Plant-based chimeric HPV-virus-like particles bearing amyloid- β epitopes elicit antibodies able to recognize amyloid plaques in APP-tg mouse and Alzheimer's disease brains

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Abstract The main amyloid-beta ($A\beta$) variants detected in the human brain are full-length $A\beta$ 1–40 and $A\beta$ 1–42 peptides; however, a significant proportion of AD brain $A\beta$ consists also of N-terminal truncated/modified species. The majority of the previous immunotherapeutic strategies targeted the N-terminal immunodominant epitope of the full-length $A\beta$; however, most of the pathological N-truncated forms of $A\beta$ lack this critical B cell epitope. Recently, virus-like particles (VLPs), self-assembled structures with highly ordered repetitive patterns on their surface and capable of inducing robust immune responses, were applied as a promising platform for various antigen expressions. In this study, we expressed in plants two chimeric HPV16 L1 capsid proteins obtained by introduction of the β -amyloid 11–28 epitope ($A\beta$ 11–28) into the h4 helix or into the coil regions of the L1 protein. The $A\beta$ 11–28 epitope was chosen because it is present in the full-length $A\beta$ 1–42 as well as in the truncated/modified amyloid peptide species. After expression, we assembled the chimerical L1/ $A\beta$ 11–28 into a VLP in which the $A\beta$ 11–28 epitope is exposed at very high density (360 times) on the surface of the VLP. The chimeric VLPs elicited in mice $A\beta$ -specific antibodies binding to β -amyloid plaques in APP-tg mouse and AD brains. Our study is the first to demonstrate a successful production in plants and

immunogenic properties in mice of chimeric HPV16 L1 VLPs bearing $A\beta$ epitope that may be of potential relevance for the development of multivalent vaccines for a multifactorial disease such as AD.

Keywords Alzheimer disease · Virus-like particles · Molecular pharming · Amyloid- β epitopes · Vaccine · Antibodies

Introduction

Extracellular and intracellular accumulation of amyloid-beta ($A\beta$) peptide in the brain has been hypothesized to play a central role in the neuropathology of Alzheimer's disease (AD) (Masters et al. 1985; Tseng et al. 2004; Selkoe and Hardy 2016). The main $A\beta$ variants detected in the human brain are full-length $A\beta$ 1–40 and $A\beta$ 1–42 peptides; however, a significant proportion of AD brain $A\beta$ consists also of N-terminal truncated/modified species that represent highly desirable and abundant therapeutic targets (Russo et al. 2001; Perez-Garmendia and Gevorkian 2013; Bayer and Wirths 2014).

Immunotherapy approaches, both active immunization with $A\beta$ peptide, or passive transfer of anti- $A\beta$ antibodies, have shown therapeutic efficacy in several amyloid precursor protein transgenic (APP/Tg) mouse models, which develop AD-like amyloid plaque pathology (Schenk et al. 1999; Bard et al. 2000; Town et al. 2001; Brody and Holtzman 2008), as well as canine and primates models of amyloidosis (Lemere et al. 2004; Head et al. 2008). Importantly, the majority of the previous studies used mainly full-length $A\beta$ 1–42 peptide or various immunogens based on the N-terminal immunodominant epitope for active immunization, which induced antibodies binding to

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the EFRH epitope of A β . However, most of the pathological N-truncated forms of the A β lack this critical B cell epitope and, consequently, cannot be recognized by anti-N-terminus antibodies. Efforts have been made to generate antibodies recognizing other linear or conformational epitopes within different amyloid- β peptides and a number of clinical studies are underway (Perez-Garmendia et al. 2010; Piechotta et al. 2017; <http://clinicaltrials.gov>).

