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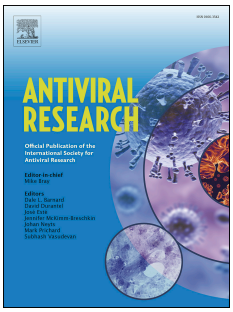

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Design of TLR2-ligand-synthetic long peptide conjugates for therapeutic vaccination of chronic HBV patients

Yingying Dou[1*], Diahann T.S.L. Jansen[1*], Aniek van den Bosch[1], Robert A. de Man[1], Nadine van Montfoort[1,2], Can Araman[5], Sander I. van Kasteren[5], Gijs G. Zom[4], Willem-Jan Krebber[4], Cornelis J.M. Melief[4], Andrea M. Woltman[1,3] and Sonja I. Buschow[1#]

Affiliations:

[1] *Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, The Netherlands*

[2] *Current address: Department of Medical Oncology, Leiden University Medical Center, Leiden, the Netherlands.*

[3] *Current address: Institute of Medical Education Research Rotterdam, Erasmus MC, University Medical Center Rotterdam.*

[4] *ISA Pharmaceuticals BV, Leiden, the Netherlands.*

[5] *Leiden Institute of Chemistry and the Institute for Chemical Immunology, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands.*

[*] *Authors contributed equally*

[#] *Corresponding author:*

Sonja I. Buschow,

Na1007, Department of Gastroenterology and Hepatology,

Erasmus MC, University Medical Center Rotterdam,

Wytemaweg 80, 3015 CN Rotterdam, The Netherlands.

Email: s.buschow@erasmusmc.nl

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GG Zom receive a salary from ISA Pharmaceuticals B.V., CJM Melief is in possession of stock appreciation rights. In addition, CJM Melief and WJ Krebber are inventors on numerous patents that are licensed to or owned by ISA Pharmaceuticals B.V. dealing with synthetic long peptide vaccines or dealing with the proprietary TLR ligand used in this paper.

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Abstract

Synthetic long peptide (SLP) vaccination is a promising new treatment strategy for patients with a chronic hepatitis B virus (HBV) infection. We have previously shown that a prototype HBV-core protein derived SLP was capable of boosting CD4+ and CD8+ T cell responses in the presence of a TLR2-ligand in chronic HBV patients *ex vivo*. For optimal efficacy of a therapeutic vaccine *in vivo*, adjuvants can be conjugated to the SLP to ensure delivery of both the antigen and the co-stimulatory signal to the same antigen-presenting cell (APC). Dendritic cells (DCs) express the receptor for the adjuvant and are optimally equipped to efficiently process and present the SLP-contained epitopes to T cells. Here, we investigated TLR2-ligand conjugation of the prototype HBV-core SLP. Results indicated that TLR2-ligand conjugation reduced cross-presentation efficiency of the SLP-contained epitope by both monocyte-derived and naturally occurring DC subsets. Importantly, cross-presentation was improved after optimization of the conjugate by either shortening the SLP or by placing a valine-citrulline linker between the TLR2-ligand and the long SLP, to facilitate endosomal dissociation of SLP and TLR2-ligand after uptake. HBV-core SLP conjugates also triggered functional patient T cell responses *ex vivo.* These results provide an import step forward in the design of a therapeutic SLP-based vaccine to cure chronic HBV.

1. Introduction

Synthetic long peptide (SLP®)-based vaccines show favourable results for the treatment of human papilloma virus (HPV) induced cancers by triggering HPV-specific T cell responses (Dijkgraaf et al., 2015; Kenter et al., 2009; Leffers et al., 2012; van Poelgeest et al., 2016; Vermeij et al., 2012; Welters et al., 2016). These results render an SLP vaccine platform of high interest to treat patients suffering from chronic hepatitis B virus (HBV) infection, that is characterized by an ineffective virus-directed T cell response (reviewed in (Bertoletti and Ferrari, 2016)). Worldwide about 250 million people are affected by chronic HBV infection (CHB). CHB patients are prone to develop cirrhosis and liver cancer, leading to approximately 1 million deaths each year (Schweitzer et al., 2015). Currently, no effective treatment for CHB exists and SLP-based vaccination represents a promising treatment strategy. We have recently demonstrated the potency of a prototype HBV-based SLP to boost patient HBV-directed T cell responses *ex vivo* (Dou et al., 2018).

SLPs are linear amino acid (AA) sequences that are a more effective source of peptides for HLA antigen presentation than whole protein, and sufficiently large to cover multiple CD4- and CD8-epitopes for several HLA-types (Melief and van der Burg, 2008; Rosalia et al., 2013; Zhang et al., 2009). Upon vaccination, SLP-contained epitopes are most efficiently presented by dendritic cells (DCs) (Bijker et al., 2008). Only DCs are equipped with an efficient intracellular machinery to liberate SLP-contained epitopes for loading onto both HLA I and II to trigger CD8+ cytotoxic T cells and CD4+ helper T cells respectively (reviewed by (Joffre et al., 2012; Rock et al., 2016)). The unique capacity of DCs to load exogenous antigens on HLA I, termed cross-presentation, is essential to mount cytotoxic CD8+ T cells responses against virus infected cells (van Montfoort et al., 2014). CD4+ T help cell further empowers CD8+ T cell function and aids their migration into infected tissue and the development of lasting responses (reviewed by (Borst et al., 2018)). Furthermore, to ensure induction of immunogenic rather than tolerogenic T cells, DCs need to express co-stimulatory receptors and secrete immune activating cytokines during antigen presentation (Steinman, 2007). For this, DCs require proper activation by adjuvants such as Toll Like Receptor (TLR)-ligands (Melief et al., 2015). Previous studies demonstrated that the TLR2-ligand Amplivant can potently activate DCs and facilitate the induction of CD4+ and CD8+ T cell responses by SLPs *in vitro* and *in vivo* (Dou et al., 2018; Khan et al., 2009; Gijs G Zom et al., 2014; Zom et al., 2016).

Recently, we designed a 37AA prototype SLP based on the HBV core protein and demonstrated this SLP could boost T cell responses against an SLP-contained epitope *ex vivo* (Dou et al., 2018). In that study, soluble TLR-ligands were added as adjuvants to the SLPs in the same culture dish. By this method SLP and adjuvant have no problem to reach the same DC,

ensuring that antigen presentation occurs only in an immunogenic context. *In vivo*, however, this is more challenging as each compound will rapidly diffuse after injection (G G Zom et al., 2014). A solution for this is conjugation of SLP and adjuvant. This strategy can also improve vaccine safety by reducing the required dose of adjuvant (Khan et al., 2007; Gijs G Zom et al., 2014). In mice, TLR2-ligand-SLP conjugates have superior CD8+ and CD4+ T-cell priming capacity and anti-tumor activity compared to free SLPs injected together with free TLR2-ligand (Gijs G Zom et al., 2014). Importantly, TLR2-ligand-SLP conjugates are currently being tested in a phase I clinical trial to treat HPV16+ cancer patients (NCT02821494).

As a next step towards the development of an SLP-based vaccine for CHB, we set out to design a TLR2-ligand-HBV SLP conjugate. Our results show that, in contrast to previous studies, conjugation was associated with hampered cross-presentation *in vitro*. To overcome this, we generated TLR2-ligand-SLP conjugates of different sizes or with an endosomal protease-sensitive linker sequence. Our data indicate that conjugate size and TLR2-ligand-SLP linking strategy influence the efficiency of T cell activation by *in vitro* generated human monocyte-derived DCs (moDC) as well as naturally occurring myeloid DC (mDC) subsets (BDCA1+ (mDC2) and BDCA3+ (mDC1)) that are important for SLP processing *in vivo* (van Montfoort et al., 2014). Finally, we demonstrate that TLR2-ligand-SLP conjugates can boost patient HBV-specific T cell responses *ex vivo.* These data aid the design of a therapeutic SLP-based vaccine to cure CHB and are valuable for SLP-based vaccine development in general.

