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Linking T cell epitopes to a common linear B cell epitope: A targeting and adjuvant strategy to improve T cell responses

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ABSTRACT

Immune complexes are potent mediators of cellular immunity and have been extensively studied for their disease mediating properties in humans and for their role in anti-cancer immunity. However, a viable approach to use antibody-complexed antigen as vehicle for specific immunotherapy has not yet reached clinical use. Since virtually all people have endogenous antibodies against tetanus toxoid (TTd), such commonly occurring antibodies are promising candidates to utilize for immune modulation. As an initial proof-of-concept we investigated if anti-tetanus IgG could induce potent cross-presentation of a conjugate with SIINFEKL, a MHC class I presented epitope of ovalbumin (OVA), to TTd. This protein conjugate enhanced OVA-specific CD8+ T cell responses when administrated to seropositive mice. Since TTd is poorly defined, we next investigated whether a synthetic peptide–peptide conjugate, with a chemically defined linear B cell epitope of tetanus toxin (TTx) origin, could improve cellular immune responses. Herein we identify one linear B cell epitope, here after named MTTE thru a screening of overlapping peptides from the alpha and beta region of TTx, and by assessment of the binding of pooled IgG, or individual human IgG from high-titer TTd vaccinated donors, to these peptides. Subsequently, we developed a chemical protocol to synthesize defined conjugates containing multiple copies of MTTE covalently attached to one or more T cell epitopes of choice. To demonstrate the potential of the above approach we showed that immune complexes of anti-MTTE antibodies with MTTE-containing conjugates are able to induce DC and T cell activation using model antigens.

1. Introduction

Specific immunotherapy by therapeutic vaccination has gained a lot of attention since identification of relevant cancer specific peptide antigens including mutated neo-epitopes has progressed significantly (Melief et al., 2015). Synthetic long peptide (SLP) therapeutic vaccines for induction of tumor-specific T cells have been explored both pre-clinically and clinically with mixed results (Kenter et al., 2009; Leffers et al., 2009). The advantage of the long peptide strategy, and specifically also using multiple peptides in a pooled mix, is that this allows for the incorporation of multiple HLA-fitting peptides into the longer peptide stretch, i.e. not relying on only one short epitope within a HLA defined population. Of specific interest is that the long-peptide vaccine approach was effective as monotherapy in premalignant HPV16-Induced lesions, but not in disseminated malignant disease (Kenter et al., 2009; van Poelgeest et al., 2013). This is likely to improve by additional co-treatment that addresses the immunosuppressive cancer micro-environment, but can conceivably also be achieved by further improvements in vaccine adjuvant as well as dendritic cell (DC) targeting. Standard adjuvants are delivered together with the antigen as a mixture (Kenter et al., 2009; Sabbatini et al., 2012). The unlinked adjuvant/antigen delivery may lead to activation of DC that have not been loaded

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with antigen and loading with antigen of DC that have not been activated by adjuvant. Therefore a methodology that leads to efficient antigen loading and DC activation of the same DC, includes a conjugation of antigen and adjuvant (Abdel-Aal et al., 2014; Liu et al., 2015; Stergiou et al., 2017; Zom et al., 2016) for efficient uptake and activation. Targeted delivery via DEC2015 (Birkholz et al., 2010) also displays improved antigen uptake by DCs through the mannose receptor (Morse et al., 2011), and other strategies exists and can target delivery to a given cell type (Tacken et al., 2007). In the case of DEC205 targeting, an adjuvant is needed along with the targeting strategy, as antigen delivery thru DEC205 will not induce DC activation (Cheong et al., 2010).

We have developed a strategy facilitating both targeting of the antigen to DCs as well as inducing DC activation, using a peptide–peptide conjugate technology. The link between the DC targeting strategy and the antigen ensures that antigen uptake and activation takes place in the same antigen-presenting cell to ensure adequate T cell activation. Immune complexes are powerful mediators of immune activation and are known facilitators of cross-presentation (Boross et al., 2014; van Montfoort et al., 2009; van Montfoort et al., 2012). We have previously demonstrated the potency of immune-complexes both by loading of dendritic cells with pre-formed complexes (Schuurhuis et al., 2006), as well as by *in vivo* formed complexes (van Montfoort et al., 2012). To translate this into clinical use to improve synthetic long peptide (SLP) vaccination, we aimed to identify a method to allow for immune complex formation with peptides as targets and to which endogenous IgG is present. Tetanus toxoid (TTd), formalin-treated tetanus toxin (TTx), is a protein to which virtually all human individuals have antibodies due to the general vaccination program in many countries. TTd, a robust antigen could potentially be used as a vehicle, but this strategy may endure GMP limitations due to that the protein-peptide conjugate will be poorly defined with a high degree of batch-to-batch variation. Along with this, the heterogeneous immune complex formation using a protein carrier can, upon repeated administration with close intervals as performed in cancer vaccination, cause unwanted side-effects such as serum sickness. An alternative approach would be to use a defined peptide sequence from TTx that could be coupled to a SLP and in which the antibody binding sites are better defined. Herein we describe the identification of such a linear TTx-derived peptide B cell epitope, and the use of it to generate defined immune complexes, improve DC activation and T cell responses.

2. Material and methods

2.1. Mice and reagents

All mouse studies were approved by the Leiden University Medical Center (LUMC) Institutional Review Board or Uppsala animal ethical committee. Wild type C57BL/6 mice were purchased while OT-I and pmel (CD90.1 +) mice (CD8 + T cell transgenic mice expressing a TCR recognizing OVA257-264 SIINFEKL or the gp100 epitope in H2-Kb or H2-Db) are bred at LUMC or Uppsala University respectively. Hybridoma cell lines producing mouse anti-MTTE IgG1 and IgG2a were made by conjugating FIGITELKKLESKINKVFC-amide to KLH and thru immunization of AIP-3 mice. When sufficient IgG1 and IgG2a titers were established isolated spleen cells were fused with NS-1 myeloma cells. Primary clones and sub clones were analyzed for reactivity and two clones (one IgG1 and one IgG2a clone) were chosen for further antibody isolation. To isolate antibodies the hybridomas were cultured in CELLline 1000 bioreactors (INTEGRA). Supernatants were harvested and spun down at 2500 rpm for 5 min and frozen at −80. Antibody purification (prot G purified) and endotoxin measurements were performed by Capra science (Sweden). The peptides and MTTE-conjugates are all produced at LUMC.

