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Modular MLV-VLPs co-displaying ovalbumin peptides and GM-CSF effectively induce expansion of CD11b$^+$ APC and antigen-specific T cell responses *in vitro*

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ABSTRACT

The development of novel vaccination strategies is a persistent challenge to provide effective prophylactic treatments to encounter viral infections. In general, the physical conjugation of selected vaccine components, *e.g.* antigen and adjuvant, has been shown to enhance the immunogenicity and hence, can increase effectiveness of the vaccine. In our proof-of-concept study, we generated non-infectious, replication deficient Murine Leukemia Virus (MLV)-derived virus-like particles (VLPs) that physically link antigen and adjuvant in a modular fashion by co-displaying them on their surface. For this purpose, we selected the immunodominant peptides of the model antigen ovalbumin (OVA) and the cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) as non-classical adjuvant. Our results show that murine GM-CSF displayed on MLV-VLPs mediates expansion and proliferation of CD11b$^+$ cells within murine bone marrow and total spleen cells. Moreover, we show increased immunogenicity of modular VLPs co-displaying OVA peptides and GM-CSF by their elevated capacity to induce OVA-specific T cell-activation and -proliferation within OT-I and OT-II splenocyte cultures. These enhanced effects were not achieved by using an equimolar mixture of VLPs displaying either OVA or GM-CSF. Taken together, OVA and GM-CSF co-displaying MLV-VLPs are able to target and expand antigen presenting cells which in turn results in enhanced antigen-specific T cell activation and proliferation *in vitro*. These data suggest MLV-VLPs to be an attractive platform to flexibly combine antigen and adjuvant for novel modular vaccination approaches.

1. Introduction

Modular vaccines physically conjugating the components antigen and adjuvant possess a drastically enhanced immunogenicity (Chiu et al., 2015; Khan et al., 2007; Schulke et al., 2010). Most likely, this is achieved by the improved ability to direct these two components to the same antigen presenting cell (APC) simultaneously (Khan et al., 2007). This co-delivery results in efficient antigen-loading and concurrent activation of the APC, thereby enabling strong T cell responses and antibody production (Khan et al., 2007; Noh et al., 2014). A fusion protein combining the model antigen ovalbumin (OVA) and the Toll-like receptor (TLR)-agonist flagellin A as adjuvant has been shown to induce innate and antigen-specific adaptive immune responses in a murine experimental allergy model. This immune activation was significantly enhanced when compared to an equimolar mixture of both non-conjugated components (Schulke et al., 2010; Schulke et al., 2011). The usage of cell type-specific ligands as a component of modular vaccines can even direct the type of adaptive immune response that will be induced. For example, a fusion protein consisting of an antibody targeting the costimulatory molecule CD40 and an influenza virus-specific immunodominant peptide (FluNP) was able to increase FluNP-specific cytotoxic T cell responses. In contrast, FluNP fused to an antibody targeting Dectin-1, expressed on APC-subsets, rather induced T helper cell-activation (Yin et al., 2016). Being able to shift immune responses to a specific T cell subset provides one further step towards tailored vaccines.

The cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) has been successfully used and approved by the FDA and

Abbreviations: GM-CSF, granulocyte macrophage-colony stimulating factor; MLV, Murine Leukemia Virus; VLP, virus-like particle; OVA, ovalbumin; DC, dendritic cell; APC, antigen-presenting cell; MOP, multiplicity of particles; HPV, Human Papillomavirus

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EMA (Food and Drug Administration in the United States and the European Medicines Agency) as component of a fusion protein for generation of Provenge®, an autologous cell preparation licensed for treatment of prostate cancer patients (Gardner et al., 2012). Within an experimental approach, also soluble GM-CSF applied at the same injection site as a recombinant hepatitis B virus vaccine resulted in significantly enhanced immune responses and vaccine efficacy when compared to the approach where GM-CSF was omitted (Tarr et al., 1996). Accordingly, the immunogenicity of Hantaan virus-like particles (VLPs) was enhanced by incorporation of GM-CSF into the VLP membrane (Cheng et al., 2016). These findings indicate that GM-CSF as a component of vaccines is able to enhance antigen-specific immune responses, particularly when incorporated into VLPs. At the same time and in contrast to commonly used adjuvants such as aluminum hydroxide, oil-in-water emulsions or TLR-ligands, GM-CSF is not acting as a danger signal *per se* which could diminish the risk of side effects.