2. Materials and Methods

2.1 Peptides and adjuvants

Three different HBV core SLPs were designed based on HBV sequence genotype A (UniProtKB - P0C625) SLP37 *MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASAL*; SLP26: *TVELLSFLPSDFFPSVRDLLDTASAL*; SLP16 *TVELLSFLPSDFFPSV* and conjugated with TLR2-ligand Amplivant® (AV) to obtain AV-SLP37, AV-SLP26 and AV-SLP16 respectively. The reported immunodominant HLA-A*02:01-restricted CD8+ T cell epitope $HBcAg^{18-27}$ (*FLPSDFFPSV*) was part of each SLP and also used as short peptide control (SP10) (Bertoletti et al., 1993). SP10 was purchased from Peptide 2.0. All SLPs, conjugates and Amplivant were obtained from ISA Pharmaceuticals BV, Leiden, the Netherlands. SLPs were generated using solid phase Fmoc/tBu chemistry on an Advanced ChemTech TETRAS peptide synthesizer and purified on a Gilson preparative HPLC system to at least 95% purity. The identity and purity of the peptides was confirmed with UPLC-MS on a Waters ACQUITY UPLC/TQD system. Mass spectrometry demonstrated that no small fragments representing the minimal CD8+ T cell epitope $HBcAg^{18-27}$ were detectable in our SLP samples. SLPs were dissolved in DMSO and stored at -20°C.

Dynamic light scattering spectroscopy (DLS, Zetasizer Nano S, Malvern Instruments) was used to assure no aggregates formed after dilution in medium (data not shown).

2.2 Flow cytometry

Staining with fluorescent primary antibodies (supplementary information) was performed in PBS, 1% BSA, 1% normal human serum and 0.02% sodium azide. HLA-A*02:01/HBcAg$^{18-27}$ (Biolegend) staining was performed according to manufacturer's instructions. Fluorescence was measured using a FACS Canto II (BD Biosciences) and analyzed using FlowJo (version 7.2.2, Tree Star, Inc.).

2.3 Cell culture, isolation and cross-presentation assays

HBcAg$^{18-27}$-TCR engineered T cells were generated and cultured as before (Dou et al., 2018). For DCs, healthy donor buffy coats were collected from the local blood bank. All donors gave written informed consent. PBMC were isolated by using Ficoll-Paque (GE Healthcare) density gradient centrifugation. Monocytes were isolated using anti-CD14 microbeads (Miltenyi Biotec) and cultured for 6-7 days in DC medium (RPMI 1640 Glutamax (Lonza), supplemented with 8% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich), penicillin/streptomycin (Invitrogen) with 10 ng/ml GM-CSF (Leukine) and 10ng/ml IL-4 (eBioscience) to obtain moDC. mDC2 and mDC1 were sorted from PBMC using Dynabeads (Life Technologies) based on BDCA1 and BDCA3 expression respectively, using a FACSAria (BD Biosciences). For cross-presentation assays, moDC and primary DC subsets were incubated with Amplivant and/or SLPs at concentrations indicated for 20 hours unless otherwise stated, washed and cultured with HBcAg$^{18-27}$-TCR transduced T cells overnight as before (Dou et al., 2018). Synthetic inhibitors epoxomicin (1µM; Cayman, US) or NH_4CL (5mM; Merck) were present 1 hour before and during the peptide pulse in the corresponding samples. IFN-γ secretion by T cells was measured by ELISA (eBioscience).

2.4 Patient-derived HBV-specific T cell proliferation and function

Blood was collected from 9 HLA-A2-positive CHB patients with low serum viral load (HBV DNA ≤ 1000 IU/ml), serum ALT levels below the upper limit of normal (56 IU/ liter) and low to moderate fibrosis (fibroscan F0-F2). For this, written informed consent from each patient and approval by the Medical Ethics Committee of Erasmus MC was obtained. 78% of the patients received nucleos(t)ide analogues. Plasma HBV DNA, HBsAg and HBeAg were determined as before (Dou et al., 2018). All patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus. See Table 1 for HBV patient characteristics. MoDCs were generated as above and loaded with 10µM AV-SLP37 or AV-VC-SLP for 20 hours. Subsequently, either autologous B cell depleted PBLs (as before; (Dou et al., 2018)) or isolated T cells (by pan T cell isolation

kit; Miltenyi Biotech), as indicated for individual patients, were added to moDCs in a ratio of 10:1. Cells were incubated at 37°C for 12 days as before (Dou et al., 2018). On day 12, the frequency of HBV-specific CD8+ T cells was determined by HLA-A*02:01/HBcAg$^{18-27}$ tetramer (Biolegend) staining on viable (LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit, Life technologies) CD8+ T cells. To assess cytokine production, day 12 T cells were re-stimulated with HBcAg$^{18-27}$-peptide (5µg/ml) or SLP-loaded (10µM) autologous moDC as indicated for 6 hours. Brefeldin A (10µg/ml, Sigma-Aldrich) was added during the last 5 hours. Subsequently, cells were fixed with 2% formaldehyde, permeabilized in 0.5% saponin and stained for CD4, CD8, IFN-γ and TNF-α and analyzed by flow cytometry.

2.5 Statistical analysis

Non-parametric Wilcoxon matched pairs tests and paired t-tests (one-tailed) were performed using the GraphPad Prism 5 software (Graph-Pad Prism Software, Inc.) as appropriate. P-values <0.05 were considered statistically significant.

3. Results

3.1 Conjugation of TLR2-ligand to the HBV-SLP reduces cross-presentation efficiency.

We conjugated our prototype 37AA HBVcore$^{1-37}$ SLP (SLP37) directly to the TLR2-ligand Amplivant® (AV-SLP37) and evaluated its cross-presentation efficiency compared to free mixed compounds (AV+SLP37; Figure 1A). To this end we assessed the capacity of moDCs and mDC2 to activate engineered HBcAg$^{18-27}$-epitope specific T cells (Dou et al., 2018). Although the conjugate was still able to activate HBcAg$^{18-27}$-specific T cells, conjugation reduced CD8+ T cell activation (i.e. IFNγ production) compared to admixed compounds by both DC subtypes (Figure 1B & 1C). Previously, we found that SLP37 is processed rather slowly (i.e. over the course of days) and relies on the proteasome to efficiently release the HBcAg$^{18-27}$-epitope (Dou et al., 2018). Similarly, in the presence of AV, presentation of the HBcAg$^{18-27}$-epitope from SLP37 was slow and did not level off after 20 hours of antigen loading as measured by T cell activation. In contrast, from the AV-SLP37 conjugate, epitope presentation was already close to maximal after 3 hours and did not increase much further with time (Figure 1D). Epitope presentation from the mixture was, similar to SLP alone, partially blocked by the proteasome inhibitor epoxomicin (Figure 1E; (Dou et al., 2018)). Surprisingly, epoxomicin had no effect on cross-presentation of the HBcAg$^{18-27}$-epitope from conjugated AV-SLP37. Importantly, inhibition of lysosomal acidification by NH_4CL strongly augmented epitope presentation from AV-SLP37 while this not at all affected cross-presentation of AV+ SLP37 (Figure 1E). Presentation of SP10 that does not require

internally processing by moDCs, was unchanged by either time (Figure 1D) or inhibitors (Figure 1E). These data suggest that by conjugating SLP37 to AV, the SLP is re-routed towards rapid lysosomal degradation, impairing cross-presentation.

3.2 Shortening of SLPs within conjugates enhances cross-presentation.

The inefficient cross-presentation from AV-SLP37 is not in line with previous reports for other SLP-TLR2-ligand conjugates (Khan et al., 2007; Gijs G Zom et al., 2014; Zom et al., 2016). These, however, were mostly 24AA and only in one case 32AA long (Khan et al., 2007; Gijs G Zom et al., 2014; Zom et al., 2016). For the latter, cross-presentation of the mixture and conjugate was not directly compared (Zom et al., 2016). We hypothesized that TLR2-ligand conjugation could render SLP37 too long/bulky. If so, shortening of the SLP may recover cross-presentation efficiency. To test this, we generated two shorter conjugates of 26AA (AV-SLP26) and 16AA (AV-SLP16; Figure 2A). Cross-presentation of the $HBcAg^{18-27}$-epitope from the AV-SLP16 conjugate by both moDCs and mDC2 was significantly enhanced compared to AV-SLP37, demonstrating that shortening the SLP in the conjugate indeed was effective (Figure 2B). For moDCs, but not mDC2, AV-SLP16 even outperformed the mixture. Of note, performance of AV-SLP16 was not affected by the position of the epitope within the SLP (Supplemental Figure 1). In moDC, but not mDC2, also AV-SLP26 performed better than AV-SLP37 and similar to the mixture.

