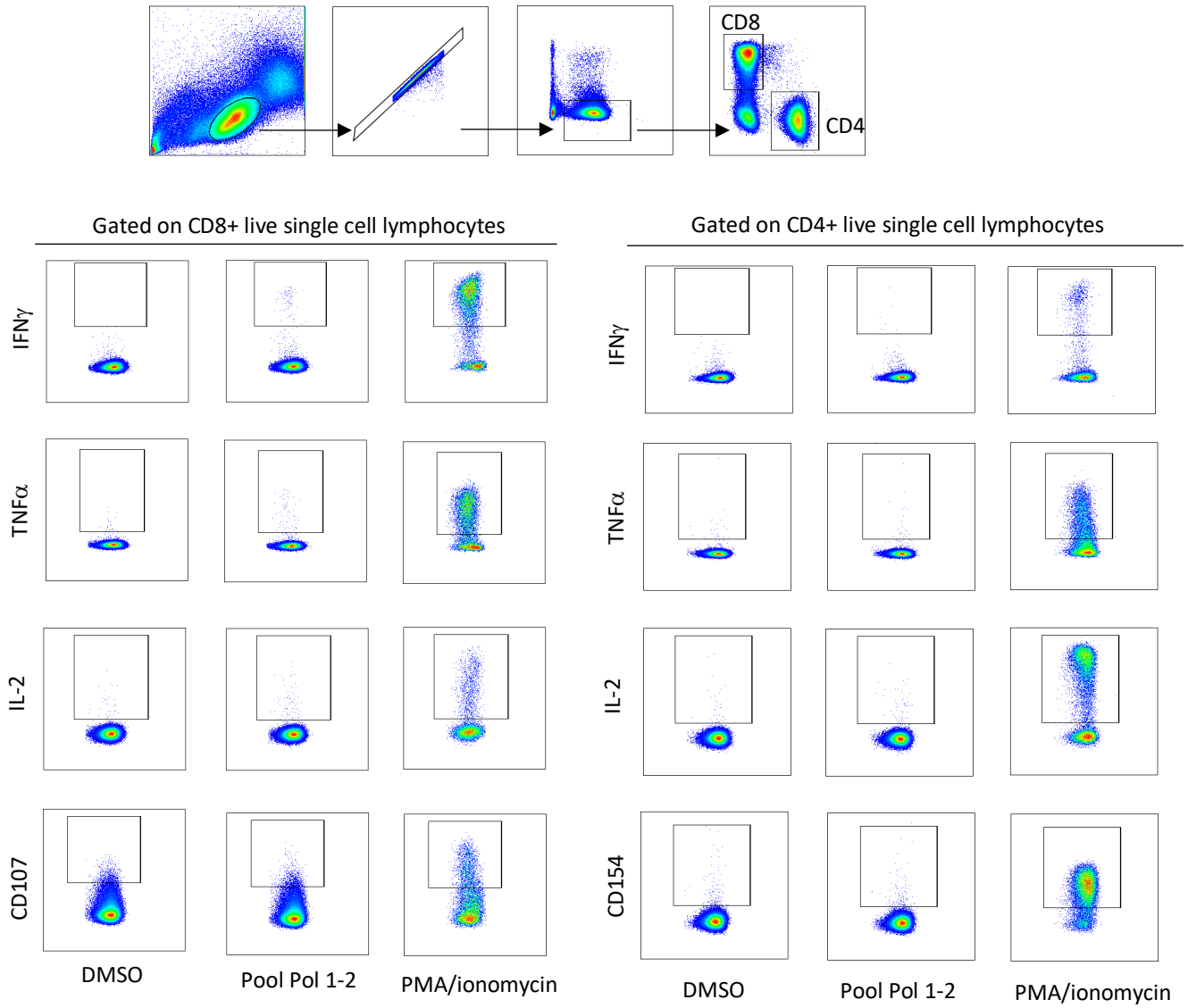


Figure 5.7 The detection of cytokine production by vaccine induced T cells by flow cytometry

Gating strategy to detect live, singlet CD8 or CD4 lymphocytes is shown in the top panel. Gating strategy to detect the percentage live singlet lymphocytes producing IFNγ, TNFα or IL2 after stimulation with DMSO, peptide pool Pol 1-2 or PMA/ionomycin bottom left (CD8 T cells) and bottom right (CD4 T cells).

ICS assays were performed on PBMCs taken at the timepoint at, or closest to, the peak magnitude of total responses to the HBV immunogen on ELISpot (day 14, 28 or 56 after vaccination, dependent on availability of sufficient cell numbers, Table 5.3). Peptide pools were tested only where responses on ELISpot exceeded 150 SFU per million PBMCs, as responses with lower magnitudes were unlikely to be detectable in an ICS assay.

Table 5.3: Timepoint of peripheral blood mononuclear cell evaluation in intracellular staining assay for healthy volunteers in HBV001

Participant	Timepoint of peak total response magnitude (day)	Timepoint tested in ICS (day)
401	28	28
402	14	14
403	28	56
404	28	28
406	28	14
405	14	14
407	14	14
408	28	56
411	28	28
412	14	56

For each peptide pool, between 88 and 100 percent of participants produced at least one detectable cytokine (IFNγ, TNFα, IL-2 or CD107a+ for CD8 T cells and IFNγ, TNFα, IL-2 or CD154+ for CD4 T cells, Table 5.4). CD8 T cells were most likely to produce IFNγ (n=22/34, 65% positive assays) or IL-2 (n=21/34, 65% positive assays) in response to a

peptide pool (Table 5.4). In contrast CD107a was detectable on CD8 T cells in 21% (n=7/34) of ICS assays. CD4 T cells were most likely to produce IL-2 (n=23/34, 68% positive assays) or be positive for CD154 (n=22/34, 64% positive assays) in response to a peptide pool (Table 5.4).

Table 5.4: Detectable cytokine responses of CD8 and CD4 T cells towards HBV peptide pools in ICS assays

	Detectable response/Total tested n (%)									
Peptide pool	**CD8**					**CD4**				
	IFNγ	**TNFα**	**IL-2**	**CD107a**	**Any cytokine**	**IFNγ**	**TNFα**	**IL-2**	**CD154**	**Any cytokine**
Core	3/8 (38)	2/8 (25)	6/8 (75)	1/8 (13)	8/8 (100)	4/8 (50)	3/8 (38)	6/8 (75)	6/8 (75)	7/8 (88)
Pol1-2	4/4 (100)	2/4 (50)	3/4 (75)	1/4 (25)	4/4 (100)	2/4 (50)	0/4 (0)	4/4 (100)	2/4 (50)	4/4 (100)
Pol3-4	6/10 (60)	6/10 (60)	5/10 (50)	2/10 (20)	10/10 (100)	7/10 (70)	5/10 (50)	6/10 (60)	3/10 (30)	9/10 (90)
PreS1/S2/surface	7/10 (70)	3/10 (30)	5/10 (50)	1/10 (20)	10/10 (100)	3/10 (30)	5/10 (50)	6/10 (60)	9/10 (90)	10/10 (100)
sIi	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	1/2 (50)	1/2 (50)	1/2 (50)	2/2 (100)	2/2 (100)
Any positive peptide pool	22/34 (65)	15/34 (44)	21/34 (62)	7/34 (21)	34/34 (100)	17/34 (50)	14/34 (41)	23/34 (68)	22/34 (64)	32/34 (94)

The percentage of CD8 T cells producing cytokines in response to stimulation with peptide pools ranged from 0 to 0.38% (Figure 5.8). The highest percentage of CD8 T cells to produce IFNγ was in response to the sIi peptide pool (median 0.039% of CD8 T cells, Figure 5.8.A). The highest percentage of CD8 T cells to produce TNFα (median 0.004% of CD8 T cells, Figure 5.8.B) or IL-2 (median 0.033 % of CD8 T cells, Figure 5.8.C) were in response to the pol 1-2 peptide pool. The median CD107a expression across all samples was 0, although 3 of the 10 volunteers had positive responses to one or more peptide pool (Figure 5.8.D). Most cytokine producing CD8 T cells were single cytokine producers, although over half of responders to the sIi peptide pool were double or triple producers (Figure 5.8.E).

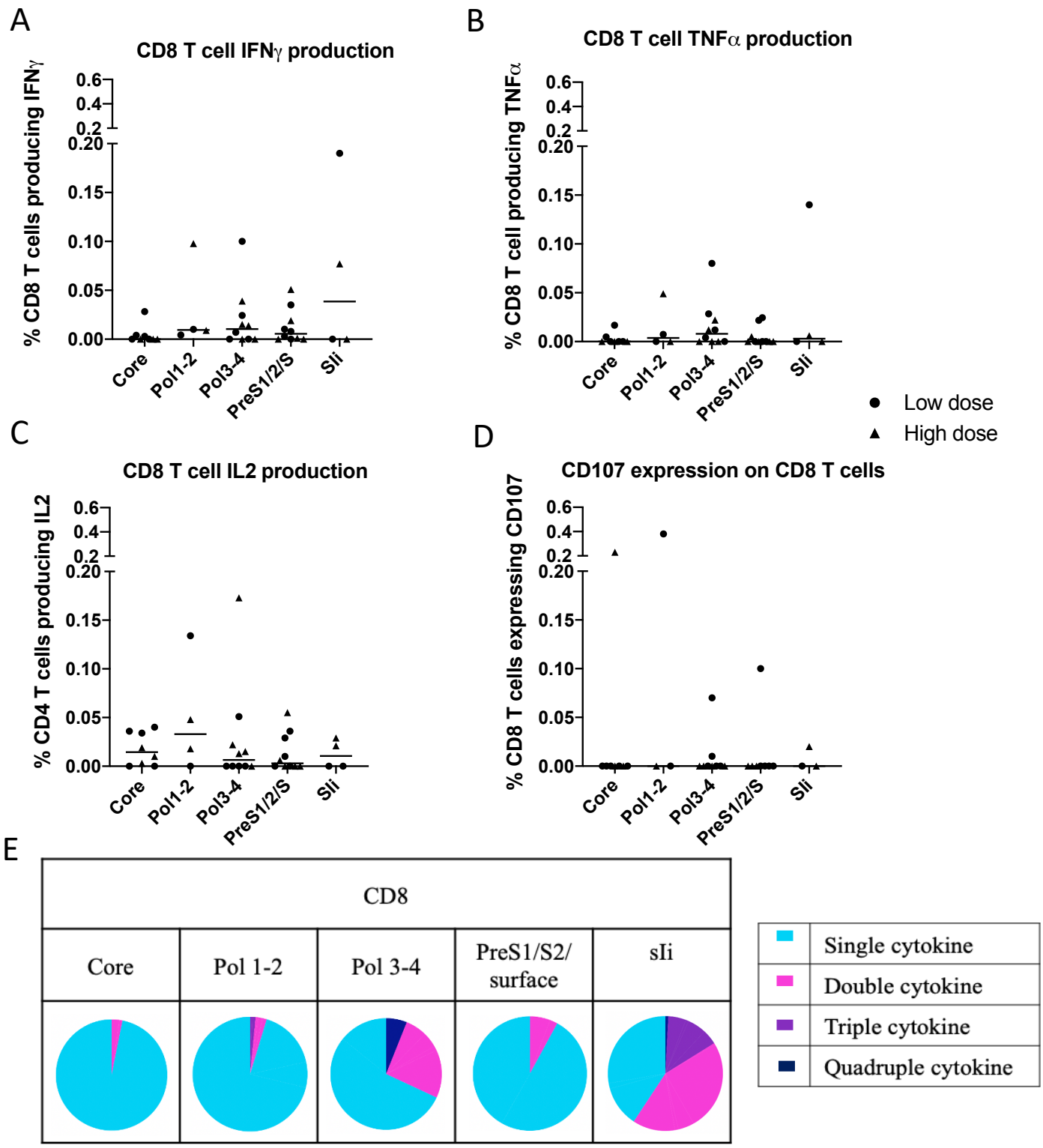


Figure 5.8 Cytokine production by CD8 T cells after ChAdOx1-HBV vaccination in healthy volunteers

Peripheral blood mononuclear cells (PBMC) from healthy participants enrolled in HBV001 at peak magnitude of total response after vaccination or the closest timepoint with available cells (day 14, day 28 or day 56) were plated with pooled peptides covering the HBV vaccine immunogen, DMSO (negative control) or PMA/ionomycin (positive control) in intracellular staining assays. From left to right peptide pools corresponded to HBV core (core), HBV polymerase (pol 1-2 and pol 3-4) HBV preS1 and pre-S2 (preS1/S2), HBV surface (surface) and truncated shark invariant chain (sIi). Cytokine production by CD8 T cells was detected by flow cytometry. Percentage of CD8 T cells producing **A:** IFNγ, **B:** TNFα, **C:** IL-2 or **D:** expressing CD107a in response to stimulation with each peptide pool. **E:** The proportion of IFNγ, TNFα, IL2 or CD107a positive CD8 T cells positive for single (blue), double (pink), triple (purple) or quadruple (navy) cytokines in response to stimulation with each peptide pool. Base of pie chart = median. All results shown after DMSO background subtraction. Median and interquartile range displayed.

The percentage of CD8 T cells producing cytokines in response to peptide pools ranged from 0 to 0.55% (Figure 5.9). The highest percentage of CD4 T cells to produce IFNγ (0.007% of CD4 T cells, Figure 5.9.A) or TNFα (0.032% of CD4 T cells, Figure 5.9.A) were in response to the sIi peptide pool and the highest percentage to produce IL-2 (median 0.032 of CD4 T cells, Figure 5.9.A) were in response to the pol 1-2 peptide pool. The highest proportion of CD154 positive CD4 cells (median 0.280 of CD4 T cells, Figure 5.9.A) was in response to the pre S1/S2/S peptide pool. Up to quarter of cytokine producing CD4 T cells in response to core, pol1-2 or pol3-4 peptide pools produced were double, triple or quadruple cytokines, whilst the remainder were largely single cytokine producers (Figure 5.9.E).

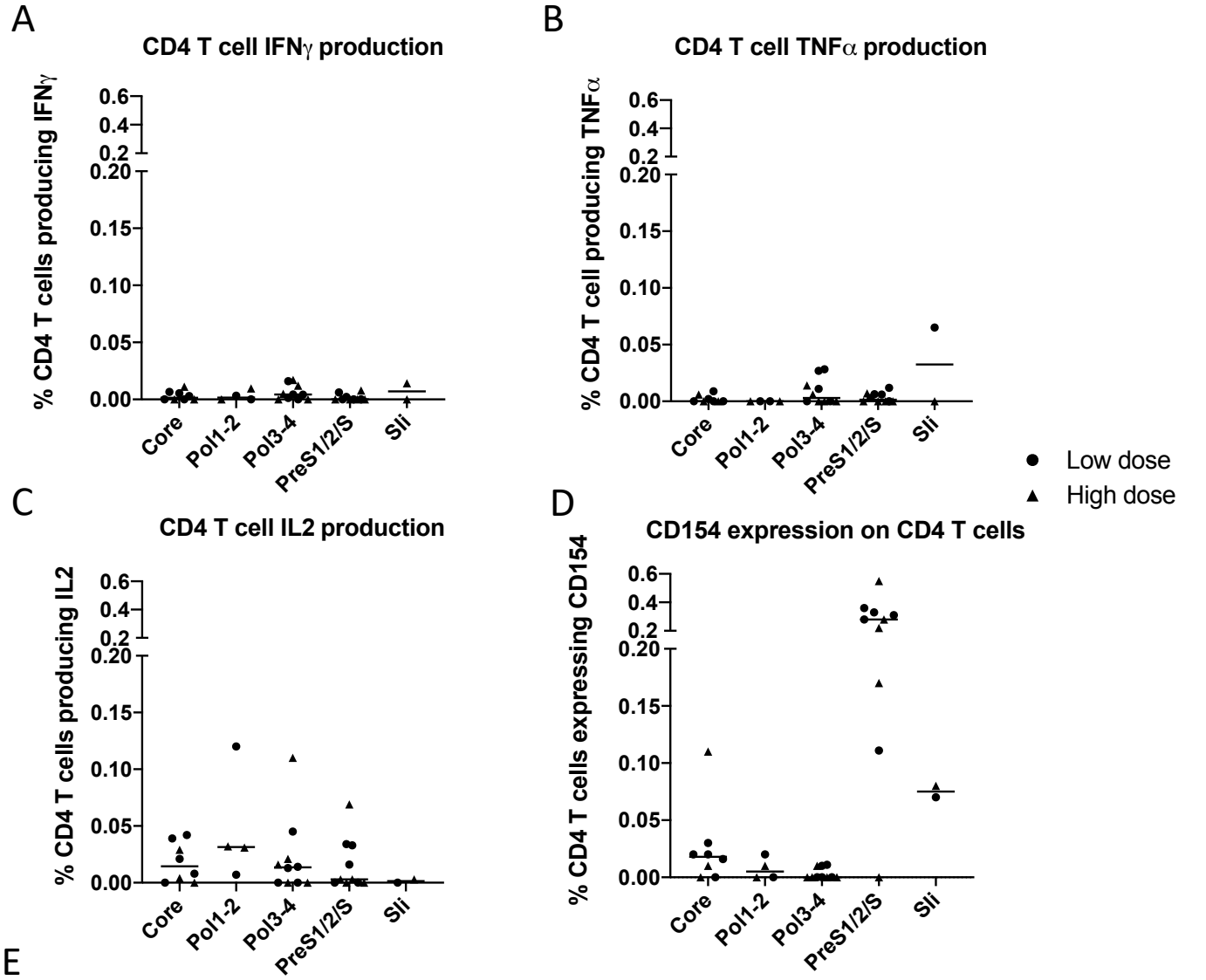


Figure 5.9 Cytokine production by CD4 T cells after ChAdOx1-HBV vaccination in healthy volunteers

Peripheral blood mononuclear cells (PBMC) from healthy participants enrolled in HBV001 at peak magnitude of total response after vaccination or the closest timepoint with available cells (day 14, day 28 or day 56) were plated with pooled peptides covering the HBV vaccine immunogen, DMSO (negative control) or PMA/ionomycin (positive control) in intracellular staining assays. From left to right peptide pools corresponded to HBV core (core), HBV polymerase (pol 1-2 and pol 3-4) HBV preS1 and pre-S2 (preS1/S2), HBV surface (surface) and truncated shark invariant chain (sIi). Cytokine production by CD4 T cells was detected by flow cytometry. Percentage of CD4 T cells producing **A:** IFNγ, **B:** TNFα, **C:** IL-2 or **D:** expressing CD154 in response to stimulation with each peptide pool. **E:** The proportion of IFNγ, TNFα, IL2 or CD107a positive CD4 T cells positive for single (blue), double (pink), triple (purple) or quadruple (navy) cytokines in response to stimulation with each peptide pool. Base of pie chart = median. All results shown after DMSO background subtraction. Median and interquartile range displayed.

5.5.5 T cell responses towards Hepatitis B surface antigen are present in some healthy volunteers, who have not received ChAdOx1-HBV vaccination

Unexpectedly, at baseline 25% (n=2) of healthy volunteers enrolled in HBV001 had T cell responses towards the HBV surface peptide pool. In order to test for any background reactivity to the peptide pools corresponding to the ChAdOx1-HBV immunogen in other unvaccinated healthy volunteers, peptide pools were incubated with peripheral blood mononuclear cells (PBMC) from 10 unvaccinated healthy volunteers recruited into the Oxford BRC GI biobank in IFNγ ELISpot assays. Responses were detectable in 60% (n=6) of these healthy volunteers to the peptide pools corresponding to the HBV surface region (median 81, range 2 - 946 IFNγ SFU per million PBMCs, Figure 5.10).

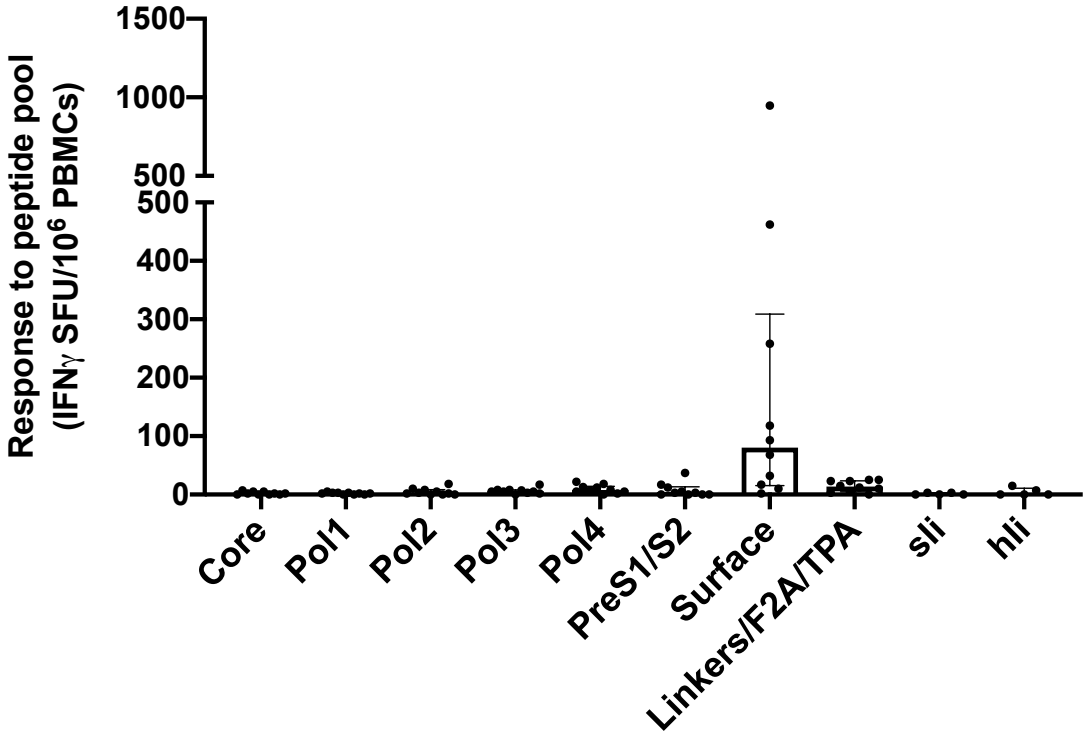


Figure 5.10 T cell responses towards Hepatitis B peptide pools in healthy volunteers who have not received ChAdOx1-HBV vaccination

Peripheral blood mononuclear cells (PBMC) from healthy volunteers, who had not received ChAdOx1-HBV vaccination, were incubated with pools of peptides corresponding to the entire vaccine immunogen. From left to right peptide pools corresponded to HBV core (core), HBV polymerase (pol 1-4), HBV preS1 and pre-S2 (preS1/S2), HBV surface (surface), linkers F2A and TPA, truncated shark invariant chain (sIi) and human invariant chain (hIi). The magnitude of responses to vaccination were recorded as IFNγ spot-forming units per million PBMC (SFU/10^6 PBMC). Median and interquartile range shown.

To determine the antigen specificity of T cell responses to HBV surface, PBMCs from the 5 donors with positive responses were stimulated with peptide minipools (G1-G7) containing 8 or 9 peptides making up the HBV surface pool. Responses were detected in PBMCs stimulated with the G1 minipool (median 73, IQR 8-122 SFU per million PBMC) and the G7 minipool (median 133, IQR 54-259 SFU per million PBMC, Figure 5.11.A). PBMCs from 3 volunteers with positive responses were then stimulated with individual peptides (15 amino acids in length) comprising the G1 and G7 minipools. Responses were detectable in the G1 minipool towards peptide numbers 346-348 with amino acid sequence FLGPLLVLQAGFFLLTRILTIPQ (median response 38, IQR 8-45 SFU per million PBMCs). In the G7 minipool responses were detectable towards peptide number 389 with amino acid sequence FLPLLPIFFCLWVYI (median 100, IQR 20-188 SFU per million PBMCs) and peptide number 396 with amino acid sequence VGLSPTVWLSVIWMM (median 28, IQR 0-50 SFU per million PBMCs, Figure 5.11.B).