2.2. Cells

B3Z is a T cell hybridoma, specific for SIINFEKL in H-2Kb, which carries a β-galactosidase construct driven by NF-AT elements from the IL-2 promoter (Sanderson and Shastri, 1994). Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (BioWhittaker, Verviers, Belgium) with 8% heat-inactivated FCS (Greiner), 100IU/ml penicillin/streptavidin, 2 mM L-glutamine, and 50 μM 2-ME (complete medium). Complete medium was supplemented with Hygromycin B (Invivogen Life Technologies, Rockville, MD) for culturing of B3Z to select for clones with the β-galactosidase construct. D1 cell-line, a long-term growth factor-dependent immature splenic DC line derived from C57BL/B6 mice, was kindly provided by P. Ricciardi-Castagnoli (University of Milano- Bicocca, Milan, Italy). D1 cells were cultured as described (Winzler et al., 1997) with the exception of supplementing with GM-CSF (20 ng/ml) instead of R1 supernatant. D1 cells were collected by detaching, using 3 mM EDTA.

2.3. Screen of antibodies against TTd-derived linear peptides in human sera

The various peptides were synthesized by normal Fmoc-based solid phase chemistry. All peptides were tested using ELISA assays. Biotinylated peptides were coated on streptavidin plates O/N with 100 μl 5 μg/ml of the peptides in coating buffer at room temperature (RT). After incubation and washing the plate was blocked with 200 μl PBS/0.05%BSA for 1 h at RT, and subsequently diluted sera or Tetaquin (100 and 200 times diluted respectively) was added to the wells. Plates were washed and incubated with 100 μl HRP conjugated anti-human IgG monoclonal (G18-145, BD) diluted 1:1000 in PBS/1%BSA for 1hr at RT. ABTS was added 50 μl/well. Absorption was measured at 415 nm. The same approach was used for initial identification and later for mimotope identification.

2.4. ELISA detecting anti-tetanus antibodies in mice sera

Antibody titers in the sera of mice were assessed with ELISA. Nunc 96-wells microtiter plates were coated with 2 lf/ml TTd. Plates were blocked for 1hr with PBS containing 0.05% Tween and 1% BSA and subsequently washed with 100 μl/well PBS-0.05%Tween. Plates were incubated 2 h at 37 °C with 50 μl/well serum diluted in PBS-0.05%Tween. Serum dilutions started at 1:100. Subsequently, plates were incubated for 1hr with 50 μl/well HRP-conjugated goat-anti-mouse IgG diluted 1:1000 in PBS-0.05%Tween at room temperature in the dark. Substrate ABTS (Sigma Aldrich) was added 50 μl/well and reaction was stopped with 50 μl/well 1 M H2SO4. Absorption was measured at 415 nm.

2.5. In vitro cellular uptake and presentation experiments

MTTE-immune complexes (MTTE-ICs), ETTM-immune complexes (ETTM-ICs) and OVA-immune complexes (OVA-ICs) were formed by incubating different concentrations of soluble MTTE-conjugates, ETTM-conjugates or soluble OVA (grade V; Sigma-Aldrich) with a fixed concentration of either purified mIgG1, mIgG2a αMTTE (CapraScience) or rIgGαOVA (ICN Biomedicals) for 30 min at 37 °C in 96-well round-bottom plates. Soluble OVA or MTTE-conjugates alone or with control purified mouse IgG1 and IgG2a, and SIINFEKL short peptide were used as controls. Concentrations shown in the figures are the final concentrations after addition of the DCs. ICs were performed in 3-fold higher concentrations in 150 μl. After 30 min pre-incubation, 100 μl containing preformed ICs were added to 50 μl $2.5*10^4$ D1 cells and incubated for 24 h 37 °C in a 96-well flat-bottom plate. After incubation, supernatants were collected and $5*10^4$ B3Z T cells were added to each well and incubated for another 24 h at 37 °C. Presentation of SIINFEKL in H-2Kb was measured by the activation of B3Z cells, measured by a colorimetric assay using chlorophenol red-β D-

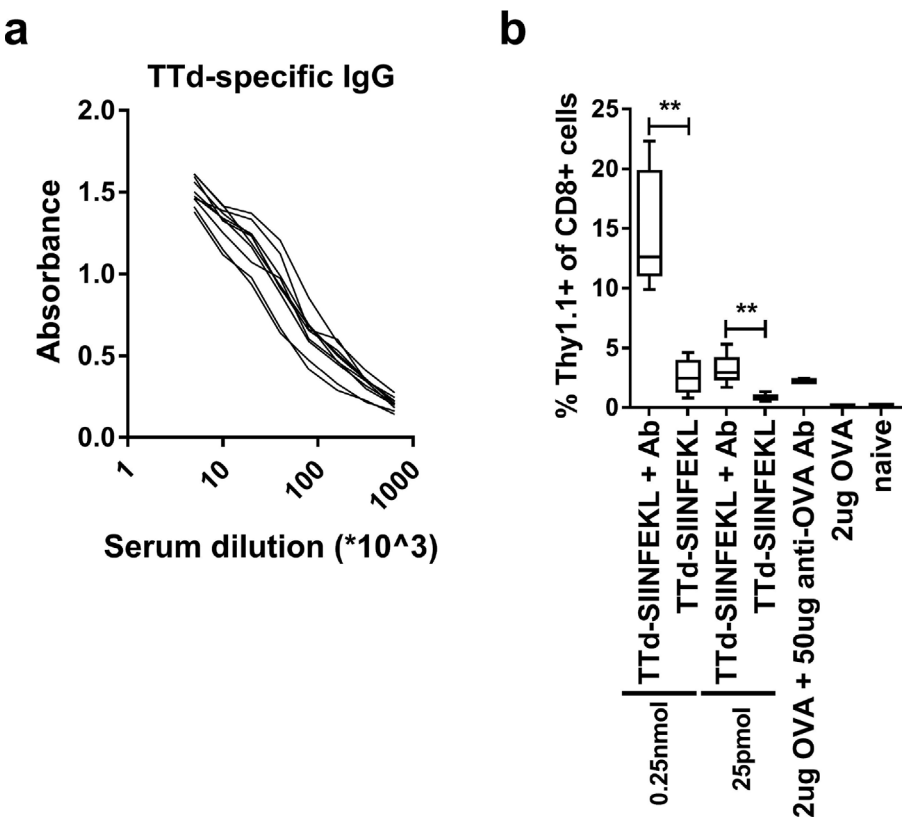

Fig. 1. Antibody titers of mice vaccinated with TTd and the subsequent accumulation of SIINFEKL-specific T cells when challenged with TTd-SIINFEKL conjugates.
Mice were immunized with TTd and antibody titers were confirmed by ELISA (a) (see ELISA section for detailed description). Seropositive (+Ab) and negative mice were adoptively transferred (i.v) with 2.7 million OT1 cells (CD8+ T cells) and were challenged with TTd-SIINFEKL conjugate (250 or 25 pmol/mouse) or OVA (2 μg) with or without OVA-specific rabbit IgG (50 μg/mouse), 1 day post OT1 transfer. 4 days post OT1 injection, spleens were harvested and single cell suspensions were stained with surface antibodies. Transferred cells were identified by the congeneic marker Thy1.1. Accumulation of Thy1.1+ CD8+ T cells (%Thy1.1+ out of all CD8+ T cells) in the spleen is displayed in (b). Statistical analyses were calculated with the Mann-Whitney test ** $p < 0.01$. n = 5/group for the TTd-SIINFEKL groups, n = 3 for the OVA groups and n = 2 for the naive group.