On the other hand, it is well documented that the beneficial effect of immunotherapy may be counteracted by the exacerbation of the chronic inflammatory environment in AD brain (Heneka et al. 2014; Pasqualetti et al. 2015). Early clinical trials of A β immunization resulted in the development of brain inflammation in participants and were halted (Munch and Robinson 2002). There is an urgent need for development of an adjuvant or immunization protocol capable of inducing a strong A β -specific humoral immune response without accompanying pro-inflammatory Th1 response. Alum, being the only adjuvant approved for use in humans in the USA, is not the most suitable choice for AD patients because of known neurotoxic effect (Savory et al. 2006). Many clinical trials using saponin-based adjuvants (QS21, IMX or GPI-0100) in healthy adults as well as patients with progressive prostate or renal cancer, metastatic breast cancer, influenza or AD are underway or have been concluded recently (<http://www.clinicaltrials.gov>). However, saponin-based adjuvants mainly induce a pro-inflammatory Th1 response (Wu et al. 1992).

One approach that is gaining more acceptance in vaccine development is the production of virus-like particles (VLPs). VLPs are self-assembled structures with highly ordered repetitive patterns on their surface, which are capable of stimulating an adaptive immune response by cross-linking B cell receptors (Bachmann et al. 1993). Also, they can be incorporated by antigen-presenting cells and have self-adjuncting properties (Lua et al. 2014). One of the most well-known VLPs is that of the human papilloma virus (HPV), which is composed of the major capsid protein, L1 (Li et al. 2004). HPV has a non-enveloped icosahedral structure consisting of 72 pentameric capsomers of L1. Many studies have shown that L1 protein is one of the most promising carriers of foreign epitopes (Paz de la Rosa et al. 2009). Importantly, it has been shown previously that a VLP-based vaccine expressing a construct with a tandem repeat of M2e sequences derived from human, swine and avian influenza A viruses confers cross protection against various subtypes of influenza virus and lowers inflammatory cytokine levels in experimental animals (Kim et al. 2013). Also, VLPs bearing the N-terminal immunodominant epitope of amyloid- β peptide had been evaluated in immunization protocols in mice and rabbits and had been demonstrated to induce protective antibody

responses without concomitant T cell response and inflammation (Chackerian et al. 2006; Zamora et al. 2006; Chakerian 2010). However, the immunogens described in those studies would not bind to amino-truncated/modified amyloid peptides present in human brain, as discussed above.

In this study, we expressed in plants, considered an ideal platform for VLPs production, two chimeric L1 capsid proteins obtained by introduction of the β -amyloid 11–28 epitope (A β 11–28) into the h4 helix or into the coil regions of the L1 protein (Matić et al. 2011; Kushnir et al. 2012). The A β 11–28 epitope was chosen as a target because it is present in the full-length A β (A β 1–42) protein as well as in the truncated/modified amyloid peptide species. After expression, we assembled the chimerical L1/A β 11–28 into a VLP in which the A β 11–28 epitope is exposed at very high density (360 times) on the surface of the VLP. The chimeric VLPs were purified and administered into mice, and the elicited antibodies were employed to detect β -amyloid plaques in transgenic mice and AD brains.

Materials and methods

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Synthetic human A β 1–42, A β 1–16 and A β 12–28 as well as N-pyroglutamate-modified peptides pyroGluA β 3–42 and pyroGluA β 11–42 were purchased from AnaSpec (San Jose, CA, USA). A monoclonal anti-A β antibody binding to the central region of A β 1–42 (BAM90.1) was from Sigma. HRP-conjugated anti-mouse IgG1, IgG2b and IgG3 were from Zymed (San Francisco, CA, USA). HRP-conjugated goat anti-mouse IgG2c was from Thermo Fisher Scientific (San Jose, CA, USA). AlexaFluor 594 or AlexaFluor 488 goat anti-mouse IgG were from Invitrogen (Carlsbad, CA, USA). Super Signal West Dura Extended Duration Substrate kit was from Pierce (Rockford, IL, USA).