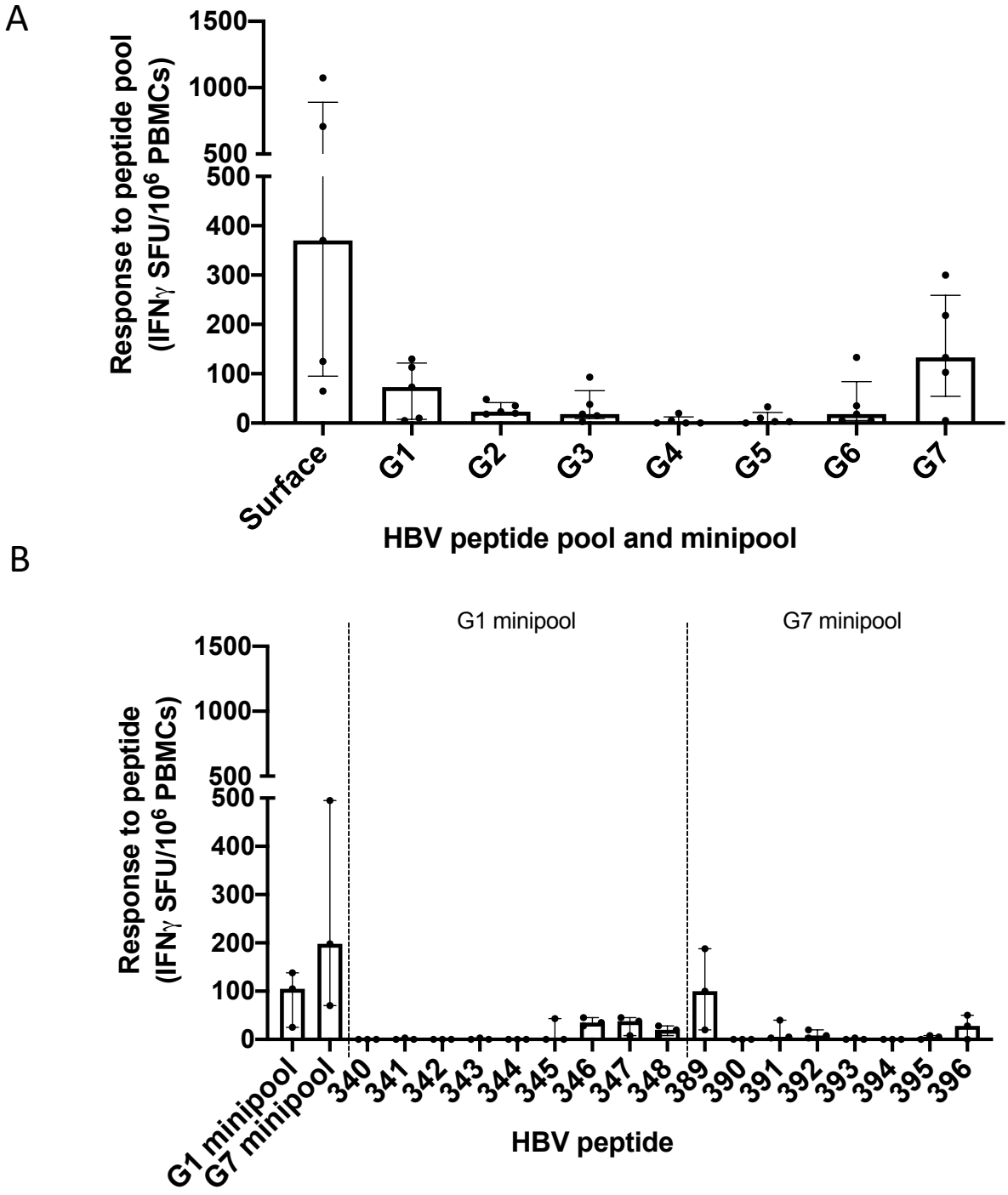


Figure 5.11 Fine specificities of HBV surface specific T cells in healthy volunteers, who have not received ChAdOx1-HBV vaccination

Peripheral blood mononuclear cells (PBMC) from healthy volunteers, who had not received ChAdOx1-HBV vaccination, were incubated with **A:** the HBV surface peptide pool and minipools G1-G7 or **B:** HBV peptides (15 amino acids in length) numbers 340-348 (corresponding to the G1 minipool) and 389-396 (corresponding to the G7 minipool) in interferon-gamma (IFNγ) ELISpot assays. The magnitude of responses to vaccination were recorded as IFNγ spot-forming units per million PBMC (SFU/10^6 PBMC). Median and interquartile range shown.

PBMCs were then stimulated with the HBV surface peptide pool in intracellular staining assays and cytokine production from CD8 and CD4 T cells was detected by flow cytometry (gating strategy Figure 5.12.A). The highest responses were detected in donor KW-21, in whom 0.5% of CD4 T cells produced IL-2 (Figure 5.12.B).

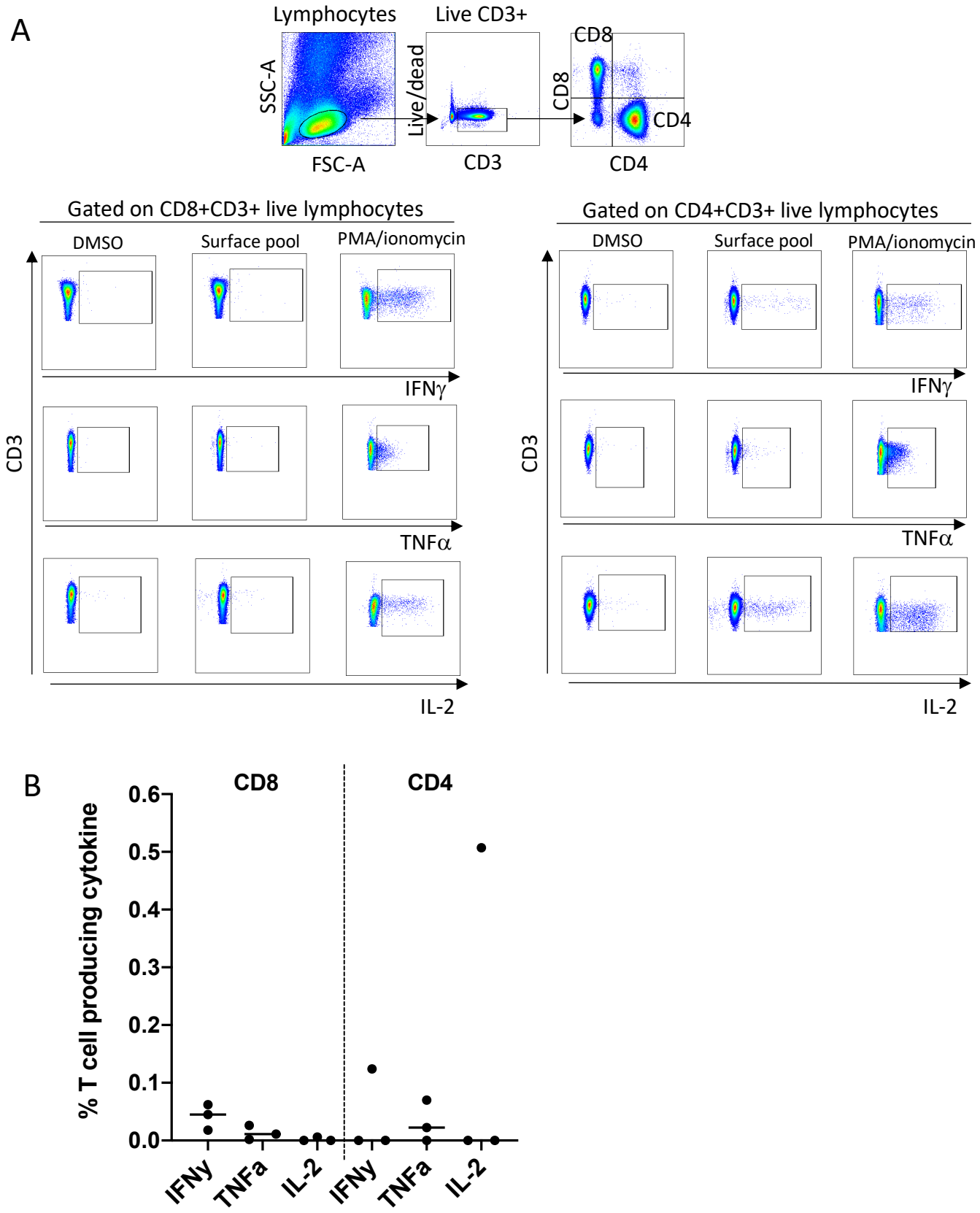


Figure 5.12 Cytokine production of HBV surface specific T cells in healthy volunteers, who have not received ChAdOx1-HBV vaccination

Peripheral blood mononuclear cells (PBMC) from healthy volunteers, who had not received ChAdOx1-HBV vaccination, were incubated with the HBV surface peptide pool, DMSO (negative control) or PMA/ionomycin (positive control) in intracellular staining assays. Cytokine production by CD8 and CD4 T cells were detected by flow cytometry. **A:** Representative gating strategy for cytokine detection. **B:** Percentage of cytokines IFNγ, TNFα or IL-2 produced by CD8 or CD4 T cells in response to HBV surface peptide pools. Median and interquartile range shown.

The regions of the HBV surface antigen able to induce T cell responses without prior ChAdOx1-HBV vaccination, were investigated for known MHC class II restricted epitopes using Immune Epitope Database and Analysis Resource (IEDB) (445) and further literature searches of relevant articles. This revealed several MHC class II epitopes within these regions have been reported to induce CD4 T cell responses towards Hepatitis B surface antigen (Table 5.2). The majority of the epitopes were detected in people after vaccination with the recombinant HBsAg vaccine (286–288,517–523), however in some cases, CD4 T cell responses were detected in people with current or previous HBV infection (524–527). MHC class I restricted epitopes have also been reported within these regions of HBsAg, however these have all been described in the context of HBV infection rather than after vaccination with recombinant HBsAg (174,468,528–530).

Table 5.5: Known MHC class II restricted epitopes that induce CD4 T cells specific for Hepatitis B surface antigen

Peptide	Amino acid sequence																																					HLA restriction	Reference
345 - 347					**F**	**L**	**G**	**P**	**L**	**L**	**V**	**L**	**Q**	**A**	**G**	**F**	**F**	**L**	**L**	**T**	**R**	**I**	**L**	**T**	**I**	**P**	**Q**														
	I	T	S	G	F	L	G	P	L	L	V	L	Q	A	G	F	F	L	L	T	R	I	L	T	I	P	Q	S	L	D									HLA-DR/DP/DQ	(517)
			S	G	F	L	G	P	L	L	V	L	Q	A	G	F	F	L	L	T	R																		-	(286)
					F	L	G	P	L	L	V	L	Q	A	G	F	F	L	L	T	R	I	L	T	I	P	Q														HLA-DR	(524)
											V	L	Q	A	G	F	F	L	L	T	R	I	L	T	I	P	Q	S	L	D										-	(517)
													Q	A	G	F	F	L	L	T	R	I	L	T	I	P	Q	S													HLA-DR/DQ	(288,521,525)
													Q	A	G	F	F	L	L	T	R	I	L	T	I	P	Q	S	L	D											-	(519,523)
														A	G	F	F	L	L	T	R	I	L	T	I	P	Q	S													HLA-DR	(520,526)
															G	F	F	L	L	T	R	I	L	T	I	P	Q														-	(522)
																F	F	L	L	T	R	I	L	T	I	P	Q	S	L	D											HLA-DR/DQ	(286,517)
																F	F	L	L	T	R	I	L	T	I																HLA-DP/DP/DQ	(517,518,525,527)
																		L	L	T	R	I	L	T	I																HLA-DR/DP	(287)
																		L	L	T	R	I	L	T	I	P	Q	S	L	D	S	W	W	T	S	L	N			HLA-DR/DP	(287)
																			L	T	R	I	L	T	I	P	Q														HLA-DR/DQ	(525)
																				T	R	I	L	T	I	P	Q	S	L	D	S	W	W	T	S	L	N	F	-	(286)
																							L	T	I	P	Q	S	L	D	S									HLA-DR/DQ	(525)
389													**F**	**L**	**P**	**L**	**L**	**P**	**I**	**F**	**F**	**C**	**L**	**W**	**V**	**Y**	**I**														
								S	I	L	S	P	F	L	P	L	L	P	I	F	F	C	L	W	V	Y	I														-	(287)
													F	L	P	L	L	P	I	F	F	C	L	W	V	Y	I														-	(287)
														L	P	L	L	P	I	F	F	C	L	W	V	Y	I														-	(519,523)

| | | |

5.5.6 Baseline characteristics of patients with chronic HBV in HBV001

The baseline characteristics of the 7 patients with chronic HBV enrolled in HBV001 to date are shown in Table 5.6. Cohort 3 received low dose (2.5 x10^9 vp) and cohort 4 received high dose (2.5 x10^{10} vp) ChAdOx1-HBV. The median age was 40 (range 31-48), 57% (n=4) were male and 57% (n=4) were of Asian ethnicity. All patients had HBV DNA of under 40 IU/mL and in 86% (n=8) HBV DNA was undetectable. HBeAg was positive in 29% (n=2). Median baseline ALT was 24 IU/L (range 14-66) and in all patients it was under 1.5x the upper limit of normal. Median HBsAg was 2421 IU/mL (range 146-5207).

Table 5.6 Baseline characteristics of patients with chronic HBV enrolled in HBV001.

ND = Not detected, D = detected.

Volunteer ID	Cohort	Age	Gender	Ethnicity	ALT (IU/L)	HBV DNA (IU/mL)	HBeAg	HBsAg
02202	3	33	F	Asian	14	ND	D	2421
02203	3	35	F	Caucasian	23	ND	ND	146
02204	3	48	M	Caucasian	20	ND	ND	908
02209	3	41	M	Asian	45	ND	ND	2567
02210	3	47	M	Caucasian	66	ND	ND	4178
02212	3	31	M	Asian	24	D	D	1896
02211	4	40	F	Asian	41	ND	ND	5207

5.5.7 Vaccination with ChAdOx1-HBV in patients with chronic Hepatitis B infection is well tolerated

Vaccination with ChAdOx1-HBV was well tolerated in all participants with chronic HBV infection receiving either low dose or high dose vaccine (cohorts 3 and 4). No local adverse reactions were recorded (Figure 5.13.A) and mild systemic adverse events were recorded in 29% (n=2) participants (Figure 5.13.B). One adverse event was dizziness after vaccination, which resolved within 24 hours. The other adverse event was a planned admission for a day-case operative procedure, not deemed to be vaccine related. Alanine aminotransferase levels were all under 1.5x the upper limit of normal (ULN) at baseline and remained so throughout study follow up (Figure 5.13.C).

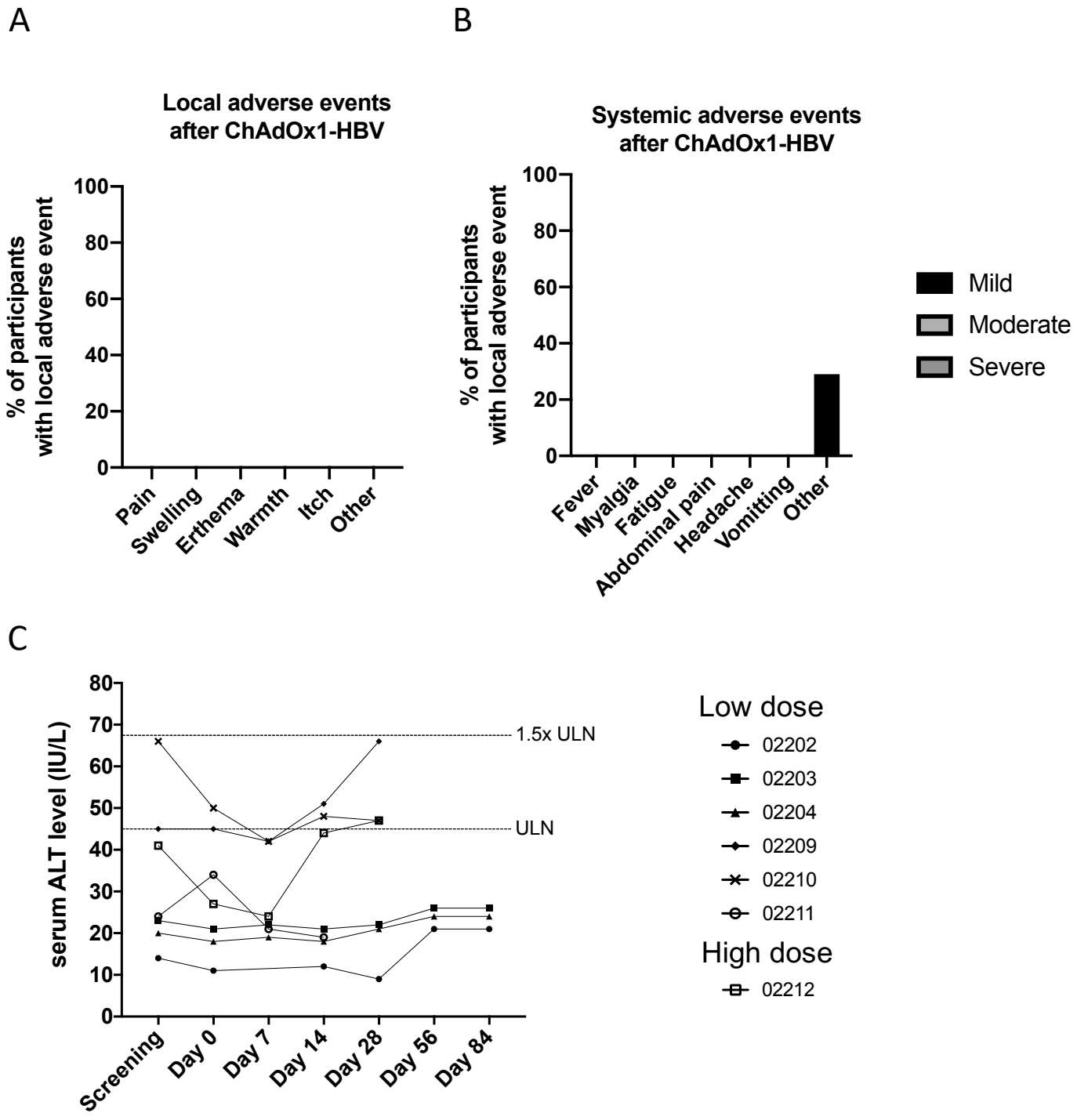


Figure 5.13 Adverse events after vaccination with ChAdOx1-HBV in patients with chronic HBV

A: Local and **B:** systemic adverse reactions within 7 days as a percentage of all patients with chronic HBV infection receiving ChAdOx1-HBV enrolled in cohorts 3 and 4 of HBV001. Mild, moderate, or severe grading shown. Other = dizziness after vaccination and planned day-case admission for laparoscopic procedure. C: Longitudinal serum alanine aminotransferase (ALT) levels (IU/L) from day 0-84 post vaccination. Upper limit of normal (ULN) and 1.5x ULN indicated.

5.5.8 Vaccination with ChAdOx1-HBV induces or boosts HBV specific T cells responses in some patients with chronic HBV infection

PBMCs from 7 patients with chronic HBV infection enrolled in HBV001 were collected at day 0, 14, 28, 56 and 84 after vaccination with ChAdOx1-HBV and were tested for T cell responses in IFNγ ELISpot assays. The total T cell response towards the HBV components of the vaccine immunogen were calculated by the summation of responses towards the HBV peptide pools core, pol1-pol4, preS1/S2 and surface. At baseline (timepoint day 0), responses were detectable in some participants towards the pol4 peptide pool (median 10, range 0-60 SFU per million PBMC) and surface peptide pools (median 20, range 0-163 SFU per million PBMC, Figure 5.14).

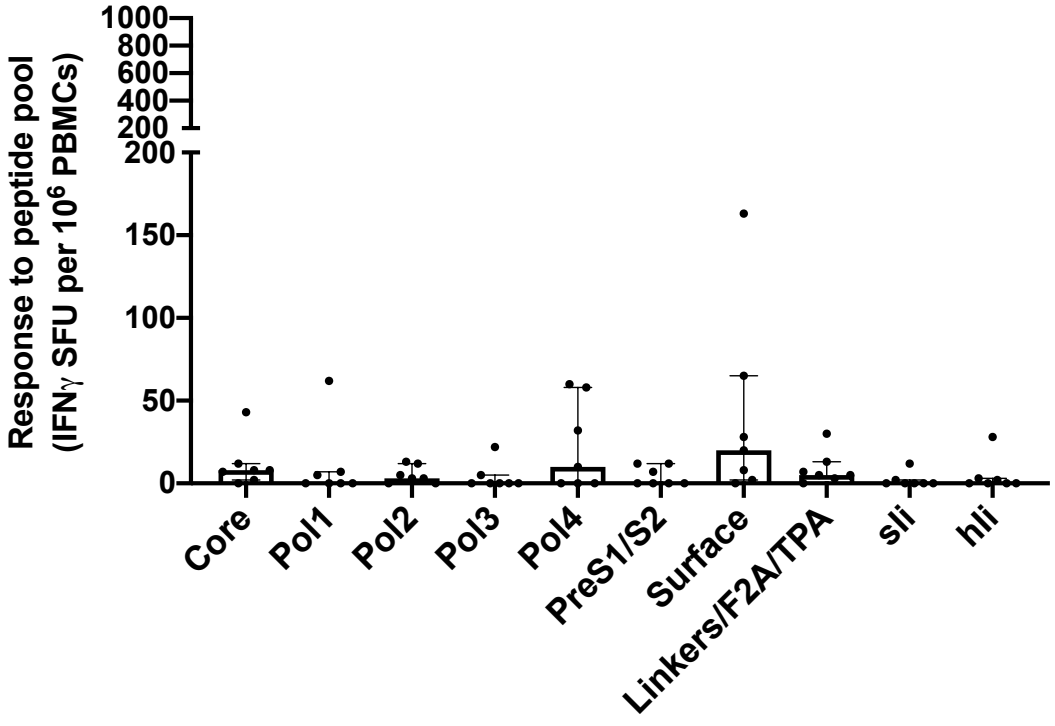


Figure 5.14 Baseline responses to HBV peptide pools in patients with chronic HBV enrolled into HBV001

Peripheral blood mononuclear cells (PBMC) from individuals with chronic HBV enrolled in HBV001 were isolated timepoint day 0 prior to vaccination. PBMCs were incubated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. From left to right peptide pools corresponded to HBV core (core), HBV polymerase (pol 1-4), HBV preS1 and pre-S2 (preS1/S2), HBV surface (surface) and truncated shark invariant chain (sIi). The magnitude of responses to vaccination were recorded as IFNγ spot-forming units per million PBMC (SFU per 10^6 PBMC). Median and interquartile range shown.

After vaccination with ChAdOx1-HBV, positive responses (responses equal to or exceeding 50 IFN-γ spot forming units per million PBMC, over 2-fold greater than the background well and at least 2 times higher than baseline) were detected in 4 of the 7 participants (Table 5.7 and Figure 5.15). Participant 02204 received low dose ChAdOx1-HBV and total response increased from 277 to 822 SFU/million PBMCs between day 0 and day 28. Participant 02210 received low dose ChAdOx1-HBV and total response increased from 25 to 97 SFU/million PBMCs between day 0 and day 14, declining thereafter. Participant 02212 received low dose ChAdOx1-HBV and total response increased from 2 to 288 SFU/million PBMCs day 0 and day 14 but decreased at day 28. Finally, participant 02211 received high dose ChAdOx1-HBV and total response increased from 163 to 398 SFU/million PBMCs between day 0 and day 28. Overall, there was no significant difference in the magnitude of total responses between day 0 and any timepoint after vaccination.

