Lipid Rafts, Pseudotyping, and Virus-Like Particles: Relevance of a Novel, Configurable, and Modular Antigen-Presenting Platform

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Key Words

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Abstract

Antigen presentation by professional antigen-presenting cells (APC) is the first step towards the initiation of an adaptive immune response carried out by naïve T lymphocytes. For this purpose, antigens are presented in the form of peptide/major histocompatibility complexes (pMHC) on APC to the antigen receptor of T cells. For sustained T cell activation to occur, numerous additional molecules specifically expressed on the surface of APC have to synergize with pMHC to stimulate a given T lymphocyte. Moreover, soluble factors such as cytokines critically contribute to the specific milieu during T cell activation. APC 'talk' to T cells only when they engage in intimate physical interaction. The cell biological correlate of this APC-T cell interaction is commonly referred to as the formation of an immunological synapse. In this review we aim to provide an overview of a novel cell-free antigen-presenting platform, i.e. virus-like particles (VLP) decorated with immune receptors of choice, which was devised to overcome the molecular and cell biological complexity of the APC side of the immunological synapse. In the past we have demonstrated that immune receptor-decorated VLP are able to activate, modulate, or abrogate antigen-specific

T lymphocyte responses. Thus, antigen-specific VLP represent a valuable tool which might help to explore and understand the function of antigen-specific T lymphocytes in more detail and might thus open new avenues for the modulation of pathologic T lymphocyte responses, e.g. for the treatment of allergic diseases.

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Introduction

Immunity is maintained by the proper function and interaction of the innate and adaptive immune systems [1]. The innate immune system controls the induction of adaptive immune responses by opsonization, uptake, processing, presentation, and transport of antigens and by regulating the provision of cosignals for lymphocyte activation. It furthermore instructs lymphocytes to develop particular effector responses by releasing key cytokines [2]. The initial transfer of information towards lymphocytes is governed by professional antigen-presenting cells (APC), i.e. dendritic cells (DC) [3]. DC are particularly important to prime major histocompatibility complex (MHC) class II-restricted CD4⁺ T helper cells [4] as well as MHC class I-restricted CD8⁺ cytotoxic T cells [5]. T lymphocytes which have been instructed by DC play a pivotal role especially during recall responses against

pathogenic microorganisms and malignancies [6–10]; however, they are also the driving force in autoimmune disorders [11, 12] and allergic diseases [13].

Over the last 15 years, a multitude of protocols for the *ex vivo* generation and expansion of professional APC have been established [14–17] and immunotherapies based on DC have been developed as promising tools to fight infectious diseases and cancer [18, 19]. However, the high number of molecular interactions identified between APC and T cells makes the functional net effect of DC as T cell stimulants not easy to predict and direct [20–22]. Moreover, the limited availability of autologous DC as well as the time-consuming and cost-intensive maturation and differentiation processes required to generate high numbers of DC are additional points to be considered when planning to use DC to modulate T cell function for therapeutic purposes.

For these reasons, and to the benefit of both basic and applied research, we have developed an inducible and reductionist antigen-presenting platform for the modulation of T lymphocyte function which is based on a natural assembly system, i.e. virus-like particles (VLP). We aimed to keep the variables low while still creating a fully functional and modular T cell stimulatory unit consisting of a flexible composition of T cell receptor (TCR) ligands, costimulatory and adhesion molecules, as well as cytokines, all displayed within a physiological plasma membrane context.

The objective of this review is to describe the cell and molecular biological principles on which this novel platform is built as well as the spectrum of possible applications of the platform to induce, modulate, or abrogate defined T cell-driven immune responses.

The Immunological Synapse – A Link between Innate and Adaptive Immune Systems

The immunological synapse formed between APC and T lymphocytes translates information about endogenous and foreign protein antigens to the adaptive immune system. Three categories of signals are considered to be important for optimal T cell activation. The first and most central interaction, ‘signal 1’, is initiated upon TCR binding to the peptide/MHC complex (pMHC) [23, 24], which defines the antigen specificity of the immune response [25–27]. Costimulatory signals may be delivered via cell-to-cell interaction, referred to as ‘signal 2’, or alternatively via soluble factors such as cytokines and growth factors collectively called ‘signal 3’ [28].

Specific subdomains of the plasma membrane, known as lipid rafts, are considered to play a crucial role during the sorting of molecules contributing to the formation of the immunological synapse [29–32]. In fact, the efficiency of antigen presentation is greatly improved by the enrichment of MHC molecules in lipid rafts of APC [33]. Likewise, upon binding to pMHC, the TCR and its coreceptors become concentrated in lipid rafts eventually leading to the formation of a supramolecular activation cluster [34]. When the number of engaged TCRs binding to pMHC reach a certain threshold, within a given period of time T lymphocyte signaling and activation will occur [35].

Costimulatory signals play essential roles during T lymphocyte activation. In fact, molecular interactions between CD80/CD28, CD86/CD28, CD58/CD2, OX40-L/OX40 (CD252/CD134), and ICOS-L/ICOS (CD275/CD278) have been demonstrated to provide activating T cell signals [36–39]. They either enhance the activation state of T cells by amplifying TCR signals themselves or by reducing the threshold for T cell activation [40]. The output of such cosignals is increased cell survival, proliferation, and/or differentiation towards effector cells [41, 42]. Another set of costimulators delivers inhibitory T cell signals; CD80/CTLA-4, CD86/CTLA-4, PD-L1/PD-1, and PD-L2/PD-1 usually inhibit T lymphocyte activation and thereby dampen ongoing immune responses [43–45]. In the complete absence of costimulation T lymphocytes usually enter into an anergic/tolerogenic state by default [46, 47].