Prediction of the L1 chimeric CP tertiary and VLP quaternary molecular structures

We built three-dimensional molecular models of the two chimeric protein sequences, L1a and L1b, based on the structure of the wild-type L1 protein from HPV16, following the methodology previously reported for *in silico* prediction of protein tertiary structures (Bonneau et al. 2001, 2002; Cabrera et al. 2012; Noda-García et al. 2013, 2015). Currently, Rosetta (Leaver-Fay et al. 2011) is one of the best available methods for homology structure prediction. We used the following protocol in both cases. The primary amino acid sequence of the chimeric CP was used as the initial input to the Robetta public server (Kim

et al. 2004) to generate a fragment library comprising three- and nine-residue three-dimensional segments of the protein chain. This library was used locally to produce a large number of possible structures (10,000 decoys) by a Monte Carlo procedure on a high-performance computing facility. We used the structure of the HPV16 L1 capsid protein as the molecular template (PDBID: 1DZL), downloaded from VIPERdb (Carrillo-Tripp et al. 2009). Such big sampling of the tertiary conformational space for protein structure prediction is the minimal size recommended in the literature. The generated decoy space was then clustered using the C α RMSD pairwise values as a metric to produce a smaller set of models, ranked by the size of the cluster and an energy function that favors compact structures with well-formed secondary structure motifs and buried hydrophobic residues. We selected the optimal model in each case by identifying the structure on the biggest cluster with the lowest Rosetta energy. As a final step, we constructed the chimeric full capsid models by a three-dimensional structure alignment and multiple rotation + translation of the predicted chimeric CP structures on top of the wild-type HPV16 capsid template, giving rise to the quaternary protein–protein interactions.

Plasmid construction

Full-length sequences of L1a and L1b were synthesized by GenScriptTM. In L1a, the A β 11–28 epitope is cloned in the h4 helix (TLEDTYRFVTQAI), and in L1b the epitope is cloned in the coil region, replacing the sequence KHTPPAP-KEDDDPLKK (Matić et al. 2011). The 1462 pb fragment was subcloned into the expression vector pICH31070 (Icon GeneticsTM) to obtain pICH31070–L1a and pICH31070–L1b. Both vectors and the modules for the Magniffection[®] System were then transferred to *Agrobacterium tumefaciens* GV3101 by electroporation. Colonies of transformed *Agrobacterium tumefaciens* were cultured 48 h at 28 °C in solid YEB media with rifampicin (50 mg/L) and kanamycin (25 mg/L). Agroinfiltration and incubation of the plants were performed as described by Uribe-Campero et al. (2015).

Total soluble protein extraction and Western blot

Seven days post-agroinfiltration, the leaves were cut and immediately frozen in liquid nitrogen. The frozen tissue was macerated with mortar and pestle and mixed with extraction buffer (1% sodium bisulfite), using 1.5 mL of extraction buffer for each gram of fresh tissue. The extract was incubated in a 45 °C water bath for 20 min, cooled for 40 min at RT and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was collected and stored at 4 °C. The pellet was resuspended in PBS, pH 7.4 (500 μ L of PBS for each gram of fresh tissue) and centrifuged at

10,000 rpm for 15 min at 4 °C. The supernatant was filtered through 0.45 and 0.22 μ m cellulose acetate filters successively (Coconi-Linares et al. 2013). Protein quantification in total soluble extract was performed using Bradford assay (Bradford 1976). For Western blot analysis, proteins were separated by electrophoresis on 12% SDS-PAGE gels and transferred onto nitrocellulose membranes. Blocking of the membrane was performed with 5% skimmed milk (DifcoTM) in PBS supplemented with 0.05% Tween-20. Membranes were incubated with mouse anti-L1 antibodies (Santa Cruz Biotechnology, IncTM Catalog Number sc-53324) at 1:2000 dilution. Detection was performed with goat anti-mouse IgG. Immunoreactive bands were detected using NBT Alkaline Phosphatase Substrate (Sigma-AldrichTM Catalog Number B5655) diluted in 10 mL of PBS.