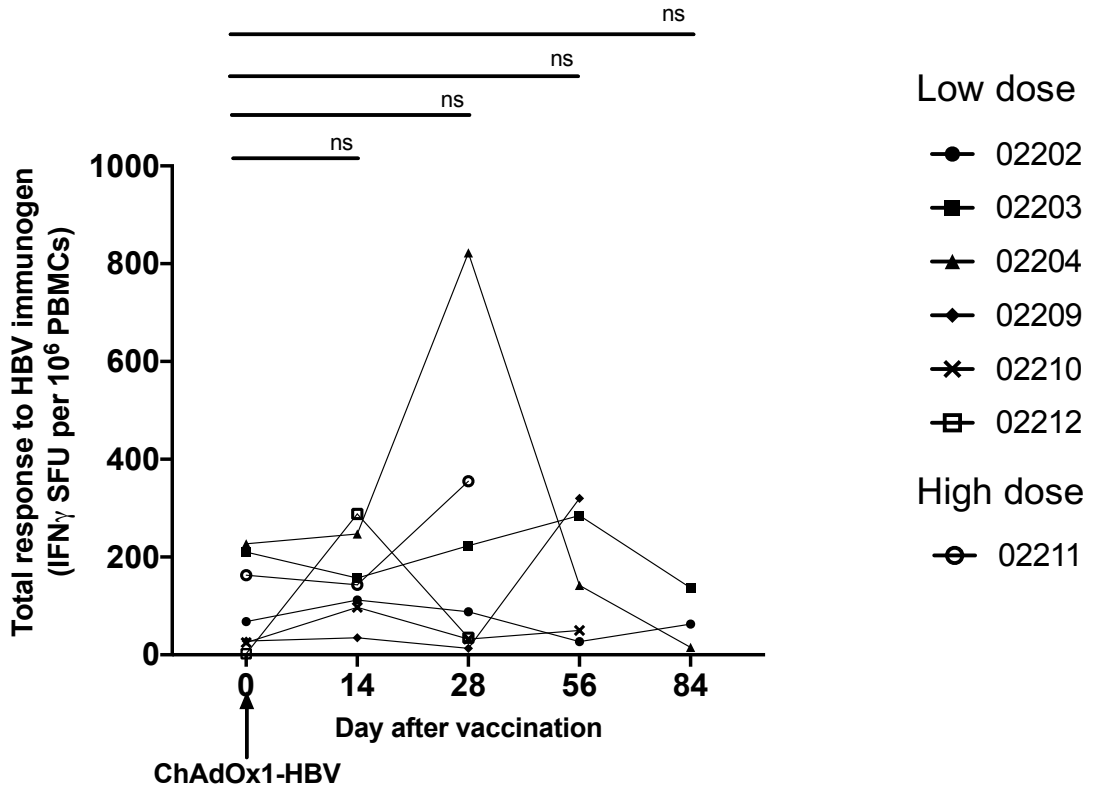


Figure 5.15 Total T cell responses after ChAdOx1-HBV vaccination in healthy volunteers

Peripheral blood mononuclear cells (PBMC) from individuals with chronic HBV enrolled in HBV001 were isolated timepoint day 0 and then days 14, 28, 56, 84 after intramuscular vaccination with ChAdOx1-HBV at either low dose (2.5 x10^9 viral particles(vp)) or high dose (2.5 x10^{10} vp, participant 02011). PBMCs were incubated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. Total vaccine response magnitude at all timepoints (summation of the response to all peptide pools corresponding to HBV components of the vaccine immunogen including core, pol1, pol2, pol3, pol4, preS1/S2 and surface) were recorded as IFNγ spot-forming units per million PBMC (SFU per 10^6 PBMC). Kruskal-Wallis test with Dunn's multiple comparisons used to compare multiple groups. ns = not significant

Table 5.7: Positive IFNγ ELISpot responses in patients with chronic HBV at day 14 and 28 post vaccination with ChAdOx1-HBV

A positive assay was defined as a response equal to or exceeding 50 IFN-γ spot forming units per million PBMC, over 2-fold greater than the background well and at least 2 times higher than baseline.

Participant	Day 14 total response magnitude SFU/10^6 PBMC	Day 14 Positive/negative	Day 28 total response magnitude SFU/10^6 PBMC	Day 28 Positive/negative
02202	112	Negative	88	Negative
02203	157	Negative	223	Negative
02204	247	Negative	822	Positive
02209	27	Negative	13	Negative
02210	97	Positive	32	Negative
02212	288	Positive	35	Negative
02211	143	Negative	355	Positive

Longitudinal T cell responses towards HBV core specific increased in participant 02204 from 0 SFU/million PBMC at day 0 to 198 SFU/million PBMC at day 28 and in participant 02211 from 62 SFU/million PBMC at day 0 to 156 SFU/million PBMC at day 28 (Figure 5.16.A). Responses towards HBV polymerase also increased in participant 02204 from 50 SFU/million PBMC at day 0 to 472 SFU/million PBMC at day 28 and in participant 02211 from 65 SFU/million PBMC at day 0 to 177 SFU/million PBMC at day 28 (Figure 5.16.B). T cell responses to HBV pre-S1/S2 and surface remained low throughout follow up (Figure 5.16.C and D). Overall, responses were not significantly increased from baseline towards any

HBV region at any timepoint after vaccination. There were no responses towards non-HBV vaccine components (Figure 5.17A-C). No decline in HBsAg levels occurred during follow up of any participant (Figure 5.17.D).

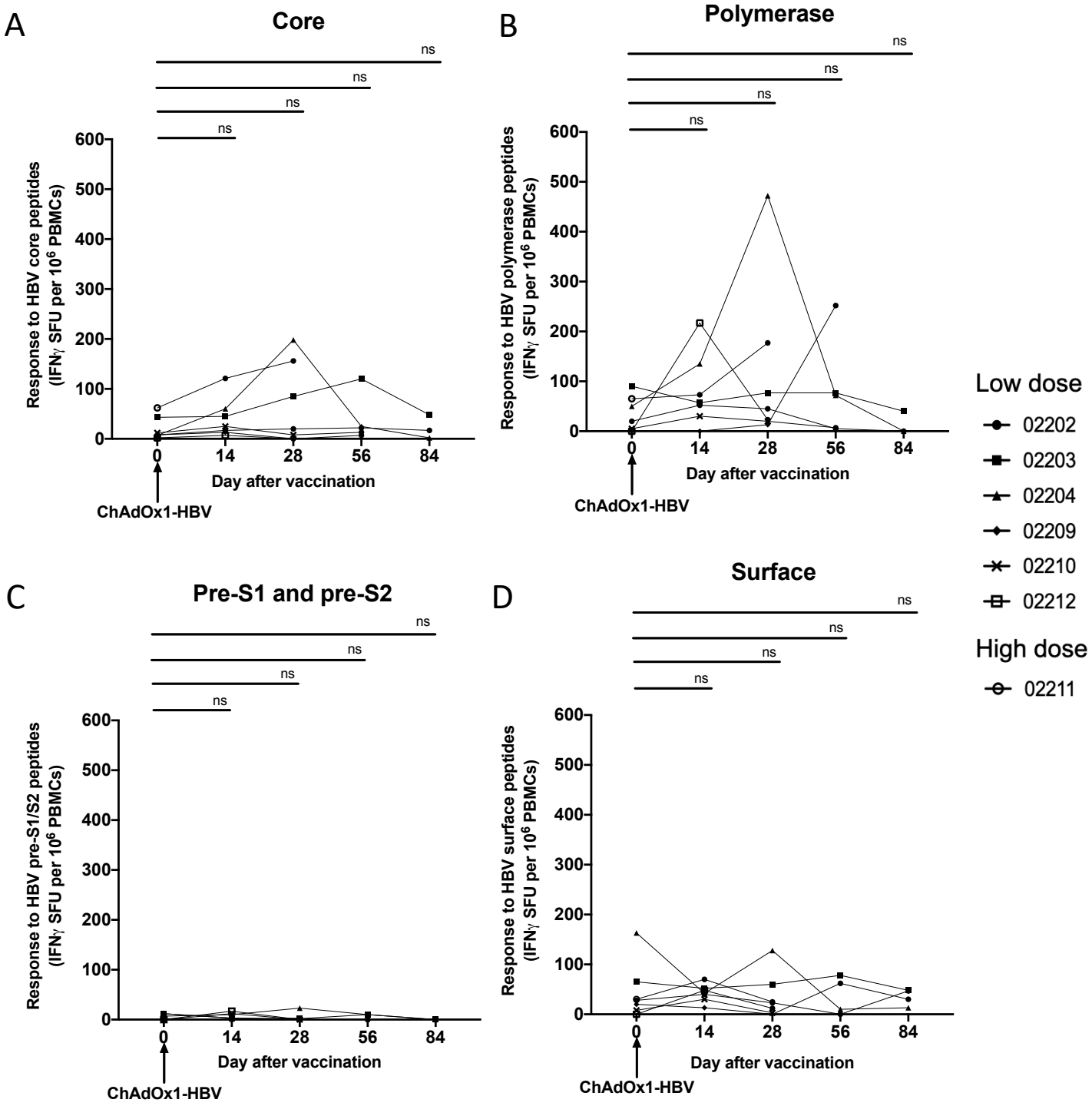


Figure 5.16 HBV specific T cell responses after vaccination in patients with chronic HBV

Peripheral blood mononuclear cells (PBMC) from individuals with chronic HBV enrolled in HBV001 were isolated timepoint day 0 and then days 14, 28, 56, 84 after intramuscular vaccination with ChAdOx1-HBV at either low dose (2.5 x10^9 viral particles(vp)) or high dose (2.5 x10^{10} vp, participant 02011). PBMCs were incubated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. Longitudinal vaccine response magnitude towards **A:** HBV core (peptide pool core), **B:** HBV polymerase (peptide pools pol1-pol4), **C:** HBV pre S1 and pre S2 (peptide pools preS1/S2), **D:** HBV surface (surface peptide pool) as IFNγ spot-forming units per million PBMC (SFU per 10^6 PBMC). Kruskal-Wallis test with Dunn's multiple comparisons used to compare multiple groups. ns = not significant

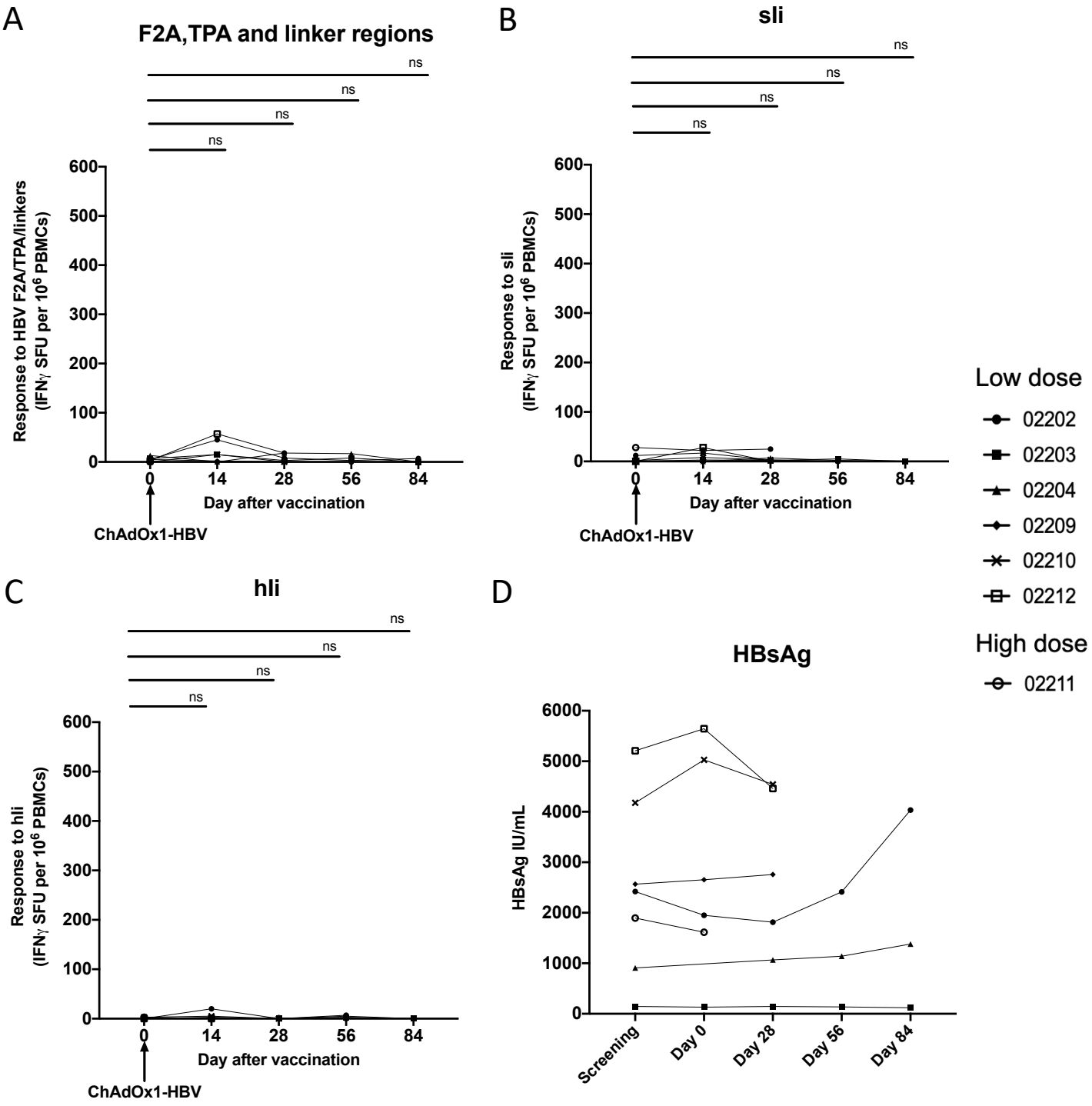


Figure 5.17 non-HBV specific T cell responses and HBsAg levels after vaccination in patients with chronic HBV

Peripheral blood mononuclear cells (PBMC) from individuals with chronic HBV enrolled in HBV001 were isolated timepoint day 0 and then days 14, 28, 56, 84 after intramuscular vaccination with ChAdOx1-HBV at either low dose (2.5 x10^9 viral particles(vp)) or high dose (2.5 x10^{10} vp, participant 02011). PBMCs were incubated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. Longitudinal vaccine response magnitude towards **A:** vaccine linker regions, F2A and TPA (peptide pool linker/F2A/TPA), **B:** truncated shark invariant chain (peptide pool sIi) and **C:** non-vaccine component human invariant chain (peptide pool hIi), as IFNγ spot-forming units per million PBMC (SFU per 10^6 PBMC). Kruskal-Wallis test with Dunn's multiple comparisons used to compare multiple groups. ns = not significant. **D:** Longitudinal quantitative HBsAg levels (IU/mL).

5.5.9 T cells induced by vaccination recognise HBV genotype C and D peptides

Nine genotypes of the HBV genome have been described (A-I), which differ in amino acid sequences and geographical distribution (47). As genotype D infectious are estimated to account for the second highest number of HBV infections after genotype C (47), the cross-reactivity of ChAdOx1-HBV vaccine induced genotype C T cell responses with genotype D responses were investigated. PBMCs from vaccinated individuals enrolled in HBV001 were plated in ELISpot assays with HBV peptide pools based on genotype C and corresponding genotype D sequences.

In the two healthy volunteers tested, genotype C and genotype D responses were comparable at day 28 after vaccination (median genotype C total response 1432 and genotype D total response 814 SFU per million PBMCs, (Figure 5.18).

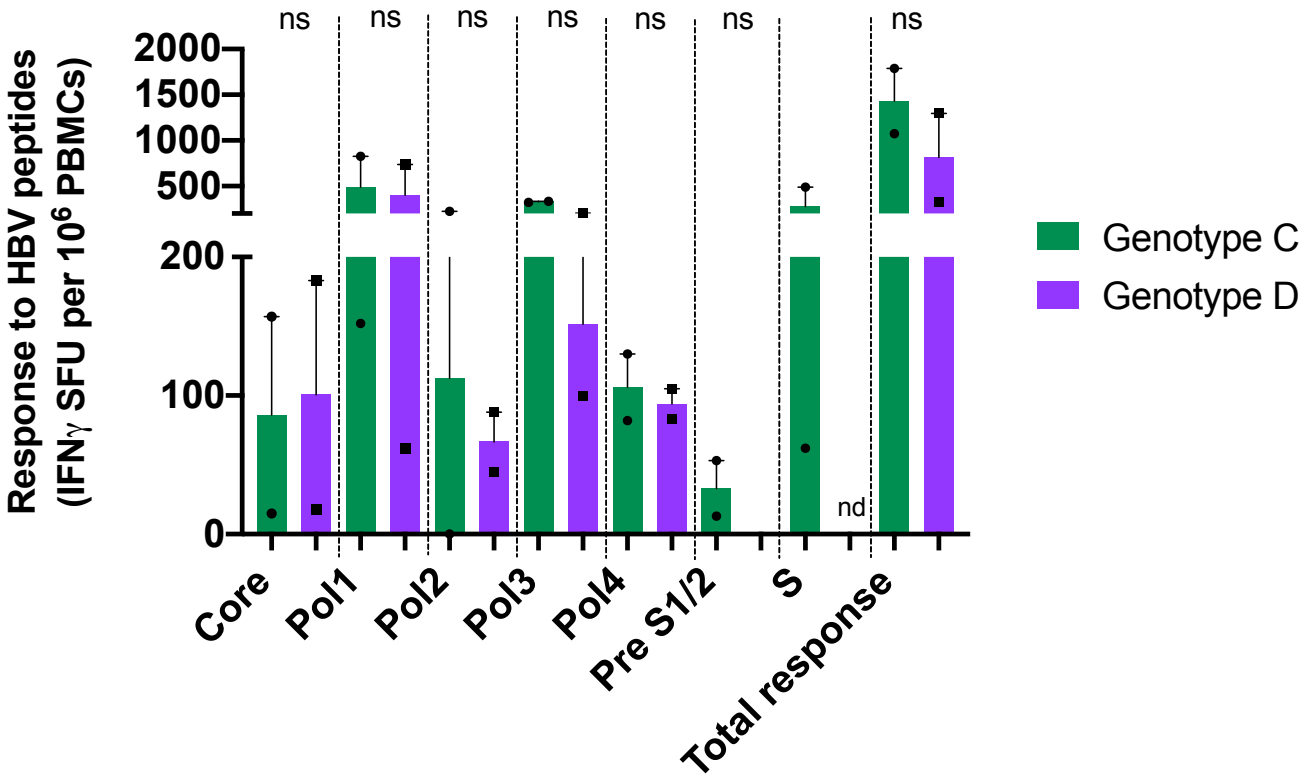


Figure 5.18 T cell responses to genotype C and genotype D HBV peptides after ChAdOx1-HBV vaccination in healthy volunteers

Peripheral blood mononuclear cells (PBMC) from 2 healthy volunteers enrolled in HBV001 were isolated at day 28 post ChAdOx1-HBV vaccination and were incubated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. From left to right peptide pools corresponded to genotype C (green) or genotype D (purple) HBV core (core), HBV polymerase (pol 1-4), HBV preS1 and pre-S2 (preS1/S2), HBV surface (surface). Total response is the summation of all responses to each peptide pool, were recorded as IFNγ spot-forming units per million PBMC (SFU/10^6 PBMC). Median and interquartile range shown. 2-way ANOVA with Sidak's multiple comparisons used to compare multiple groups. ns = not significant.

In patients with chronic HBV infection, total T cell responses to genotype D HBV peptides were significantly higher than genotype C peptides (median genotype C total response 68 and genotype D total response 247 SFU per million PBMCs, Figure 5.19.A). This difference was accounted for by strong positive responses to the genotype D polymerase 3 (pol3) peptide pool in participants 02209 and 02212, (1570 and 1380 SFU/million PBMCs respectively), without corresponding response to the genotype C pol3 peptide pool. By day 28 after vaccination there were no significant differences in the magnitude of responses towards genotype C compared to genotype D HBV peptide pools (Figure 5.19.B). Participants 02209 and 02212 still had strong responses to the genotype D polymerase 3 (pol3) peptide pool (1452 and 829 SFU per million PBMCs respectively).

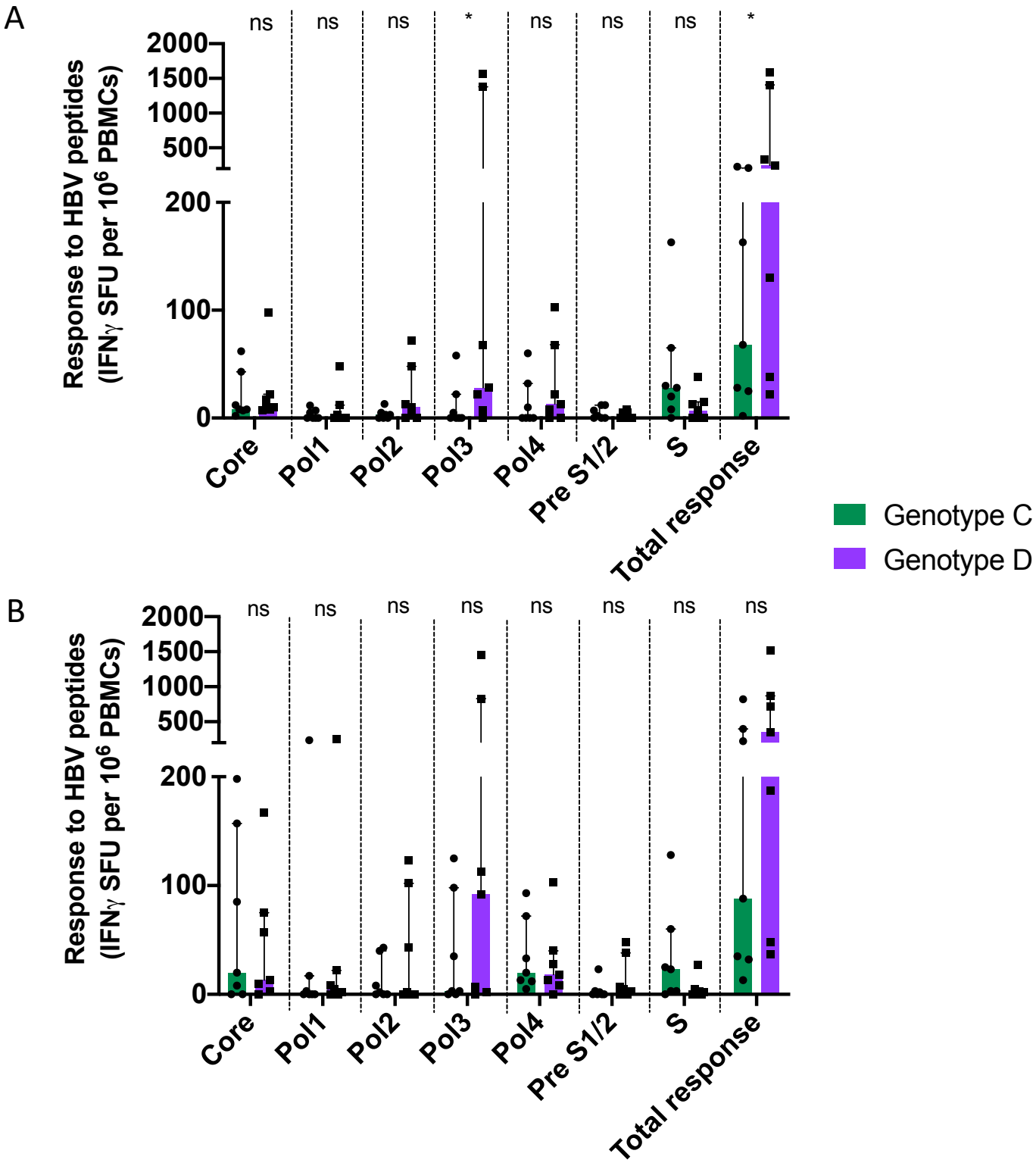


Figure 5.19 T cell responses to genotype C and genotype D HBV peptides after ChAdOx1-HBV vaccination in patients with chronic HBV

Peripheral blood mononuclear cells (PBMC) from 7 patients with chronic HBV enrolled in HBV001 were isolated at day 0 (**A**) and day 28 (**B**) post ChAdOx1-HBV vaccination and were incubated with pools of peptides corresponding to the entire vaccine immunogen in interferon-gamma (IFNγ) ELISpot assays. From left to right peptide pools corresponded to genotype C (green) or genotype D (purple) HBV core (core), HBV polymerase (pol 1-4), HBV preS1 and pre-S2 (preS1/S2), HBV surface (surface). Total response is the summation of all responses to each peptide pool, were recorded as IFNγ spot-forming units per million PBMC (SFU/10^6 PBMC). Median and interquartile range shown. 2-way ANOVA with Sidak's multiple comparisons used to compare multiple groups. * $p<0.05$, ns = not significant.

5.6 DISCUSSION

The findings in this chapter support the primary hypothesis that vaccination with ChAdOx1-HBV is well tolerated and can induce HBV specific T cells in healthy volunteers and in some patients with chronic HBV.

Vaccination with intramuscular ChAdOx1-HBV at either low or high dose in healthy volunteers is tolerated well, with nearly all recorded adverse reactions mild in severity. This is consistent with large studies of healthy volunteers vaccinated with the ChAdOx1 vectored severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) vaccine ChAdOx1-nCov19, in whom the majority of vaccine associated adverse events were mild (344,345,413).

ELISpot assays revealed that in some healthy volunteers HBV surface specific T cell responses are present prior to vaccination with ChAdOx1-HBV. Flow cytometry and intracellular staining revealed these to be CD4 T cells directed towards regions within the HBV surface antigen, previously described as MHC class II epitopes induced after receiving prophylactic recombinant HBsAg vaccine (286–288,517–523). As all the healthy volunteers enrolled into HBV001 were HBsAg and anti-HBc Ab negative at baseline, previous HBV infection or exposure is unlikely. However, they were all HBsAb positive at recruitment and therefore it is likely that the baseline HBV surface specific T cell responses were induced by previous vaccination with prophylactic HBsAg vaccine.