The GM-CSF receptor is expressed on various APCs, including dendritic cells (DCs) which are the most efficient antigen presenters to naïve T cells (Delamarre et al., 2003; Jung et al., 2002). Upon GM-CSF binding and receptor-mediated signaling, cells differentiate and/or proliferate (Inaba et al., 1992; Metcalf, 1991). Moreover, the GM-CSF receptor is internalized upon ligand-binding (Walker and Burgess, 1987) which might further facilitate the uptake of a co-delivered antigen in addition to phagocytosis-mediated processes. Taken together, GM-CSF can act as an effective and safe adjuvant and hence is an attractive candidate as adjuvant-component within modular vaccines.

In general, VLPs can be derived from a large variety of parental viruses resulting in non-infectious particles, unable to replicate, lacking the viral genetic information, and eventually lacking viral protein(s) necessary for host cell entry (Cheng et al., 2016; Flatz et al., 2010; Lua et al., 2014; Nikles et al., 2005; Wang et al., 2017). VLPs are already used as main components of authorized Human Papillomavirus (HPV) vaccines such as Cervarix® and Gardasil®, conferring protection from infection with the Human Papillomavirus that can result i.a. in cervical cancer or warts (Harper and DeMars, 2017). In addition, certain VLPs can be designed in a way to display desired proteins such as antigen and adjuvant on their surface.

With respect to the development of modular vaccines, physical conjugation of vaccine constituents might be realized *via* antigen and adjuvant co-displaying VLPs. In the present study we generated MLV-VLPs co-displaying GM-CSF as “non-classical” adjuvant and the immunodominant peptides of the model antigen OVA. Data presented here show that displayed GM-CSF is able to target and expand $CD11b^+$ APCs. When GM-CSF was co-displayed along with OVA, these modular VLPs induce significantly increased antigen-specific T cell responses. A non-modular mixture of VLPs displaying either OVA peptides or GM-CSF was less effective. This biotechnically engineered system enables an uncomplicated and rapid exchange of the displayed antigen by changing one plasmid during particle production. Hence, the investigated MLV-VLPs might be a promising vaccine vector-platform for any given antigen.

2. Materials and methods

2.1. Plasmids

pHit60 encoding the MLV gag and pol was described previously (Soneoka et al., 1995). pDisΔHA was derived from pDisplay ((Chesnut et al., 1996) Thermo Fisher Scientific, St. Leon-Rot, Germany) by deleting the HA-Tag, originally in frame with the signal peptide and the transmembrane region of the platelet-derived growth factor receptor. Between the transmembrane domain and the displayed protein, a Myc tag is included. Both Myc tag and transmembrane domain add approximately additional 11 kDa to each displayed protein. For generation of pDisΔHA-based expression constructs encoding murine GM-CSF (pDisΔHA-GM-CSF) or OVA peptides (pDisΔHA-OVA_{pep}), corresponding coding genes were amplified from donor plasmids pET15b-OVA (Schulke et al., 2010) or pMA-T-muGM-CSF (Life Technologies, Darmstadt, Germany) by PCR. Used oligonucleotides (Eurofins Genomics, Ebersberg, Germany), 5′GGCCCAGCCGGCCGCCCCTACCAGATCCCCC ATCACC3′ (forward SfiI-muGM-CSF), 5′AGATCTTTTCTGGCCGGGTTT CTTGCACTC3′ (reverse BglIImuGM-CSF), 5′GGCCCAGCCGGCCGATGA AGTTAGCGGTCTGGAACAG3′ (forward SfiI-OVA_{pep}), 5′AGATCTAACG CCTGCTTCTGCGCTACCCAC3′ (reverse BglII-OVA_{pep}) include a 5′SfiI and a 3′BglII restriction site for insertion into pDisΔHA.