Purification of chimeric L1 protein

The supernatants were dialyzed in a 30 kDa cellulose membrane (Sigma-Aldrich) against acetate buffer of pH 5 for 24 h at 4 °C. The dialyzed supernatant was loaded on an ionic exchange HiTrap Capto S ImpAct purification column (GE Healthcare Life Sciences) in the ÄKTA Purification SystemTM and the fractions containing the chimerical L1 a/b protein were pooled and loaded on an exclusion Superdex 75 10/300 GL column (GE Healthcare Life Sciences).

Assembly of virus-like particles and electron microscopy

To assemble the VLP–L1a and VLP L1b, the purified chimerical L1a and L1b were incubated for 1 h at room temperature in assembly buffer (PBS, 2 mM DTT, 0.03% Tween 80, 0.166 NaCl pH 8.2) and then dialyzed for 24 h at 4 °C in PBS, pH 6, and another 24 h at 4 °C in PBS, pH 7.4. For electron microscopy, fresh samples were adsorbed on formvar/carbon-coated copper grids and negatively stained with 2% phosphotungstic acid of pH 5.5 for 1 min, twice. The stained VLPs were observed by transmission electron microscopy in a transmission electron microscope TEM Morgagni M-268, Philips/FEI, The Netherlands.

Experimental animals

Young (2-month-old) male C57BL/6J mice were maintained on a 12 h light/dark cycle in a temperature-controlled room. Animal handling was approved by the Institutional Animal Care and Use Committee (CICUAL, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México; ID 209, revised and approved on 10/18/2016), which adheres to the national law and NIH rules.

Immunization protocol

Four groups of six mice were inoculated with VLP-L1a, VLP-L1b and Gardasil (MERCK & Co) or PBS as controls. Mice were inoculated intramuscularly five times at 2-week intervals with 10 µg of VLP in 40 µL of PBS or 40 µL of PBS alone.

ELISA for evaluation of anti-A β antibodies

ELISA was performed using MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) coated overnight with synthetic A β peptides at a concentration of 0.2 µg per well in carbonate buffer, pH 9.6. After washing with phosphate buffer containing 0.2% Tween-20 (PBS-Tween), plates were blocked with PBS/2% non-fat dry milk for 1 h at 37 °C. Plates were washed, then mouse sera diluted 1:100 in PBS/2% non-fat dry milk/0.2% Triton X-100 were added and incubated for 1 h at 37 °C. Plates were washed with PBS/0.2% Tween, HRP-conjugated goat anti-mouse IgG or anti-mouse IgG isotypes diluted 1:2000 in PBS/2% non-fat dry milk/0.2% Triton X-100 were added, and plates were incubated for 1 h at 37 °C. Plates were washed and 2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid (ABTS)-Zymed) single solution was added. The OD reading at 405 nm was registered using Opsys MR Microplate Reader (DYNEX Technologies, Chantilly, VA, USA).

Western blot analysis

Synthetic amyloid- β peptides diluted in loading buffer (Laemmli 4 $\times$ containing 1% β -mercaptoethanol), were separated by electrophoresis on 4–12% polyacrylamide precast NuPAGE Bis-Tris gels (Invitrogen) at 150 V for 60 min and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) using a semi dry blot system (Bio-Rad) at 25 V for 50 min. Membranes were blocked in PBS/2% non-fat dry milk/0.2% Triton X-100 overnight at 4 °C and incubated overnight at 4 °C with mouse sera diluted 1:500. After washing with PBS/0.2% Tween, the membranes were incubated with HRP-conjugated goat anti-mouse IgG at RT for 2 h. Immunoreactive bands were detected by chemiluminescence using Super Signal West Dura Extended Duration Substrate kit (Pierce), according to the manufacturer's protocol.