Beside the cell contact-dependent signals 1 and 2, T lymphocyte activation is also modulated by soluble factors, i.e. cytokines (signal 3), that in many instances serve as ‘danger signals’ in response to a large group of microbial, viral, and other stimuli as well [28, 48]. Interleukin (IL)-12 and type I interferons seem to be of particular importance for CD8⁺ T lymphocyte activation and proliferation, while proinflammatory cytokines such as tumor necrosis factor- α and IL-1 are more potent stimulatory factors for CD4⁺ T lymphocytes [28, 48–51]. Growth factors such as IL-2, IL-4, IL-7, and IL-15 contribute to T cell survival and proliferation [52–55]. In contrast, IL-10 and TGF- β direct T cells rather towards antigen nonresponsiveness and tolerance [56–58]. However, in the complex network of interactions, cytokines may have opposing functions. In particular, IL-2 contributes to the regulation of the immune response, both through promoting T lymphocyte proliferation and by sensitizing T cells for programmed cell death [59, 60]. The presence of IL-4 at the time of antigen presentation to T lymphocytes is crit-

ical for the development of T helper 2 (Th2) lymphocytes, and IL-4 – the major inducer of class switch recombination towards ϵ -immunoglobulin heavy chains in B cells – as well as the eosinophil-activating cytokines IL-5 and IL-13 which are characteristic products of such Th2 cell responses [61, 62]. In contrast, IL-12, TGF- β , and IFN- γ negatively regulate Th2 development, instead favoring the development of T cells with a Th1 phenotype [63–66]. In the past, coadministration of cytokines had been considered a valuable strategy to overcome nonresponsiveness to vaccination in immunodeficient patients and to enhance antitumor immunity [67–71]. In these applications cytokines resemble adjuvants, i.e. ‘helper substances’ [72, 73]. Thus, besides molecularly defined ligands for TCRs and accessory molecules a novel antigen-presenting platform should ideally also be apt to accommodate cytokines and growth factors known to have an impact on T lymphocyte function.

Virus-Like Particles: A Novel, Modular, and Reductionist Antigen-Presenting Platform

In this report we review a novel technology for the facile preparation of antigen-presenting platforms, i.e. VLP, that show high functional activity. Since VLP production uses some salient features of the assembly and budding processes of enveloped viruses, we will first discuss the mechanisms operative during lipid raft formation; secondly, we will refer to the attachment of proteins by GPI (glycosyl phosphatidyl inositol) anchoring to the plasma membrane, and thirdly, we will discuss the pseudotyping of VLP in more detail.

Lipid Rafts – A Plasma Membrane Sorting Compartment

It is well established that many enveloped viruses preferentially bud from plasma membrane microdomains of infected cells known as lipid rafts [74–81]. A similar budding behavior is observed with VLP, i.e. particles lacking viral nucleic acids and fusogenic envelope proteins. Lipid rafts are hydrophobic, detergent-insoluble, glycosphingolipid-enriched domains which accumulate cholesterol, sphingolipids, and gangliosides [82]. Their mobility allows them to act as mobile platforms that carry specific proteins from the trans-Golgi network to the cell surface and back to the internal membranes [83, 84]. Cholesterol depletion or the inhibition of glycosphingolipid synthesis blocks the formation of lipid rafts [85–87]. Isopycnic density gradient centrifugation in the nonionic detergent Tri-

ton X-100 allows the purification of the insoluble lipid rafts from whole plasma membrane preparations at low temperatures [88]. Due to the physical properties of their major constituents, lipid rafts are structurally highly ordered [89, 90], attracting only proteins with ‘favorable’ transmembrane sequences and/or those undergoing distinct posttranslational lipid modifications such as glycosyl phosphatidyl inositol (GPI) anchor attachment [91–93] or fatty-acid acylation [94–97]. Reciprocally, the composition, spatial dimensions, and half-lives of lipid rafts are influenced by lipid raft-resident proteins [98–100]. Overall, lipid rafts have been considered highly dynamic membrane substructures with short half-lives and small dimensions ranging from 5 to 20 nm [101–104]. In functional terms, lipid rafts have several physiological roles: (i) the sorting of membranes and associated proteins [105–107], (ii) the bidirectional intracellular trafficking of molecules to and from the eukaryotic plasma membrane [108–111], and (iii) the compartmentalization of cell surface and associated intracellular signaling molecules to enable signal transduction across the plasma membrane [112–115].

GPI-Anchored Membrane Proteins

GPI-anchored proteins are involved in a broad range of physiological processes and have diverse functions. Some of these proteins have enzymatic activity, e.g. placental alkaline phosphatase [116], acetylcholinesterase [117] or dipeptidase [118]. Others such as LFA-3 (CD58) [119] or Campath-1 (CD52) [120] influence intercellular interactions. Still others have been identified as ligand-binding receptors, e.g. LPS receptor (CD14) [121], folate receptor [122], and Fc γ RIII (CD16b) [123], or have been shown to regulate the complement activation cascade, e.g. decay-accelerating factor CD55 [117] or CD59 [124] (table 1). Previous studies have demonstrated that the genetic introduction of GPI anchor acceptor sequences at the C-terminus of a previously non-GPI-anchored protein efficiently targets the modified molecule into the lipid rafts of the plasma membrane [91, 125, 126]. Another type of lipid modification that targets proteins into lipid rafts is fatty acid acylation, which attaches either myristic acid to an N-terminal glycine or palmitic acid to cysteine residues. The prominent coreceptors CD4 and CD8 as well as a number of Src-related tyrosine kinases such as Lck or Fyn become fatty acid acylated and selectively sequestered into rafts to promote cellular signal transduction (table 1) [94–96, 127–133]. Hence, in our work the genetic introduction of GPI anchor acceptor sequences at the C-terminal ends of the ectodomains of previously

Table 1. Factors that favor the partitioning of proteins into lipid rafts

Modification	Chemical bond	Examples of proteins of immune cells	Reference No.
Myristoylation	Covalent amid bond on N-terminal glycine	Src-related tyrosine kinases, Lck, Fyn, [HIV Pr55 Gag (Gag)]	74, 127, 269–271
Palmitoylation	Covalent bond; acyl-thioester linkage on cysteins (S-acylation)	CD4, CD8, CD9, CD44, CD95 (Fas, APO-1), Lck, Fyn, LAT, trimeric G proteins, caveolin-3, [influenza (HA), MoMLV (Env)]	127, 129, 130, 272–278
GPI linkage	Covalent bond; C-terminal phosphoethanolamine bridge	CD14, CD16b, CD37, CD48, CD52, CD53, CD55, CD58, CD59 (and many others)	30, 120, 124, 279–284
Attachment of cholesterol	Covalent bond; esterification on C-terminal glycine after internal protein cleavage	Hedgehog protein (Hh)	285–288
Interaction with scaffolding proteins	Noncovalent protein-protein interactions	Caveolins, flotillin, FcγRI (CD64)	289–292
Cholesterol recognition/interaction with amino acid consensus sequence (CRAC)	Noncovalent cholesterol-protein assembly; LWYIK sequence	Flotillin-1, flotillin-2, caveolin-1, caveolin-2, peripheral-type benzodiazepine receptor (PBR), HIV gp41	293–296

non-GPI-anchored proteins can efficiently target the immunomodulatory molecules of choice into the lipid rafts of the plasma membrane [134–138].