In addition to baseline T cell responses towards the HBV surface peptide pool, one healthy volunteer had low but detectable responses towards HBV core and HBV polymerase prior to vaccination with ChAdOx1-HBV. On enrollment, this participant was HBsAg negative, anti-

HBc Ab negative and anti-HBs Ab positive, suggesting they were not previously or currently infected with HBV, but had been vaccinated with recombinant HBsAg vaccination.
None of the other healthy volunteers tested had such responses at baseline. However, it was not possible to perform full ELISpot assays on all of the samples collected at baseline, as for some only a low number of PBMCs were available. Therefore, it is not possible to confirm whether all other participants had negative responses to the HBV peptide pools prior to vaccination.

Assessing the T cell immunogenicity of therapeutic vaccines for HBV in healthy volunteers is important to ascertain their baseline performance in conditions where HBV specific T cell tolerance and exhaustion are not present. In healthy volunteers, vaccination with ChAdOx1-HBV induced high magnitude T cell responses. Both CD8 and CD4 T cells were generated producing IFNγ, TNFα or IL-2 in response to their cognate antigens. These results are of a similar magnitude and functionality to T cells induced by the ChAdOx1-nCoV-19 vaccine, which uses the same vector with a different immunogen (343,344,427).

Only one previous trial of a candidate HBV therapeutic vaccine has published ex-vivo T cell immunogenicity data towards peptides across the vaccine immunogen (325). In this trial, 3-6 doses of yeast derived vaccine GS-4774 was given to healthy volunteers at weekly or monthly intervals. A positive ELISpot response was defined similarly to HBV001, as a response of over 50 SFU/million PBMCs and 2-fold change in response magnitude compared to baseline. Using this definition, on day 29 (after 1 or 3 doses of vaccine), positive responses were detected in 51% (n=30/59) of all participants and in 74% (n=14/19) of participants receiving the highest vaccine dose (325). This is lower than the 90% of positive responses detected in HBV001 at a similar timepoint. Although direct comparison of

response magnitude should be interpreted with caution, ChAdOx1-HBV appears to induce higher magnitude responses than GS-4774, with 100% of healthy volunteers achieving a total vaccine response of over 200 SFU per million PBMCs at day 28 compared to only 12% (n=7/59) of healthy volunteers after receiving GS-4774 (325). Randomised trials of GS-4774 in patients with chronic HBV have had disappointing results, which may in part be due to its poor ability to induce HBV specific T cells reliably, even in healthy volunteers (326,327).

In healthy volunteers, ChAdOx1-HBV induced T cell responses to all the major HBV antigens within the vaccine immunogen (core, polymerase and surface). In the two participants tested, T cell responses towards HBV genotype C peptides were highly cross reactive with HBV genotype D peptides. This indicates ChAdOx1-HBV is able to induce T cells reactive to both genotype C and D HBV, which are the most prevalent genotypes globally (47).

T cell responses were also detectable towards non-HBV vaccine components. Low magnitude T cell responses were detected to the linker/F2A/TPA peptide pool, which corresponds to the vaccine linker sequences, cleavage protein Furin-2A and adjuvant TPA with their adjoining HBV immunogen regions. T cell responses towards TPA were not generated in humans on administration of ChAdOx1-nCoV-19, which also includes a TPA sequence within its immunogen (427). It is more likely that the responses detected to the linker/F2A/TPA peptide pool peptide pool are directed towards the HBV regions adjacent to the non-HBV vaccine components. However further work is necessary to understand the fine specificities of these T cell responses.

High magnitude responses were also generated in some healthy volunteers towards to the non-HBV vaccine component shark invariant chain (sIi). These responses were predominantly CD8 T cells and over 50% were polyfunctional, producing two or more cytokines on restimulation with cognate antigen. Importantly, in the two healthy volunteers with the highest magnitude of sIi specific T cell responses, HBV antigen specific T cell responses were not impaired. High magnitude sIi specific T cell responses were also demonstrated in response to ChAd-OX1-HBV and MVA-HBV vaccination in HLA-A2 restricted HHD mice (Chapter 3) and in humans with HLA type HLA-A*02 given a malaria vaccine containing sIi as a molecular adjuvant (Amy Flaxman and Adrian Hill, personal correspondence). It is likely that the healthy volunteers in HBV001 in whom ChAdOx1-HBV induced sIi specific T cell responses also have the HLA-A2*02 haplotype, but this requires confirmation by HLA phenotyping.

Responses to human invariant chain (hIi) were also assessed to ensure there were no cross-reactive responses generated after vaccination. Truncated shark invariant chain has low homology to truncated human invariant chain (431) and therefore should have a low likelihood of generating auto-reactive immune responses to hIi. However, after vaccination some low magnitude T cell responses to the hIi peptide pool were detectable. No hIi responses were detectable at baseline therefore it is plausible that these responses to hIi were induced by vaccination. However, there was no significant correlation between the magnitude of responses towards the sIi and hIi peptide pool and vaccinations encoding full length human invariant chain have been administered safely in human trials without inducing hIi responses (508). Future iterations of the ChAdOx1-HBV vaccine should consider replacement of sIi with human invariant chain as a tried and tested way of boosting vaccine induced CD8 T cell responses without inducing responses to hIi (508).

In patients with chronic HBV infection, vaccination with intramuscular ChAdOx1-HBV is well tolerated with only two recorded adverse events thus far, both mild in severity. This is in keeping with the experiences of several clinical trials of novel HBV therapeutic vaccination candidates conducted over the last decade, which all reported favorable adverse event profiles (230,309,312,318,319,321,322,326,329,531). This includes a recent randomised controlled trial in 48 adults with chronic HBV infection who received placebo or therapeutic vaccine TG1050, which uses a human adenoviral platform (human adenovirus 5) (329), a related viral vector to ChAdOx1. Similarly to HBV001, in the TG1050 trial all recorded adverse events were grade 1 or 2 (329).

No ALT flares above 2x ULN have been recorded in patients with chronic HBV, vaccinated with ChAdOx1-HBV thus far. This is reassuring as increases in ALT can indicate increased viral activity or drug induced liver injury (109,363,379). However immune mediated ALT flares can also herald viral control, so the absence of ALT flares in HBV001 may reflect of the limited immunogenicity generated by ChAdOx1-HBV in the majority of patients with chronic HBV.

Prior to vaccination, patients with chronic HBV had low magnitude total T cell responses towards the HBV peptide pools core, polymerase and surface. This is in keeping with previous reports that circulating HBV specific T cells are deleted or dysfunctional in chronic HBV. Positive responses to vaccination were detected in nearly 60% of participants with chronic HBV. HBV polymerase and core specific T cells were increased after vaccination, but responses towards HBV surface remained low. This is consistent with data that HBV surface specific T cells are the most dysfunctional and often absent in patients with chronic HBV, and therefore most difficult to recover functionally (251–253). In previous trials of

therapeutic HBV vaccination, surface specific T cell have also been the most difficult to restore (327).

Vaccine induced T cells were cross reactive to HBV genotype D peptide pools, suggesting ChAdOx1-HBV has the potential to be immunogenic across several HBV genotypes. However, the extremely high baseline responses towards the HBV polymerase pol3 pool need further investigation to understand their fine specificity. It could be these participants are infected with HBV genotype D, or that T cells specific for another antigen are cross reacting with a peptide in this region.

In the majority of patients with chronic HBV enrolled in HBV001, the longitudinal HBV specific T cell responses did not increase after ChAdOx1-HBV vaccination. These results are similar to previous trials of therapeutic vaccination for chronic hepatitis B, which have also found HBV specific T cell responses are not reliably induced by vaccination and in many cases require *in-vitro* expansion for detection (Appendix 1) (326,327,319,322,318,321,329,290,296,300,516,306,313,316,323,320,326,362). Three of these trials have assessed *ex-vivo* T cell responses to HBV vaccine candidates encoding multiple HBV antigens and have found that between 22-67% participants have increased T cell responses after vaccination (321,326,327). ChAdOx1-HBV was highly immunogenic in healthy volunteers and so it is likely that the lower T cell responses to vaccination are driven by the HBV specific T cell dysfunction and tolerance associated with chronic HBV infection. Additional steps are likely to be necessary to overcome the HBV specific T cell dysfunction associated with chronic HBV infection.

One strategy to maximise HBV T cell responses in future studies is to use heterologous prime boost vaccination strategies. We have shown previously in healthy mice that HBV specific T cell responses induced by ChAdOx1-HBV are boosted by vaccination with MVA-HBV (Chapter 3 and (346)). Clinical trial HBV-002 which has recently opened for recruitment, will assess ChAdOx1-HBV and MVA-HBV in prime-boost strategies in patients with chronic HBV infection, to assess whether the MVA-HBV boost can improve HBV specific T cell responses (348).

A further strategy that might improve the efficacy of therapeutic vaccination is to give an immune checkpoint inhibitor in parallel. This approach was successful in inducing HBsAg loss in the woodchuck model of chronic HBV infection (361). In a small pilot study using low dose PD-1 inhibitor Nivolumab with or without therapeutic vaccination in patients with chronic HBV, HBsAg was lost in one participant who received Nivolumab alone (531). The HBV002 study includes two arms in which Nivolumab will be administered alongside ChAdOx1-HBV and MVA-HBV, to assess whether this can improve the efficacy of HBV therapeutic vaccination (348).

Another approach that might improve therapeutic vaccination efficacy is to target specific populations who are most likely to respond to treatment such as those with lower HBsAg titers. Low HBsAg titers at time of nucleos(t)ide therapy withdrawal in patients with chronic HBV is associated with subsequent loss of HBsAg off therapy (357) and in murine models of chronic HBV treated with therapeutic vaccine, lowering HBsAg levels prior to vaccination improves vaccine efficacy (338,355,356). In HBV001 the participant with chronic HBV that had the largest increases in HBV specific T cell responses after vaccination did not have the lowest HBsAg titer at baseline but did have the highest baseline HBV T cell responses.

Recent work suggests patients with HBeAg positive chronic HBV infection (previously termed the immune tolerant phase) have HBV T cells that are more functional compared to later phases of infection, and therefore might respond better to immunomodulatory therapy (253). However, HBsAg burden is often very high in this phase of infection and might require pre-treatment prior to vaccination with therapies that directly target HBsAg such as small interference RNA (siRNA) or nucleic acid polymers (NAPs) (358–360). Detailed immunological and virological responses in therapeutic vaccination trials in humans will be necessary to understand these nuances further as there is currently no single reliable measure to predict which patients will respond to therapeutic vaccination at baseline.

5.7 CONCLUSION

Vaccination with ChAdOx1-HBV is safe and induces high magnitude HBV specific T cells in healthy volunteers. In patients with chronic HBV infection, ChAdOx1-HBVinduces/boosts HBV specific T cell responses is some but not all participants.

5.8 SUMMARY OF RESULTS

- Vaccination with ChAdOx1-HBV is safe in healthy volunteers and patients with chronic HBV infection
- Vaccination with ChAdOx1-HBV induces polyfunctional CD8 and CD4 T cell responses towards HBV core, polymerase and surface regions in healthy volunteers
- Vaccination with ChAdOx1-HBV induces or boosts HBV specific T cell responses in some patients with chronic HBV, but the magnitude of these responses is lower than those induced in healthy individuals

- In patients with chronic HBV, ChAdOx1-HBV induces T cells specific to HBV polymerase and core regions, whereas in healthy volunteers T cells specific to HBV surface predominate.
- T cells induced by ChAdOx1-HBV are responsive to both HBV genotype C and genotype D peptides

CHAPTER 6: Discussion

6.1 NOVEL THERAPEUTIC VACCINES FOR HBV ARE IMMUNOGENIC IN MICE AND MEN

In this thesis I describe the development of novel HBV therapeutic vaccine candidates ChAdOx1-HBV and MVA-HBV in mice, healthy humans and individuals with chronic HBV infection.

Evidence in chimpanzees infected with acute HBV infection have shown a robust CD8 T cell response is necessary for resolution of infection (178), however in chronic HBV the T cell response is dysfunctional (12). Previous therapeutic vaccine candidates for HBV therapy have not reliably induced high magnitude T cell or humoral responses nor induced functional cure in persons with chronic HBV (Appendix 1, (295,296,300,302,306,313,316–319,321–323,326,327,329,362,516)). Based on this evidence it is desirable for therapeutic vaccine candidates for chronic HBV to induce high magnitude HBV specific T cell responses, although a precise magnitude of HBV specific CD8 immune responses that predicts resolution is not known. Vaccination using heterologous prime-boost strategies with ChAd and MVA vectors have been shown to induce high magnitude, broad, polyfunctional T cell responses and humoral responses in healthy persons for infections including HCV, malaria, influenza, Ebola and TB (398,397,405,400,409). In keeping with these findings, I show that novel ChAd and MVA vectored vaccines ChAdOx1-HBV and MVA-HBV induce high magnitude functional T cell responses to in mice and healthy human subjects. In mice, the total magnitude of HBV specific T cell responses is increased after MVA-HBV boost compared to ChAdOx1-HBV prime alone. In humans, high magnitude HBV specific T cell responses are induced with ChAdOx1-HBV alone. These responses are likely to be further

improved by adding MVA-HBV boost to the vaccine schedule, however this needs investigation in future human studies.

The resolution of acute HBV infection is associated with T cell responses specific to several HBV antigenic regions including HBV core, polymerase and surface (168–175,178). In chronic infection, global dysfunction of HBV specific T cells towards all HBV antigens is described. However, HBV surface specific T cells appear more depleted and dysfunctional than HBV polymerase and core specific T cells (251–253). I show in HHD mice restricted to human HLA-A*02 expression and in healthy human volunteers, vaccination with ChAdOx1-HBV alone or in combination with MVA-HBV induces T cells specific to HBV core, polymerase and surface regions. In mice the breadth of response is increased after MVA-HBV boost compared to ChAdOx1-HBV prime alone. Adding MVA-HBV booster vaccine after ChAdOx1-HBV prime in humans is also likely to broaden the T cell responses induced to vaccination, as has been the case with other vaccine regimes using ChAd and MVA vectors (398,399). In chronic HBV infection, whether the addition of MVA-HBV will also broaden the HBV specific T cell responses primed by ChAdOx1-HBV infection and the impact this might have on vaccine efficacy requires investigation.

During the resolution of acute HBV infection, immune control through non-cytopathic mechanisms such as cytokine production predominates (164,177). In the liver, HBV specific T cells secreting cytokines including IFNγ, TNFα and IL-2 are present in persons with chronic HBV infection and in resolvers of acute HBV infection, suggesting cytokine production by T cells is important for the ongoing local immune control of the virus (18,254). I show that in mice and healthy humans, ChAdOx1-HBV alone or in combination with MVA-HBV induces functional CD8 T cells that produce IFNγ, TNFα or IL-2 alone or in

combination. This supports that vaccination with ChAdOx1-HBV and MVA-HBV can induce HBV specific T cells with relevant effector functions, important for HBV control in natural infection.

Functional cure of HBV is defined by the sustained loss of HBsAg (9). Neutralising anti-HBs Abs are an important component in HBV immune control, but HBs specific B cells are dysfunctional in chronic HBV (192,268,269). A therapeutic HBV vaccine should ideally induce functional HBs B cells that produce anti-HBs Abs. ChAd vector vaccines, including ChAdOx1 are able to induce antibody responses toward the vaccine immunogen (400,399,427). However, I found vaccination with ChAdOx1-HBV and MVA-HBV was unable to reproducibly induce high tires of anti-HBs Abs in mice. This was despite the *in-vitro* secretion of the small surface protein into the supernatants of ChAdOx1-HBV infected HEK293A cells (346). CD4 T cells specific to pooled HBV pre-S1/Pre-S2 and surface peptides were induced by vaccination, suggesting the T helper support for the differentiation of B cell responses is present. However, the fine specificities of the CD4 T cell responses to this region need further investigation, as it might be that the dominant responses lie within the HBV pre-S1 or pre-S2 regions, as opposed to the surface region. The response of surface specific T follicular helper cells, necessary for B cell differentiation and antibody production also require investigation to further understand why anti-HBs Abs are not readily detected after vaccination. It is possible that in mice, antibody responses towards other HBV regions such as HBV pre-S1 or HBV core are induced in preference to the HBV surface region. Future studies should therefore measure the production of antibodies towards these regions in addition to anti-HBs Abs.

In humans it has not been possible to assess whether ChAdOx1-HBV can induce anti-HBs Abs, as all healthy human trial participants vaccinated with ChAdOx1-HBV had detectable anti-HBs Abs at baseline, presumably due to previous prophylactic vaccination with a recombinant HBV vaccine. In the future, ChAdOx1-HBV needs to be tested in persons that do not already have anti-HBs Abs at baseline and/or be detected quantitively to understand whether vaccination can induce or boost anti-HBs Ab responses in humans.

The immunogenicity of ChAdOx1-HBV and MVA-HBV vaccines might be further optimised for example by using different regimes or boost intervals. The addition of a second ChAd vaccination has been shown to improve immunogenicity of ChAd and MVA vectored malaria vaccines (399) and a longer interval between ChAdOx1 vectored vaccines for SARS-CoV-2 improved antibody titres (428). Other vaccine platforms might also be efficacious. For example, a vaccine approach that uses particulate HBsAg and HBcAg to prime the immune response followed by and MVA boost vaccination, has been successful at inducing immune control in a murine model of chronic HBV infection (338). mRNA vaccines, which have shown success in inducing protective immune responses specific to SARS-CoV-2 (337), might be alternative vectors for future HBV therapeutic vaccine candidates.

Together, I show that ChAdOx1-HBV and MVA-HBV alone or in combination are immunogenic in both mice and men. In particular, vaccination induces HBV specific T cell responses towards all major HBV antigens within the vaccine immunogen at high magnitude that secrete cytokines relevant in the immune control of HBV. However, these vaccines do not induce neutralising anti-HBs Ab reliably in mice and whether they can in humans remains to be determined.

6.2 ALTERING THE ROUTE OF VACCINATION CAN IMPROVE VACCINE RESPONSES IN THE LIVER

Intrahepatic HBV specific T cells are necessary for the resolution of acute HBV, viral containment during chronic HBV and long-term immune control of HBV after resolution of infection (18,178,254,509). HBV therapeutic vaccine strategies to optimise the induction and retention of T cells in the liver are therefore desirable, to improve the likelihood of vaccine efficacy.

Previous studies have shown the intravenous route of vaccination, compared to intramuscular or intranasal routes, can increase the efficacy of vaccines for the prevention of liver stage malaria in mice, or pulmonary TB in non-human primates (353,494). In particular, a malaria vaccine strategy using Ad5 or ChAd63 and MVA vector vaccines in heterologous prime-boost strategies showed the magnitude of intrahepatic vaccine induced T cell responses was improved when the MVA boost vaccination was given intravenously compared to intramuscularly (353). In keeping with these findings, I show that the magnitude of vaccine induced HBV specific T cells is increased in the liver after intravenous as opposed to intramuscular MVA-HBV vaccination, in mice primed with intramuscular ChAdOx1-HBV.

I found that HBV specific T cell responses in the liver were durable for at least 60 days after MVA-boost vaccination. However, there was no longer any difference between the intravenous and intramuscular routes when assessing the magnitude of T cell responses at this later timepoint. Previous work has not reported the magnitude of T cell responses to vaccination at such a late time point. However, protection against liver stage malaria was durable in mice that had received intravenous as opposed to intramuscular MVA booster vaccination for up to 2 months after vaccination (353). This suggests that even if responses

do wane, the increased liver T cells at peak response after intravenous MVA boost vaccination might be enough to confer efficacy. ChAdOx1-HBV prime followed by intravenous or intramuscular MVA-HBV booster vaccination need to be investigated in mouse models of chronic HBV, to assess whether the route of vaccination enhances immune control of HBV in this setting.

In both chronic and resolved HBV, the majority of HBV specific T cells have a liver tissue resident memory phenotype characterised by the expression in humans of CD69 with or without CD103 or CXCR6 (18,254,509). I investigated the phenotype of intrahepatic HBV specific T cells in mice vaccinated with ChAdOx1-HBV and MVA-HBV and found the expression of markers associated with liver residency in the mouse, including CD69, CD11a and CXCR6 (532,533), were upregulated in the liver compared to blood derived T cells. This is in keeping with previous studies of ChAd/MVA vaccine strategies, which induced T cells in the liver with a liver resident memory phenotype (353). Previous studies of ChAd/MVA vector vaccines have shown blood derived T cells induced by vaccination have a T effector memory phenotype and produce proinflammatory cytokines including IFNγ (398). In keeping with this, I show vaccination with ChAdOx1-HBV and MVA-HBV in mice induces HBV specific T cells with an effector memory phenotype in the blood and solid organs. Together these findings support that vaccination with ChAdOx1-HBV and MVA-HBV induce HBV specific T cells with effector memory and liver resident memory phenotypes, that have been shown to be important for HBV control in natural infection.

Overall, I show that vaccination with ChAdOx1-HBV and MVA-HBV induces HBV specific T cells with an effector memory phenotype and liver resident memory phenotype in mice. The phenotype of vaccine induced T cells in the liver should be assessed by for example

FNA in human trials ChAdOx1-HBV and MVA-HBV vaccines to understand whether these vaccines induce liver resident memory T cells in humans. Intravenous as compared to intramuscular boost vaccination with MVA-HBV increases the magnitude of HBV specific T cells in the liver in mice primed with ChAdOx1-HBV. As HBV antigen is already expressed in the liver in chronic HBV infection, vaccination primed HBV specific T cells might be pulled to the liver by this antigen signal without the need for intravenous boost vaccination. Therefore, this vaccine strategy needs to be further evaluated to assess its efficacy in chronic HBV mouse models.

6.3 T CELL RESPONSES TO TRUNCTED SHARK INVARIANT CHAIN SIi CAN BE ALMELIORATED USING TRUNCATED HUMAN INVARIENT CHAIN

Unexpectedly I found vaccination with ChAdOx1-HBV and MVA-HBV in HLA-A*02 restricted HHD mice induced a strong CD8 T cell response to sIi, used as a molecular adjuvant in the vaccine. In addition, 2 of the 10 healthy volunteers vaccinated with ChAdOx1-HBV had high magnitude T cell responses to the sIi region. Collaborators using sIi adjuvanted ChAd/MVA vectored vaccines for malaria have also detected T cell responses to sIi in HLA-A*02 positive individuals (Amy Flaxman and Adrian Hill, personal correspondence). Low magnitude T cell responses towards human invariant chain peptides were present in some human volunteers vaccinated with ChAdOx1-HBV, which needs further investigation to exclude the possibility of cross reactivity. The presence of responses to sIi was not associated with lower magnitude responses to HBV antigens in the two individuals that developed a sIi response, but it is possible that the immunodominance of the sIi responses might ‘out-compete’ subdominant epitopes within the HBV immunogen, preventing their proliferation and differentiation.

I found that *in-silico*, I was unable to easily disrupt the sIi HLA-A*02 restricted epitope without creating other putative epitopes. Therefore, a vaccine replacing sIi with truncated human invariant chain (hIi) was designed. Truncated human invariant chain has been shown previously to be a good molecular adjuvant when multiple invariant chain constructs were compared for immunogenicity in the ChAd63 vectored malaria vaccine (431). In HHD mice vaccination with ChAdOx1-HBV with hIi removed the T cell response directed at sIi, whilst maintaining the total magnitude of HBV specific T cell responses. An HCV vaccine using ChAd/MVA vectors incorporating full length human invariant chain has been shown to significantly improve the magnitude of vaccine induced T cell responses, whilst not inducing T cells reactive to the human invariant chain (508). Using hIi as a molecular adjuvant is therefore a suitable option for future iterations of the ChAdOx1-HBV vaccines.

6.4 REDUED VACCINE IMMUNOGENICICTY IN INDIVIDUALS WITH CHRONIC HBV

Despite vaccination with ChAdOx1-HBV inducing high magnitude T cell responses in healthy persons, when given to individuals with chronic HBV vaccination induced HBV specific T cells in a minority. This is in keeping with results from previous studies of therapeutic HBV vaccine candidates that have been poorly immunogenic in the setting of chronic HBV even when they induce immune responses in healthy persons (286–288,314,325,330–333). From investigation in both mice and healthy humans, I have shown that ChAdOx1-HBV induces high magnitude functional HBV specific T cell responses. Therefore, the blunted immune responses observed in individuals with chronic HBV is likely to be secondary to the dysfunctional immune response associated with chronic infection (12).