Immunohistochemistry

All postmortem brain samples were from the first Latin American Brain Bank established at the Center of Research and Advanced Studies, Mexico, in 1994, in compliance with all applicable laws and requirements of the

Institutional Review Board, and aimed to study neurological disorders. Anonymous brain tissue samples from AD patients and from cognitively normally aging (NA) elderly subjects were used in this study.

30 µm-thick free-floating 3xTg-AD mouse or AD and control human brain sections were processed essentially as described previously (Hernandez-Zimbron et al. 2012). After antigen retrieval by incubating in citrate buffer (0.01 M citric acid, 0.05% Tween 20, pH 6.0) at 70 °C for 30 min, samples were thoroughly washed several times, incubated in TBS containing 0.1% Triton X-100 (TBS-Tx) for 15 min and blocked in a solution of 2% IgG free-albumin (Sigma) in TBS-Tx for 20 min at RT. Then, brain sections were incubated overnight at 4 °C with experimental mouse sera and control monoclonal anti-amyloid- β antibody (BAM90.1) diluted in TBS-Tx containing 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA). After washing, sections were incubated for 1 h at RT with AlexaFluor 594 or AlexaFluor 488 goat anti-mouse IgG diluted in TBS-Tx plus 5% normal goat serum. Samples were mounted onto glass slides in Vectashield medium (Vector Laboratories) with DAPI for nuclei imaging. Staining was visualized on an Olympus Ix51 microscope equipped with a DP71 camera (Nikon Instruments Inc., Melville, NY, USA).

Results

Analysis of the position of the A β 11–28 epitope on the chimeric VLP molecular structure

The use of VLPs as carriers for epitope presentation is becoming more and more popular. The VLPs from HPV have been intensively studied and several exposed regions have been identified that potentially allow the insertion of foreign epitopes. However, not all of these regions can be substituted because their absence prevents assembly of VLPs (Matić et al. 2011). The h4 helix has been suggested as a potential site for insertion of foreign epitopes without disrupting VLP assembly (Matić et al. 2011). For that reason, we decided to insert the A β 11–28 epitope into this helix and additionally into the coil region, which is located a few amino acids downstream, yielding two chimeric molecules. Since it was important that the VLPs should assemble and that the epitope should be fully exposed, we built three-dimensional molecular models of the two chimeric protein sequences. As can be seen in Fig. 1, the epitope was fully exposed in both constructs according to our models. When the wild-type sequence was subjected to the same analysis, it revealed that both the h4 and coil regions were also exposed, as expected.

Fig. 1 Full capsid molecular model of the **a** wild-type human papilloma virus 16 and the chimeric capsids formed by the proteins **(b)** L1a and **(c)** L1b. The 60 protein subunits forming the capsid are represented by gray lines. One of the five capsid proteins forming one

fivefold unit is highlighted in blue. The protein segment replaced with the β -amyloid peptide sequence (aa 11–28) is shown in red. A zoomed in view on the protein–protein interface formed at the quasi sixfold is presented in the insets

Purification and analysis of the chimeric VLP

Different groups, including ours, have expressed chimeric HPV molecules in plants. In this study, we were able to express our chimeric constructs in plant cells successfully. We purified both chimeric proteins and were able to detect them using specific anti-L1 monoclonal antibodies (Fig. 2a). The expected molecular weight of both chimeric proteins was around 55 kDa and a band of that size was clearly detectable in both cases. The commercial antibody was able to recognize these same bands but, interestingly, it also recognized additional bands albeit of smaller molecular weight (Fig. 2). Examination of the purified proteins by transmission electron microscopy revealed the presence of VLPs in both preparations, indicating that insertion of the foreign epitope either into the h4 or coil regions of L1 did not disrupt VLP assembly (Fig. 2b).