Lipid Rafts Are Pivotal Membrane Compartments for the Assembly of Enveloped Viruses and the Site of Viral Pseudotyping

The biology of many enveloped viruses is closely associated with lipid raft biology. In fact, several enveloped RNA and DNA viruses use lipid rafts as a matrix for their assembly and/or budding, even though the exact mechanisms responsible for budding and vesiculation remain a matter of speculation [139]. There is a consensus, however, that during the release of enveloped viruses from host cells viral core and surface glycoproteins become enriched in lipid rafts [79, 140, 141]. Consequently, the specific lipid composition of viruses can differ considerably from that of the respective host cells [142]. Enveloped viruses currently known to use lipid rafts for their assembly and/or budding are listed in table 2.

Experimental VLP production has mostly relied on the Gag protein, which is the major structural protein of *Retroviridae*. The Gag protein contains 3 conserved regions with distinct functions: (i) the matrix protein (MA) [143], (ii) the capsid protein [144], and (iii) the nucleic acid-binding nucleocapsid protein [145]. Significantly, Gag assembles even in the absence of any other viral factor in the lipid raft regions of transduced cells and leads to the budding of noninfectious VLP [146]. Lipid raft association is primarily achieved by myristoylation of the N-terminal glycine residue of the MA subunit within Gag pre-

cursors (Pr55 Gag and Pr65 Gag) and becomes stabilized by subsequent Gag-Gag interactions [147, 148]. Consequently, the replacement of the myristic acid attachment site of MA results in a loss of association of Gag with lipid rafts associated with a loss of function [149, 150]. Moreover, recent studies have suggested a critical role for Gag L domains interacting with cellular factors during the assembly and budding processes [151]. Additional cleavage products arising from the regions between the MA and the capsid protein, e.g. the p12 domain of Moloney murine leukemia virus (MoMLV), are generally believed to facilitate virus budding [152, 153]. In addition to the direct budding from the plasma membrane, retroviruses have been shown to bud into late endosomal compartments such as multivesicular bodies (MVB) by recruiting cellular factors from the endosomal sorting complexes required for transport [154–157]. Thus, retroviruses have developed several elaborate mechanisms for exiting from their host cells, which in fact has dramatic consequences on their molecular composition and function.

Along those lines, it was first described in the early 1970s that the coinfection of a host cell by 2 unrelated enveloped viruses can lead to the formation of virus particles that contain the genome of one virus packaged within the envelope proteins of the other or both viruses. These chimeric viral particles were named pseudotypes [158, 159]. The subsequent observation that ubiquitously expressed, lipid raft-targeted molecules such as GPI-anchored proteins, e.g. CD55 and CD59, the coreceptor CD4, Src-related tyrosine kinases but also antigen-presenting human leukocyte antigen (HLA)-DR molecules,

Table 2. Enveloped viruses budding from lipid raft-containing membrane regions

Virus	Family	Proteins involved in VLP forming	Raft association of viral proteins demonstrated by	Authors	Reference No.
<i>Negative-sense ssRNA viruses</i>					
Newcastle disease virus (NDV)	Paramyxoviridae	Matrix protein (M) Hemagglutinin-neuraminidase (HN)	Cholesterol depletion (cyclodextrin) Detergent resistance (Triton X-100) Incorporation of raft-associated proteins into virions (e.g. Caveolin-1) Exclusion of nonraft associated proteins from virions (e.g. CD71)	Takimoto and Portner, 2004; Laliberte et al., 2006; Pantua et al., 2006; Shnyrova et al., 2007	76, 297–299
Respiratory syncytial virus (RSV)	Paramyxoviridae	Fusion protein (F) Matrix protein (M)	Detergent resistance (Triton X-100) Colocalization with CD44, Caveolin-1 DiI _{C16} (3) Electron microscopy Confocal microscopy (GM1 staining)	McCurdy et al., 2003; Marty et al., 2004; Takimoto and Portner, 2004; Fleming et al., 2006; Oomens et al., 2006; Batonick et al., 2008	297, 300–304
Measles virus (MV)	Paramyxoviridae	Matrix protein (M) Fusion protein (F)	Detergent resistance (Triton X-100)	Manie et al., 2000; Takimoto and Portner, 2004; Pohl et al., 2007	77, 297, 305
Sendai virus (SeV)	Paramyxoviridae	Matrix protein (M) Fusion protein (F)	Detergent resistance (Triton X-100)	Takimoto et al., 2001; Schmitt et al., 2002; Gosselin-Grenet et al., 2006; Irie et al., 2008	306–309
Vesicular stomatitis virus (VSV)	Rhabdoviridae	Matrix protein (M)	Immunogold electron microscopy	Brown et al., 2003; Irie et al., 2004; Swintek et al., 2008	310–312
Marburg virus (MARV)	Filoviridae	Matrix protein (VP40)	Detergent resistance (Triton X-100) Confocal microscopy (GM1 staining)	Bavari et al., 2002; Kolesnikova et al., 2004; Kolesnikova et al., 2009	313–315
Ebola virus	Filoviridae	Matrix protein (VP40)	Detergent resistance (Triton X-100) Confocal microscopy (GM1 staining)	Bavari et al., 2002; Freed, 2002; Aman et al., 2003; Dolnik et al., 2008	313, 316–318
Influenza virus type A	Orthomyxoviridae	Hemagglutinin (HA) Neuraminidase (NA) Matrix protein (M1)	Cholesterol depletion (Cyclodextrin) Detergent resistance (Triton X-100) Electron microscopy Dynamic light scattering	Scheifferle et al., 1999; Gomez-Puertas et al., 2000; Leser et al., 2005; Chen et al., 2007; Radyukhin et al., 2008	142, 319–322
<i>RNA reverse transcribing viruses</i>					
MoMLV	Retroviridae	Group-specific antigens (Gag)	Detergent resistance (Triton X-100) Electron microscopy	Henderson et al., 1983; Rein et al., 1986; Hansen et al., 1990; Pickl et al., 2001; Derdak et al., 2006; Kueng et al., 2007	79, 134, 135, 144, 149, 323
Human immunodeficiency virus (HIV-1, HIV-2)	Retroviridae	Pr55 Gag (Gag)	Detergent resistance (Triton X-100)	Bryant et al., 1990; Spearman et al., 1994; Zhou et al., 1994; Morikawa et al., 1996; Nguyen et al., 2000; Bhattacharya et al., 2006; Urano et al., 2008	74, 78, 270, 324–327