To my knowledge, this is the first time that the immunogenicity of the intervention vaccine, in this case ChAdOx1-HBV, has been studied in both healthy persons and individuals with chronic HBV infection in the same trial. It clearly shows that ChAdOx1-HBV is an immunogenic vaccine, but the environment of chronic HBV infection constraints its ability to induce new or recover existing HBV specific T cell responses. Furthermore, in patients with chronic HBV, vaccine induced T cell responses in those that do respond to vaccination are predominantly specific for HBV polymerase and HBV core rather than HBV surface. This is in keeping with evidence suggesting HBV surface specific T cells are the most functionally exhausted subset in chronic infection and most resistant to recovery (251,252).

HBsAg levels did not change after vaccination with ChAdOx1-HBV in any participant, even those who developed a T cell response to vaccination. Of the two individuals with an HBsAg titre of under 1000 IU/mL at baseline, one of them developed a T cell response to ChAdOx1-HBV but the other did not. There is evidence supporting the observation that persons with lower HBsAg titres are more likely to go onto develop functional cure after nucleos(t)ide withdrawal (357) and that persons who go onto to develop HBsAg loss after Nivolumab have the highest functional T cell responses (362). However, in the studies of therapeutic vaccine candidates where quantitative HBsAg has been measured, most participants have not lost HBsAg during the study and no difference have been found between HBsAg loss rates in vaccine compared to control groups (294,298,318,326,327,329,362). With such a low incidence of HBsAg loss in these trials, it is difficult to find associations between the immune response and HBsAg loss in that might predict the efficacy of therapeutic vaccination at present.

In those that did respond to ChAdOx1-HBV vaccination, HBV specific T cells were reactive to both genotype C and genotype D peptides. Genotype C and D are the most common HBV genotypes, together accounting for over 50% of worldwide infections (52). This suggests ChAdOx1-HBV could be suitable for patients with both Genotype C and genotype D HBV infection.

6.5 FUTURE DIRECTIONS OF THE HEPATITIS B THEREPEUTIC VACCINATION PROGRAMME

The data in this thesis support that therapeutic vaccination with ChAdOx1-HBV is safe in people with chronic HBV and healthy volunteers, which is important to go onto proceed with larger clinical trials using this vaccine. However, the results also show that ChAdOx1-HBV is likely to require additional interventions in chronic HBV to improve efficacy. This was expected, as data from previous trials suggests therapeutic vaccines are less immunogenic when given to those with chronic HBV than others with healthy immune systems (286–288,314,325,330–333).

I show in healthy mice that HBV specific T cell responses primed by ChAdOx1-HBV can be increased by giving a booster vaccine of MVA-HBV(346). Furthermore, I show that intravenous as compared with intramuscular MVA-HBV boost increases the intrahepatic T cell response to vaccination. These two strategies could be used in future vaccine trials of the ChAdOx1-HBV vaccine to improve efficacy. HBV-002, a phase II clinical trial that has recently opened for recruitment, is assessing ChAdOx1-HBV and MVA-HBV in prime-boost strategies in persons with chronic HBV infection (NCT04778904) (348).

If the combination of ChAdOx1-HBV with MVA-HBV does not prove to be more immunogenic than ChAdOx1-HBV alone, several other novel therapies might be useful in combination with vaccination (12,13). Options include directly reducing HBsAg levels with small interference RNA (siRNA) or nucleic acid polymers (NAPs) (358–360) or concurrent therapy with immune checkpoint inhibitors. In the Woodchuck model of HBV, the combination of therapeutic vaccination with a PD-1 inhibitor Nivolumab improved HBsAg loss (361). More recently it has been shown that T cell responses induced by ChAdOx1-HBV can be improved by concurrent administration of a PD-1 inhibitor Nivolumab in a mouse model of chronic HBV infection (Mariana Diniz and Mala Maini, personal correspondence and manuscript in submission). HBV-002 is also trialling therapeutic vaccination with ChAdOx1-HBV and MVA-HBV together with Nivolumab, to assess this strategy in humans (summary in Appendix 8, (348)).

Beyond this, the continuing development of immunological strategies for HBV therapy is required. Firstly, we need to support further research into the immune response during acute, chronic and resolved HBV infection. Several other immune cells and processes have been found to be impaired in chronic HBV infection including toll-like receptor signalling and the functions of NK cells, B cells and T cells (reviewed in chapter 1). The identification of new molecular targets and novel technologies to manipulate some of these pathways can be built on through further research into the immune response. Secondly, we need to comprehensively evaluate the immune response to novel therapeutic agents under development including therapeutic vaccination, CAR-T cells and checkpoint inhibition, to inform the iterative development of more efficacious strategies. The liver itself is the most important site of immune control in chronic HBV infection, and therapeutic trials of any novel treatments for chronic HBV should consider routinely assessing the immune response

to therapy in the liver via FNA (254,509). Finally HBV therapy development is just one strand in the bid to eradicate viral hepatitis by 2030 (4). Sustained political pressure, infrastructure and research are all important prevent new HBV infections, and strengthen the diagnosis and treatment of existing HBV going forward (2,5).

APPENDIX 1: Previous clinical trials of HBV therapeutic vaccine candidates

Table 1 Recombinant vaccines

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Pol et al. 1994 (289)	Non-randomised	32	Gen Hevac B	No control group	Pre-S2, S	Adult	Chronic hepatitis, unknown HBeAg status	26	n=10/32 (31%) of vaccinated	NR	NR	NR	NR	NR
Pol et al. 2001 (290) Couillin et al 1999 (295)	Randomised	170 40	Gen Hevac B/ Recombivax	No treatment	Pre-S2, S	Adult	HBeAg positive or negative chronic infection or chronic hepatitis	52	No significant difference	No significant difference	No significant difference	NR	Increased in n= 7/27 (26%) after vaccination, undetectable if unvaccinated	Positive CD4 T cell responses in n=5/27 (19%) after vaccination, undetectable if unvaccinated.
Pata et al. 2002 (291)	Non-randomised	29	Gen Hevac B/ Recombivax	No treatment	Pre-S2, S	Adult	HBeAg positive or negative chronic infection or chronic hepatitis	52	Significant reduction between BL and EOS in vaccinated but not unvaccinated	NR	n=4/11 (36%) in vaccinated	NR	NR	NR
Yalcin et al. 2003 (292)	Randomised	47	Gen Hevac B	No treatment	Pre-S2, S	Adult	HBeAg positive chronic infection	52	No significant difference	No significant difference	No significant difference	Nil	NR	NR
Dikici et al. 2003a (293)	Randomised	74	Gen Hevac B	No treatment	Pre-S2, S	Child	HBeAg positive chronic infection	52	No significant difference	n= 0/43 (0%) in vaccinated, n=1/31 (3%) in control group	Nil	NR	NR	NR
Dikici et al. 2003b (294)	Randomised	51	Gen Hevac B	No treatment	Pre-S2, S	Child	HBeAg positive chronic infection	52	No significant difference	No significant difference	Nil	n=1/23 (4%) in vaccinated, n= 1/28 (4%) in control group		
Dahmen et al. 2002 (296)	Non-randomised	25	ENGERIX-B with NUCs +/- IL-2	NUC	Pre-S2, S	Adult	HBeAg positive or negative chronic hepatitis	26	With normal ALT n=4/14 (29%) vaccinated, n=2/11 (18%) in control group	n=1/11 (9%) in vaccinated n=2/11 (18%) in control group	n=1/11 (9%) in vaccinated group and n=2/11(18%) control group	NR	BL responses to HBV core and surface increased in vaccination group after therapy	CD4 responses n=10/14 (71%) at BL, n=8/12 (67%) after vaccination, n=4/11 (36%) in unvaccinated

Table 1 Recombinant vaccines (continued)

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Demirtürk et al. 2002 (297)	Non-randomised	29	Gen Hevac B with IFN	NUC + IFN	Pre-S2, S	Adult	HBeAg positive or negative chronic infection or chronic hepatitis	52	With ALT normalisation n=3/10 (30%) in vaccinated, n=11/14 (79%) in combined control groups	n=3/3 (100%) in vaccinated group and n=1/9 (11%) in combined control groups	NR	Nil	NR	NR
Helvaci et al. 2004 (298)	Randomised	50	Gen Hevac B with IFN	IFN	Pre-S2, S	Child	HBeAg positive chronic infection or chronic hepatitis	60	No significant difference	NR	No significant difference	n=1/25 (4%) in both vaccinated and control group	NR	NR
Horiike et al. 2005 (299)	Non-randomised	72	HBsAg with NUC	NUC	S	Adult	HBeAg positive or negative chronic hepatitis	52	In HBeAg positive subgroup, significantly higher HBV DNA suppression in vaccinated (n=9/9, 100%) compared to control group (n=15/31, 48%)	NR	In HBeAg positive subgroup, significantly higher HBeAg seroconversion in vaccinated (n=5/9, 56%) compared to control group (n=5/31, 15%)	NR	NR	NR
Vandepapelière et al. 2007 (300)	Randomised	195	HBsAg with NUC	NUC	S	Adult	HBeAg positive chronic hepatitis	52	No significant difference	No significant difference	No significant difference	NR	Detectable in 83% of vaccination group	No detectable responses at BL. In vaccinated group, cellular responses peaked at week 7 after vaccination

Table 1 Recombinant vaccines (continued)

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Ishikawa 2007 (301)	Randomised	53	HBsAg with NUC	NUC	Pre-S2, S	Adult	HBeAg positive or negative chronic hepatitis	52	No significant difference	No significant difference	No significant difference	NR	NR	NR
Hoa et al. 2009 (302)	Randomised	180	Sci-B-Vac +/- NUC	NUC	PreS1, Pre-S2, S	Adult	HBeAg positive chronic hepatitis	78	Significantly increased in groups treated with NUC	No significant difference	Significantly increased in groups treated with NUC	No significant difference	Detectable in n=50/120 (42%) in combine vaccination groups and n=0/60 (0%) in NUC monotherapy group.	NR
Lee et al. 2015 (303)	Non-randomised	32	Hepavax-Gene TF	No treatment	S	Adult	HBeAg negative chronic infection	52	No significant difference	NR	NR	No significant difference	NR	NR
Lai et al. 2018 (304)	Non-randomised	20	ENGERIX-B	No control group	Pre-S2, S	Adult and child	HBeAg negative chronic infection	96	NR	NR	NR	n=4/19 (21%) of vaccinated	NR	NR
Al-Mahtab et al. 2013 (516)	Non-randomised	20	HBsAg + HBcAg	No treatment	S, C	Adult	Chronic	48	n=9/20 (45%) of vaccinated	n=7/11 (64%) of vaccinated	n=2/11 (18%) of vaccinated	NR	NR	Cytokine ELISA (culture supernatant after in-vitro expansion): increased cytokine production to HBV antigen stimulation in vaccinated compared to unvaccinated
Al-Mahtab et al. 2018 (312)	Randomised	160	NASVAC (HBsAg +HBcAg)	IFN	S, C	Adult	HBeAg positive or negative chronic hepatitis	72	Significantly lower mean DNA in vaccinated vs control group at 24 week FU after treatment completion	NR	n=5/14 (36%) of vaccinated group and n=3/16 (19%) of control group	NR	NR	NR

Table 2 Immune complex vaccines

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Wen et al. 1995 (305)	Non-randomised	14	HBVac + HBIG	No control group	S	Adult	HBeAg positive chronic infection or chronic hepatitis	26	n=9/14 (64%) vaccinated	n=6/14 (43%) vaccinated	n=2/14 (14%) vaccinated	Nil	NR	NR
Yao et al. 2007 (306)	Randomised	26	YIC (HBsAg+ HBIG + allum adjuvant)	Placebo or no treatment	S	Adult	HBeAg positive chronic hepatitis	44	NR	With 2 log reduction in HBV DNA titre in n=5/10 (50%) vaccinated, n=0/10 (0%) controls	n=2/10 (20%) vaccinated and n=0/10 (0%) controls	NR	detectable in n=2/10 (20%) vaccination group and n=0/10 (0%) control group	Detectable cytokine responses to HBsAg in n=4/5 (80%) of vaccine responders (HBeAg loss and HBV DNA reduction) and n=2/5 (40%) of vaccine non-responders
Xu et al. 2008 (307) Wang et al. 2010 (308)	Randomised	237/156	YIC (HBsAg-HBIG + allum adjuvant)	Placebo	S	Adult	HBeAg positive chronic hepatitis	44	Significantly lower in vaccine group if HBeAg seroconverted compared to non-seroconverted or control group	No significant difference	Significantly higher in vaccinated compared to unvaccinated group	Significantly greater HBsAg decline if vaccinated and HBeAg seroconverted compared to non-seroconverted or control groups	NR	NR
Xu et al. 2013 (309)	Randomised	450	YIC (HBsAg-HBIG + allum adjuvant)	Placebo	S	Adult	HBeAg positive chronic hepatitis	76	No significant difference	No significant difference	No significant difference	NR	NR	NR

Table 3 Lipopeptide epitope-based vaccines

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Heathcote et al. 1999 (313)	Non-randomised	90	CY-1899	No control group	Core (18-25)*	Adult	HLA-A*02 positive HBeAg positive or negative chronic infection or chronic hepatitis	52	No significant difference between baseline and after vaccination	NR	NR	NR	NR	Cytotoxicity assay: specific lysis increased in response to vaccination in a dose responsive way

Table 4 DNA vaccines

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Mancini-Bourgine et al. 2004 (316) Mancini-Bourgine et al. 2006 (317)	Non-randomised	10	pCMV.PS2.S	No control group	Pre-S2, S	Adult	HBeAg positive chronic infection or chronic hepatitis	44	n=2/10 (20%) of vaccinated	NR	NR	NR	NR	**ELISpot (*ex-vivo)*:** BL HBs responses in n=1/10 (10%). **ELISpot (after 14 days *in-vitro* expansion):** BL HBs responses in n=2/10 (20%), increased to n=5/8 (63%) month 5 after vaccine **Proliferation assay:** No BL responses. Responses in n=2/10 (20%) after vaccination.
Fontaine et al. 2015 (318)	Randomised	70	pCMV-S2.S with NUC	NUC	Pre-S2, S	Adult	HBeAg positive or negative chronic infection	72	No significant difference	NR	n=0/3 (0%) in vaccinated group and n=1/5 (20%) in control group	n=2/34 (6%) in vaccinated group and n=1/36 (28%) in control group	NR	**ELISpot (ex-vivo)**: BL responses in 6-24% of participants. Increase in responses towards HBs after vaccination, but no significant difference between vaccinated and control groups.

Table 4 DNA vaccines (continued)

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Cavenaugh et al. 2011 (319)	Randomised	64	DNA pSG2.HBs and MVA vaccine (MVA.HBs). Vaccination alone or with NUC.	NUC or placebo (rabies vaccine)	Pre-S2, S	Adult	HBeAg positive or negative chronic infection	48	No significant difference	No significant difference	Nil	Nil	NR	**ELISpot (*ex-vivo*):** low numbers of BL IFNγ producing cells, did not increase after vaccination
Yang et al. 2012 (322)	Randomised	39	ED-DNA.PS2.S with NUC	NUC + placebo	Pre-S2, S	Adult	HBeAg positive chronic hepatitis	72	decrease of >2 log in significantly more in vaccinated compared to control group	No significant difference	No significant difference	Nil	NR	**ELISpot (ex-vivo):** Positive responses in vaccinated group compared to control group at week 52 and 72 of study. **Flow cytometry (ex-vivo, HBV specific tetramers):** IFNγ producing T cells in n=6/9 (22%) vaccine+ NUC compared to n=2/7 (29%) in placebo + NUC
Yang et al. 2017 (323)	Randomised	225	ED-DNA.PS2.S with NUC	NUC + placebo	Pre-S2, S	Adult	HBeAg positive chronic hepatitis	72	No significant difference	NR	in participants with HBV DNA <1000 copies/mL at BL, n=6/11 (55%) compared to n=3/19 (16%) seroconverted HBe	Nil	NR	**ELISpot (after 9 day in-vitro expansion):** detectable responses significantly increased n=19/22 (86%) in vaccinated vs. n=13/23 (57%) in control group. **ICS:** Detectable CD8+ T cell responses significantly increased in vaccinated n=17/22 (77%) vs. n=10/23 (44%) in control group

Table 4 DNA vaccines (continued)

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Yang et al. 2006 (320)	Non-randomised	12	HB-100 with NUC	No control group	Pre-S1/Pre-S2/X, S, C, P	Adult	HBeAg positive or negative chronic hepatitis	104	n=6/12 (50%) at EOT (week 52)	NR	n=4/6 (67%) vaccinated	n=1/12 (8%) vaccinated	NR	**ELISpot (ex-vivo):** No detectable responses at BL. At EOT (week 52) responses to at least one HBV antigen present in n=7/7 (100%) subjects tested. Responses significantly higher in viral responders compared to non-responders. **ELISpot (after in-vitro expansion):** Detectable responses 40 weeks after EOT in viral responders. Most responses CD4+
Yoon et al. 2015 (321)	Randomised	27	HB-110 with NUC	NUC	Pre-S2, S, C, P	Adult	HBeAg negative chronic hepatitis	48	No significant difference	NR	n= 0/7 (0%) vaccinated, n=4/18(22%) controls	NR	NR	**ELISpot (ex-vivo):** Detectable responses in n=5/18 (28%) of vaccinated, n=0/6 (0%) control group. **Proliferation assay:** Vaccine induced T cells were predominantly CD8 and polyfunctional

Table 5 Yeast derived vaccines

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Lok et al. 2016 (326)	Randomised	178	GS-4774 with NUC	NUC	S, C, X	Adult	HBeAg positive or negative chronic infection	48	No HBV DNA breakthrough in any group	n=5/37 (14%) of vaccinated, n=0/7 (0%) of control group	n=4/37 (11%) of vaccinated, n=0/7 (0%) of control group	Nil	NR	**ELISpot (ex-vivo):** positive response to HBcAg in n=53/94 (56%) in vaccinated groups and n=5/14 (36%) in control group
Gane et al. 2019 (362)	Non-randomised	24	GS-4774 with NUC	Nivolumab with NUC	S, C, X	Adult	HBeAg negative chronic infection	24	NR	NR	NR	n=0/10 (0%) vaccinated group and n=1/14 (7%) control group	NR	**ELISpot (ex-vivo):** detectable responses at BL towards HBc and HBs, no change in response to vaccination.
Boni et al. 2019 (327)	Randomised	195	GS-4774 with NUC	NUC	S, C, X	Adult	HBeAg positive or negative chronic hepatitis	48	NR	n= 5/66 (8%) in vaccinated, n= 0/10 (0%) in control group	n= 3/66 (8%) in vaccinated, n= 0/10 (0%) in control group	Nil	NR	**ELISpot (ex-vivo):** Increased responses compared to BL in vaccinated but not control group **Proliferation assay and ICS:** Increased cytokine production by HBV specific CD8 T cells in vaccinated compared to unvaccinated groups

Table 6 Adenoviral vectored vaccines

Study	Study type	N	Intervention	Control group	HBV antigens within vaccine	Age group	Population	FU (weeks)	Virological assessment				Immunologic assessment	
									HBV DNA suppression	HBeAg loss	HBeAg sero-conversion	HBsAg loss	HBsAb	T cell
Zoulim et al. 2020 (329)	Randomised	48	Human adenoviral type 5 vector (TG1050) with NUC	Placebo with NUC	S, C, P	Adult	HBeAg positive or negative chronic infection	54	NR	NR	NR	Nil	NR	**ELISpot (10 day in-vitro expansion):** responses increased after vaccination compared to BL in n=9/17 (53%) of vaccinated but 0% of control group

APPENDIX 2 – Vaccine immunogen peptides for mouse experiments, synthesised 2016

ID	Sequence
1	SLLWGGVTVLAAMLI
2	GGVTVLAAMLIAGQV
3	VLAAMLIAGQVASVV
4	MLIAGQVASVVFLVK
5	GQVASVVFLVKGGGP
6	SVVFLVKGGGPGGGM
7	LVKGGGPGGGMQLFH
8	GGPGGGMQLFHLCLI
9	GGMQLFHLCLIISCS
10	LFHLCLIISCSCPTV
11	CLIISCSCPTVQASK
12	SCSCPTVQASKLCLG
13	PTVQASKLCLGWLWG
14	ASKLCLGWLWGMDID
15	CLGWLWGMDIDPYKE
16	LWGMDIDPYKEFGAS
17	DIDPYKEFGASVELL
18	YKEFGASVELLSFLP
19	GASVELLSFLPSDFF
20	ELLSFLPSDFFPSIR
21	FLPSDFFPSIRDLLD
22	DFFPSIRDLLDTASA
23	SIRDLLDTASALYRE
24	LLDTASALYREALES
25	ASALYREALESPEHC
26	YREALESPEHCSPHH
27	LESPEHCSPHHTALR
28	EHCSPHHTALRQAIL
29	PHHTALRQAILCWGE
30	ALRQAILCWGELMNL
31	AILCWGELMNLATWV
32	WGELMNLATWVGSNL
33	MNLATWVGSNLEDPA
34	TWVGSNLEDPASREL
35	SNLEDPASRELVVSY
36	DPASRELVVSYVNVN
37	RELVVSYVNVNMGLK
38	VSYVNVNMGLKIRQL
39	NVNMGLKIRQLLWFH
40	GLKIRQLLWFHISCL
41	RQLLWFHISCLTFGR
42	WFHISCLTFGRETVL
43	SCLTFGRETVLEYLV
44	FGRETVLEYLVSFGV
45	TVLEYLVSFGVWIRT
46	YLVSFGVWIRTPPAY
47	FGVWIRTPPAYRPPN
48	IRTPPAYRPPNAPIL
49	PAYRPPNAPILSTLP
50	PPNAPILSTLPETTV
51	PILSTLPETTVVRRR
52	TLPETTVVRRRGRSP
53	TTVVRRRGRSPRRRT
54	RRRGRSPRRRTPSPR
55	RSPRRRTPSPRRRRS
56	RRTPSPRRRRSQSPR
57	SPRRRRSQSPRRRRS
58	RRSQSPRRRRSQSRE
59	SPRRRRSQSRESQCG
60	RRSQSRESQCGGGSG
61	SRESQCGGGSGGGMP
62	QCGGGSGGGMPLSYQ
63	GSGGGMPLSYQHFRK
64	GMPLSYQHFRKLLLL
65	SYQHFRKLLLLDDEA
66	FRKLLLLDDEAGPLE
67	LLLDDEAGPLEEELP
68	DEAGPLEEELPRLAD
69	PLEEELPRLADEGLN
70	ELPRLADEGLNRRVA
71	LADEGLNRRVAEDLN
72	GLNRRVAEDLNLGNL
73	RVAEDLNLGNLNVSI
74	DLNLGNLNVSIPWTH
75	GNLNVSIPWTHKVGN
76	VSIPWTHKVGNFTGL
77	WTHKVGNFTGLASST
78	VGNFTGLASSTVPVF
79	TGLASSTVPVFNPEW
80	SSTVPVFNPEWQTPS
81	PVFNPEWQTPSFPHI
82	PEWQTPSFPHIHLQE
83	TPSFPHIHLQEDIIN
84	PHIHLQEDIINRCQQ
85	LQEDIINRCQQYVGP
86	IINRCQQYVGPLTVN
87	CQQYVGPLTVNEKRR
88	VGPLTVNEKRRLKLI
89	TVNEKRRLKLIMPAR
90	KRRLKLIMPARFYPN
91	KLIMPARFYPNLTKY
92	PARFYPNLTKYLPLD
93	YPNLTKYLPLDKGIK
94	TKYLPLDKGIKPYYP
95	PLDKGIKPYYPEHAV
96	GIKPYYPEHAVNHYF
97	YYPEHAVNHYFKTRH
98	HAVNHYFKTRHYLHT
99	HYFKTRHYLHTLWKA
100	TRHYLHTLWKAGILY