To confirm that the A β 11–28 epitope was incorporated into the particles, ELISA was performed on VLP–L1a and VLP–L1b using BAM 90.1, a specific monoclonal antibody that binds to A β 12–28 epitope. A β 1–42 and A β 11–28 peptides were used as positive controls and HPV VLPs from the Gardasil commercial vaccine and an unrelated peptide were used as negative controls. Both chimeric proteins and the A β 1–42 and A β 11–28 peptides were recognized by BAM 90.1. As expected, no signal was detected with the HPV commercial vaccine or the unrelated peptide samples (Fig. 2c).

Immunogenicity of the chimeric VLPs

The next step was to determine if the epitopes exposed on the surface of the chimeric proteins could elicit anti-amyloid- β antibodies. We employed ELISA to evaluate the

immune response in mice immunized with our chimeric VLPs. PBS and HPV commercial vaccine were used as negative controls for mice immunization. The antibodies obtained from mice immunized with the chimeric VLPs (VLP–L1a and VLP–L1b) were capable of recognizing both the full-length peptide and the truncated species—pyroGluA β 3–42 and pyroGluA β 11–42, whereas the serum obtained from mice immunized with the HPV commercial vaccine and PBS did not recognize any of the A β peptides (Fig. 3a–c).

To corroborate our ELISA results, we performed a Western blot experiment using anti-sera obtained from mice immunized with VLP–L1a and VLP–L1b. A β 1–42, pyroGluA β 3–42 and pyroGluA β 11–42 peptides were loaded in triplets on a gradient gel and transferred onto PVDF membranes. Each triplet was incubated with serum obtained from mice immunized with VLP–L1a or BAM 90.1 as a positive control. The Western blot results confirmed that the antibodies produced in mice were able to recognize the A β 1–42, pyroGluA β 3–42 and pyroGluA β 11–42 peptides.

To further characterize the immune response induced by chimeric VLPs, we determined the IgG isotypes of antibodies present in sera from mice immunized with VLP–L1a and VLP–L1b. As shown in Fig. 4b, predominantly IgG1 and IgG2b antibodies were detected that indicates an anti-inflammatory Th2 response. Interestingly, pro-inflammatory IgG2c antibodies were not detected in these sera. Mice inoculated with PBS and immunized with VLP–L1 wt did not produce anti-A β antibodies.

Fig. 2 Chimeric L1a and L1b proteins bearing the Aβ11–28 epitope (EVHHQKLVFFAEDVGSNK) at different regions (see “Materials and methods”) were loaded into an ion exchange purification column and the positive fractions obtained were loaded into an exclusion purification column (a). Aliquots of purified L1a and L1b were separated by SDS-PAGE and analyzed by Western blot using anti-L1 monoclonal antibody. In both, SDS-PAGE and Western blot, a 55 kDa band concordant to L1 protein was observed. Chimeric L1a and L1b proteins assemble into VLP-L1a and VLP-L1b, respectively (b). Aliquots of VLP-L1a and VLP-L1b were absorbed onto glow-

discharged carbon-coated copper grids, negatively stained with 5% phosphotungstic acid and visualized by TEM (magnification $\times 40,000$). VLPs are indicated by arrows. Scale bar represents 100 nm. VLP-L1a and VLP-L1b were examined by ELISA using anti-Aβ12–28 monoclonal antibody (BAM90.1). Aβ1–42 and Aβ11–28 peptides were used as positive controls (c). VLP-L1 wt and an unrelated peptide were used as negative controls. Both VLP-L1a and VLP-L1b showed mAb anti-Aβ12–28 recognition for the Aβ11–28 epitope in their surface. Bars represent mean values of three different experiments

Recognition of amyloid plaques in mouse and human brain tissue

To determine if the antibodies produced after immunization with our chimeric VLPs were able to recognize amyloid plaques in transgenic mouse brain tissue, we prepared 30 μm -thick brain tissue sections from 20-month-old 3xTg-AD mice. The brain tissue was immunostained as described above. BAM 90.1 was used as positive control to detect amyloid aggregates (Fig. 5a). Serum of VLP-L1 wt-immunized mouse was used as negative control (Fig. 5b). As expected, sera from VLP-L1a- and VLP-L1b-immunized mice were able to recognize amyloid aggregates in the hippocampus of 3xTg-AD mice (Fig. 5c, d).