or the adhesion molecule ICAM-1 (CD54) were efficiently incorporated into virus particles along with viral Gag proteins allowed the formation of a first basic concept for the intentional production of noninfectious VLP decorated with lipid-modified molecules of choice [79, 160–164].

In summary, 2 requirements are crucial for the successful generation of VLP as a modular antigen-present-

ing platform: (i) the application of viral core proteins, which efficiently trigger VLP budding and release from lipid raft regions of producer cells, and (ii) the modification of immunomodulatory molecules of choice by sequences inducing their posttranslational lipid modification, e.g. GPI anchor attachment, leading to their concentration in lipid rafts of producer cells and the subsequent pseudotyping of emerging VLP. The specific composi-

Table 3. Methodology of VLP production

VLP production is performed according to the following standardized protocol:	
1	Semiconfluent culture and passage HEK-293 cells (American Type Culture Collection, Manassas, Va., USA) in Iscove's modified Dulbecco's medium plus 10% fetal calf serum at 37°C and 5% CO ₂ in a humidified atmosphere.
2	Day 1: Trypsinize HEK-293 cells at 37°C for 5 min, wash twice with the medium, and resuspend to obtain a single cell suspension. Seed 3 × 10 ⁶ cells per 100-mm dish and culture in a total volume of 13 ml of Iscove's modified Dulbecco's medium overnight.
3	Day 2 (cell confluence should be 30–50%): Tilt plate by 10°, remove the medium, and carefully add 13 ml of fresh medium by pointing the pipette tip to the wall of the tissue culture plate, preventing the detachment of adherent cells.
4	Rest cells by incubating for at least 1 h (37°C, 5% CO ₂). Prewarm the transfection reagents to 37°C.
5	Transfection procedure [328]: Dilute 30 µg of plasmid DNA (e.g. encoding GPI-anchored T cell ligand) in 900 µl of ddH ₂ O (including 7 µg of pMD-gag-pol to induce VLP production). Add 100 µl of 2.5 M CaCl ₂ . While vortexing the DNA/CaCl ₂ solution, add 1 ml of 2 × HEPES-buffered saline. Incubate the transfection mix for exactly 1 min at room temperature. Subsequently, use a 1-ml pipette to quickly spread the transfection mix drop-wise onto HEK-293 cells and culture overnight.
6	Day 3: Change medium as described above and culture cells for the next 48 h.
7	Day 5: Collect the VLP-containing supernatant into a 15-ml tube. Remove cellular debris by centrifugation (900 g for 10 min) and filtration (0.45 µm). Use VLP directly (crude) or upon concentration/purification by ultracentrifugation (100,000 g for 1 h) [134, 135].
8	Determine and adjust the protein concentration of the purified VLP.
9	Use at a concentration of 1–10 µg/ml for stimulation assays.
10	Store the VLP at –80°C for later use [136].

tion, functional capabilities, and possible applications of such pseudotyped VLP, called 'immunosomes', 'anergosomes', and 'fluorosomes' (FS) [134–137], are shown in figure 1.

Immunosomes: Immunostimulatory VLP Decorated with Ligands for TCR and Costimulatory Molecules
The T lymphocyte stimulatory potential of VLP decorated with immunomodulatory molecules, called 'immu-

Table 4. VLP – facts and figures

Yield	9.8 ± 4.5 µg/ml supernatant [135, 136]
Size	diameter of ~100 nm (transmission electron microscopy) [134, 135]
Mass	assumed mass of 6.5 × 10 ⁷ Da (C-type retrovirus particles) [329]
Targeted molecules, n	8–26 per particle [135, 136]
Biological activity	6 × 10 ⁴ units of IL-2 per mg of VLP (human IL-2:GPI) [135]
Purification	ultracentrifugation at 100,000 g for 1 h, high physical stability and recovery [134]
Stability	stable at 4°C for up to 4 days (crude VLP), stable at –80°C for prolonged periods of time, i.e. >30 days (purified VLP) [136]
Safety	noninfectious and nonrenewable [134]

nosomes', was first demonstrated by a polyclonal approach in vitro [134] (the method for the production of VLP is shown in table 3). For this purpose a GPI-modified surrogate TCR ligand was targeted to lipid rafts of human embryonic kidney (HEK)-293 epithelial cells to subsequently decorate VLP induced by the cotransfection of MoMLV core proteins (original *gag-pol*; OGP). For the delivery of the polyclonal signal 1, a single-chain variable fragment (scFv) of the OKT3 hybridoma recognizing the CD3 epsilon subunit of the TCR/CD3 complex was generated and fused to the GPI-anchored molecule CD14 (OKT3scFv::GPI) [165]. Similarly, and in order to also provide signal 2 for T cell activation on VLP, the costimulatory molecule CD80 as well as the intercellular adhesion molecule ICAM-1 (CD54) were modified at their C-terminus with the GPI anchor acceptor sequence of the low-affinity FcγRIII (CD16b). The colocalization of immune receptors on immunosomes was confirmed by capture enzyme-linked immunosorbent assays (ELISA). [³H]-thymidine uptake experiments showed that VLP-induced T cell proliferation was strictly dependent on the coexpression of both TCR/CD3 ligands (signal 1) and costimulatory molecules (signal 2). Interestingly, immunosomes were found to be able to stimulate T lymphocytes in vitro directly and independently of accessory cells. This was in clear contrast to several studies performed with a related form of microvesicles, i.e. exosomes, showing that their function critically depends on cross presentation by DC [134, 166]. In contrast to constitutively released exosomes [166], MoMLV Gag neoexpression