galactopyranoside (CPRG) as substrate to detect lacZ activity in B3Z lysates. CPRG was mixed with a lysing solution (100 mM β-mercaptoethanol, 0.125% IGEPAL CA-630, 9 mM $MgCl_2$, and 1.8 μg/ml CPRG) and incubated with the B3Z cells at 37 °C for 6 h and subsequently absorbance was measured at 595 nm.

2.6. Detection of cytokine production and flow cytometry

Harvested supernatants were tested for IL12p40 content using a standard sandwich ELISA. Coating Ab: rat anti-mouse IL-12p40 mAb (clone C15.6, Biolegend). Detection Ab: biotinylated rat anti-mouse IL12p40 mAb (clone C17.8; Biolegend). Streptavidin-HRP and TMB (Dako) were used as enzyme and substrate, respectively. Single cell suspension of the D1 cells or spleen (after erythrocyte lysis) were stained with several of the following detection antibodies; anti-CD3ε FITC (clone 145-2C11), anti-Thy1.1 (CD90.1) APC (clone HIS51), from eBiosciences; anti-CD8b PE (clone YTS156.7.7), I-A/I-E (Clone M5/114.15.2) and anti-CD40 PE/Cy7 (clone 3/23) from Biolegend.

2.7. In vivo assays in mice

In vivo TTd-LEQLESIINFEKLAAAAAK: C57BL/6 mice were immunized with TTd in a prime/boost setting. Seropositive and negative mice were adoptively transferred (i.v) with 2.7 million OT1 CD8+ T cells (enriched thru MACS separation protocol) and were 1 day later challenged i.v with TTd- LEQLESIINFEKLAAAAAK. This specific long peptide, harboring the SIINFEKL epitope was chosen as it can be produced with a high yield. On day 4 post T cell transfer, spleens were harvested and single stain suspensions were stained for Thy1.1+, CD8+ T cells and analyzed by flow cytometry.

In vivo $[MTTE]_3$-hgp100: C57BL/6 mice were adoptively transferred (i.v) with 10 million splenocytes from pmel mice (Containing T cells with a TCR specific for human gp100 in H-$2D^b$). After 1 day mice were injected s.c in the footpad with $[MTTE]_3$-hgp100 (1 nmol/mouse) premixed with MTTE-specific rabbit antibodies (250 μg/mouse) or with an irrelevant rabbit IgG faction (250 μg/mouse/mouse). After another 3 days the draining popliteal lymph nodes were harvested and single cell suspensions were stained for CD3, CD8, Vβ13 (antibody specific for the pmel TCR) and the congenic marker Thy1.1, and analyzed by flow cytometry. In a separate experiment mice adoptively transferred with pmel splenocytes were injected i.p with $[MTTE]_3$-hgp100 (1 nmol/mouse); pre-mixed with MTTE-specific rabbit antibodies (1 mg/mouse) or with an irrelevant rabbit IgG faction (1 mg/mouse), or alone. After another 3 days the draining mesenteric lymph nodes were harvested and single cell suspensions were stained for CD3, CD8, the congenic marker Thy1.1 and CD107a, and analyzed by flow cytometry.

2.8. Synthesis of the core structure

The synthesis of the conjugates was performed according to Fig. 4 and as described in supplementary material and methods and a more detailed analytical analysis of compound 10 and 14 is given in Fig. S1.

2.9. Statistics

Statistical analyses were performed using Graphpad Prism version 7.02 software (Graphpad software). Statistical analysis was calculated using the Mann-Whitney test or one way ANOVA with Tukey's multiple comparison test. * $p < 0.05$ and ** $p < 0.01$ (ns = not significant).

3. Results

3.1. Circulating TTd-specific antibodies improve T cell priming

We have previously demonstrated that circulating specific antibodies can improve the induction of T cell immunity via cross-presentation by CD11c+ DCs when employing OVA-TNP haptenated protein as the target antigen (van Montfoort et al., 2012). To assess if TTd could induce the same effect we immunized mice with TTd resulting in high anti-TTd IgG titers (Fig. 1a). Seropositive and negative