In vivo, especially mDC1 are considered important for cross-presentation (Borst et al., 2018; Crozat et al., 2011; Jongbloed et al., 2010; Poulin et al., 2010; van der Aa et al., 2014). This subset, however, can only be isolated in low numbers from blood. We obtained sufficient cells to compare cross-presentation of the differentially sized conjugates but lacked cells for additional controls or references. Indeed, also when using mDC1, shorter SLP length seemed to favor cross-presentation (Figure 2B).

Linking the TLR2-ligand to the SLP can be envisaged to physically impair TLR2 binding and thereby hinder DC maturation and subsequent T cell activation. Upregulation of costimulatory markers CD80, CD86 and CD83 on DC however was similar for AV alone and all conjugates, indicating conjugated AV was not hindered to bind TLR2 by conjugation (Figure 2C). Concordantly, addition of excess AV to mDC2 during antigen loading did not increase T cell activation by AV-SLP37 (or any of the shorter conjugates; (Figure 2D).

Taken together, we demonstrate that conjugation of HBV-SLP37 to AV reduces cross-presentation of the SLP-contained epitope by DCs and that this can be improved by reducing SLP length.

3.3 Inserting a protease-sensitive linker between Amplivant and SLP improves cross-presentation.

Although shortening of the SLP in the conjugates can recover antigen cross-presentation, longer SLPs are preferred since these can include more epitopes. For this reason, we ventured to improve cross-presentation without reducing length. We designed a conjugate in which a Valine-Citrulline (VC) linker was placed between the TLR2-ligand and the SLP (Figure 3A; AV-VC-SLP37). This linker provides a cleavage site for endosomal proteases to facilitate dissociation of SLP and AV after uptake (Gene M. Dubowchik et al., 2002). A consistent superior cross-presentation of AV-VC-SLP37 was observed over the original AV-SLP37 conjugate in all donors and DC-subtypes tested (Figure 3B). Despite this consistent pattern, this was only statistically significant for moDC, likely due to a large variation in T cell IFNγ production between donors. The superiority of AV-VC-SLP37 using mDC2 was independent of SLP/AV concentration (Figure 3C). Similar mDC2 maturation was achieved by AV-SLP37, AV-VC-SLP37 and AV alone, indicating that enhanced TLR2 activation did not explain the better performance of AV-VC-SLP37 (Figure 3D). Thus placing a VC-linker between the TLR2-ligand and the SLP improves cross-presentation without reducing SLP length.

3.4 Induction of HBV-specific T cell response in CHB patients by Amplivant-SLP conjugates.

So far all experiments used TCR engineered T cells as a read-out for cross-presentation. To further translate our work towards treating CHB patients, we evaluated the capacity of AV-SLP conjugates to activate patient T cells *ex vivo*. Using patient T cells, SLPs may now also trigger responses against other SLP-contained CD8+ T cell epitopes and importantly also against CD4+ T cell epitopes. To match future treatment candidates best, we selected patients with low serum HBV DNA and low liver damage (ALT<37) as these are expected to suffer least from HBV induced immune suppression/T cell dysfunction (Table 1; (Bertoletti and Ferrari, 2011; Maini et al., 2000; Shi et al., 2012; Webster et al., 2004; Woltman et al., 2010)). In addition, treatment of low viremic patients is likely safest considering liver damage that may result from vaccine induced HBV specific immune responses. The limited material available did not allow us to test the full set of mixtures and conjugates. We restricted our experiments to AV-SLP37 and AV-VC-SLP37 as these have most potential in the clinic to treat a large population of patients. We co-cultured T cells from HLA-A2 positive chronic HBV patients for 12 days with autologous, conjugate-loaded moDC. Both conjugates induced significantly more $HBcAg^{18-27}$-specific CD8+ T cells compared to AV alone. 5 out of 13 patients were clear responders to the vaccine (defined as >2-fold increase in $HBcAg^{18-27}$-specific CD8+ T cell numbers) (Figure 4B). Proliferation of $HBcAg^{18-27}$-specific CD8+ T cells by AV-VC-SLP37 overall was not significantly better than by AV-SLP37 (Figure 4B). For the five responders, AV-SLP37 and AV-VC-SLP37 -induced $HBcAg^{18-27}$-specific CD8+ T cells produced cytokines after re-stimulation with $HBcAg^{18-27}$ short peptide (SP10), indicating that in both cases the induced T cells were functional (Figure 4C). SLP37 also contains CD4+ T cell epitopes (Dou et al., 2018). Because donors were matched for HLA-A2 but not HLA-DP/DQ/DR, specific

CD4+ T cell responses could only be assessed by cytokine production assay and only for part of the patient samples because of the limited number of cells available. However, upon re-stimulation with SLP, cytokine production was detected in 12-day SLP-conjugate expanded CD4+ T cells. Despite high background this was higher upon stimulation with AV-VC-SLP37 compared to AV-SLP37 (Supplemental figure 2). Overall, these experiments demonstrate that Amplivant-SLP conjugates are able to induce functional T cells from patient blood *ex vivo* but a clear advantage for one of the two conjugates was thus far not observed..

4. Discussion

We have previously shown that a prototype HBV-core protein derived SLP was capable of boosting CD4+ and CD8+ T cell responses in the presence of TLR2-ligand Amplivant in CHB patients *ex vivo*. Other studies indicated that conjugation of the TLR2-ligand to SLPs may lead to superior results *in vivo* by assuring delivery of both antigen and costimulatory signal to the same cell (Khan et al., 2009, 2007). In the present study, we are the first to design TLR2-ligand-HBV core SLP conjugates and assess their effectiveness *in vitro* and *ex vivo* on patient material. We showed that conjugation of the prototype SLP to TLR2-ligand reduced cross-presentation by DC of an SLP contained HBc epitope, which could be (partially) overcome by shortening SLP-conjugates or inclusion of a valine-citrulline linker. Furthermore, TLR2-ligand-SLP conjugates with and without the VC-linker were able to trigger CHB patients' T cell responses *ex vivo*. With this study, we provide important and elaborate data on the effect of SLP conjugation using an HBV-derived epitope and importantly we do this in a human setting using moDCs as well as primary myeloid DCs.

Studies assessing the efficacy of SLP-TLR2-ligand conjugation in T cell activation thus far demonstrated more effective T cell activation by conjugates compared to mixtures *in vitro* and *in vivo* (Khan et al., 2009, 2007; Gijs G Zom et al., 2014). The effect of conjugation has been most elaborately tested using SLPs based on the well-known murine ovalbumin (OVA) CD8+ T cell epitope SIINFEKL *in vitro* and *in vivo* (Khan et al., 2009, 2007; Gijs G Zom et al., 2014). In addition, 2 HPV16-based SLP-TLR2 conjugates have been assessed on their ability to activate a CD8+ and a CD4+ T cell clone obtained from HPV16+ cervical cancer patients (Zom et al., 2016). In this later human epitope-based study, however, conjugates and mixtures were not directly compared. Interestingly, recent work shows the *in vivo* superiority of Amplivant (used in this study) conjugates compared to TLR2-ligand conjugates in a mouse HPV+ tumor therapeutic vaccination model (Zom et al., 2018).