Fig. 1. Constructs, production scheme, and application of VLP decorated with molecules of choice. Coding sequences of immunomodulatory molecules were modified at their C-termini by the CD16b GPI anchor acceptor sequence in the presence or absence of 2 CD16b Ig-like domains (Ig) (left). Molecular flexibility was provided by a polyglycine linker (G). MoMLV matrix protein (MA p15) was fused to the N-terminus of green fluorescent protein. Three different systems for the provision of TCR ligands were established: (i) for polyclonal activation the variable heavy and light chains of an α CD3 antibody were linked and expressed as fusion with the GPI-anchored molecule CD14, (ii) for the antigen-specific activation of MHC class I-restricted CD8+ T cells a peptide of LCMV GP₃₃₋₄₁ was employed as minigene expressed in combination with H-2D^b and β_2 -microglobulin ($\beta 2m$), and (iii) for the antigen-specific activation of MHC class II-restricted CD4+ T helper cells an Ii-based antigen delivery system was applied allowing the exchange of CLIP for model allergen peptide sequences,

e.g. Art v₁₂₅₋₃₄ of mugwort (*Artemisia vulgaris*). The retroviral MoMLV proteins gag-pol (OGP) were used to induce VLP budding. After transfection into HEK-293 producer cells, GPI-anchored or MA-anchored molecules of interest become expressed and targeted to lipid rafts of the plasma membrane (center). Additional coexpression of MoMLV gag-pol (OGP) induces the budding and secretion of pseudotyped plasma membrane-derived VLP. To stimulate various forms of T cells, VLP were harvested from the producer cell culture supernatants and either used directly or upon purification by ultracentrifugation. Depending on the quality of the cosignals used, VLP offer the possibility to activate and expand, anergize, or polarize antigen-specific but also polyclonal T lymphocytes (right). VLP decorated with biologically active mono- and multi-subunit immune receptors of choice (e.g. cytokine receptors) and fluorescent proteins (FS) enables the visualization, targeting, and blocking of complex immune receptor-ligand interactions.

clearly induces de novo particle budding from the plasma membrane, as demonstrated by biochemical and functional experiments [134, 135] (table 4). Moreover, the molecular composition of immunosomes, in contrast to exosomes, was shown to be designable and adjustable at the level of producer cell transfection [134, 167] (table 4). As a consequence, the donor-dependent MHC allotypes as well as the overall molecular makeup of immunosomes are easily adjustable [134, 137]. Nevertheless, the expression of molecules involved in antigen presentation and T cell activation also classifies immune cell-derived exosomes as vesicular types of immunomodulators [167].

VLP Decorated with Membrane-Bound Cytokines

Apart from classical immune receptors, VLP can also be efficiently decorated with functionally active cytokines. For that purpose, membrane-bound cytokines were generated from different species and their function compared to their soluble homologs. To that end, the GPI anchor acceptor sequence of CD16b or the transmembrane domains of type I proteins were used to construct membrane-anchored variants of cytokines [135]. In biochemical experiments and immunoelectron microscopy evaluations it became apparent that lipid raft targeting and the enrichment of cytokines on VLP were strictly dependent on the type of membrane attachment. While GPI-anchored cytokines decorated VLP, cytokines fused to transmembrane regions of type I membrane proteins did not, although the expression levels of molecules on producer cells were comparable [135].

The biological properties of the soluble growth factor IL-2 during primary and recall immune responses as well as its role in the induction of immunotolerance has been well established in the past [168–170]. Thus, IL-2 seemed to be an ideal model cytokine to test membrane-attached variants functionally and in a broader context. Significantly, dose-dependent proliferation assays revealed that the specific functional activity of GPI-anchored VLP-bound human IL-2 is equal to that of its natural soluble counterpart [135]. Other GPI-anchored growth factors, such as IL-7, IL-12, and IL-15 showed a comparable, although somewhat weaker tendency to costimulate T cells. Another study demonstrated that the cytokines IL-4 and GM-CSF, when expressed in a GPI-anchored form on VLP, synergized to differentiate purified CD14⁺ monocytes towards DC similar to their soluble homologs [15, 135, 171]. These results indicated that the colocalization of more than 1 cytokine on the VLP surface does not compromise their individual functions. Nevertheless, extensive comparative studies detailing the function of

VLP-bound cytokines, e.g. as adjuvants to foster amongst others antiviral T and B cell responses, are clearly warranted in the future.

Notably, VLP can also be efficiently decorated with functionally active multimeric cytokine receptors such as the IL-2 receptor which consists of an α -, a β -, and a γ -chain (CD25, CD122, CD132) [136]. These findings seem to be of special interest since CD25⁺ regulatory CD4⁺ T cell (nTreg) subsets have been shown to exert their regulatory function on activated effector CD4⁺ T cells by – among other mechanisms – binding and neutralizing secreted IL-2 and other common γ -chain-(CD132)-associated cytokines [172]. In our studies, IL-2 receptor-decorated VLP not only bound but also neutralized soluble IL-2 [136]. IL-2 receptor-decorated VLP thereby displayed cytokine deprivation capabilities commonly associated with regulatory cells [136]. This may open new avenues to exploit ‘regulatory’ VLP for therapeutic purposes.

Peptide/MHC-Decorated VLP Elicit Effector Programs in Antigen-Specific CD8⁺ T Lymphocytes

In a further study, the replacement of the surrogate TCR ligand by bona fide MHC molecules was assessed by peptide loading and presentation, e.g. of viral antigens, by VLP to activate antigen-specific effector programs in CD8⁺ cytotoxic T lymphocytes (CTL). CTL have the specific ability to destroy pathogenic cells, such as those infected with viruses or presenting tumor antigens.