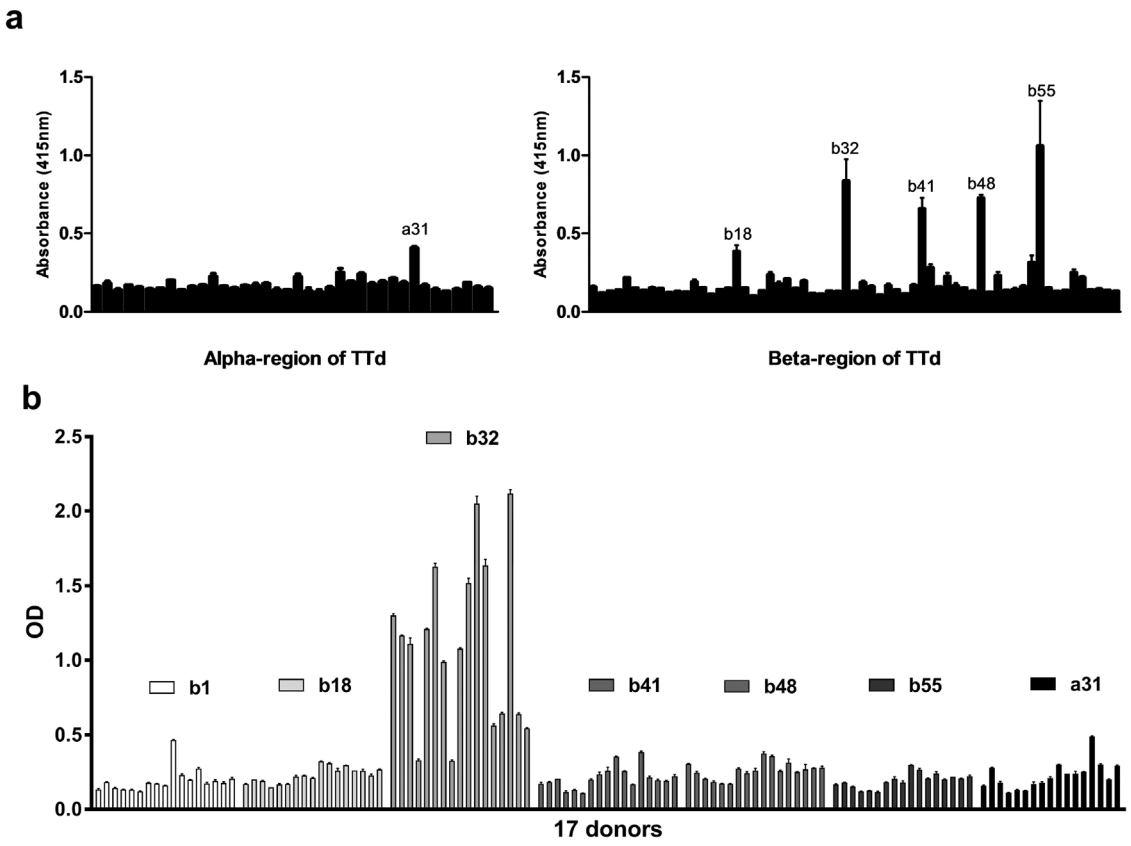

Fig. 2. Screen of IgG antibody responses against TTd-derived linear peptides in human individuals. Different linear TTd peptides were synthesized, biotinylated and coated on streptavidin plates. An anti-human IgG-HRP conjugated antibody was used to detect peptide-specific IgG antibodies in Tetaquin®. All natural linear peptides from the alpha- and beta-region of TTd were analyzed (a). The 6 peptides recognized by Tetaquin® in (a) (a31/b18/b32/b41/b48/b55) were subsequently tested for recognition by sera from 17 high-titer donors (b). These experiments were performed 2–3 times with similar results.

mice were adoptively transferred with OVA-specific T cells (OT1) and subsequently challenged (1 day later) with a TTd-LEQLESIINFEKLAAAAAK conjugate. Day 4 post OT1 transfer a strong accumulation of Thy1.1+ CD8+ T cells could be seen in the spleen of seropositive mice (Fig. 1b) indicating effective antibody-dependent cross-presentation *in vivo*.

3.2. Identification of a linear tetanus toxin-derived peptide

As large antigen/antibody-based complexes are unlikely to be acceptable for use in humans we wished to identify a linear tetanus toxin epitope that could be linked to SLPs to be able to move from pre-clinical to clinical testing of this concept. We therefore constructed overlapping 22-mer linear peptides from both TT alfa and beta chain and tested them for binding to TTd-specific antibodies using Tetaquin® as an antibody source. Tetaquin® is a registered product containing anti-tetanus IgG, among other specificities and is used clinically for passive immunization. In total 6 peptides were recognized by the human IgG in Tetaquin® (Fig. 2a). These 6 peptides, designated as a31/b18/b32/b41/b48/b55, were all tested for recognition by sera from 17 high-titer donors, which had been vaccinated against tetanus in the past with the aim to increase their anti-TTd titers and the use of their IgG to establish pooled IgG that can be used for passive transfer therapy (Fig. 2b). Only one linear peptide (peptide b32 from the beta region) was recognized by all except two donors. Subsequently, several sequence variations of the b32 peptide were synthesized and analyzed to identify the shortest and best antibody-binding B cell epitope.

3.3. Epitope identification

Tetaquin® and sera were used to determine the minimal and optimal epitope. Trimming of the identified peptide at the C-terminus and the N-terminus was performed. This yielded an 18-mer peptide as the shortest best binding peptide (data not shown). Immobilization of the peptide to a streptavidin coated ELISA plate via a C-terminal or N-terminal biotin group in the peptide revealed that only the C-terminally biotinylated peptide was able to bind antibodies, highlighting the importance of a free N-terminus of the peptide (Fig. 3a and Table 1).

Both an Ala-scan and a conserved amino acid scan were performed on the 18-mer peptide. Although some substitutions were shown to negatively influence the antibody binding, no improved peptides could be detected in this way (Fig. 3b and c). The 18-mer peptide FIGITELKKLESKINKVF came out as the best candidate for subsequent studies. This 18-mer peptide is referred to as Minimal Tetanus Toxoid Epitope (MTTE) (Table 2). A scrambled peptide sequence, ETTM, without antibody binding properties was used as control (see Table 2 for the ETTM sequence).

To address the presence of specific anti-MTTE antibody levels in healthy individuals, the MTTE peptide reactivity was tested using sera from a random set of healthy volunteers. Seven out of 10 healthy individuals had detectable IgG levels against MTTE. In none of the healthy individuals anti-MTTE IgM Ab levels could be detected (data not shown). In healthy individuals it is also possible to boost anti-MTTE titers by administrating a TTd containing vaccine boost (data not shown).

3.4. Linear or globular peptide structure

As more than one MTTE per SLP requires coupling chemistry, the technique must also provide an opportunity to be used in the next step of drug-development. In order to synthesize peptide–peptide conjugates in a clean and efficient fashion, a chemical procedure was developed in