TLR2-ligand conjugation reduced (but not aborted) cross-presentation efficiency of the HBcAg$^{18-27}$-epitope which was not due to impairment of TLR2-mediated DC activation but likely involved re-routing of the SLP for rapid lysosomal destruction. Based on our data we believe that a cause for the discrepancy between the OVA-based-SLP conjugates and the HBV-conjugate here tested may lie in the length of the HBVcore SLP that was 37AA long, while reported OVA-based SLP conjugates had been 18-25AA (Khan et al., 2007; Gijs G Zom et al., 2014; Zom et al., 2016). Concordantly, we observed that size reduction of the SLP conjugate to 16AA greatly augmented cross-presentation efficiency by both moDC and primary mDCs. For non-conjugated SLPs, increasing SLP length to 32AA did not reduce cross-presentation efficiency but addition of the TLR2-ligand may be a game-changer (Rosalia et al., 2013). TLR2-ligand conjugation will not only increase compound size but will also change its electrostatic properties which may affect cross-presentation by their own merit. Alternatively, the discrepancy with reported conjugates may be explained by differences between human and murine systems, the APCs used, and/or other proteolytic requirement releasing or destroying the different epitopes and others. The importance of the APC type was underscored by our observation that moDCs but not primary mDCs efficiently cross-presented the HBcAg$^{18-27}$-epitope from the intermediately sized AV-SLP26 conjugate. This may reflect a difference in endosomal proteolytic milieu between these cell types and/or other requirements for antigens to be transported to the proteasome for processing (McCurley and Mellman, 2010). More research is required to elucidate exactly how the length of conjugated SLP, conjugate size as well as its physical properties determine epitope release for cross-presentation in different murine and human DC types.

As an alternative strategy to size reduction, we show that an endosomal protease-sensitive linker can be used. This strategy ensures uptake of both SLP and adjuvant by the same cell, presumably followed by subsequent unhindered processing and transport of the SLP (Gene M. Dubowchik et al., 2002). Placement of the VC-linker indeed improved cross-presentation of the HBcAg$^{18-27}$-epitope by both moDC and mDC subsets. Using patient T cells, both conjugates increased expansion of functional CD8+ T cells in responding donors, but inclusion of the VC-linker did not overtly yield more T cell responses. Our patient cohort, however, also contained several HBcAg$^{18-27}$-epitope non-responsive patients which could mask differences between the compounds. The number of responders in this study (5/13) was comparable to our previous study using AV+SLP37 (6/19; (Dou et al., 2018)). All responders were of Caucasian origin. Because we selected patients using pan-HLA-A2 antibodies, non-responding patients may have expressed certain HLA-A2 subtypes prevalent among Asians (e.g. HLA-A*02:03) less able to bind the HBcAg$^{18-27}$-epitope or the HLA-A2:01 tetramer (Tan et al., 2008). We have no clear explanation for the different results from the *ex vivo* experiments with patient T cells compared to our *in vitro* model system with engineered HBcAg$^{18-27}$ specific T cells. Of influence could be the fact that patients' SLP-induced T cell responses may have been polyclonal, of different affinity and potentially directed against other CD4 and CD8 epitopes, while our read out was only for the HBcAg$^{18-27}$-

epitope and patient selection only for HLA-A*2. Possibly, competition between T cells recognizing different SLP-nested epitopes within our co-culture influenced the response of $HBcAg^{18-27}$ cognate T cells. Future studies would benefit from a read out method for more/all SLP-contained epitopes to better appreciate the total response induced by our SLP-conjugates. Making this possible with the limited material at hand is currently focus of our research.

Here we investigated the conjugation of a TLR2-ligand to a prototype HBV-core derived SLP. With this, we explored a novel promising vaccination strategy, which could further be improved for HBV vaccine development. Besides TLR2-ligands that have been proven effective *in vitro* and *in vivo*, other adjuvants may also be of interest to conjugate to SLPs (Khan et al., 2007; Gijs G Zom et al., 2014; Zom et al., 2016). Thus far, conjugation of SLPs to TLR7- and TLR9-ligands has been assessed (Khan et al., 2007; Weterings et al., 2006). For TLR7 this resulted in abrogation of receptor binding, but for TLR9 this proved a valid strategy. At present, TLR2 is preferred over TLR9 because of its broad expression on myeloid cells while TLR9 is expressed predominantly on plasmacytoid DCs, which are poorer APCs (Schreibelt et al., 2010; See et al., 2017; Villani et al., 2017). Likewise, conjugates containing TLR3 or TLR8 ligands are of interest as these also act on mDCs (Schreibelt et al., 2010).

In conclusion, we have demonstrated that SLP-TLR2-ligand conjugation is not always beneficial for cross-presentation, but may be improved by altering SLP size and by VC-linker inclusion. Furthermore, we have demonstrated that TLR2-SLP conjugates are able to trigger HBV-specific T cells responses *ex vivo*. These data and derived insights will benefit SLP-based vaccine development for the treatment of CHB and other infectious diseases, as well as cancer in general.

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Table 1. Characteristics of Chronic HBV Patients Included in the Study

Patient number	viral load (IU/ml)	ALT (IU/l)	HBeAg	HBsAg	Fibrosis	Genotype	Ethnicity	Current treatment	Gender	Age (yr)
pt1	<2.00E1	32	neg	pos	F0-F1	C	Asian	entecavir	M	46
pt2	<2.00E1	13	pos	pos	F0-F1	n.d.	Asian	entecavir	F	62
pt3	<2.00E1	37	neg	pos	F0-F1	n.d.	Asian	entecavir	M	35
pt4	<2.00E1	28	neg	pos	F2	n.d.	Caucasian	tenofovir	M	50
pt5	<2.00E1	27	neg	neg*	F0-F1	n.d.	Caucasian	entecavir	M	41
pt6	<2.00E1	21	neg	pos	F0-F1	n.d.	Caucasian	entecavir	M	32
pt7	5.5E2	29	neg	pos	F0-F1	n.d.	Caucasian	none	M	48
pt8	8.3E2	25	neg	pos	F0-F1	B	Asian	none	M	53
pt9	2.0E1	22	neg	pos	F2	C	Asian	entecavir	F	48
pt10	3.0E1	33	neg	pos	F0-F1	n.d.	Caucasian	tenofovir	M	28
pt11	4.9E1	25	neg	pos	F2	n.d.	Asian	none	F	42
pt12	<2.00E1	19	neg	pos	F0-F1	n.d.	Caucasian	none	F	42
pt13	9.0E1	31	neg	pos	F0-F1	n.d.	Caucasian	none	M	67

M, male; F, female; n.d., not determined; ALT, alanine aminotransferase. Typically the range for normal ALT is between 7 and 56 units per liter. * patient proved to have cleared HBsAg and seroconverted to anti-HBs at time of inclusion.

Figure legends

Figure 1. Conjugation of TLR2-ligand to the HBV-SLP reduces cross-presentation efficiency.

(**A**) Schematic representation of Amplivant (AV)-SLP mixture (AV+SLP37) and conjugate (AV-SLP37). The $HBcAg^{18-27}$ CD8+ T cell epitope is indicated in red. (**B**) Dose titration of compounds on moDC, that after antigen/ adjuvant loading were co-cultured for 20 hours with engineered $HBcAg^{18-27}$-specific CD8+ T cells. IFN-γ production by T cells was measured by ELISA. Representative result of 2 experiments & donors. (**C**) Experiment as in B but using freshly isolated mDC2. Representative result of 2 experiments & donors. (**D**) Kinetics of antigen presentation by moDC loaded with 2µM of specified compounds for indicated times, read out by $HBcAg^{18-27}$-specific CD8+ T cell activation by IFN-γ production. Mean+SEM. n=4. (**E**) IFN-γ production by $HBcAg^{18-27}$-specific T cells in response to moDC pulsed with indicated compounds in the presence or absence of epoxomicin (1µM) or NH_4CL (5mM). moDC were fixed with 0.2% PFA prior to the start of co-culture. Mean+SEM. n=4 donors. For visualization IFN-γ levels were normalized to the amount induced by medium control (no inhibitor) in each experiment. Statistical analysis based on raw data. *$p<0.05$ by two-sided paired t-test.

Figure 2. Shortening of SLPs within conjugates enhances cross-presentation.