Although numerous reports demonstrated that peptide/HLA complexes might accumulate in APC lipid rafts and thus in the immunological synapse during antigen presentation [33, 173, 174], the functionality of artificially GPI-anchored pMHC has remained a matter of controversy [175, 176]. Since endogenous HLA class I molecules of HEK-293 cells are only weakly targeted to lipid rafts [177], immunosomes could be decorated nearly exclusively with heterologous HLA class I molecules of choice modified by GPI anchor acceptor sequences and expressed in VLP producer cells. In proof-of-principle studies, VLP decorated with the GPI-anchored murine (m) MHC molecule H-2D^b along with m β_2 -microglobulin, mCD54::GPI, mCD80::GPI, and a peptide minigene coding for the immunodominant CD8⁺ T cell epitope of lymphocytic choriomeningitis virus glycoprotein (LCMV-GP_{33–41}) induced a strong activation of antigen-specific T lymphocytes in vitro [134] which was comparable to bone marrow-derived (BM) DC pulsed with LCMV peptide. Furthermore, the coculture of splenocytes from P14 TCR transgenic mice specific for LCMV-GP_{33–41} [178] with antigen-specific immunosomes

induced the surface expression of CD107a (LAMP-1) on the majority of the LCMV-GP_{33–41}-specific CD8⁺ T cell subset, which indicated cytotoxic granule exocytosis [179]. Additionally, the cell population expanded in the presence of LCMV-specific immunosomes was cytotoxic and lysed syngeneic EL4 target cells that were pulsed with LCMV-GP_{33–41} peptide with a high specificity [134]. Again, the cytotoxic potential of T lymphocytes (CTL) activated by immunosomes was comparable to CTL activated by BM DC and superior to BM DC pulsed with exosomes in vitro [134]. In vivo studies further corroborated the in vitro findings. In fact, upon intravenous injection LCMV_{33–41}-specific immunosomes activated antigen-specific transgenic T lymphocytes as assessed by the up-regulation of CD69. In those studies, a colocalized second signal on VLP was strictly required to induce T cell activation [135]. It will be interesting to explore whether and how the immune responses against whole viral envelope proteins and peptide/MHC class II complexes expressed on VLP might benefit from coexpressed membrane-bound cytokines displayed on VLP in future experiments.

The antigen-specific priming and activation of virus- or tumor-specific CD8⁺ T cells could lead to the development of new strategies for the treatment of viral diseases and cancer [180–182].

Peptide/MHC Class II-Decorated VLP Modulate the Function of Antigen-Specific CD4⁺ T Lymphocytes

CD4⁺ T helper lymphocytes are key elements on the one hand for the optimal activation of CD8⁺ cytotoxic T lymphocytes [183] and on the other for the activation and provision of helper signals for B lymphocytes during antigen recognition and immunoglobulin class switching [184–186]. Interferon- γ (IFN- γ)-secreting Th1 cells are important for the clearance of intracellular pathogens and were shown to drive antibody-mediated responses, in particular IgG2a, but are also involved in cell-mediated immune responses leading to tissue damage [62]. In contrast, Th2 lymphocytes drive mainly IgE-mediated antibody responses and thus play a central role in the defense against helminthes and in the induction and maintenance phase of type I allergies [13, 187, 188]. Th17 cells, characterized as preferential producers of IL-17, IL-21, and IL-22, are important for the clearance of extracellular pathogens and are known to have potent proinflammatory functions [189, 190], while regulatory CD4⁺ T cell subsets, CD25⁺ nTregs, IL-10-producing type 1 regulatory T (Tr1) cells, and TGF- β -expressing T regulatory type 3 (Tr3) cells play critical roles for the maintenance of immune homeostasis and peripheral tolerance to self an-

tigens [191–194]. Substantial evidence suggests that these cell types also control the immune responses to allergens and transplantation antigens [13, 195, 196].

Thus, it was thought that the antigen-specific VLP-based modulation of CD4⁺ T helper lymphocytes in general and of allergen-specific T cells in particular might represent a promising approach to influence the undesired Th2-biased immune response to allergens. To address this question, HLA class II/allergen peptide-decorated VLP have been generated expressing the immunodominant epitope of a model allergen, i.e. Art v 1_{25–36}, the major mugwort pollen allergen (*Artemisia vulgaris*), together with HLA-DRA*0101 and HLA-DRB1*0101, respectively [197–200], to deliver the allergen-specific signal 1 [138]. The loading of HLA class II molecules with Art v 1_{25–36} peptide was achieved by an invariant-chain (Ii)-based approach [138, 201] which replaces the CLIP sequence of Ii with the sequence coding for the allergen peptide. Significantly, VLP displayed transmembrane HLA class II molecules organized as $\alpha\beta$ -heterodimers with expression levels comparable to related types of vesicles, i.e. exosomes derived from immortalized B cell lines. VLP expressing HLA-DR1/Art v 1_{25–36} complexes and coexpressing CD80::GPI induced the allergen-specific proliferation of Art v 1-specific T cell clones from allergic individuals and PB T cells from nonallergic individuals transduced with an allergen-specific TCR [138]. T cell activation and clonal expansion in vitro were found to be independent of accessory cells. Moreover, primary cultures of allergen-specific T lymphocytes cocultivated with VLP presenting signal 1 in the absence of costimuli, proliferated neither upon primary stimulation nor upon secondary stimulation with anti-CD3/CD28-coated microbeads. Significantly, this form of T cell unresponsiveness could be overcome by the addition of high concentrations of IL-2, indicating that the T cells were nonresponsive but still alive [137], which was perfectly in line with the functional definition of anergic cells [47, 202, 203]. However, recent reports imply increasing complexity within the field of anergic T cell phenotypes warranting further in vitro and in vivo investigations to clarify whether the VLP-induced anergy is long lasting and whether altered peptide ligand/MHC complexes would further affect anergic T cell phenotypes [204–207].

The coexpression of the CD28 ligands CD80::GPI or CD86 on allergen-specific VLPs led to the clear-cut secretion of IFN- γ , IL-2, and IL-13 [137], which is compatible with previous reports [208, 209]. In marked contrast, CD58::GPI coexpression induced IL-10/IFN- γ -secreting T cells rendering IL-2, IL-4, and IL-13 levels low. The cy-

tokine profile observed upon CD58 costimulation was intriguingly reminiscent of T-regulatory cells 1 (Tr1) described by Groux et al. [192, 210]. Of the utmost clinical importance in that context are the reports describing the induction of IL-10 and/or IFN- γ -secreting T cells upon allergen-specific immunotherapy [211–213]. These findings have been further corroborated by reports demonstrating the predominance of IL-10 and/or IFN- γ -secreting T cells in healthy individuals without allergy. The results obtained with allergen-presenting VLPs coexpressing CD58 suggest an important role of the CD58-CD2 signaling axis in the induction of allergen-specific IL-10/IFN- γ -secreting T cells. Similar T cell phenotypes were observed upon the triggering of CD46 by its natural ligands as well as upon T cell activation in a milieu rich in IL-27 [214, 215].