ID	Sequence
101	LHTLWKAGILYKRET
102	WKAGILYKRETTRSA
103	ILYKRETTRSASFCG
104	RETTRSASFCGSPYS
105	RSASFCGSPYSWEQE
106	FCGSPYSWEQELQHG
107	PYSWEQELQHGRLVF
108	EQELQHGRLVFQTST
109	QHGRLVFQTSTRHGD
110	LVFQTSTRHGDESFC
111	TSTRHGDESFCSQSS
112	HGDESFCSQSSGILS
113	SFCSQSSGILSRSPV
114	QSSGILSRSPVGPCV
115	ILSRSPVGPCVRSQL
116	SPVGPCVRSQLKQSR
117	PCVRSQLKQSRLGLQ
118	SQLKQSRLGLQPQQG
119	QSRLGLQPQQGSLAR
120	GLQPQQGSLARGKSG
121	QQGSLARGKSGRSGS
122	LARGKSGRSGSIRAR
123	KSGRSGSIRARVHPT
124	SGSIRARVHPTTRRS
125	RARVHPTTRRSFGVE
126	HPTTRRSFGVEPSGS
127	RRSFGVEPSGSGHID
128	GVEPSGSGHIDNSAS
129	SGSGHIDNSASSTSS
130	HIDNSASSTSSCLHQ
131	SASSTSSCLHQSAVR
132	TSSCLHQSAVRKTAY
133	LHQSAVRKTAYSHLS
134	AVRKTAYSHLSTSKR
135	TAYSHLSTSKRQSSS
136	HLSTSKRQSSSGHAV
137	SKRQSSSGHAVELHN
138	SSSGHAVELHNIPPS
139	HAVELHNIPPSSARS
140	LHNIPPSSARSQSEG
141	PPSSARSQSEGPIFS
142	ARSQSEGPIFSAWWL
143	SEGPIFSAWWLQFRN
144	IFSAWWLQFRNSKPA
145	WWLQFRNSKPASDYA
146	FRNSKPASDYALTHI
147	KPASDYALTHIVNLL
148	DYALTHIVNLLEDWG
149	THIVNLLEDWGPATE
150	NLLEDWGPATEHGEH
151	DWGPATEHGEHNIRI
152	ATEHGEHNIRIPRTP
153	GEHNIRIPRTPARVT
154	IRIPRTPARVTGGVF
155	RTPARVTGGVFLVDK
156	RVTGGVFLVDKNPHN
157	GVFLVDKNPHNTTES
158	VDKNPHNTTESRLVV
159	PHNTTESRLVVDFSQ
160	TESRLVVDFSQFSRG
161	LVVDFSQFSRGSTHV
162	FSQFSRGSTHVSWPK
163	SRGSTHVSWPKFAVP
164	THVSWPKFAVPNLQS
165	WPKFAVPNLQSLTNL
166	AVPNLQSLTNLLSSN
167	LQSLTNLLSSNLSWL
168	TNLLSSNLSWLSLDV
169	SSNLSWLSLDVSAAF
170	SWLSLDVSAAFYHIP
171	LDVSAAFYHIPLHPA
172	AAFYHIPLHPAAMPH
173	HIPLHPAAMPHLLVG
174	HPAAMPHLLVGSSGL
175	MPHLLVGSSGLPRYV
176	LVGSSGLPRYVARLS
177	SGLPRYVARLSSTSR
178	RYVARLSSTSRNINY
179	RLSSTSRNINYQHGT
180	TSRNINYQHGTMQDL
181	INYQHGTMQDLHDSC
182	HGTMQDLHDSCSRNL
183	QDLHDSCSRNLYVSL
184	DSCSRNLYVSLLLLY
185	RNLYVSLLLLYKTFG
186	VSLLLLYKTFGRKLH
187	LLYKTFGRKLHLYSH
188	TFGRKLHLYSHPIIL
189	KLHLYSHPIILGFRK
190	YSHPIILGFRKIPMG
191	IILGFRKIPMGVGLS
192	FRKIPMGVGLSPFLL
193	PMGVGLSPFLLAQFT
194	GLSPFLLAQFTSAIC
195	FLLAQFTSAICSVVR
196	QFTSAICSVVRRAFP
197	AICSVVRRAFPHCLA
198	VVRRAFPHCLAFSYM
199	AFPHCLAFSYMDDVV
200	CLAFSYMDDVVLGAK

ID	Sequence
201	SYMDDVVLGAKSVQH
202	DVVLGAKSVQHLESL
203	GAKSVQHLESLFTSI
204	VQHLESLFTSITNFL
205	ESLFTSITNFLLSLG
206	TSITNFLLSLGIHLN
207	NFLLSLGIHLNPNKT
208	SLGIHLNPNKTKRWG
209	HLNPNKTKRWGYSLN
210	NKTKRWGYSLNFMGY
211	RWGYSLNFMGYVIGS
212	SLNFMGYVIGSWGTL
213	MGYVIGSWGTLPQEH
214	IGSWGTLPQEHIVLK
215	GTLPQEHIVLKIKQC
216	QEHIVLKIKQCFRKL
217	VLKIKQCFRKLPVNR
218	KQCFRKLPVNRPIDW
219	RKLPVNRPIDWKVCQ
220	VNRPIDWKVCQRIVG
221	IDWKVCQRIVGLLGF
222	VCQRIVGLLGFAAPF
223	IVGLLGFAAPFTQCG
224	LGFAAPFTQCGYPAL
225	APFTQCGYPALMPLY
226	QCGYPALMPLYACIQ
227	PALMPLYACIQSKQA
228	PLYACIQSKQAFTFS
229	CIQSKQAFTFSPTYK
230	KQAFTFSPTYKAFLC
231	TFSPTYKAFLCKQYL
232	TYKAFLCKQYLNLYP
233	FLCKQYLNLYPVARQ
234	QYLNLYPVARQRSGL
235	LYPVARQRSGLCQVF
236	ARQRSGLCQVFADAT
237	SGLCQVFADATPTGW
238	QVFADATPTGWGLAI
239	DATPTGWGLAIGHRA
240	TGWGLAIGHRAMRGT
241	LAIGHRAMRGTFVAP
242	HRAMRGTFVAPLPIH
243	RGTFVAPLPIHTAEL
244	VAPLPIHTAELLAAC
245	PIHTAELLAACFARS
246	AELLAACFARSRSGA
247	AACFARSRSGAKLIG
248	ARSRSGAKLIGTDNS
249	SGAKLIGTDNSVVLS
250	LIGTDNSVVLSRKYT
251	DNSVVLSRKYTSFPW
252	VLSRKYTSFPWLLGC
253	KYTSFPWLLGCAANW
254	FPWLLGCAANWILRG
255	LGCAANWILRGTSFV
256	ANWILRGTSFVYVPS
257	LRGTSFVYVPSALNP
258	SFVYVPSALNPAADP
259	VPSALNPAADPSAGR
260	LNPAADPSAGRLGLY
261	ADPSAGRLGLYRPLL
262	AGRLGLYRPLLHLPF
263	GLYRPLLHLPFRPTT
264	PLLHLPFRPTTGRTS
265	LPFRPTTGRTSLYAV
266	PTTGRTSLYAVSPSV
267	RTSLYAVSPSVPSHL
268	YAVSPSVPSHLPDRV
269	PSVPSHLPDRVHFAS
270	SHLPDRVHFASPLHV
271	DRVHFASPLHVAWRP
272	FASPLHVAWRPPRKR
273	LHVAWRPPRKRRAPV
274	WRPPRKRRAPVKQTL
275	RKRRAPVKQTLNFDL
276	APVKQTLNFDLLKLA
277	QTLNFDLLKLAGDVE
278	FDLLKLAGDVESNPG
279	KLAGDVESNPGPMGG
280	DVESNPGPMGGWSSK
281	NPGPMGGWSSKPRQG
282	MGGWSSKPRQGMGTN
283	SSKPRQGMGTNLSVP
284	RQGMGTNLSVPNPLG
285	GTNLSVPNPLGFFPD
286	SVPNPLGFFPDHQLD
287	PLGFFPDHQLDPAFG
288	FPDHQLDPAFGANSN
289	QLDPAFGANSNNPDW
290	AFGANSNNPDWDFNP
291	NSNNPDWDFNPNKDH
292	PDWDFNPNKDHWPEA
293	FNPNKDHWPEANQVG
294	KDHWPEANQVGAGAF
295	PEANQVGAGAFGPGF
296	QVGAGAFGPGFTPPH
297	GAFGPGFTPPHGGLL
298	PGFTPPHGGLLGWSP
299	PPHGGLLGWSPQAQG
300	GLLGWSPQAQGILTT

ID	Sequence
301	WSPQAQGILTTVPAA
302	AQGILTTVPAAPPPA
303	LTTVPAAPPPASTNR
304	PAAPPPASTNRQSGR
305	PPASTNRQSGRQPTP
306	TNRQSGRQPTPISPP
307	SGRQPTPISPPLRDS
308	PTPISPPLRDSHPQA
309	SPPLRDSHPQAMQWN
310	RDSHPQAMQWNSTTF
311	PQAMQWNSTTFHQAL
312	QWNSTTFHQALLDPR
313	TTFHQALLDPRVRGL
314	QALLDPRVRGLYFPA
315	DPRVRGLYFPAGGSS
316	RGLYFPAGGSSSGTV
317	FPAGGSSSGTVNPVP
318	GSSSGTVNPVPTTAS
319	GTVNPVPTTASPISS
320	PVPTTASPISSIFSR
321	TASPISSIFSRTGDP
322	ISSIFSRTGDPAPNM
323	FSRTGDPAPNMENTT
324	GDPAPNMENTTSGFL
325	PNMENTTSGFLGPLL
326	NTTSGFLGPLLVLQA
327	GFLGPLLVLQAGFFL
328	PLLVLQAGFFLLTRI
329	LQAGFFLLTRILTIP
330	FFLLTRILTIPQSLD
331	TRILTIPQSLDSWWT
332	TIPQSLDSWWTSLNF
333	SLDSWWTSLNFLGGA
334	WWTSLNFLGGAPTCP
335	LNFLGGAPTCPGQNS
336	GGAPTCPGQNSQSPT
337	TCPGQNSQSPTSNHS
338	QNSQSPTSNHSPTSC
339	SPTSNHSPTSCPPIC
340	NHSPTSCPPICPGYR
341	TSCPPICPGYRWMCL
342	PICPGYRWMCLRRFI
343	GYRWMCLRRFIIFLF
344	MCLRRFIIFLFILLL
345	RFIIFLFILLLCLIF
346	FLFILLLCLIFLLVL
347	LLLCLIFLLVLLDYQ
348	LIFLLVLLDYQGMLP
349	LVLLDYQGMLPVCPL
350	DYQGMLPVCPLLPGT
351	MLPVCPLLPGTSTTS
352	CPLLPGTSTTSTGPC
353	PGTSTTSTGPCKTCT
354	TTSTGPCKTCTIPAQ
355	GPCKTCTIPAQGTSM
356	TCTIPAQGTSMFPSC
357	PAQGTSMFPSCCCTK
358	TSMFPSCCCTKPSDG
359	PSCCCTKPSDGNCTC
360	CTKPSDGNCTCIPIP
361	SDGNCTCIPIPSSWA
362	CTCIPIPSSWAFARF
363	PIPSSWAFARFLWEW
364	SWAFARFLWEWASVR
365	ARFLWEWASVRFSWL
366	WEWASVRFSWLSLLV
367	SVRFSWLSLLVPFVQ
368	SWLSLLVPFVQWFVG
369	LLVPFVQWFVGLSPT
370	FVQWFVGLSPTVWLS
371	FVGLSPTVWLSVIWM
372	SPTVWLSVIWMMWYW
373	WLSVIWMMWYWGPSL
374	IWMMWYWGPSLYNIL
375	WYWGPSLYNILSPFL
376	PSLYNILSPFLPLLP
377	NILSPFLPLLPIFFC
378	PFLPLLPIFFCLWVY
379	FLPLLPIFFCLWVYI
380	ASVVFLVKGGGPGGG
381	VFLVKGGGPGGGMQL
382	KGGGPGGGMQLFHLC
383	SQSRESQCGGGSGGG
384	ESQCGGGSGGGMPLS
385	GGGSGGGMPLSYQHF

APPENDIX 3a – Vaccine immunogen peptides for mouse experiments, synthesised 2019, genotype C

ID	Sequence
1	SLLWGGVTVLAAMLI
2	GGVTVLAAMLIAGQV
3	VLAAMLIAGQVASVV
4	MLIAGQVASVVFLVK
5	GQVASVVFLVKGGGP
6	SVVFLVKGGGPGGGM
7	LVKGGGPGGGMQLFH
8	GGPGGGMQLFHLCLI
9	GGMQLFHLCLIISCS
10	LFHLCLIISCSCPTV
11	CLIISCSCPTVQASK
12	SCSCPTVQASKLCLG
13	PTVQASKLCLGWLWG
14	ASKLCLGWLWGMDID
15	CLGWLWGMDIDPYKE
16	LWGMDIDPYKEFGAS
17	DIDPYKEFGASVELL
18	YKEFGASVELLSFLP
19	GASVELLSFLPSDFF
20	ELLSFLPSDFFPSIR
21	FLPSDFFPSIRDLLD
22	DFFPSIRDLLDTASA
23	SIRDLLDTASALYRE
24	LLDTASALYREALES
25	ASALYREALESPEHC
26	YREALESPEHCSPHH
27	LESPEHCSPHHTALR
28	EHCSPHHTALRQAIL
29	PHHTALRQAILCWGE
30	ALRQAILCWGELMNL
31	AILCWGELMNLATWV
32	WGELMNLATWVGSNL
33	MNLATWVGSNLEDPA
34	TWVGSNLEDPASREL
35	SNLEDPASRELVVSY
36	DPASRELVVSYVNVN
37	RELVVSYVNVNMGLK
38	VSYVNVNMGLKIRQL
39	NVNMGLKIRQLLWFH
40	GLKIRQLLWFHISCL
41	RQLLWFHISCLTFGR
42	WFHISCLTFGRETVL
43	SCLTFGRETVLEYLV
44	FGRETVLEYLVSFGV
45	TVLEYLVSFGVWIRT
46	YLVSFGVWIRTPPAY
47	FGVWIRTPPAYRPPN
48	IRTPPAYRPPNAPIL
49	PAYRPPNAPILSTLP
50	PPNAPILSTLPETTV
51	PILSTLPETTVVRRR
52	TLPETTVVRRRGRSP
53	TTVVRRRGRSPRRRT
54	RRRGRSPRRRTPSPR
55	RSPRRRTPSPRRRRS
56	RRTPSPRRRRSQSPR
57	SPRRRRSQSPRRRRS
58	RRSQSPRRRRSQSRE
59	SPRRRRSQSRESQCG
60	RRSQSRESQCGGGSG
61	SRESQCGGGSGGGMP
62	QCGGGSGGGMPLSYQ
63	GSGGGMPLSYQHFRK
64	GMPLSYQHFRKLLLL
65	SYQHFRKLLLLDDEA
66	FRKLLLLDDEAGPLE
67	LLLDDEAGPLEELP
68	DEAGPLEELPRLAD
69	PLEELPRLADEGLN
70	ELPRLADEGLNRRVA
71	LADEGLNRRVAEDLN
72	GLNRRVAEDLNLGNL
73	RVAEDLNLGNLNVSI
74	DLNLGNLNVSIPWTH
75	GNLNVSIPWTHKVGN
76	VSIPWTHKVGNFTGL
77	WTHKVGNFTGLASST
78	VGNFTGLASSTVPVF
79	TGLASSTVPVFNPEW
80	SSTVPVFNPEWQTPS
81	PVFNPEWQTPSFPHI
82	PEWQTPSFPHIHLQE
83	TPSFPHIHLQEDIIN
84	PHIHLQEDIINRCQQ
85	LQEDIINRCQQYVGP
86	IINRCQQYVGPLTVN
87	CQQYVGPLTVNEKRR
88	VGPLTVNEKRRLKLI
89	TVNEKRRLKLIMPAR
90	KRRLKLIMPARFYPN
91	KLIMPARFYPNLTKY
92	PARFYPNLTKYLPLD
93	YPNLTKYLPLDKGIK
94	TKYLPLDKGIKPYYP
95	PLDKGIKPYYPEHAV
96	GIKPYYPEHAVNHYF
97	YYPEHAVNHYFKTRH
98	HAVNHYFKTRHYLHT
99	HYFKTRHYLHTLWKA
100	TRHYLHTLWKAGILY

ID	Sequence
101	LHTLWKAGILYKRET
102	WKAGILYKRETTRSA
103	ILYKRETTRSASFCG
104	RETTRSASFCGSPYS
105	RSASFCGSPYSWEQE
106	FCGSPYSWEQELQHG
107	PYSWEQELQHGRLVF
108	EQELQHGRLVFQTST
109	QHGRLVFQTSTRHGD
110	LVFQTSTRHGDESFC
111	TSTRHGDESFCSQSS
112	HGDESFCSQSSGILS
113	SFCSQSSGILSRSPV
114	QSSGILSRSPVGPCV
115	ILSRSPVGPCVRSQL
116	SPVGPCVRSQLKQSR
117	PCVRSQLKQSRLGLQ
118	SQLKQSRLGLQPQQG
119	QSRLGLQPQQGSLAR
120	GLQPQQGSLARGKSG
121	QQGSLARGKSGRSGS
122	LARGKSGRSGSIRAR
123	KSGRSGSIRARVHPT
124	SGSIRARVHPTTRRS
125	RARVHPTTRRSFGVE
126	HPTTRRSFGVEPSGS
127	RRSFGVEPSGSGHID
128	GVEPSGSGHIDNSAS
129	SGSGHIDNSASSTSS
130	HIDNSASSTSSCLHQ
131	SASSTSSCLHQSAVR
132	TSSCLHQSAVRKTAY
133	LHQSAVRKTAYSHLS
134	AVRKTAYSHLSTSKR
135	TAYSHLSTSKRQSSS
136	HLSTSKRQSSSGHAV
137	SKRQSSSGHAVELHN
138	SSSGHAVELHNIPPS
139	HAVELHNIPPSSARS
140	LHNIPPSSARSQSEG
141	PPSSARSQSEGPIFS
142	ARSQSEGPIFSAWWL
143	SEGPIFSAWWLQFRN
144	IFSAWWLQFRNSKPA
145	WWLQFRNSKPASDYA
146	FRNSKPASDYALTHI
147	KPASDYALTHIVNLL
148	DYALTHIVNLLEDWG
149	THIVNLLEDWGPATE
150	NLLEDWGPATEHGEH
151	DWGPATEHGEHNIRI
152	ATEHGEHNIRIPRTP
153	GEHNIRIPRTPARVT
154	IRIPRTPARVTGGVF
155	RTPARVTGGVFLVDK
156	RVTGGVFLVDKNPHN
157	GVFLVDKNPHNTTES
158	VDKNPHNTTESRLVV
159	PHNTTESRLVVDFSQ
160	TESRLVVDFSQFSRG
161	LVVDFSQFSRGSTHV
162	FSQFSRGSTHVSWPK
163	SRGSTHVSWPKFAVP
164	THVSWPKFAVPNLQS
165	WPKFAVPNLQSLTNL
166	AVPNLQSLTNLLSSN
167	LQSLTNLLSSNLSWL
168	TNLLSSNLSWLSLDV
169	SSNLSWLSLDVSAAF
170	SWLSLDVSAAFYHIP
171	LDVSAAFYHIPLHPA
172	AAFYHIPLHPAAMPH
173	HIPLHPAAMPHLLVG
174	HPAAMPHLLVGSSGL
175	MPHLLVGSSGLPRYV
176	LVGSSGLPRYVARLS
177	SGLPRYVARLSSTSR
178	RYVARLSSTSRNINY
179	RLSSTSRNINYQHGT
180	TSRNINYQHGTMQDL
181	INYQHGTMQDLHDSC
182	HGTMQDLHDSCSRNL
183	QDLHDSCSRNLYVSL
184	DSCSRNLYVSLLLLY
185	RNLYVSLLLLYKTFG
186	VSLLLLYKTFGRKLH
187	LLYKTFGRKLHLYSH
188	TFGRKLHLYSHPIIL
189	KLHLYSHPIILGFRK
190	YSHPIILGFRKIPMG
191	IILGFRKIPMGVGLS
192	FRKIPMGVGLSPFLL
193	PMGVGLSPFLLAQFT
194	GLSPFLLAQFTSAIC
195	FLLAQFTSAICSVVR
196	QFTSAICSVVRRAFP
197	AICSVVRRAFPHCLA
198	VVRRAFPHCLAFSYM
199	AFPHCLAFSYMDDVV
200	CLAFSYMDDVVLGAK

ID	Sequence
201	SYMDDVVLGAKSVQH
202	DVVLGAKSVQHLESL
203	GAKSVQHLESLFTSI
204	VQHLESLFTSITNFL
205	ESLFTSITNFLLSLG
206	TSITNFLLSLGIHLN
207	NFLLSLGIHLNPNKT
208	SLGIHLNPNKTKRWG
209	HLNPNKTKRWGYSLN
210	NKTKRWGYSLNFMGY
211	RWGYSLNFMGYVIGS
212	SLNFMGYVIGSWGTL
213	MGYVIGSWGTLPQEH
214	IGSWGTLPQEHIVLK
215	GTLPQEHIVLKIKQC
216	QEHIVLKIKQCFRKL
217	VLKIKQCFRKLPVNR
218	KQCFRKLPVNRPIDW
219	RKLPVNRPIDWKVCQ
220	VNRPIDWKVCQRIVG
221	IDWKVCQRIVGLLGF
222	VCQRIVGLLGFAAPF
223	IVGLLGFAAPFTQCG
224	LGFAAPFTQCGYPAL
225	APFTQCGYPALMPLY
226	QCGYPALMPLYACIQ
227	PALMPLYACIQSKQA
228	PLYACIQSKQAFTFS
229	CIQSKQAFTFSPTYK
230	KQAFTFSPTYKAFLC
231	TFSPTYKAFLCKQYL
232	TYKAFLCKQYLNLYP
233	FLCKQYLNLYPVARQ
234	QYLNLYPVARQRSGL
235	LYPVARQRSGLCQVF
236	ARQRSGLCQVFADAT
237	SGLCQVFADATPTGW
238	QVFADATPTGWGLAI
239	DATPTGWGLAIGHRA
240	TGWGLAIGHRAMRGT
241	LAIGHRAMRGTFVAP
242	HRAMRGTFVAPLPIH
243	RGTFVAPLPIHTAEL
244	VAPLPIHTAELLAAC
245	PIHTAELLAACFARS
246	AELLAACFARSRSGA
247	AACFARSRSGAKLIG
248	ARSRSGAKLIGTDNS
249	SGAKLIGTDNSVVLS
250	LIGTDNSVVLSRKYT
251	DNSVVLSRKYTSFPW
252	VLSRKYTSFPWLLGC
253	KYTSFPWLLGCAANW
254	FPWLLGCAANWILRG
255	LGCAANWILRGTSFV
256	ANWILRGTSFVYVPS
257	LRGTSFVYVPSALNP
258	SFVYVPSALNPAADP
259	VPSALNPAADPSAGR
260	LNPAADPSAGRLGLY
261	ADPSAGRLGLYRPLL
262	AGRLGLYRPLLHLPF
263	GLYRPLLHLPFRPTT
264	PLLHLPFRPTTGRTS
265	LPFRPTTGRTSLYAV
266	PTTGRTSLYAVSPSV
267	RTSLYAVSPSVPSHL
268	YAVSPSVPSHLPDRV
269	PSVPSHLPDRVHFAS
270	SHLPDRVHFASPLHV
271	DRVHFASPLHVAWRP
31610001	FASPLHVAWRPPKSP
31610002	LHVAWRPPKSPNSNN
31610003	WRPPKSPNSNNPDWD
291	NSNNPDWDFNPNKDH
292	PDWDFNPNKDHWPEA
293	FNPNKDHWPEANQVG
294	KDHWPEANQVGAGAF
295	PEANQVGAGAFGPGF
296	QVGAGAFGPGFTPPH
297	GAFGPGFTPPHGGLL
298	PGFTPPHGGLLGWSP
299	PPHGGLLGWSPQAQG
300	GLLGWSPQAQGILTT
301	WSPQAQGILTTVPAA
302	AQGILTTVPAAPPPA
303	LTTVPAAPPPASTNR
304	PAAPPPASTNRQSGR
305	PPASTNRQSGRQPTP
306	TNRQSGRQPTPISPP
307	SGRQPTPISPPLRDS
308	PTPISPPLRDSHPQA
309	SPPLRDSHPQAMQWN
310	RDSHPQAMQWNSTTF
311	PQAMQWNSTTFHQAL
312	QWNSTTFHQALLDPR
313	TTFHQALLDPRVRGL
314	QALLDPRVRGLYFPA
315	DPRVRGLYFPAGGSS
316	RGLYFPAGGSSSGTV