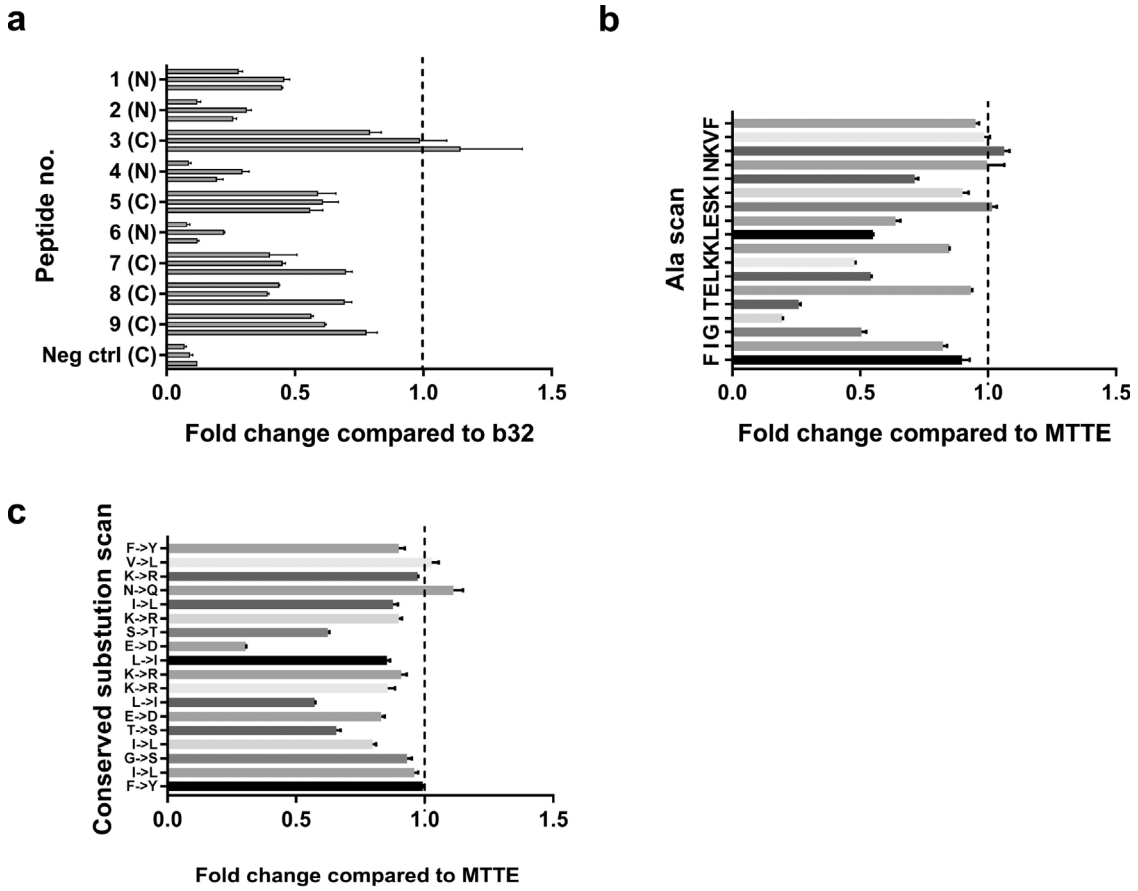

Fig. 3. MTTE peptides linked via the N- or C-terminus and variants created by single amino acid substitutions were screen for IgG antibody binding (human serum was used as the source of IgG).

Different length variants of the minimal 18-mer long B cell epitope were biotinylated and linked to an ELISA streptavidin plate via the N- or C-terminus (See Table 1 for peptide sequences). Tetaquin® and serum from two donors were used for the screen and specific IgG antibodies were detected with an anti-human IgG-HRP conjugated antibody. Different variants of the 18-mer long minimal B cell epitope of TTd were made, in which the peptides were substituted with Ala or with a similar amino acid. The graph in b shows the Ala-substituted peptides and the recognition of each peptide by high-titer donor sera. Conserved amino acids were substituted and the recognition of these peptides by the high-titer donor sera was screened (c).

Table 1
Variants of the b32 peptide sequence linked via the N-terminus or C-terminus.

1(N)	X Z Z S K **F I G I T E L K K L E S K I** N K V F
2(N)	X Z S K **F I G I T E L K K L E S K I** N K V F
3(C)	**F I G I T E L K K L E S K I** N K V F Z Z O
4(N)	X Z Z **F I G I T E L K K L E S K I** N K V F
5(C)	**F I G I T E L K K L E S K I** N K V Z Z O
6(N)	X Z **F I G I T E L K K L E S K I** N K V F
7(C)	**F I G I T E L K K L E S K I** N K Z Z O
8(C)	**F I G I T E L K K L E S K I** N Z Z O
9(C)	**F I G I T E L K K L E S K I** Z Z O
B32	Q Y I K A N S K **F I G I T E L K K L E S K I**

Abbreviations: Z = aminohexanoic acid, X = biotin and O = Lys(biotin).

Table 2
Peptide conjugates.

Abbreviation	Peptide/conjugate description
MTTE	FIGITELKKLESKINKVF
ETTM	EKLINKLSKIFKGTIEVF
[MTTE]$_1$-*SIINFEKL*****	[MTTE]$_1$-core-LEQLESIINFEKLAAAAAK
[MTTE]$_2$-*SIINFEKL*****	[MTTE]$_2$-core-LEQLESIINFEKLAAAAAK
[MTTE]$_3$-*SIINFEKL*****	[MTTE]$_3$-core-LEQLESIINFEKLAAAAAK
[ETTM]$_3$-*SIINFEKL*****	[ETTM]$_3$-core-LEQLESIINFEKLAAAAAK
[MTTE]$_3$-hgp100	[MTTE]$_3$-core-AVGALKVPRNQDWLGVPRQL

Abbreviations: MTTE = minimal tetanus toxin epitope, SLP = synthetic long peptide, hgp100 = human glycoprotein100, The ***SIINFEKL*** illustrate that the T cell epitope (SIINFEKL) in the conjugates is flanked by the amino acids listed in the right hand side column. The underlined sequence is the CD8 T cell epitope. The MTTE and ETTM epitope is synthesized with a linker sequence on the C-terminal side that is connected to the core, see sequence in Fig. 4.

which the MTTE-sequence and the SLP sequence were coupled to a central core unit by two orthogonal procedures. A central core unit was synthesized containing one, two or three maleimido groups and one cyclo-octyn click handle. First a spacer-containing C-terminally thiolated MTTE was coupled to the maleimido group(s) of the core, after which an azido-OVA-SLP (azido-LEQLESIINFEKLAAAAAK or another SLP of choice) was attached via a click reaction. Classical click reactions are performed in the presence of a copper catalyst. It is known that copper can strongly bind to a variety of peptide sequences from which it is hard to remove by purification. Due to its toxicity the presence of copper in a vaccine is not desirable. We therefore chose to utilize a copper-free click protocol based on a cyclo-octyn moiety as has been reported by (Ning et al., 2008). A chemical protocol was developed in which the two peptide coupling reactions can be performed as a one-pot reaction, without the need for intermediate purification. The most challenging generation of a conjugate containing three MTTE sequences is illustrated in Fig. 4. See Table 2 for a list of conjugate peptides made.