(**A**) Schematic representation of AV-SLP length variants and mixture. The $HBcAg^{18-27}$ CD8+ T cell epitope is indicated in red. (**B**) IFN-γ production by $HBcAg^{18-27}$-specific T cells in response to different DC subsets pulsed with indicated compounds after 20 hours of coculture. Each symbol represents a donor. Bar represents the mean. (moDC, n=3; mDC2, n=6; mDC1, n=2(AV-SLP26)-4(AV-SLP37 & AV-SLP16). *$p<0.05$ by two-sided paired t-test. (**C**) moDC or mDC2 were stimulated with 2µM of indicated compounds for 20 hours. Shown are representative histograms for the cell surface expression of CD80, CD86 and CD83 on moDC (n=3) and mDC2 (n=4) as determined by flow cytometry. (**D**) IFN-γ production by $HBcAg^{18-27}$-specific CD8+ T cells in response to mDC2 pulsed with indicated compounds with or without excess AV (2uM). Each symbol represents a donor. n.s., not significant by two-sided paired t-test.

Figure 3. Inserting a protease-sensitive linker between Amplivant and SLP improves cross-presentation.

(**A**) Schematic representation of the conjugates with (AV-VC-SLP37) and without (AV-SLP37) the Valine-Citruline (VC) linker. (**B**) IFN-γ production by $HBcAg^{18-27}$-specific CD8+ T cells in response to DC subsets pulsed with indicated compounds after 20 hours of coculture. Each symbol represents a donor. Bars represent the mean. (moDC, n=3; mDC2, n=6; mDC1, n=4). *$p<0.05$ by two-sided paired t-test. (**C**) Dose titration of conjugates on mDC2 and IFN-γ production by $HBcAg^{18-27}$-specific CD8+ T cells in response to these cells. Mean+SEM. n=2. (**D**) Shown are representative histograms of CD80, CD86 and CD83

cell surface expression on mDC2 in response to indicated compounds after 20 hours of incubation as measured by flow cytometry. n=4

Figure 4. Induction of HBV-specific CD8+ T cell response in CHB patients by Amplivant-SLP conjugates.

(**A**) Representative tetramer staining of $HBcAg^{18-27}$-specific CD8+ T cells in PBLs from a CHB patient after 12-day culture with autologous moDC loaded with 10uM of indicated conjugates. (**B**) Left panel: absolute number of $HBcAg^{18-27}$-specific CD8+ T cells after 12-day culture as described in A for 13 patients. Bars indicate mean of absolute number of $HBcAg^{18-27}$-specific CD8+ T cells of 13 patients. Responding patients are marked in red (defined by a >2-fold increase in $HBcAg^{18-27}$-specific CD8+ T cell numbers). 3 patients in grey symbols used autologous B cell depleted PBLs for co-culture (see Material and Method). Right panel: fold increase of $HBcAg^{18-27}$-specific CD8+ T cells after 12-day culture normalized to AV alone. Samples from one individual are connected by a line. N=13. (C) Cytokine production of 12-day CD8+ T cell cultures (with indicated compounds) from responding patients, upon overnight re-stimulation with $HBcAg^{18-27}$ short peptide (SP10). Samples from one individual are connected by a line. n.s., not significant, *$p<0.05$ by Wilcoxon signed rank test.

Supplementary Figure 1. Effect of epitope position within AV-SLP16 on cross-presentation by human DC.

(**A**) Schematic representation of AV-SLP16 as used in this study and an alternative AV-SLP16-mid (where "mid" indicates the epitope is positioned in the middle of the SLP sequence). (**B**) IFN-γ production by engineered $HBcAg^{18-27}$-specific CD8+ T cells in response to different DC subsets pulsed with indicated compounds (2μM) for 20 hours. Each symbol represents a donor. Bar represents the mean. (n=4). n.s., not significant by paired-t test, two-sides.

Supplementary Figure 2. Induction of functional CD4+ T cells in CHB patients by AV-SLP conjugates.

Summary of the mean+SEM of absolute cell numbers of cytokine-producing CD4+ T cells from CHB patients upon re-stimulation of with SLP-loaded autologous moDC after an initial 12-day expansion culture by indicated compounds (n=9). * $p<0.05$, ** $p<0.01$ by Wilcoxon signed rank test (two-tailed) on raw data.

Supporting Information

Design of TLR2 ligand-synthetic long peptide conjugates for therapeutic vaccination of chronic HBV patients

Supplementary methods

Antibodies used to detect indicated antigens by flow cytometry were: HLA-A*02 (BB7.2, Serotec), CD3 (UCHT1, eBioscience), CD8 (RPA-T8, BD Biosciences), CD4 (RPA-T4, eBioscience), CD19 (HIB19, eBioscience), CD14 (61D3, eBioscience), CD1a (HI149, eBioscience), NGFR (ME20.4, BioLegend), BDCA1 (AD5-8E7, Miltenyi), BDCA3 (AD5-14H12, Miltenyi), Interferon gamma (IFN-γ) (25723.11, BD FastImmune), tumor necrosis factor α (TNF-α) (MAb11, eBioscience) and the live/dead marker Aqua (LifeTechnologies).

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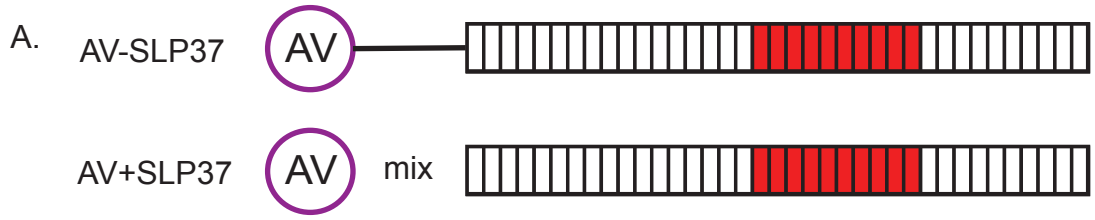
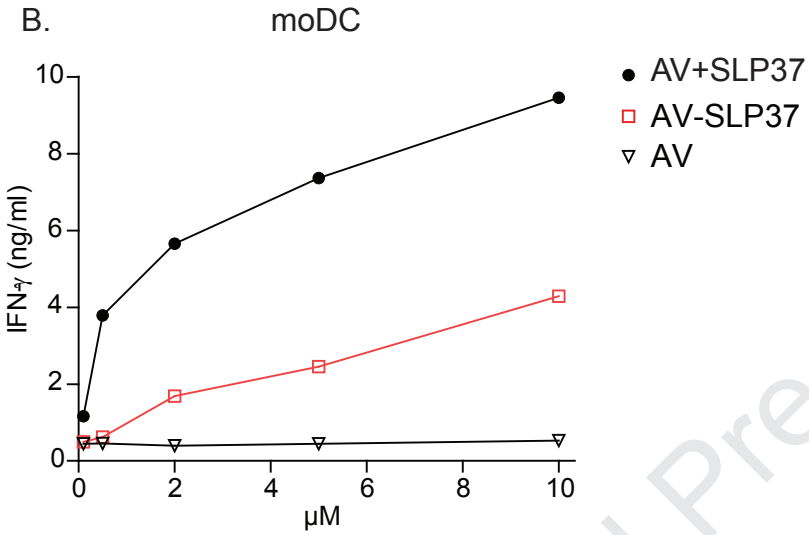
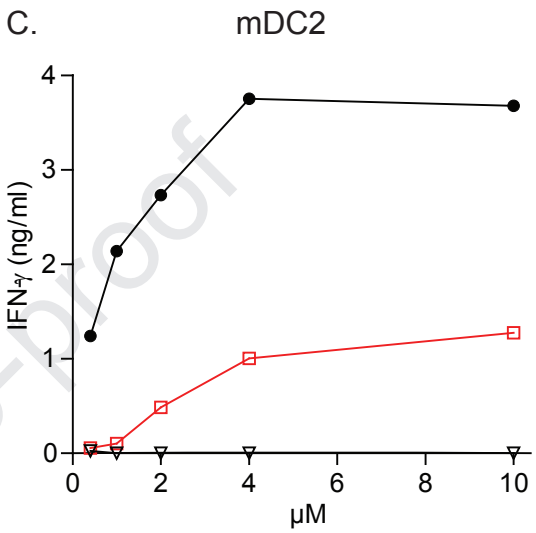
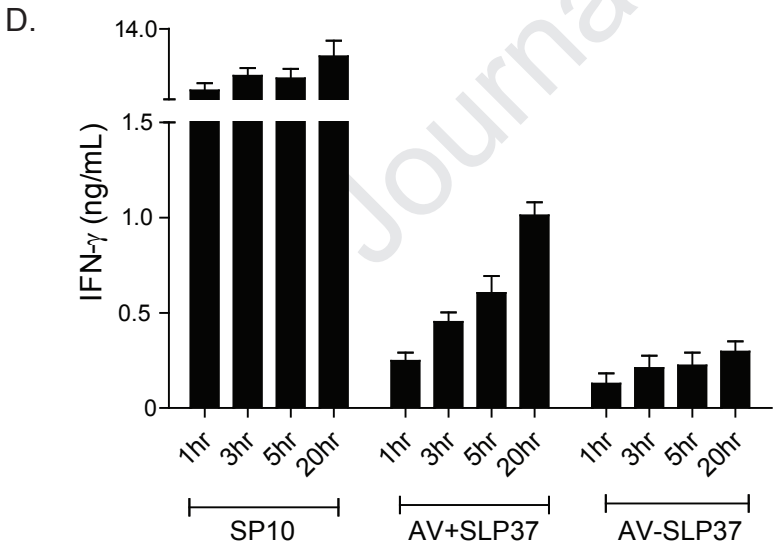
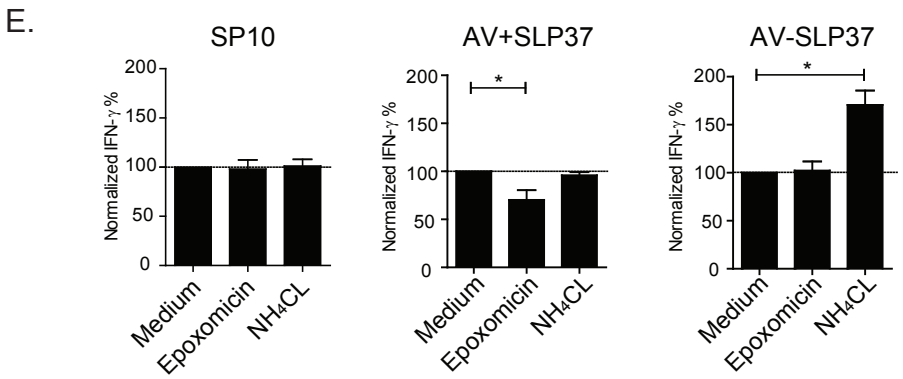
F1. Conjugation of TLR2-ligand to the HBV-SLP reduces cross-presentation efficiency.