ID	Sequence
317	FPAGGSSSGTVNPVP
318	GSSSGTVNPVPTTAS
319	GTVNPVPTTASPISS
320	PVPTTASPISSIFSR
321	TASPISSIFSRTGDP
31610004	ISSIFSRTGDPAPNG
31610005	FSRTGDPAPNGSKGK
31610006	GDPAPNGSKGKRKRR
31610007	PNGSKGKRKRRAPVK
275	RKRRAPVKQTLNFDL
276	APVKQTLNFDLLKLA
277	QTLNFDLLKLAGDVE
278	FDLLKLAGDVESNPG
31610008	KLAGDVESNPGPMDA
31610009	DVESNPGPMDAMKRG
31610010	NPGPMDAMKRGLCCV
31610011	MDAMKRGLCCVLLLC
31610012	KRGLCCVLLLCGAVF
31610022	CCVLLLCGAVFVSPS
31610014	LLCGAVFVSPSQEIH
31610015	AVFVSPSQEIHARFR
31610016	SPSQEIHARFRRMGG
282	MGGWSSKPRQGMGTN
283	SSKPRQGMGTNLSVP
284	RQGMGTNLSVPNPLG
285	GTNLSVPNPLGFFPD
286	SVPNPLGFFPDHQLD
287	PLGFFPDHQLDPAFG
288	FPDHQLDPAFGANSN
289	QLDPAFGANSNNPDW
290	AFGANSNNPDWDFNP
291	NSNNPDWDFNPNKDH
292	PDWDFNPNKDHWPEA
293	FNPNKDHWPEANQVG
31610017	KDHWPEANQVGLEGG
31610018	PEANQVGLEGGSGGM
31610019	QVGLEGGSGGMENTT
31610020	EGGSGGMENTTSGFL
31610021	GGMENTTSGFLGPLL
326	NTTSGFLGPLLVLQA
327	GFLGPLLVLQAGFFL
328	PLLVLQAGFFLLTRI
329	LQAGFFLLTRILTIP
330	FFLLTRILTIPQSLD
331	TRILTIPQSLDSWWT
332	TIPQSLDSWWTSLNF
333	SLDSWWTSLNFLGGA
334	WWTSLNFLGGAPTCP
335	LNFLGGAPTCPGQNS
336	GGAPTCPGQNSQSPT
337	TCPGQNSQSPTSNHS
338	QNSQSPTSNHSPTSC
339	SPTSNHSPTSCPPIC
340	NHSPTSCPPICPGYR
341	TSCPPICPGYRWMCL
342	PICPGYRWMCLRRFI
343	GYRWMCLRRFIIFLF
344	MCLRRFIIFLFILLL
345	RFIIFLFILLLCLIF
346	FLFILLLCLIFLLVL
347	LLLCLIFLLVLLDYQ
348	LIFLLVLLDYQGMLP
349	LVLLDYQGMLPVCPL
350	DYQGMLPVCPLLPGT
351	MLPVCPLLPGTSTTS
352	CPLLPGTSTTSTGPC
353	PGTSTTSTGPCKTCT
354	TTSTGPCKTCTIPAQ
355	GPCKTCTIPAQGTSM
356	TCTIPAQGTSMFPSC
357	PAQGTSMFPSCCCTK
358	TSMFPSCCCTKPSDG
359	PSCCCTKPSDGNCTC
360	CTKPSDGNCTCIPIP
361	SDGNCTCIPIPSSWA
362	CTCIPIPSSWAFARF
363	PIPSSWAFARFLWEW
364	SWAFARFLWEWASVR
365	ARFLWEWASVRFSWL
366	WEWASVRFSWLSLLV
367	SVRFSWLSLLVPFVQ
368	SWLSLLVPFVQWFVG
369	LLVPFVQWFVGLSPT
370	FVQWFVGLSPTVWLS
371	FVGLSPTVWLSVIWM
372	SPTVWLSVIWMMWYW
373	WLSVIWMMWYWGPSL
374	IWMMWYWGPSLYNIL
375	WYWGPSLYNILSPFL
376	PSLYNILSPFLPLLP
377	NILSPFLPLLPIFFC
378	PFLPLLPIFFCLWVY
379	FLPLLPIFFCLWVYI

APPENDIX 3b – Vaccine immunogen peptides for mouse experiments, synthesised 2019, genotype D (left) compared to genotype C (right)

ID (genotype D)	Sequence (genotype D)	ID (genotype C)	Sequence (genotype C)
400	DPASRDLVVSYVNTN	36	DPASRELVVSYVNVN
401	RDLVVSYVNTNMGLK	37	RELVVSYVNVNMGLK
402	VSYVNTNMGLKFRQL	38	VSYVNVNMGLKIRQL
403	NTNMGLKFRQLLWFH	39	NVNMGLKIRQLLWFH
404	GLKFRQLLWFHISCL	40	GLKIRQLLWFHISCL
405	WFHISCLTFGRETVI	42	WFHISCLTFGRETVL
406	SCLTFGRETVIEYLV	43	SCLTFGRETVLEYLV
407	FGRETVIEYLVSFGV	44	FGRETVLEYLVSFGV
408	TVIEYLVSFGVWIRT	45	TVLEYLVSFGVWIRT

APPENDIX 4 – HBV001 inclusion and exclusion criteria

Inclusion Criteria

All participants (cohorts 1-4):

1. Adult males or females aged ≥18 to ≤65 years at screening
2. Body Mass Index ≤30kg/m^2
3. Able to provide informed consent indicating they understand the purpose of, and procedures required, for the study and are willing to participate
4. If female, willing not to become pregnant up to 8 weeks after last dose of study vaccine, not breast feeding
5. If female: Not pregnant and one of the following:
   - Of non-childbearing potential
   - Of childbearing potential but agrees to practice highly effective contraception for 4 weeks prior to study vaccine and 8 weeks after study vaccine.

Additionally, for healthy volunteers (cohorts 1 and 2):

6. Considered to be healthy with no current conditions that may significantly impair participant safety or influence study results, in the opinion of the Investigator

Additionally, for patients with chronic HBV (cohorts 3 and 4):

7. Documented evidence of chronic HBV infection (HBsAg positive ≥6 months with detectable HBsAg levels at screening)
8. Receipt of only either entecavir or tenofovir for at least 12 months before screening
9. Virally suppressed (HBV DNA <40 IU/mL for ≥6 months)
10. HBsAg <4000IU/mL

Exclusion Criteria

All participants (cohorts 1-4):

1. Presence of any significant acute or chronic, uncontrolled medical/psychiatric illness
2. Hepatitis C virus (HCV) antibody positive.
3. HIV antibody positive
4. History or evidence of autoimmune disease or known immunodeficiency of any cause

5. Prolonged therapy with immunomodulators (e.g. corticosteroids) or biologics (e.g. monoclonal antibodies, IFN) within 3 months of screening
6. Receipt of immunoglobulin or other blood products within 3 months prior to screening
7. Receipt of any investigational drug or vaccine within 3 months prior to screening
8. Any history of receipt of any adenoviral vaccine
9. Receipt of any live vaccines within 30 days prior to screening
10. Receipt of any inactivated vaccines within 14 days prior to screening
11. History of allergic disease or reactions likely to be exacerbated by any component of the vaccine
12. Any history of anaphylaxis in reaction to vaccination
13. Malignancy within 5 years prior to screening with the exception of specific cancers that are cured by surgical resection (e.g. except basal cell skin carcinoma of the skin and cervical carcinoma). Participants under evaluation for possible malignancy are not eligible
14. Current alcohol or substance abuse judged by the Investigator to potentially interfere with participant safety and compliance
15. Significant cardiac disease or unstable uncontrolled cardiac disease
16. Any laboratory test at screening which is abnormal and which is deemed by the Investigator to be clinically significant
17. Any other finding that, in the opinion of the Investigator, deems the participant unsuitable for the study

Additionally, for healthy volunteers (cohorts 1 and 2):

18. HBsAg positive

Additionally, for patients with chronic HBV (cohorts 3 and 4):

19. Co-infection with hepatitis delta
20. Documented cirrhosis or advanced fibrosis indicated by a liver biopsy within 6 months prior to screening.

    In the absence of an appropriate liver biopsy, either 1 of the following:
    - Screening Fibroscan with a result >9 kPa within ≤6 months of screening or
    - Screening FibroTest >0.48 and AST to platelet ratio index of >1

    In the event of discordant results between non-invasive methods, the Fibroscan result will take precedence.
21. ALT >3 × ULN, INR >1.5 unless the participant was stable on an anticoagulant regimen affecting INR, albumin <35 g/L, total bilirubin >2 mg/dL, platelet count <100,000/mL
22. A history of liver decompensation (e.g. ascites, encephalopathy or variceal haemorrhage)
23. Prior or current hepatocellular carcinoma
24. Chronic liver disease of a non-HBV aetiology
25. Any herbal supplements and or other medicines with potential liver toxicity within the previous 3 months prior to enrolment into this study

APPENDIX 5 – HBV001 study procedures

	Screen	Vaccination			Follow-up						End of Study
	Day-42 to -1	Day 0			Day 1	Day 7±1	Day 14±1	Day 28±2	Day 56±3	Day 84±7	Day 168±7
		Pre-	0	Post-							
Informed consent	X										
Baseline/eligibility variables											
Demographics	X										
Inclusion and exclusion criteria	X	X									
Height and Weight	X										
Medical and disease history	X	X									
HIV, HCV, HBV, HDV serology	X										
Urinalysis	X										
Urine pregnancy test (β-hCG)	X	X						X	X	X	X
Laboratory eligibility and safety tests											
Haematology	X	X				X	X	X	X	X	X
Biochemistry	X	X				X	X	X	X	X	X
Liver function tests	X	X				X	X	X	X	X	X
Study vaccination											
Vaccination			X								
Post-vaccination observation				X							
Other Safety assessments											
Full physical examination	X	X									
Directed physical examination if required						X	X	X	X	X	X
Vital signs	X	X		X		X	X	X	X	X	X
Local/systemic reactogenicity		X		X	X	X					
Unsolicited adverse events	X	X	X	X	X	X	X	X			
Serious adverse events and adverse events of special interest	X	X	X	X	X	X	X	X	X	X	X
Prior and concomitant medications	X	X	X	X	X	X	X	X	X	X	X
Blood for HBV disease markers		X						X	X	X	X
Blood for cellular immunogenicity		X					X	X	X	X	X

APPENDIX 6a – Vaccine immunogen peptides for HBV001, genotype C

ID	Sequence
1	MSLLWGGVTVLAAML
2	WGGVTVLAAMLIAGQ
3	TVLAAMLIAGQVASV
4	AMLIAGQVASVVFLV
5	AGQVASVVFLVKGGG
6	ASVVFLVKGGGPGGG
7	FLVKGGGPGGGMQLF
8	GGGPGGGMQLFHLCL
9	GGGMQLFHLCLIISC
10	QLFHLCLIISCSCPT
11	LCLIISCSCPTVQAS
12	ISCSCPTVQASKLCL
13	CPTVQASKLCLGWLW
14	QASKLCLGWLWGMDI
15	LCLGWLWGMDIDPYK
16	WLWGMDIDPYKEFGA
17	MDIDPYKEFGASVEL
18	PYKEFGASVELLSFL
19	FGASVELLSFLPSDF
20	VELLSFLPSDFFPSI
21	SFLPSDFFPSIRDLL
22	SDFFPSIRDLLDTAS
23	PSIRDLLDTASALYR
24	DLLDTASALYREALE
25	TASALYREALESPEH
26	LYREALESPEHCSPH
27	ALESPEHCSPHHTAL
28	PEHCSPHHTALRQAI
29	SPHHTALRQAILCWG
30	TALRQAILCWGELMN
31	QAILCWGELMNLATW
32	CWGELMNLATWVGSN
33	LMNLATWVGSNLEDP
34	ATWVGSNLEDPASRE
35	GSNLEDPASRELVVS
36	EDPASRELVVSYVNV
37	SRELVVSYVNVNMGL
38	VVSYVNVNMGLKIRQ
39	VNVNMGLKIRQLLWF
40	MGLKIRQLLWFHISC
41	IRQLLWFHISCLTFG
42	LWFHISCLTFGRETV
43	ISCLTFGRETVLEYL
44	TFGRETVLEYLVSFG
45	ETVLEYLVSFGVWIR
46	EYLVSFGVWIRTPPA
47	SFGVWIRTPPAYRPP
48	WIRTPPAYRPPNAPI
49	PPAYRPPNAPILSTL
50	RPPNAPILSTLPETT
51	APILSTLPETTVVRR
52	STLPETTVVRRRGRS
53	ETTVVRRRGRSPRRR
54	VRRRGRSPRRRTPSP
55	GRSPRRRTPSPRRRR
56	RRRTPSPRRRRSQSP
57	PSPRRRRSQSPRRRR
58	RRRSQSPRRRRSQSR
59	QSPRRRRSQSRESQC
60	RRRSQSRESQCGGGS
61	QSRESQCGGGSGGGM
62	SQCGGGSGGGMPLSY
63	GGSGGGMPLSYQHFR
64	GGMPLSYQHFRKLLL
65	LSYQHFRKLLLLDDE
66	HFRKLLLLDDEAGPL
67	LLLLDDEAGPLEEEL
68	DDEAGPLEEELPRLA
69	GPLEEELPRLADEGL
70	EELPRLADEGLNRRV
71	RLADEGLNRRVAEDL
72	EGLNRRVAEDLNLGN
73	RRVAEDLNLGNLNVS
74	EDLNLGNLNVSIPWT
75	LGNLNVSIPWTHKVG
76	NVSIPWTHKVGNFTG
77	PWTHKVGNFTGLASS
78	KVGNFTGLASSTVPV
79	FTGLASSTVPVFNPE
80	ASSTVPVFNPEWQTP
81	VPVFNPEWQTPSFPH
82	NPEWQTPSFPHIHLQ
83	QTPSFPHIHLQEDII
84	FPHIHLQEDIINRCQ
85	HLQEDIINRCQQYVG
86	DIINRCQQYVGPLTV
87	RCQQYVGPLTVNEKR
88	YVGPLTVNEKRRLKL
89	LTVNEKRRLKLIMPA
90	EKRRLKLIMPARFYP
91	LKLIMPARFYPNLTK
92	MPARFYPNLTKYLPL
93	FYPNLTKYLPLDKGI
94	LTKYLPLDKGIKPYY
95	LPLDKGIKPYYPEHA
96	KGIKPYYPEHAVNHY
97	PYYPEHAVNHYFKTR
98	EHAVNHYFKTRHYLH
99	NHYFKTRHYLHTLWK
100	KTRHYLHTLWKAGIL

ID	Sequence
101	YLHTLWKAGILYKRE
102	LWKAGILYKRETTRS
103	GILYKRETTRSASFC
104	KRETTRSASFCGSPY
105	TRSASFCGSPYSWEQ
106	SFCGSPYSWEQELQH
107	SPYSWEQELQHGRLV
108	WEQELQHGRLVFQTS
109	LQHGRLVFQTSTRHG
110	RLVFQTSTRHGDESF
111	QTSTRHGDESFCSQS
112	RHGDESFCSQSSGIL
113	ESFCSQSSGILSRSP
114	SQSSGILSRSPVGPC
115	GILSRSPVGPCVRSQ
116	RSPVGPCVRSQLKQS
117	GPCVRSQLKQSRLGL
118	RSQLKQSRLGLQPQQ
119	KQSRLGLQPQQGSLA
120	LGLQPQQGSLARGKS
121	PQQGSLARGKSGRSG
122	SLARGKSGRSGSIRA
123	GKSGRSGSIRARVHP
124	RSGSIRARVHPTTRR
125	IRARVHPTTRRSFGV
126	VHPTTRRSFGVEPSG
127	TRRSFGVEPSGSGHI
128	FGVEPSGSGHIDNSA
129	PSGSGHIDNSASSTS
130	GHIDNSASSTSSCLH
131	NSASSTSSCLHQSAV
132	STSSCLHQSAVRKTA
133	CLHQSAVRKTAYSHL
134	SAVRKTAYSHLSTSK
135	KTAYSHLSTSKRQSS
136	SHLSTSKRQSSSGHA
137	TSKRQSSSGHAVELH
138	QSSSGHAVELHNIPP
139	GHAVELHNIPPSSAR
140	ELHNIPPSSARSQSE
141	IPPSSARSQSEGPIF
142	SARSQSEGPIFSAWW
143	QSEGPIFSAWWLQFR
144	PIFSAWWLQFRNSKP
145	AWWLQFRNSKPASDY
146	QFRNSKPASDYALTH
147	SKPASDYALTHIVNL
148	SDYALTHIVNLLEDW
149	LTHIVNLLEDWGPAT
150	VNLLEDWGPATEHGE
151	EDWGPATEHGEHNIR
152	PATEHGEHNIRIPRT
153	HGEHNIRIPRTPARV
154	NIRIPRTPARVTGGV
155	PRTPARVTGGVFLVD
156	ARVTGGVFLVDKNPH
157	GGVFLVDKNPHNTTE
158	LVDKNPHNTTESRLV
159	NPHNTTESRLVVDFS
160	TTESRLVVDFSQFSR
161	RLVVDFSQFSRGSTH
162	DFSQFSRGSTHVSWP
163	FSRGSTHVSWPKFAV
164	STHVSWPKFAVPNLQ
165	SWPKFAVPNLQSLTN
166	FAVPNLQSLTNLLSS
167	NLQSLTNLLSSNLSW
168	LTNLLSSNLSWLSLD
169	LSSNLSWLSLDVSAA
170	LSWLSLDVSAAFYHI
171	SLDVSAAFYHIPLHP
172	SAAFYHIPLHPAAMP
173	YHIPLHPAAMPHLLV
174	LHPAAMPHLLVGSSG
175	AMPHLLVGSSGLPRY
176	LLVGSSGLPRYVARL
177	SSGLPRYVARLSSTS
178	PRYVARLSSTSRNIN
179	ARLSSTSRNINYQHG
180	STSRNINYQHGTMQD
181	NINYQHGTMQDLHDS
182	QHGTMQDLHDSCSRN
183	MQDLHDSCSRNLYVS
184	HDSCSRNLYVSLLLL
185	SRNLYVSLLLLYKTF
186	YVSLLLLYKTFGRKL
187	LLLYKTFGRKLHLYS
188	KTFGRKLHLYSHPII
189	RKLHLYSHPIILGFR
190	LYSHPIILGFRKIPM
191	PIILGFRKIPMGVGL
192	GFRKIPMGVGLSPFL
193	IPMGVGLSPFLLAQF
194	VGLSPFLLAQFTSAI
195	PFLLAQFTSAICSVV
196	AQFTSAICSVVRRAF
197	SAICSVVRRAFPHCL
198	SVVRRAFPHCLAFSY
199	RAFPHCLAFSYMDDV
200	HCLAFSYMDDVVLGA

ID	Sequence
201	FSYMDDVVLGAKSVQ
202	DDVVLGAKSVQHLES
203	LGAKSVQHLESLFTS
204	SVQHLESLFTSITNF
205	LESLFTSITNFLLSL
206	FTSITNFLLSLGIHL
207	TNFLLSLGIHLNPNK
208	LSLGIHLNPNKTKRW
209	IHLNPNKTKRWGYSL
210	PNKTKRWGYSLNFMG
211	KRWGYSLNFMGYVIG
212	YSLNFMGYVIGSWGT
213	FMGYVIGSWGTLPQE
214	VIGSWGTLPQEHIVL
215	WGTLPQEHIVLKIKQ
216	PQEHIVLKIKQCFRK
217	IVLKIKQCFRKLPVN
218	IKQCFRKLPVNRPID
219	FRKLPVNRPIDWKVC
220	PVNRPIDWKVCQRIV
221	PIDWKVCQRIVGLLG
222	KVCQRIVGLLGFAAP
223	RIVGLLGFAAPFTQC
224	LLGFAAPFTQCGYPA
225	AAPFTQCGYPALMPL
226	TQCGYPALMPLYACI
227	YPALMPLYACIQSKQ
228	MPLYACIQSKQAFTF
229	ACIQSKQAFTFSPTY
230	SKQAFTFSPTYKAFL
231	FTFSPTYKAFLCKQY
232	PTYKAFLCKQYLNLY
233	AFLCKQYLNLYPVAR
234	KQYLNLYPVARQRSG
235	NLYPVARQRSGLCQV
236	VARQRSGLCQVFADA
237	RSGLCQVFADATPTG
238	CQVFADATPTGWGLA
239	ADATPTGWGLAIGHR
240	PTGWGLAIGHRAMRG
241	GLAIGHRAMRGTFVA
242	GHRAMRGTFVAPLPI
243	MRGTFVAPLPIHTAE
244	FVAPLPIHTAELLAA
245	LPIHTAELLAACFAR
246	TAELLAACFARSRSG
247	LAACFARSRSGAKLI
248	FARSRSGAKLIGTDN
249	RSGAKLIGTDNSVVL
250	KLIGTDNSVVLSRKY
251	TDNSVVLSRKYTSFP
252	VVLSRKYTSFPWLLG
253	RKYTSFPWLLGCAAN
254	SFPWLLGCAANWILR
255	LLGCAANWILRGTSF
256	AANWILRGTSFVYVP
257	ILRGTSFVYVPSALN
258	TSFVYVPSALNPAAD
259	YVPSALNPAADPSAG
260	ALNPAADPSAGRLGL
261	AADPSAGRLGLYRPL
262	SAGRLGLYRPLLHLP
263	LGLYRPLLHLPFRPT
264	RPLLHLPFRPTTGRT
265	HLPFRPTTGRTSLYA
266	RPTTGRTSLYAVSPS
267	GRTSLYAVSPSVPSH
268	LYAVSPSVPSHLPDR
269	SPSVPSHLPDRVHFA
270	PSHLPDRVHFASPLH
271	PDRVHFASPLHVAWR
272	HFASPLHVAWRPPKS
273	PLHVAWRPPKSPNSN
274	AWRPPKSPNSNNPDW
275	PKSPNSNNPDWDFNP
276	NSNNPDWDFNPNKDH
277	PDWDFNPNKDHWPEA
278	FNPNKDHWPEANQVG
279	KDHWPEANQVGAGAF
280	PEANQVGAGAFGPGF
281	QVGAGAFGPGFTPPH
282	GAFGPGFTPPHGGLL
283	PGFTPPHGGLLGWSP
284	PPHGGLLGWSPQAQG
285	GLLGWSPQAQGILTT
286	WSPQAQGILTTVPAA
287	AQGILTTVPAAPPPA
288	LTTVPAAPPPASTNR
289	PAAPPPASTNRQSGR
290	PPASTNRQSGRQPTP
291	TNRQSGRQPTPISPP
292	SGRQPTPISPPLRDS
293	PTPISPPLRDSHPQA
294	SPPLRDSHPQAMQWN
295	RDSHPQAMQWNSTTF
296	PQAMQWNSTTFHQAL
297	QWNSTTFHQALLDPR
298	TTFHQALLDPRVRGL
299	QALLDPRVRGLYFPA
300	DPRVRGLYFPAGGSS