3.5. Immune activation by a [MTTE]$_3$-SIINFEKL conjugate

Our targeting/adjuvant strategy relies on antibodies. The

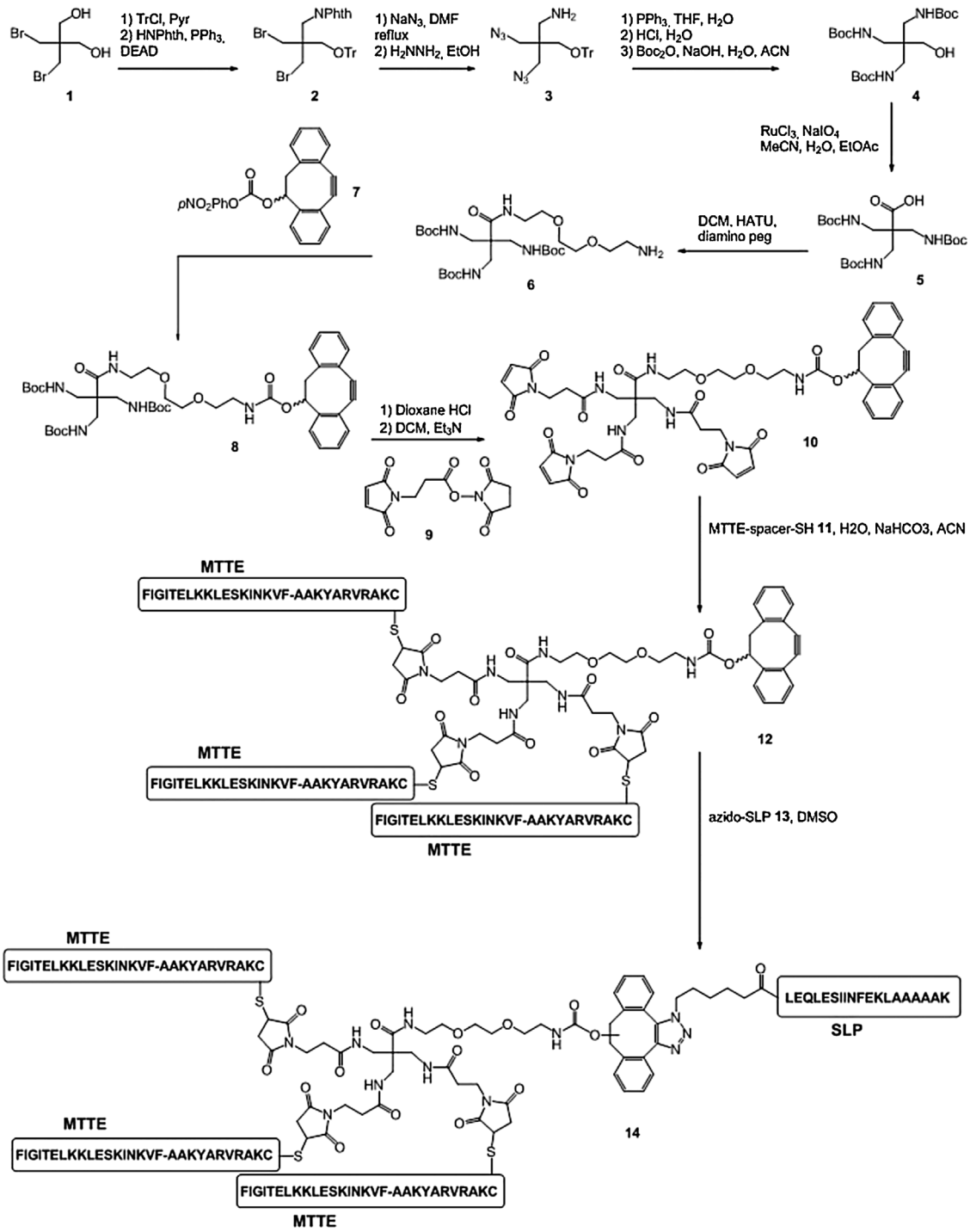

Fig. 4. An illustration of the trimer conjugate conjugation technique (see supplementary material and methods for a complete synthesis description).

knowledge that monomeric IgG do not engage low affinity Fc receptors to the same extent as IgG in an immune complex format and as activation of FcgR are known to be induced by immune complexes (as a natural adjuvant), we investigated the need for one, two or three MTTEs in a conjugate with a SLP with regards to DC activation, antigen uptake and cross-presentation. Conjugates with one MTTE sequence (pre-incubated with mouse anti-MTTE antibodies) failed to induce MHC II and CD40 expression by DCs (Fig. 5a). Furthermore, in the presence of mouse anti-MTTE IgG1 and IgG2a the conjugate with three MTTE sequences was similar to the conjugate with two in regards to induction of surface MHC II, CD40 and released IL-12p40 (Fig. 5a and b). Subsequently we assessed T cell activation thru the antigen loading capacity of IgG1 and Ig2a complexes of these DC by using the B3Z hybridoma. B3Z cells were activated upon MHC class I/SIINFEKL engagement of their specific T cell receptor. Both IgG1 and IgG2a induced efficient cross-presentation whereas the conjugate alone in the same doses did not induce T cell activation (Fig. 5c). To assess conjugates for *in vivo* antigen presentation and T cell activation a conjugate with the model antigen hgp100 and three MTTE sequences were created ($[MTTE]_3$-hgp100). Mice with adoptively transferred pmel splenocytes (with a hgp100-specific TCR) were injected in the footpad (s.c.) with $[MTTE]_3$-hgp100. In a low dose and in the presence of MTTE-specific antibodies the conjugate induced the accumulation of hgp100-specific CD8 + T cells in draining lymph nodes (Fig. 5d), whereas a higher dose

