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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-