2.2. Mice

Transgenic OT-I or OT-II mice (both C57BL/6 background) expressing an OVA-specific T cell receptor (TCR) on $CD8^+$ or $CD4^+$ T cells (Barnden et al., 1998; Hogquist et al., 2012), respectively, were bred under specific pathogen free conditions at Paul-Ehrlich-Institut. Wild-type C57BL/6 mice were purchased from Harlan (Borchen, Germany).

2.3. VLP production

4.5×10^6 293 T cells were seeded in T175 cell culture flasks in 25 ml DMEM (Th. Geyer, Renningen, Germany) supplemented with 10% fetal calf serum (FCS, Sigma Aldrich, Taufkirchen, Germany) 72 h before transfection with a total of 35 µg plasmid DNA using polyethylenimine in 15 ml medium, as described (Rasbach et al., 2013). Transfection with 35 µg pHit60 resulted in the production of “naked” particles. For generation of VLPs displaying GM-CSF or OVA peptides, 17.5 µg pHit60 were transfected along with 17.5 µg pDisΔHA-GM-CSF or pDisΔHA-OVA_{pep}. For generation of modular VLPs, 17.5 µg pHit60 were transfected along with 8.75 µg of pDisΔHA-OVA_{pep} and 8.75 µg of pDisΔHA-GM-CSF. 48 and 72 h after transfection, supernatants were collected and purified *via* ultracentrifugation through a 20% sucrose cushion in PBS for 3 h at 28,000 rpm and 4 °C. Pelleted VLPs were resuspended and stored in aliquots at −80 °C. VLP-size and concentration of VLP stocks were determined in degassed PBS using the NanoSight NS500 device (Malvern Instruments, Worcestershire, UK) (Bender et al., 2016; Uhlig et al., 2015).

2.4. SDS-PAGE and Western blot analysis

To determine individual proteins in VLP preparations, 1×10^9 particles were incubated for 5 min at 95 °C in loading buffer containing 10% SDS and 600 mM DTT, and analyzed on a 10% (for VLP-GM) or 15% (for VLP-OVA) SDS-polyacrylamide gel followed by immunoblotting on nitrocellulose membranes (GE Healthcare, Freiburg, Germany). Detection of GM-CSF was performed using a rat monoclonal antibody against GM-CSF (1:1000; clone MP1-22E9; eBioscience, Frankfurt am Main, Germany) and a goat-anti-rat-HRP secondary antibody (1:5000; Dianova, Hamburg, Germany). Detection of OVA peptides was performed using a polyclonal rabbit anti-OVA antibody (1:1000; Novus Biologicals, Littleton, USA) and a donkey-anti-rabbit-HRP secondary antibody (1:7500; Amersham, Freiburg, Germany). After immunoblotting, samples were visualized on Hyperfilm ECL (Amersham).

2.5. Cell isolation and culture

Bone marrow cells were isolated by flushing femur and tibia of mice with RPMI (Thermo Fisher Scientific, St. Leon-Rot, Germany) medium supplemented with 10% FCS (Biochrom, Berlin, Germany). Upon red blood cell lysis (RBC lysis buffer, Sigma Aldrich,), cells were washed and seeded at a density of 1×10^6 cells per 24-well in 1 ml medium.

Splenocytes were mechanically dispensed from spleens and resuspended in PBS. After red blood cell lysis (RBC lysis buffer, Sigma Aldrich), cells were washed and seeded at 5×10^5 cells in 200 µl medium supplemented with 10% FCS (Sigma Aldrich) per 96 U-bottom well.