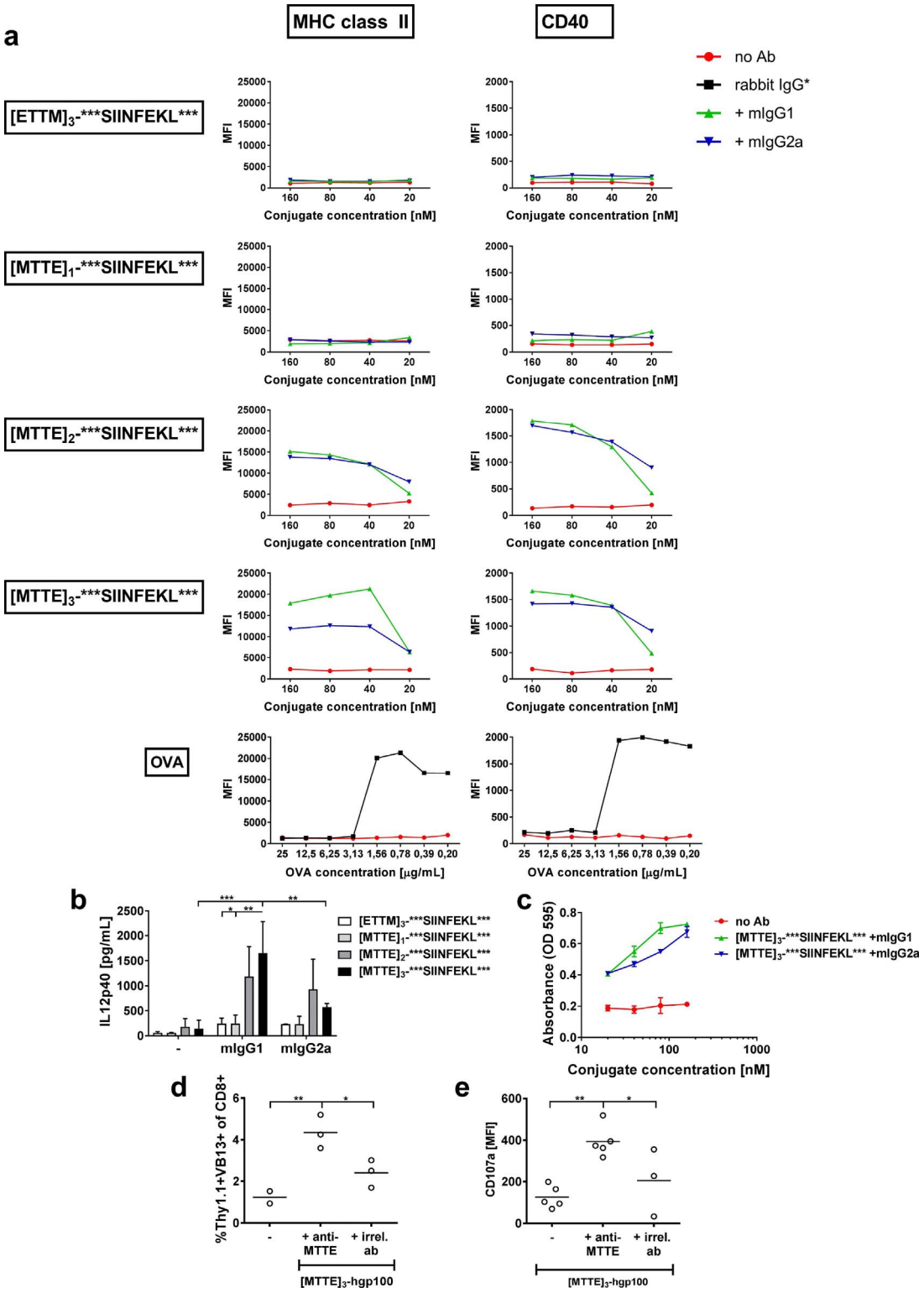

Fig. 5. [MTTE]3-SLP immune complexes mature DCs and enhance cross-presentation *in vitro* as well as promote T cell accumulation and activation *in vivo*.
Monomeric, dimeric or trimeric MTTE-conjugates were, after a pre-incubation with mouse anti-MTTE IgG1 or IgG2a for 30 min at 37 ˚C, incubated with DCs for 24 or 48 h 37 ˚C. *OVA was pre-incubated with rabbit anti-OVA (raOVA) IgG. DC activation was analyzed by staining DC after 48 h for the surface markers MHC II and CD40 (a, illustrating one representative experiment out of two) and measuring IL-12p40 in the supernatants (b, mean of four biological replicates run at two separate occasions). Transgenic CD8+ T-cell clone (B3Z) was added to DCs after they were incubated with $[MTTE]_3$-SLP-ICs for 24 h. After another 24 h the cells were washed and incubated in a lysing buffer containing the substrate CPRG of which the absorbance was read after 6 h (c). Pmel-splenocytes containing TCRs specific for human gp100 were adoptively transferred (i.v.) into C57BL/6 mice (Day 0). On Day 1, $[MTTE]_3$-hgp100 conjugate (1 nmol/mouse), alone or premixed with MTTE-specific rabbit antibodies or an irrelevant rabbit IgG fraction, was injected into the footpad (d) or intra peritoneal (e). Single cell suspensions from draining lymph nodes harvested on day 4 were analyzed for presence of gp100-specific CD8+ T cells (Thy1.1 + VB13+) (d) or their activation status (CD107a MFI of Thy1.1+VB13+CD8+ cells) (e) by flow cytometry. The experiments (a–c) were repeated 2–3 times with similar results. The ***SIINFEKL*** illustrate that the T cell epitope (SIINFEKL) in the conjugates is flanked by several amino acids (The flanking amino acids are listed in Table 2). mIgG1 = mouse IgG1, mIgG2a = mouse IgG2a and irrel. ab = irrelevant antibody. Statistical analyses were calculated with one way ANOVA with Tukey's multiple comparison test * $p < 0.05$ and ** $p < 0.01$.