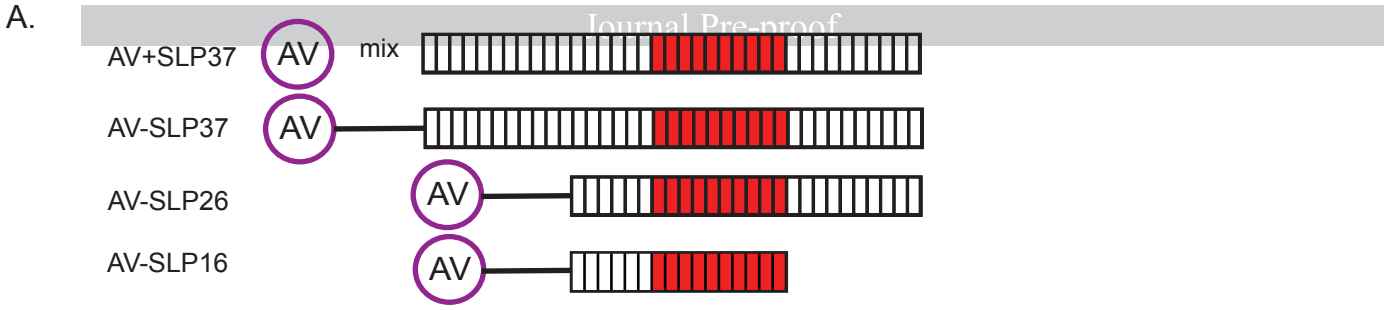


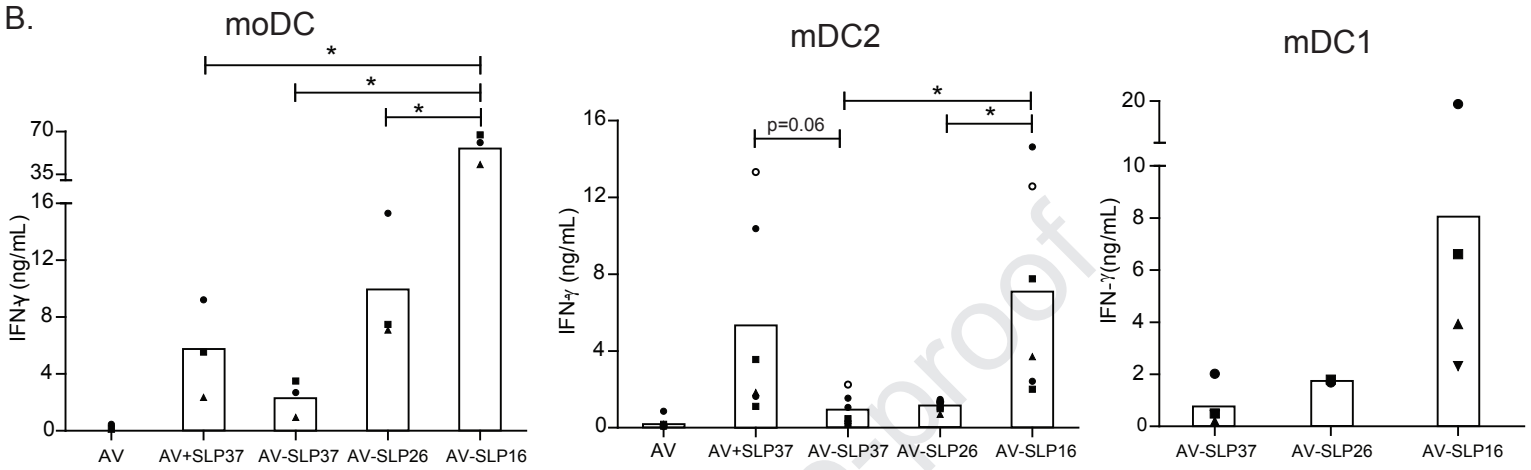


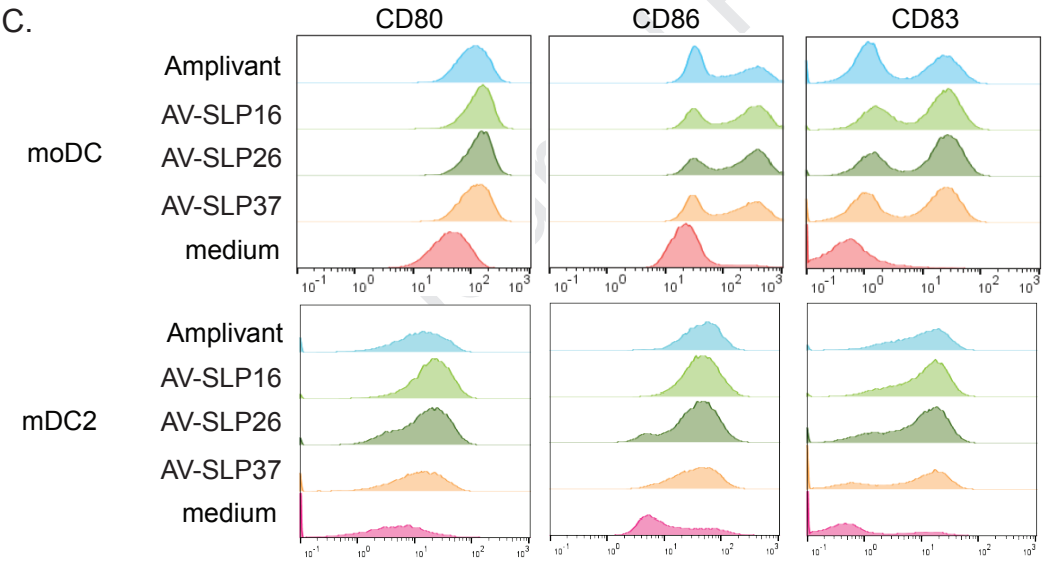


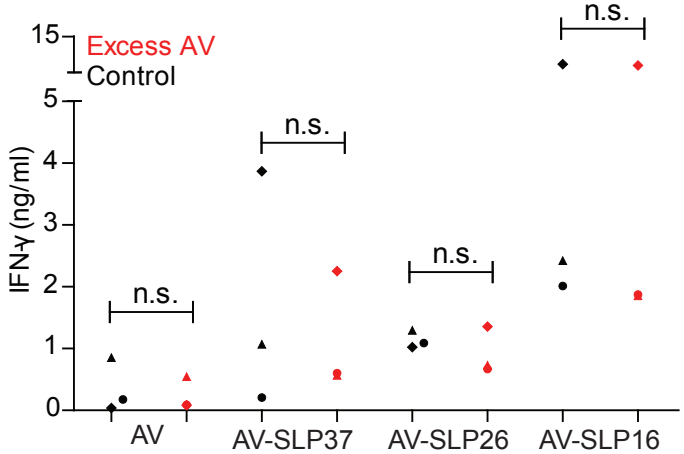


F2. Shortening of SLPs within conjugates enhances cross-presentation.

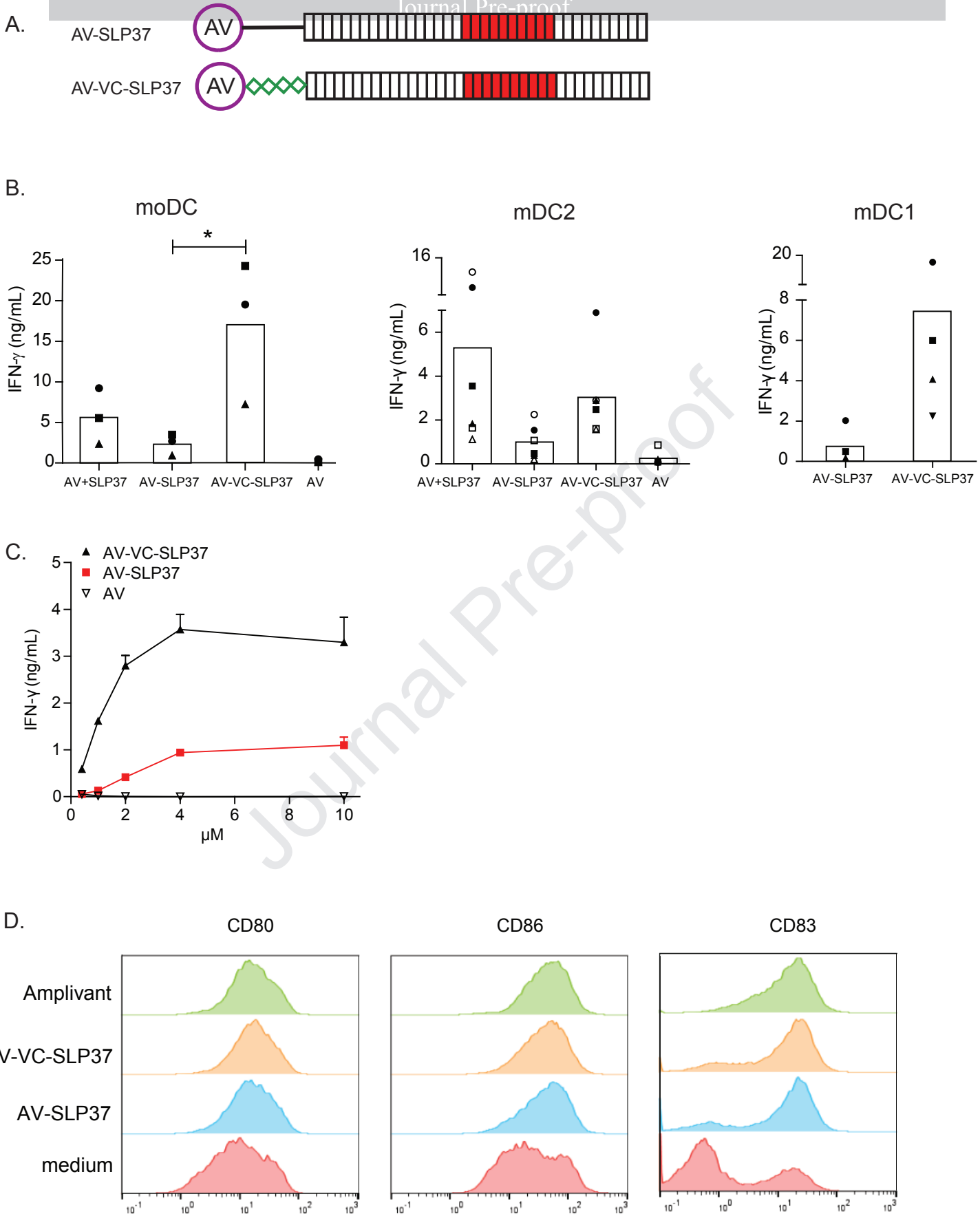