The next step was to determine if the antibodies were also capable of recognizing amyloid plaques in human

brain tissue. For that purpose, 30 μm -thick frontal cortex sections from a deceased AD patient were analyzed as described above. Control negative tissue was included. BAM90.1 monoclonal antibody was used as a positive control. Serum from VLP-L1a- and VLP-L1b-immunized mice did recognize amyloid aggregates in AD brain sections (Fig. 6d, e). Samples from a healthy brain did not show any immunofluorescence (Fig. 6c). Taken together, these results demonstrate that the antibodies produced using our chimeric VLPs were able to recognize amyloid aggregates in human and transgenic mouse brain tissue.

Fig. 3 Immune sera obtained from VLP-L1a- and VLP-L1b-immunized C57Bl/6 mice were tested by ELISA using A β 1-42 (a), pyroGluA β 3-42 (b) or pyroGluA β 11-28 (c). Serum of a mouse immunized with A β 1-42 (a), pyroGluA β 3-42 (b) or pyroGluA β 11-28 (c) served as positive control in the respective plate (shown as IgG positive control). Sera from mice inoculated with PBS or immunized with VLP-L1 wt served as negative controls. VLP-L1a

and VLP-L1b induced specific Ab against A β 11-28 epitope that were capable of recognizing the A β 1-42 (a), pyroGluA β 3-42 (b) and pyroGluA β 11-28 (c) species. Mice inoculated with PBS or immunized with VLP-L1 wt did not produced specific Ab against A β 11-28 epitope. Each bar represents the mean OD values of three different experiments

Fig. 4 Immune sera obtained from chimeric VLP-immunized C57Bl/6 mice were tested by Western blot (a). From left to right, the A β 1–42, pyroGluA β 3–42 and pyroGluA β 11–42 peptides were loaded in triplets in a 4–12% gradient gel. The gel was transferred onto PVDF membrane which was cut in triplets. Each triplet was incubated with sera obtained from VLP-immunized mice or BAM90.1 monoclonal antibody (recognizing the A β 12–28 epitope) as positive control. IgG isotypes found in immune sera obtained from VLP-L1a- and VLP-L1b-immunized mice were determined by ELISA (b). IgG1 and IgG2b antibodies, indicative of anti-inflammatory response, were found, while IgG2c, indicative of pro-inflammatory Th1 response, was not detected. Mice inoculated with PBS and immunized with VLP-L1 wt did not produce anti-A β Abs. Each bar represents the mean OD values of three different experiments

Discussion

Immunotherapeutic strategies have shown promising results in different experimental models of AD; however, there is still a need for the development of efficacious vaccines targeting different pathological amyloid species present in the human brain, without inducing inflammatory and auto-reactive T cell responses. In this study, we evaluated VLP-based immunogens, bearing an epitope from the central region of A β peptide, in mice.

VLPs are considered one of the most promising vehicles for subunit vaccines and L1 from HPV presents a number of interesting features that make it an ideal platform. The mammalian immune system is able of responding robustly to repetitive antigens present on a dense, array-like structure and that is why we decided to expose the A β 11–28 epitope on the VLP surface. It has been demonstrated that

immunization with A β peptide can elicit antibody responses, but for this to occur strong adjuvants that often lead to inflammatory Th1 responses are required. In addition, T cell responses against A β are often concurrent with humoral responses that may lead to auto-immune effects (Monsonego et al. 2003). VLP-based immunogens constructed in our study elicited antibodies against the A β 11–28 epitope at high levels without the presence of adjuvants.