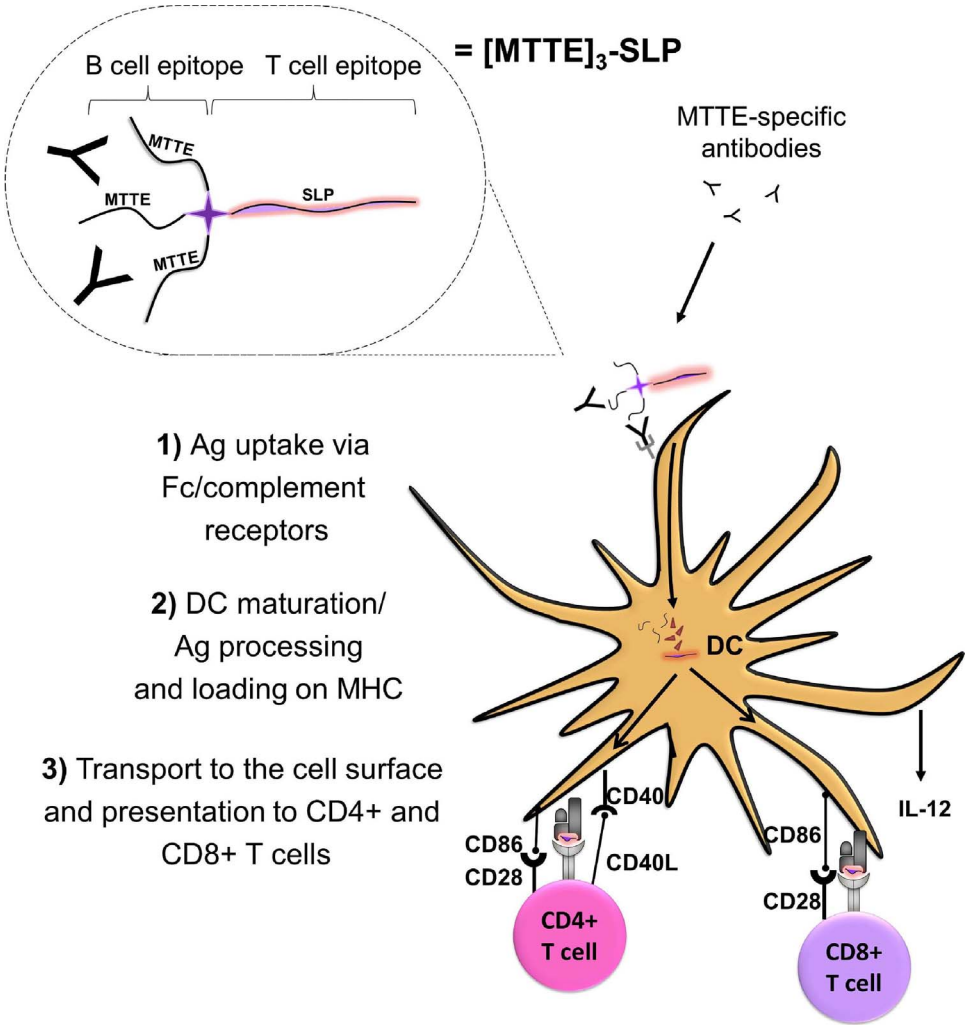

Fig. 6. Schematic mechanism of action.

induced a similar response as the low dose regardless of antibody levels (data not shown). To enable a titration of the MTTE-specific antibody dose for T cell activation analysis, the antibody stock concentration did not hold enough antibodies to increase the dose when using footpad injections. In the next experiment mice were therefore injected i.p (which allows for an injection volume of 100 µl) instead of in the footpad (which allows for an injection volume of 30 µl). Conjugates injected i.p induced activation of CD8+ T cells (surface CD107a) in draining lymph nodes in the presence of a polyclonal IgG fraction of MTTE-specific antibodies (prot A purified) at the highest dose tested 1 mg/mouse (Fig. 5e) and not 0.5 and 0.25 mg/mouse (data not shown). A schematic mechanism-of-action figure of our conjugate strategy is illustrated in Fig. 6.

4. Discussion

Antibodies are known to regulate secondary antibody responses upon re-challenge with antigen, the so called antibody feed-back regulation (Hjelm et al., 2006), but can also regulate cell-mediated immune responses. In relation to CD8 T cell responses IgG containing immune complexes of larger sizes improves cross-presentation and cellular immunity (Kalergis and Ravetch, 2002; Schuurhuis et al., 2002). In addition, it has been suggested that long-term immunity to tumors post tumor-directed antibody therapy (for example anti-CD20 therapy) could be due to cross-presentation of tumor material bound to the therapeutic antibody and the induction of CD8 T cell memory (DiLillo and Ravetch, 2015). Our aim herein is to build on the knowhow around the importance of immune-complexes in delivery of antigens to dendritic cells and the subsequent T cell activation, and translate this into a clinically applicable strategy by incorporating into synthetic peptide-based therapeutic vaccination.

Synthetic long peptide vaccination requires relatively high doses of antigen and vaccine potency can conceivably be further improved by efficient targeting to dendritic cells as well as improved DC activation. One key factor for successful DC antigen targeting and DC activation appears also to be the physical link between the adjuvant and the antigen, i.e. so that the same cell receiving the activation signal also receives the antigen material. An excellent example of this is the coupling of a TLR-2 ligand to SLPs. This greatly improves both antigen delivery and dendritic cell activation, leading to both enhanced T cell responses and a subsequent anti-tumor response (Khan et al., 2009; Zom et al., 2014) and is currently being evaluated in a clinical trial (NCT02821494).

We set out to find a clinically relevant approach for an immune complex based delivery strategy. Both food intake and vaccinations lead to circulating antibodies in our body that could be used for complex formation. We argued it would be of value to start with a protein derived target which by itself is known to elicit powerful adaptive responses as this would have a high likelihood of resulting in IgG, rather than IgM, responses to the B cell epitope. In addition, the target identified should be linear to be able to conjugate a peptide based GMP vaccine. A potent immunogenic protein is tetanus toxoid, which was chosen as the prime candidate for our further work.

We started with assessing if tetanus toxoid by itself could potentiate

CD8 T cell responses when an MHC class I epitope was linked to tetanus toxoid in seropositive mice. It was apparent that priming of OVA-specific CD8+ T cells was improved in mice with circulating antibodies to tetanus toxin, in line with what we have seen in earlier studies with other antigens (van Montfoort et al., 2012). To avoid antigen-competition in terms of T cell epitopes and to identify a defined product we set out to search for a linear B cell epitope derived from tetanus toxin. A library of peptides spanning the alpha and beta region of tetanus toxin was created and through biotin these peptides were coated on streptavidin plates. Tetaquin® along with serum from healthy donors was used to screen for candidates. Out of six identified candidates in the first screening round, only one linear peptide came out positive with extended individual sera assessment. The linear peptide identified was further analyzed to identify a possible way to improve antibody binding.

After trimming of the peptide as well as investigations of mimotopes (no improved epitope was identified) we had identified an 18-mer sequence i.e. the minimal tetanus toxoid epitope (MTTE). We also identified the crucial requirement of a free N-terminus for optimal antibody recognition. To assess if more than one antibody binding sequence was necessary to induce T cell activation, we tested conjugates harboring one, two or three tetanus sequences. Our data support that at least two MTTEs are needed for DC activation. As monomeric IgG is not known to interact with a great number of Fc receptors (Bruhns, 2012; Bruhns et al., 2009), we reasoned we would need multiple MTTEs per SLP to increase the complexity and broaden Fc receptor engagement. Recently we have also noted a requirement for at least three MTTEs per SLP in a human recall assay (Fletcher et al./Manuscript in submission). In the same system we have also established that the uncoupling of $[MTTE]_3$ from the T cell epitope, but simultaneous administration, abolishes the T cell activation.

In line with previous data, our immune complex strategy with SLPs are potent immune activators with DC marker status similar to OVA-IC stimulated DCs. T cell activation as well as IL12p40 secretion was also markedly elevated in response to the MTTE conjugate in conjunction with anti-MTTE antibodies, but not without. We could also demonstrate that the conjugates enabled both increased T cell proliferation and activation when used in conjunction with a polyclonal IgG fraction containing anti-MTTE specific antibodies. More work is needed to demonstrate the potential of this type of vaccine approach. Specifically we aim to incorporate and study epitopes from self-proteins and the potential of these conjugates to break tolerance.

Conflict of interest

SM is a founder and shareholder as well as the CSO and a board member of Immuneed AB. EF is a founder and shareholder of Immuneed AB.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.molimm.2017.11.004.

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