ID	Sequence
301	RGLYFPAGGSSSGTV
302	FPAGGSSSGTVNPVP
303	GSSSGTVNPVPTTAS
304	GTVNPVPTTASPISS
305	PVPTTASPISSIFSR
306	TASPISSIFSRTGDP
307	ISSIFSRTGDPAPNG
308	FSRTGDPAPNGSKGK
309	GDPAPNGSKGKRKRR
310	PNGSKGKRKRRAPVK
311	KGKRKRRAPVKQTLN
312	KRRAPVKQTLNFDLL
313	PVKQTLNFDLLKLAG
314	TLNFDLLKLAGDVES
315	DLLKLAGDVESNPGP
316	LAGDVESNPGPMDAM
317	VESNPGPMDAMKRGL
318	PGPMDAMKRGLCCVL
319	DAMKRGLCCVLLLCG
320	RGLCCVLLLCGAVFV
321	CVLLLCGAVFVSPSQ
322	LCGAVFVSPSQEIHA
323	VFVSPSQEIHARFRR
324	PSQEIHARFRRMGGW
325	IHARFRRMGGWSSKP
326	FRRMGGWSSKPRQGM
327	GGWSSKPRQGMGTNL
328	SKPRQGMGTNLSVPN
329	QGMGTNLSVPNPLGF
330	TNLSVPNPLGFFPDH
331	VPNPLGFFPDHQLDP
332	LGFFPDHQLDPAFGA
333	PDHQLDPAFGANSNN
334	LDPAFGANSNNPDWD
335	FGANSNNPDWDFNPN
336	SNNPDWDFNPNKDHW
337	DWDFNPNKDHWPEAN
338	NPNKDHWPEANQVGL
339	DHWPEANQVGLEGGS
340	EANQVGLEGGSGGME
341	VGLEGGSGGMENTTS
342	GGSGGMENTTSGFLG
343	GMENTTSGFLGPLLV
344	TTSGFLGPLLVLQAG
345	FLGPLLVLQAGFFLL
346	LLVLQAGFFLLTRIL
347	QAGFFLLTRILTIPQ
348	FLLTRILTIPQSLDS
349	RILTIPQSLDSWWTS
350	IPQSLDSWWTSLNFL
351	LDSWWTSLNFLGGAP
352	WTSLNFLGGAPTCPG
353	NFLGGAPTCPGQNSQ
354	GAPTCPGQNSQSPTS
355	CPGQNSQSPTSNHSP
356	NSQSPTSNHSPTSCP
357	PTSNHSPTSCPPICP
358	HSPTSCPPICPGYRW
359	SCPPICPGYRWMCLR
360	ICPGYRWMCLRRFII
361	YRWMCLRRFIIFLFI
362	CLRRFIIFLFILLLC
363	FIIFLFILLLCLIFL
364	LFILLLCLIFLLVLL
365	LLCLIFLLVLLDYQG
366	IFLLVLLDYQGMLPV
367	VLLDYQGMLPVCPLL
368	YQGMLPVCPLLPGTS
369	LPVCPLLPGTSTTST
370	PLLPGTSTTSTGPCK
371	GTSTTSTGPCKTCTI
372	TSTGPCKTCTIPAQG
373	PCKTCTIPAQGTSMF
374	CTIPAQGTSMFPSCC
375	AQGTSMFPSCCCTKP
376	SMFPSCCCTKPSDGN
377	SCCCTKPSDGNCTCI
378	TKPSDGNCTCIPIPS
379	DGNCTCIPIPSSWAF
380	TCIPIPSSWAFARFL
381	IPSSWAFARFLWEWA
382	WAFARFLWEWASVRF
383	RFLWEWASVRFSWLS
384	EWASVRFSWLSLLVP
385	VRFSWLSLLVPFVQW
386	WLSLLVPFVQWFVGL
387	LVPFVQWFVGLSPTV
388	VQWFVGLSPTVWLSV
389	VGLSPTVWLSVIWMM
390	PTVWLSVIWMMWYWG
391	LSVIWMMWYWGPSLY
392	WMMWYWGPSLYNILS
393	YWGPSLYNILSPFLP
394	SLYNILSPFLPLLPI
395	ILSPFLPLLPIFFCL
396	FLPLLPIFFCLWVYI

APPENDIX 6b – Vaccine immunogen peptides for HBV001, genotype D

ID	Sequence
17	MDIDPYKEFGATVEL
18	PYKEFGATVELLSFL
19	FGATVELLSFLPSDF
20	VELLSFLPSDFFPSV
21	SFLPSDFFPSVRDLL
22	SDFFPSVRDLLDTAS
23	PSVRDLLDTASALYR
30	TALRQAILCWGELMT
31	QAILCWGELMTLATW
32	CWGELMTLATWVGGN
33	LMTLATWVGGNLEDP
34	ATWVGGNLEDPISRD
35	GGNLEDPISRDLVVS
36	EDPISRDLVVSYVNT
37	SRDLVVSYVNTNMGL
38	VVSYVNTNMGLKFRQ
39	VNTNMGLKFRQLLWF
40	MGLKFRQLLWFHISC
41	FRQLLWFHISCLTFG
43	ISCLTFGRETVIEYL
44	TFGRETVIEYLVSFG
45	ETVIEYLVSFGVWIR
64	GGMPLSYQHFRRLLL
65	LSYQHFRRLLLLDDE
66	HFRRLLLLDDEAGPL
77	PWTHKVGNFTGLYSS
78	KVGNFTGLYSSTVPV
79	FTGLYSSTVPVFNPH
80	YSSTVPVFNPHWKTP
81	VPVFNPHWKTPSFPN
82	NPHWKTPSFPNIHLH
83	KTPSFPNIHLHQDII
84	FPNIHLHQDIIKKCE
85	HLHQDIIKKCEQFVG
86	DIIKKCEQFVGPLTV
87	KCEQFVGPLTVNEKR
88	FVGPLTVNEKRRLQL
89	LTVNEKRRLQLIMPA
90	EKRRLQLIMPARFYP
91	LQLIMPARFYPNVTK
92	MPARFYPNVTKYLPL
93	FYPNVTKYLPLDKGI
94	VTKYLPLDKGIKPYY
95	LPLDKGIKPYYPEHL
96	KGIKPYYPEHLVNHY
97	PYYPEHLVNHYFQTR
98	EHLVNHYFQTRHYLH
99	NHYFQTRHYLHTLWK
100	QTRHYLHTLWKAGIL
102	LWKAGILYKRETTHS
103	GILYKRETTHSASFC
104	KRETTHSASFCGSPY
105	THSASFCGSPYSWEQ
109	LQHGRLVFQTSTRHA
110	RLVFQTSTRHADESF
111	QTSTRHADESFHQQS
112	RHADESFHQQSSGIL
113	ESFHQQSSGILSRPP
114	QQSSGILSRPPVGSS
115	GILSRPPVGSSLQSK
116	RPPVGSSLQSKHRKS
117	GSSLQSKHRKSRLGL
118	QSKHRKSRLGLQSQQ
119	RKSRLGLQSQQGHLA
120	LGLQSQQGHLARRQQ
121	SQQGHLARRQQGRSW
122	HLARRQQGRSWSIRA
123	RQQGRSWSIRAGIHP
124	RSWSIRAGIHPTARR
125	IRAGIHPTARRPCGV
126	IHPTARRPCGVEPSG
127	ARRPCGVEPSGSGHN
128	CGVEPSGSGHNTNLA
129	PSGSGHNTNLASKSA
130	GHNTNLASKSASCLY
131	NLASKSASCLYQSPV
132	KSASCLYQSPVRKAA
133	CLYQSPVRKAAYPAV
134	SPVRKAAYPAVSTFE
135	KAAYPAVSTFEKHSS
136	PAVSTFEKHSSSGHA
137	TFEKHSSSGHAVELH
138	HSSSGHAVELHNLPP
139	GHAVELHNLPPNSAR
140	ELHNLPPNSARAQSE
141	LPPNSARAQSERPVF
142	SARAQSERPVFPCWW
143	QSERPVFPCWWLQFR
144	PVFPCWWLQFRNSKP
145	CWWLQFRNSKPCSDY
146	QFRNSKPCSDYCLSH
147	SKPCSDYCLSHIVNL
148	SDYCLSHIVNLLEDW
149	LSHIVNLLEDWGPCA
150	VNLLEDWGPCAEHGE
151	EDWGPCAEHGEHHIR
152	PCAEHGEHHIRIPRT
153	HGEHHIRIPRTPARV
154	HIRIPRTPARVTGGV
157	GGVFLVDKNPHNTAE

ID	Sequence
158	LVDKNPHNTAESRLV
159	NPHNTAESRLVVDFS
160	TAESRLVVDFSQFSR
161	RLVVDFSQFSRGNYR
162	DFSQFSRGNYRVSWP
163	FSRGNYRVSWPKFAV
164	NYRVSWPKFAVPNLQ
170	LSWLSLDVSAAFYHL
171	SLDVSAAFYHLPLHP
172	SAAFYHLPLHPAAMP
173	YHLPLHPAAMPHLLV
175	AMPHLLVGSSGLSRY
176	LLVGSSGLSRYVARL
177	SSGLSRYVARLSSNS
178	SRYVARLSSNSRIFN
179	ARLSSNSRIFNHQHG
180	SNSRIFNHQHGTMQN
181	IFNHQHGTMQNLHDS
182	QHGTMQNLHDSCSRN
183	MQNLHDSCSRNLYVS
185	SRNLYVSLLLLYQTF
186	YVSLLLLYQTFGRKL
187	LLLYQTFGRKLHLYS
188	QTFGRKLHLYSHPII
203	LGAKSVQHLESLFTA
204	SVQHLESLFTAVTNF
205	LESLFTAVTNFLLSL
206	FTAVTNFLLSLGIHL
210	PNKTKRWGYSLHFMG
211	KRWGYSLHFMGYVIG
212	YSLHFMGYVIGCYGS
213	FMGYVIGCYGSLPQD
214	VIGCYGSLPQDHIIQ
215	YGSLPQDHIIQKIKE
216	PQDHIIQKIKECFRK
217	IIQKIKECFRKLPVN
218	IKECFRKLPVNRPID
234	KQYLNLYPVARQRPG
235	NLYPVARQRPGLCQV
236	VARQRPGLCQVFADA
237	RPGLCQVFADATPTG
238	CQVFADATPTGWGLV
239	ADATPTGWGLVMGHQ
240	PTGWGLVMGHQRMRG
241	GLVMGHQRMRGTFLA
242	GHQRMRGTFLAPLPI
243	MRGTFLAPLPIHTAE
244	FLAPLPIHTAELLAA
247	LAACFARSRSGANIL
248	FARSRSGANILGTDN
249	RSGANILGTDNSVVL
250	NILGTDNSVVLSRKY
258	TSFVYVPSALNPADD
259	YVPSALNPADDPSRG
260	ALNPADDPSRGRLGL
261	ADDPSRGRLGLSRPL
262	SRGRLGLSRPLLRLP
263	LGLSRPLLRLPFRPT
264	RPLLRLPFRPTTGRT
265	RLPFRPTTGRTSLYA
266	RPTTGRTSLYADSPS
267	GRTSLYADSPSVPSH
268	LYADSPSVPSHLPDR
273	PLHVAWRPPKSPNTA
274	AWRPPKSPNTANPDW
275	PKSPNTANPDWDFNP
276	NTANPDWDFNPNKDT
277	PDWDFNPNKDTWPDA
278	FNPNKDTWPDANKVG
279	KDTWPDANKVGAGAF
280	PDANKVGAGAFGLGF
281	KVGAGAFGLGFTPPH
282	GAFGLGFTPPHGGLV
283	LGFTPPHGGLVGWSP
284	PPHGGLVGWSPQAQG
285	GLVGWSPQAQGIIQT
286	WSPQAQGIIQTLPAN
287	AQGIIQTLPANPPPA
288	IQTLPANPPPASTNR
289	PANPPPASTNRQSGR
291	TNRQSGRQPTPLSPP
292	SGRQPTPLSPPLRNT
293	PTPLSPPLRNTHPQA
294	SPPLRNTHPQAMQLN
295	RNTHPQAMQLNSTTF
296	PQAMQLNSTTFHQTL
297	QLNSTTFHQTLQEPR
298	TTFHQTLQEPRVRGL
299	QTLQEPRVRGLYFPA
300	EPRVRGLYFPAGGSS
303	GSSSGTVNPVPTTVS
304	GTVNPVPTTVSPISS
305	PVPTTVSPISSIFSR
306	TVSPISSIFSRIGDP
307	ISSIFSRIGDPALNG
308	FSRIGDPALNGSKGK
309	GDPALNGSKGKRKRR
310	LNGSKGKRKRRAPVK
327	GGWSSKPRQGMGQNL
328	SKPRQGMGQNLSTSN

ID	Sequence
329	QGMGQNLSTSNPLGF
330	QNLSTSNPLGFFPDH
331	TSNPLGFFPDHQLDP
332	LGFFPDHQLDPAFRA
333	PDHQLDPAFRANTAN
334	LDPAFRANTANPDWD
335	FRANTANPDWDFNPN
336	TANPDWDFNPNKDTW
337	DWDFNPNKDTWPDAN
338	NPNKDTWPDANKVGL
339	DTWPDANKVGLEGGS
340	DANKVGLEGGSGGME
341	VGLEGGSGGMENITS
342	GGSGGMENITSGFLG
343	GMENITSGFLGPLLV
344	ITSGFLGPLLVLQAG
351	LDSWWTSLNFLGGTT
352	WTSLNFLGGTTVCLG
353	NFLGGTTVCLGQNSQ
354	GTTVCLGQNSQSPTS
355	CLGQNSQSPTSNHSP
357	PTSNHSPTSCPPTCP
358	HSPTSCPPTCPGYRW
359	SCPPTCPGYRWMCLR
360	TCPGYRWMCLRRFII
367	VLLDYQGMLPVCPLI
368	YQGMLPVCPLIPGSS
369	LPVCPLIPGSSTTST
370	PLIPGSSTTSTGPCR
371	GSSTTSTGPCRTCTT
372	TSTGPCRTCTTPAQG
373	PCRTCTTPAQGTSMY
374	CTTPAQGTSMYPSCC
375	AQGTSMYPSCCCTKP
376	SMYPSCCCTKPSDGN
380	TCIPIPSSWAFGKFL
381	IPSSWAFGKFLWEWA
382	WAFGKFLWEWASARF
383	KFLWEWASARFSWLS
384	EWASARFSWLSLLVP
385	ARFSWLSLLVPFVQW
392	WMMWYWGPSLYSILS
393	YWGPSLYSILSPFLP
394	SLYSILSPFLPLLPI

APPENDIX 7 – The phenotype of CD8 and HBV specific T cells in mice vaccinated with ChAdOx1-HBV and MVA-HBV

Table 1 CD44 expression on bulk CD8 T cells

Significant increase, significant decrease, no significant difference compared to unvaccinated

	Blood		Liver		Spleen	
	Median CD44 expression (%)	Trend (p-value)	Median CD44 expression (%)	Trend (p-value)	Median CD44 expression (%)	Trend (p-value)
Unvaccinated	30.1		17.4		16.2	
i.m.i.m. day 14	72.2	0.0052**	60.3	0.0003***	30.4	ns
i.m.i.v. day 14	95.5	0.0050**	67.6	<0.0001****	33.4	0.0153*
i.m.i.m. day 60	54.9	ns	57.0	0.0169*	30.6	0.0262*
i.m.i.v day 60	55.5	ns	60.6	<0.0001****	39.1	<0.0001****

Table 2 KLRG1 expression on bulk CD8 T cells

Significant increase, significant decrease, no significant difference compared to unvaccinated

	Blood		Liver		Spleen		Lung	
	Median KLRG1 expression (%)	Trend (p-value)	Median KLRG1 expression (%)	Trend (p-value)	Median KLRG1 expression (%)	Trend (p-value)	Median KLRG1 expression (%)	Trend (p-value)
Unvaccinated	4.68		2.47		1.1		4.7	
i.m.i.m. day 14	46.8	0.0010***	23.0	0.0006***	15.0	0.0012***	25.8	0.0003***
i.m.i.v. day 14	74.6	<0.0001****	25.5	0.0001***	18.1	<0.0001****	42.9	<0.0001****
i.m.i.m. day 60	22.8	ns	17.8	0.0046**	11.1	0.0243*	28.1	0.0008***
i.m.i.v day 60	25.6	ns	1.8	ns	16.9	<0.0001****	14.0	0.0407*

Table 3 CD127 expression on bulk CD8 T cells

Significant increase, significant decrease, no significant difference compared to unvaccinated

	Blood		Liver		Spleen		Lung	
	Median expression CD127 (%)	Trend (p-value)	Median expression CD127 (%)	Trend (p-value)	Median expression CD127 (%)	Trend (p-value)	Median expression CD127 (%)	Trend (p-value)
Unvaccinated	52.9		75.2		54.2		70.1	
i.m.i.m. day 14	23.7	ns	27.2	<0.0001****	41.2	ns	58.8	ns
i.m.i.v. day 14	29.1	ns	40.9	0.0002***	50.8	ns	51.0	ns
i.m.i.m. day 60	59.4	ns	67.2	ns	70.1	ns	51.5	ns
i.m.i.v day 60	49.7	ns	59.8	ns	33.0	ns	39.2	ns

Table 4 T_{CM} (CD8+CD127+KLRG1-CD62L+) CD8 T cells

Significant increase, significant decrease, no significant difference compared to unvaccinated

	Blood		Liver		Spleen		Lung	
	Median T_{CM} (%)	Trend (p-value)	Median T_{CM} (%)	Trend (p-value)	Median T_{CM} (%)	Trend (p-value)	Median T_{CM} (%)	Trend (p-value)
Unvaccinated	49.0		95.8		97.6		96.8	
i.m.i.m. day 14	46.1	ns	68.4	0.0067**	88.0	0.0115*	81.5	0.0016**
i.m.i.v. day 14	20.5	0.0010***	65.1	0.0006***	85.2	<0.0001****	86.2	0.0042**
i.m.i.m. day 60	47.1	ns	76.0	ns	91.9	ns	83.7	ns
i.m.i.v day 60	42.9	ns	65.3	0.0016**	84.9	<0.0001****	77.1	0.0004***

Table 5 T_{EM} CD8 (CD8+CD127+KLRG1-CD62L-) T cells
Significant increase, significant decrease, no significant difference compared to unvaccinated

	Blood		Liver		Spleen		Lung	
	Median T_{EM} (%)	Trend (p-value)	Median T_{EM} (%)	Trend (p-value)	Median T_{EM} (%)	Trend (p-value)	Median T_{EM} (%)	Trend (p-value)
Unvaccinated	51		4.15		11.1		9.15	
i.m.i.m. day 14	53.9	ns	31.6	0.0067**	12	ns	18.5	ns
i.m.i.v. day 14	79.5	0.0010***	34.9	0.0006***	14.7	ns	13.8	ns
i.m.i.m. day 60	52.9	ns	24	ns	8.12	ns	16.3	ns
i.m.i.v day 60	57.1	ns	34.7	0.0016**	15.1	ns	22.9	ns

Table 6 CX3CR1+ expression on bulk CD8 T_{EM} cells
Significant increase, significant decrease, no significant difference compared to unvaccinated

	Blood		Liver		Spleen		Lung	
	Median expression (%)	Trend (p-value)	Median expression (%)	Trend (p-value)	Median expression (%)	Trend (p-value)	Median expression (%)	Trend (p-value)
Unvaccinated	4.24		12.9		4.96		5.715	
i.m.i.m. day 14	29	0.0435*	35.6	ns	18.2	0.0314*	44.5	0.0140*
i.m.i.v. day 14	45	<0.0001****	33.8	ns	15	ns	29.5	ns
i.m.i.m. day 60	9.4	ns	62.15	0.0036**	16.7	0.0108*	66.2	0.0005***
i.m.i.v day 60	18.9	ns	55.8	<0.0001****	24.3	0.001***	56.7	0.0001***

Table 7 CD44 expression on pentamer positive HBV specific CD8 T cells
Significant increase, significant decrease, no significant difference

	Blood		Liver		Spleen	
	Median expression CD44 (%)	Trend (p-value)	Median expression CD44 (%)	Trend (p-value)	Median expression CD44 (%)	Trend (p-value)
i.m.i.m. day 14	94.9		98.9		88.4	
i.m.i.v. day 14	87.6	ns	96.9	0.0119*	86.6	ns
i.m.i.m. day 60	97.8		97.5		86.3	
i.m.i.v day 60	83	0.0202*	96.8	ns	93.2	0.0236*

Table 8 KLRG1 expression on pentamer positive HBV specific CD8 T cells
Significant increase, significant decrease, no significant difference

	Blood		Liver		Spleen		Lung	
	Median expression KLRG1 (%)	Trend (p-value)	Median expression KLRG1 (%)	Trend (p-value)	Median expression KLRG1 (%)	Trend (p-value)	Median expression KLRG1 (%)	Trend (p-value)
i.m.i.m. day 14	66.7		33.4		55.5		59.8	
i.m.i.v. day 14	61.7	ns	41.8	ns	48.3	ns	44.9	ns
i.m.i.m. day 60	76.8	ns	40.6	ns	56.4	ns	78.7	ns
i.m.i.v day 60	64.0	ns	35.1	ns	54.9	ns	41.0	0.0486*

Table 9 CD127 expression on pentamer positive HBV specific CD8 T cells
Significant increase, significant decrease, no significant difference

	Blood		Liver		Spleen		Lung	
	Median expression CD127 (%)	Trend (p-value)	Median expression CD127 (%)	Trend (p-value)	Median expression CD127 (%)	Trend (p-value)	Median expression CD127 (%)	Trend (p-value)
i.m.i.m. day 14	21.9		25.0		22.7		25.9	
i.m.i.v. day 14	70.7	0.0024**	57.1	0.0003***	70.6	0.0255*	41.0	ns
i.m.i.m. day 60	31.5	ns	28.6	ns	26.7	ns	21.3	ns
i.m.i.v day 60	47.6	ns	46.8	0.0155*	39.2	0.0383*	27.8	ns

Table 10 T_{CM} (CD8+CD127+KLRG1-CD62L+) pentamer positive T cells
Significant increase, significant decrease, no significant difference compared to unvaccinated

	Blood		Liver		Spleen		Lung	
	Median T_{CM} (%)	Trend (p-value)	Median T_{CM} (%)	Trend (p-value)	Median T_{CM} (%)	Trend (p-value)	Median T_{CM} (%)	Trend (p-value)
i.m.i.m. day 14	12		5.91		22.6		21.4	
i.m.i.v. day 14	21.4	ns	8.41	ns	33.5	ns	27.6	ns
i.m.i.m. day 60	6.67	ns	8.05	ns	17.3	ns	16.7	ns
i.m.i.v day 60	22.5	0.0148*	7.59	ns	21.1	ns	16.3	ns

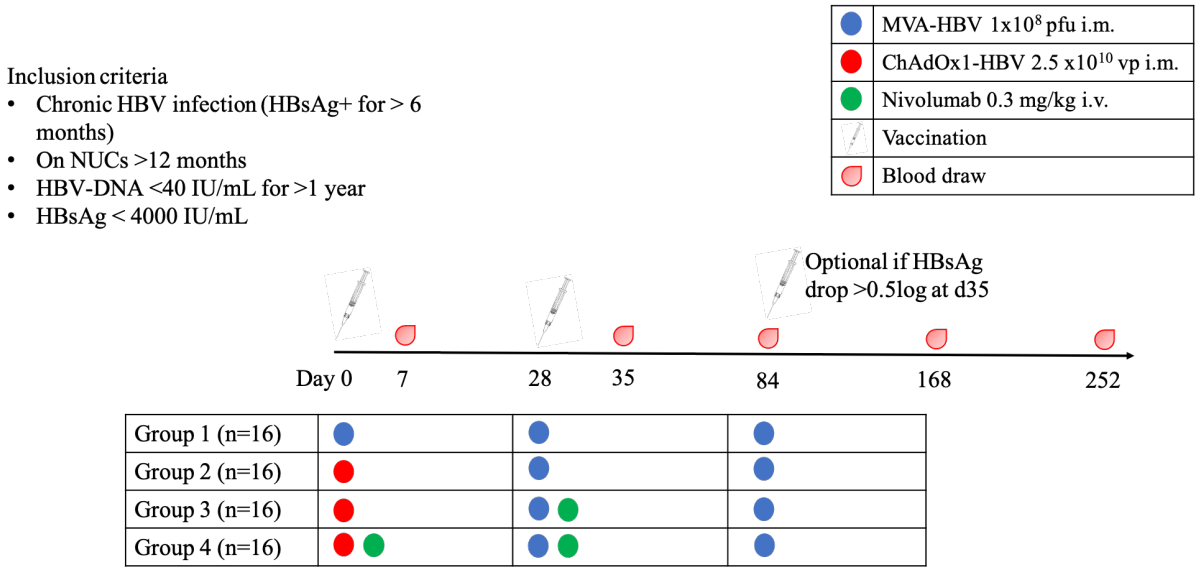
Table 11 T_{EM} (CD8+CD127+KLRG1-CD62L-) pentamer positive T cells
Significant increase, significant decrease, no significant difference compared to unvaccinated

	Blood		Liver		Spleen		Lung	
	Median expression (%)	Trend (p-value)	Median expression (%)	Trend (p-value)	Median expression (%)	Trend (p-value)	Median expression (%)	Trend (p-value)
i.m.i.m. day 14	88		94.1		77.4		75.0	
i.m.i.v. day 14	78.6	ns	91.6	ns	66.55	ns	72.5	ns
i.m.i.m. day 60	93.3	ns	91.9	ns	82.7	ns	83.3	ns
i.m.i.v day 60	77.5	0.0126*	92.4	ns	78.9	ns	83.7	ns

Table 12 CX3CR1+ expression on pentamer positive CD8 T_{EM} cells
Significant increase, significant decrease, no significant difference compared to unvaccinated

	Blood		Liver		Spleen		Lung	
	Median expression CX3CR1 (%)	Trend (p-value)	Median expression CX3CR1 (%)	Trend (p-value)	Median expression CX3CR1 (%)	Trend (p-value)	Median expression (%)	Trend (p-value)
i.m.i.m. day 14	55.7		45.3		23.3		57.9	
i.m.i.m. day 60	47.3	ns	71.2	ns	29.2	ns	69.1	ns
i.m.i.v. day 14	48.4	ns	30.7	ns	14.3	ns	37.5	ns
i.m.i.v day 60	34.5	ns	54	0.0009***	17.7	ns	50	ns

APPENDIX 8 – HBV002 summary



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