Importantly, our VLPs bearing the A β 11–28 epitope elicited predominantly anti-inflammatory IgG1 and IgG2b antibodies whereas pro-inflammatory IgG2c isoform was not detected. It has been demonstrated previously that immunization with full-length A β 1–42 in the presence of common adjuvants predominantly elicits IgG2c antibodies, indicating a pro-inflammatory Th1 response (Petrushina et al. 2003; Chackerian et al. 2006). Interestingly, immunization with VLPs bearing full-length A β 1–40 or

Fig. 5 30 μ m-thick tissue sections from 20-month-old 3xTg-AD mouse were used. Amyloid aggregates are stained in red and cellular nucleus in blue. The mAb BAM 10 was used as positive control of amyloid aggregate recognition (a). Serum of VLP-L1 wt-immunized

mouse was used as negative control of amyloid aggregate recognition (b). Sera of VLP-L1a- and VLP-L1b-immunized mice were able to recognize amyloid aggregates in the hippocampus (c, d). Scale bar represents 500 μ m

N-terminal epitope A β 1–9 induced mainly IgG1 antibodies in concordance with our results (Chackerian et al. 2006). However, in the latter study, the authors did not observe any antibody response after immunization with VLPs bearing a central-domain A β epitope, in contrast to our results. These differences may be explained by the application of different strategies for VLPs generation. We cloned A β 11–28 epitope in two predicted surface-exposed sites of HPV16 L1 protein, whereas in the previous study biotinylated HPV16 VLPs were linked to biotinylated A β peptides using streptavidin (Chackerian et al. 2006). Three-dimensional molecular modeling studies confirmed our results. We believe that the A β 11–28 epitope in our constructs is more accessible for antigen-presenting cells and this may explain the robust antibody response mounted against the epitope.

Importantly, the antibodies elicited by our chimeric VLPs were able to recognize not only three main

pathological A β species, A β 1–42, pyroGluA β 3–42 and pyroGluA β 11–42, but also amyloid aggregates in APP-tg mouse brain as well as in postmortem brain tissue from individuals diagnosed with AD. Previously, Zamora et al. demonstrated that VLPs displaying A β 1–9 epitope were able to induce antibodies inhibiting amyloid fibril formation in vitro and in vivo and binding to amyloid aggregates in mouse and human brain, indicating the effectiveness of this strategy in AD vaccine development (Zamora et al. 2006; Chackerian 2010). A VLP-based candidate vaccine (CAD 106) that combines multiple copies of A β 1–6 is currently undergoing clinical trials (Vandenberghe et al. 2017). However, N-terminal immunodominant epitope is not present in N-truncated/modified amyloid species, and in our opinion the failure of bapineuzumab (a humanized antibody raised against the N-terminus of A β 1–42) immunotherapy to meet its clinical end points in numerous clinical trials may be explained, in part, by the presence of

Fig. 6 30 µm-thick frontal cortex sections from human brain showing amyloid aggregates in red. A section of healthy human brain incubated with BAM90.1 and a section of AD human brain-incubated serum of VLP-L1 wt-immunized mouse were used as negative controls (a, c). A section of AD brain incubated with BAM90.1 was

used as positive control of amyloid aggregate recognition (b). Sera of VLP-L1a- and VLP-L1b-immunized mice were able to recognize amyloid aggregates in AD brain sections (d, e). Scale bar represents 500 µm

N-truncated/modified A β in most AD cases (Roher et al. 2011).

Finally, despite VLPs bearing A β epitopes were obtained in mammals, yeasts or insect cells, we believe that plant-based production of VLPs represents a safe, cost-effective and fast way to produce biotherapeutics in a flexible and scalable manner (Marsian and Lomonosoff 2016). To the best of our knowledge, our study is the first to demonstrate a successful production in plants and immunogenic properties in mice of chimeric HPV16 L1 VLPs bearing A β epitope that may be of potential relevance for the development of multivalent vaccines for a multifactorial disease such as AD.

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