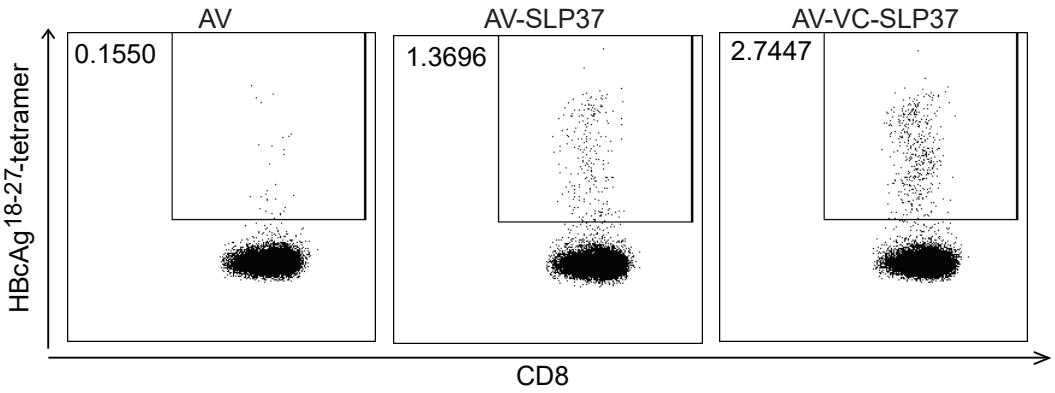


F3. Inserting a protease-sensitive linker between Amplivant and SLP improves cross-presentation.

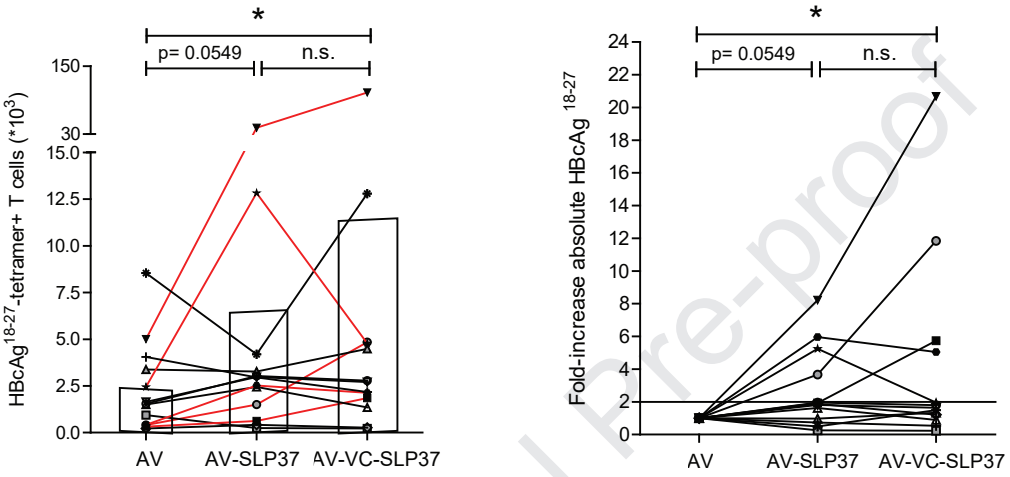


F4. Induction of HBV-specific CD8+ T cell response in CHB patients by Amplivant-SLP conjugates.