The data imply that colocalization on allergen-specific VLPs of distinct T cell ligands, either in the form of ligands for costimulatory molecules or as classical cytokines, might be a valid strategy to modulate the function of antigen-specific T cells in future immunotherapy protocols.

Comparison of Immunomodulatory VLP to Other Antigen-Presenting Systems

VLP represent versatile tools for the modulation of antigen-specific T lymphocytes. Thus, VLP unite several beneficial features of already established antigen-presenting and T cell stimulatory systems (table 5).

Ligand-Decorated VLP Compared to Purified Protein Ligands

T lymphocytes can be stimulated by matrix-immobilized ligands or antireceptor monoclonal antibodies [88, 115, 216]. In fact, receptor aggregation by multivalent ligands represents a fundamental mechanism of immune cell activation, which has been analyzed rigorously [217–222]. As described above, the VLP platform supports the display of multivalent TCR ligands in the form of pMHC complexes [134–137]. Its valency and free combination capability with costimulatory molecules of choice represents, however, a considerable advantage over T cell-stimulatory approaches using purified proteins.

VLP Compared to Planar Phospholipid Membranes and Liposomes

In the past, synthetic model membrane surfaces such as glass-supported lipid bilayers (SLB) were developed

[223]. Importantly, lipid composition in SLB can be varied and individual lipid molecules are highly mobile [224, 225]. Although molecularly well defined, their limitations should nevertheless be discussed: (i) ligand mobility on natural APC seems to be hardly reproducible by SLB, (ii) freely diffusing ligands do not support tension and may thus affect ligand clustering, and (iii) liposome preparation includes several steps that may not be compatible with fragile proteins; consequently, such proteins must be purified first and introduced upon vesicle formation or bilayer deposition [226, 227]. In contrast, VLP are direct derivatives of the plasma membrane of ‘producer cells’. They represent a natural membrane makeup regarding lipid composition, morphological membrane curvatures, and ligand mobility. Moreover, VLP production is highly reproducible and requires neither the purification of the proteins to be introduced nor the eventual painting of VLP with receptors of interest [92, 228, 229]. Finally, while VLP activate T lymphocytes directly in vitro and in vivo, activation by pMHC-decorated liposomes seems to depend on additional supporting membrane surfaces [230–232].

VLP Compared to Coated Magnetic Beads

Magnetic beads coated with anti-CD3 and anti-CD28 monoclonal antibodies were used by Levine et al. [233] to efficiently expand polyclonal CD4⁺ T lymphocytes ex vivo and in the absence of feeder cells. Moreover, pMHC/anti-CD28-coated magnetic beads were successfully applied to expand antigen-specific T cells ex vivo [234]. After 3 rounds of stimulation, the majority of T cells were found to be antigen-specific (>85%) which was comparable to data obtained with VLP (84%) [137]. Despite the clear-cut potential for the ex vivo expansion of T cells, e.g. for adoptive transfer purposes, the physical composition of microbeads certainly does not allow their systemic application.

VLP Compared to Exosomes

Exosomes represent another cell-free system for antigen presentation and T cell activation. Exosomes are microvesicles of 60–90 nm in size that are contained within MVB and released into the extracellular environment upon the fusion of the MVB with the cellular plasma membrane [166]. Along with reticulocytes [235], epithelial cells [236], neurons [237], immune cells such as B and T lymphocytes [238, 239], mast cells [240], and DC [241] but also distinct tumor cells [242] have been shown to secrete exosomes. The molecular composition of exosomes is a result of their cells of origin. Exosomes are released

Table 5. Antigen-presenting systems

System	Antigen specificity	Antigen processing	T cell expansion	Clinical application	Authors	Reference No.
<i>Acellular antigen-presenting systems</i>						
Immobilized mono-clonal antibody	no	no	+++	yes	Van Wauwe et al., 1980; Meuer et al., 1983; Tax et al., 1984; Riddell and Greenberg, 1990	216, 330–332
Coated magnetic beads	no/yes	no	+++	yes	Levine et al., 1997; Oelke et al., 2003; Maus et al., 2003	233, 333, 334
Liposomes	yes	no	+/-	no	Delbrück et al., 1968; Herrmann et al., 1982; Mescher, 1992; Prakker et al., 2000	230–232, 335
Supported planar lipid bilayers	yes	no	+/-	no	Brian and McConell, 1984; Groves et al., 1997; Grakoui et al., 1999; Groves et al., 2003; Mossman et al., 2005	219, 222, 224, 336, 337
Exosomes	yes	yes	++/-	yes	Raposo et al., 1996; Zitvogel et al., 1998; Vincent-Schneider et al., 2002; Segura et al., 2005; Admyre et al., 2007; Viaud et al., 2009	238, 241, 245, 247, 249, 338
Tolerogenic exosomes	yes	yes	–	no	Kim et al., 2005; Bianco et al., 2009	265, 266
Painted viral particles	no	no	–	no	Metzner et al., 2008	339
Immunosomes (VLP)	yes	yes	+++	no	Derdak et al., 2006; Kueng et al., 2007; Leb et al., 2009	134, 135, 137
Anergosomes (tolerogenic VLP)	yes	yes	–	no	Leb et al., 2009; Kueng et al., 2010	136, 137
<i>Cellular antigen-presenting systems</i>						
Autologous ‘feeder’ cells	yes	no	+++	yes	Dudley et al., 2002, 2003, 2005	340–342
Monocyte-derived dendritic cells	yes	yes	+++	yes	Bender et al., 1996; Pickl et al., 1996; Lutz et al., 1999; Robinson et al., 2001; Davis et al., 2003; Gilboa et al., 2007	15, 250, 251, 343–345
Autologous progenitor-derived DC	yes	yes/no	+++	yes	Bancherau et al., 2001; Lu et al., 2004; Palucka et al., 2005; Fay et al., 2006; Palucka et al., 2008	253, 346–349
Tolerogenic DC	yes	yes	+++	yes	Jonuleit et al., 2000; Dhocapkar et al., 2001; Steinman et al., 2003; Sato et al., 2003	255, 260, 261, 350
Insect cells (<i>Drosophila melanogaster</i>)	yes	no	+++	yes	Jackson et al., 1992; Cai et al., 1996; Mitchell et al., 2002	40, 351–353
Mouse fibroblasts	yes	yes	+++	no	Latouche et al., 2002; Papanicolaou et al., 2003	354, 355
K562/K32 cells	no	no	+++	no	Maus et al., 2002; Thomas et al., 2002	356, 357
Daudi cells	yes	no	+++	no	Obermann et al., 2007	358
BW 5147 cells	no	no	+++	no	Pfistershammer et al., 2006; Kober et al., 2008	165, 359