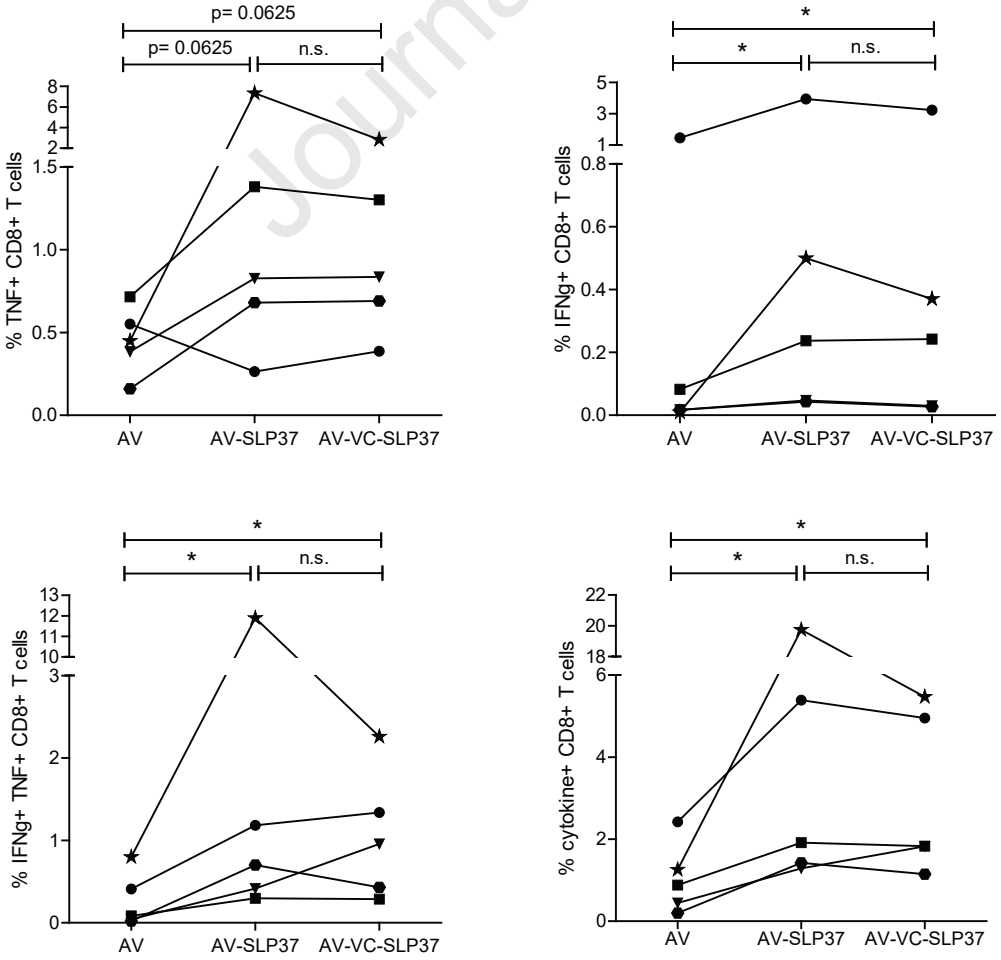
A.



B.

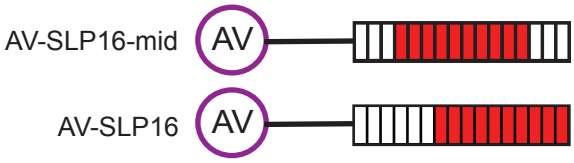


C.

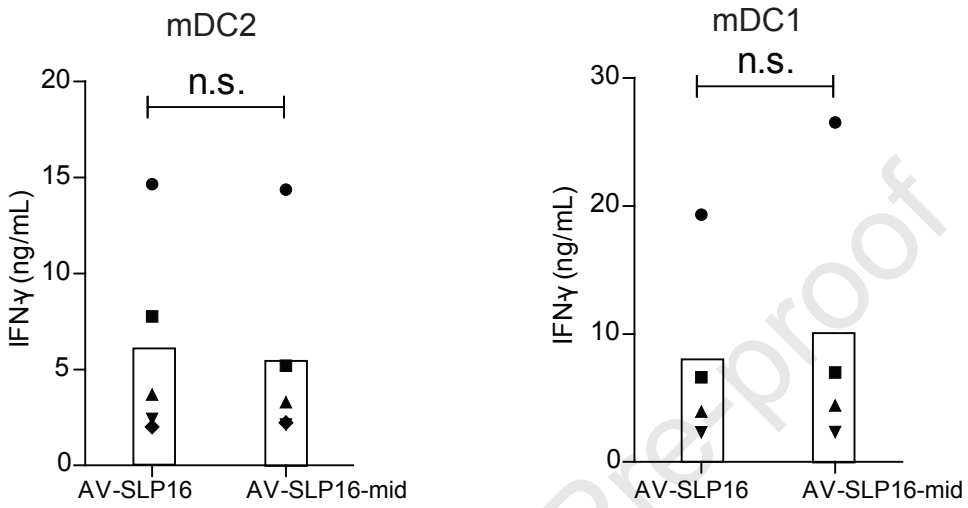


Supplementary F1. Effect of epitope position within AV-SLP16 on cross-presentation by human DC.

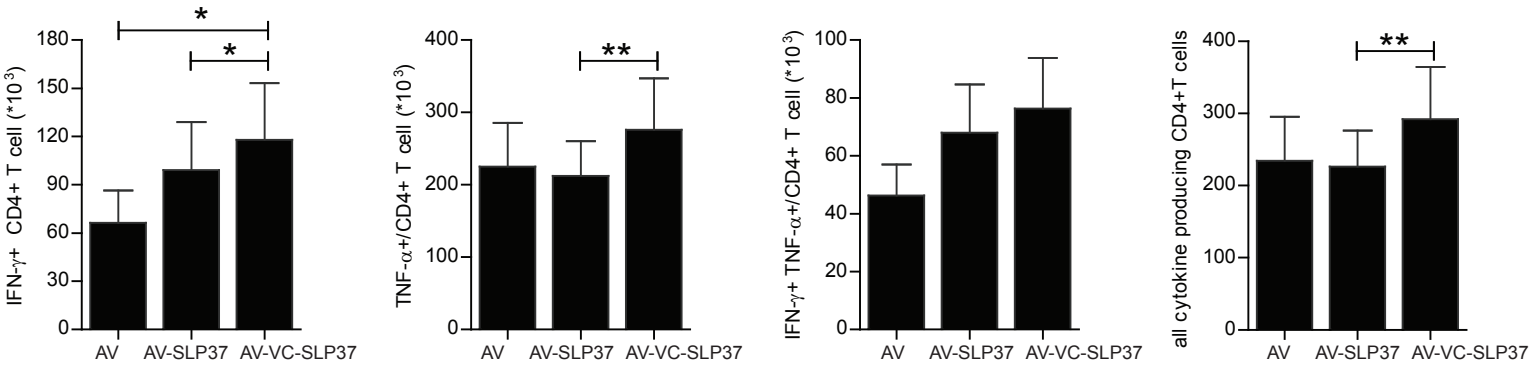
A.



B.



Supplementary F2. Induction of functional CD4+ T cells in CHB patients by AV-SLP conjugates.



Highlights

- We generated TLR2-ligand HBV-SLP conjugates to ensure delivery of SLP and TLR2-ligand to the same DC upon vaccination.
- TLR2-ligand HBV-SLP conjugates were less efficiently cross-presented by monocyte-derived and primary DC subsets *in vitro*.
- Cross-presentation of TLR2-ligand HBV-SLP conjugates was improved by reducing SLP length.
- Cross-presentation of TLR2-ligand HBV-SLP conjugates was improved by inclusion of a protease sensitive linker sequence.
- TLR2-ligand HBV-SLP conjugates boosted chronic HBV patient-derived HBV-directed T cell responses *ex vivo*.