as a consequence of a broad range of physiological and pathological processes such as cellular activation and differentiation [242–244], among others. Due to the expression of pMHC, costimulatory and adhesion molecules, B cell- and DC-derived exosomes have the ability to induce antigen-specific T cell responses in vivo [241] and, to a lesser extent, also in vitro [238], although the exact mechanism for their functional activity is still enigmatic. While some studies demonstrated that exosome function required the help of APC [245–247], others indicated that exosomes directly induced T lymphocyte stimulation [248]. Since exosomes can be efficiently loaded with antigenic peptides by pulse labeling, they are also capable of inducing antigen-specific cellular responses [238, 249]. However, since VLP are amenable to decoration with molecules of choice, VLP seem to represent a more versatile antigen-presenting platform than exosomes [134–137].

VLP Compared to Cellular APC Systems

Antigen-specific T cell activation is best achieved by antigen-loaded professional APC, e.g. DC, macrophages, or B cells, the gold standard for interventional studies being monocyte-derived dendritic cells (mdDC) [250, 251]. Antigen-pulsed mdDC are currently used as vaccines in cancer patients and several clinical trials demonstrated an induction of immunity to tumor antigens, although objective tumor regression still seems to be limited [68, 252, 253]. The culture and differentiation of mdDC entails the isolation of sufficient numbers of mononuclear cells from patients or histocompatible healthy donors by leukapheresis [250]. Such individual medicine is time-consuming, inherently expensive, and production-wise often inefficient, e.g. for patients undergoing cytotoxic chemotherapy [254]. Moreover, inadequately matured DCs might even downregulate immunity, generate CD4⁺CD25⁺ Treg cells, and thus induce peripheral tolerance to the putative tumor antigen [255].

The apparently broad tolerogenic potential of in vitro-induced DC by (i) pharmacological drugs such as aspirin, rapamycin, dexamethasone, and vitamin D3 [256–259]; (ii) the transgenic overexpression of immunosuppressive molecules, or (iii) the administration of exogenous cytokines (e.g. TGF- β , IL-10, GM-CSF, and IL-4) opens, however, new and appealing ways for the induction of Tregs [260, 261]. Such tolerance-inducing protocols might become useful for the treatment of autoimmune disorders, chronic inflammation, allergic diseases, and as graft rejection [262–264]. It is important to note that recent studies have demonstrated that purified exosomes from IL-

10-treated BM DCs were able to suppress delayed-type hypersensitivity reactions [265], and indoleamine 2,3-dioxygenase (IDO) overexpressing DC-derived exosomes displayed immunosuppressive and anti-inflammatory features [266]. Thus, in comparison to tolerogenic VLP [136, 137], regulatory DC-derived exosomes may represent another cell-free therapy for the treatment of allergy or autoimmune diseases. However, the clinical use of in vitro-generated tolerogenic DC and DC-derived exosomes might bear substantial inherent disadvantages such as the requirement of a large number of HLA-matched DC and the achievement of a relatively stable, immature, or regulatory phenotype of DC in culture.

Fluorosomes: A Convenient New Reagent to Detect and Block Multivalent and Complex Receptor-Ligand Interactions

In the past, the translational fusion of viral proteins with green fluorescent protein has been used to elucidate the cellular pathways of infectious viruses [267, 268]. Since lipid rafts serve as a meeting point for GPI-anchored surface molecules and the viral core proteins, fluorescent proteins N-terminally linked to the viral matrix protein MA of MoMLV generate fluorescently labeled VLP that can be used to track receptor-ligand interactions in different settings. In a recent report, VLP labeled with recombinant fluorescent proteins of cnidarian origin and decorated with IL-2 specifically identified normal and malignant IL-2R⁺ lymphocytes by flow cytometry [136]. Notably, the fluorescent VLP platform could also accommodate multi-subunit ligands or receptors which are otherwise not easy to express in the appropriate configuration. In further applications, FS might facilitate the detection of low-affinity interactions between monomeric receptor-ligand pairs, and fluorescently labeled VLP could serve as a versatile tool to track immunomodulatory VLP in vivo. Due to their simple generation and broad applicability, FS might serve as valuable tools in different fields of basic immunological research as well as for diagnostic purposes.

Conclusion and Perspectives

In this study we discuss the advantages and shortcomings of different approaches for the antigen-nonspecific and antigen-specific activation of T lymphocytes by a novel immunomodulatory tool, i.e. VLP decorated with immunomodulatory molecules of choice. VLP are derived from common producer cells, i.e. HEK-293 epithelial

cells, which are suitable for GMP protocols and should perse – due to their human origin – only show minor immunogenicity in future therapeutic applications in humans. VLP production is induced by simple transfection. At this level, VLP can be selectively decorated with preformed pMHC complexes, costimulatory molecules, membrane-bound cytokines, and cytokine receptors, respectively, if desired in a fluorescent form. Consequently, VLP can be regarded as ‘packages of information’ with the capacity to efficiently target antigen-specific T lymphocytes by the delivery of antigen-specific and cosignals of choice. Depending on the quality of the cosignals used, VLP offer the possibility to activate and expand, anergize, or polarize antigen-specific T lymphocytes. Given their high versatility in basic studies, the detailed analysis of the therapeutic usefulness of VLP in more clinically oriented studies is clearly warranted in the future. Along those lines,

one could envision a much broader range of applications with VLP serving as packages transporting specific cargo – e.g. in the form of nonintegrating genetic information or a pharmaceutical compound – to modulate the function/survival of target cells which they specifically recognize by surface ligand-receptor interactions.

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