Orchestrating the immune system to initiate adaptive anti-tumor immunity

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Chapter 8

Discussion Part I - Vaccination

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8.1 Components of vaccines

Just like pathogens, vaccines can assume many forms and contain many different components. Similarly, different pathogens elicit different immune responses at the molecular, cellular and systems level. We will discuss vaccine components and the associated immune responses.

8.1.1 The innate immune component

Innate immune cells can be activated in a multitude of ways, with each resulting in a specific response. In vaccines, the use of bacterial products provides several benefits. Bacteria and their products carry specific pathogen-associated molecular patterns (PAMPs) needed for activation of PRRs on innate immune cells, such as dendritic cells (DCs)[1]. DCs that sense PAMPs, are the primary activators of adaptive immunity and key to the success of active vaccination strategies[2]. We have tested the innate stimulatory capacity of two bacterial products for vaccine design; outer membrane vesicles (OMVs; chapter 2) and inclusion bodies (IBs; Chapter 3). OMVs are bilayered lipid membrane vesicles that are naturally produced by Gram-negative bacteria via the budding off of the membrane[3]. As a result, OMVs contain many of the components that are found in the parent bacterium, although in a non-replicative form. Among immunogenic PAMPs, OMVs are enriched in membrane-resident lipopolysaccharide (LPS), DNA, RNA and peptidoglycan[4]. As such, OMVs have been considered excellent candidates as vaccine-delivery system or adjuvants[5]. We have investigated the immunogenicity of engineered OMVs on dendritic cells. OMVs were shown to be highly potent activators DC maturation, dependent on accessible OMV-derived LPS and intact MyD88 signaling (Chapter 2). The effect of OMVs on DCs and macrophages has been investigated before and showed cytosolic translocation of LPS after uptake[6]. Cytosolic LPS is subsequently sensed by caspase 11, leading to a highly inflammatory form of cell death called pyroptosis[6]. However, this was not observed in our experimental setup using both bone marrow-derived dendritic cells (BMDCs) and $CD11c^+$ splenic dendritic cells. In fact, OMV-derived LPS provided an additional survival benefit over non-stimulated DCs, providing encouraging data for future OMV-based vaccine design. Still, OMVs were only tested with purified DCs *in vitro*. Pilot experiments showed that OMVs were not well tolerated *in vivo* with systemic and local pathology (data not shown). Off-target effects because of PRR-independent cell entry mechanisms in other cell types like epithelial cells may bypass TLR4-expressing DCs *in vivo* leading to pathology[4]. Hence, targeting the OMVs specifically to dendritic cells using DC-specific endocytic receptors could be an approach to significantly reduce the off-target effects and allow lowered injected dosages.

Bacteria engineered to express a recombinant protein, often show intracellular aggregates of misfolded form of the protein, called Inclusion bodies (IBs). IBs showed similar MyD88-dependent maturation of dendritic cells[7], although the role for LPS in this response is less certain. Indeed, in unpublished experiments polymyxin B (inactivates LPS) did not completely abrogate DC maturation, suggesting that other components of inclusion bodies may mediate MyD88-dependent maturation. Inclusion body formation is an endogenous adaptation of bacteria to prevent increases of soluble dysfunctional or misfolded protein that may interfere with cellular processes[8]. Inclusion bodies consist of misfolded proteins in partly β-pleated secondary conformation, closely resembling amyloidosis[9]. Interestingly, self-assembling peptides into β-sheet fibrils showed self-adjuvating effects, suggesting that the β-sheet conformation is immunogenic[10]. Additionally, proteins like heat-shock proteins and chaperones can also be found in IBs derived from E. coli and have shown immunogenic capacity[11–13]. Surprisingly, inclusion bodies without adjuvants did not induce detectable levels of $CD8^+$ T cells. More research needs to be done to evaluate the inherent IB immunogenicity *in vivo*, as well as the appropriate IB-adjuvant combinations.

Although bacterial products like OMVs and IBs have inherent immunogenic capacities, the undefined nature of the particle makes rational vaccine design and prevention of unwanted side effects more difficult. Defined ligands for PRRs are now being evaluated for clinical use in combination with antigens like proteins that lack natural innate activation capacities[14]. For example, Pam3CysSerLys4 (Pam3CSK4) is a tripalmitoylated lipopeptide that closely mimics the lipoproteins found in bacteria and activates

TLR2, leading to activation of NF-κB and cellular activation[15]. Pam3CSK4 has therefore been used as conjugate for antigen with low inherent immunogenicity, resulting in antigenic lipopeptides with both antigen and adjuvants in a single compound[16]. We have found no such adjuvant effect in mono-palmitoylated peptides, which instead directly incorporated in the lipid bilayer of cells (Chapter 5).
For the induction of $CD8^+$ T cell responses *in vivo* in mice, we describe that the preferred adjuvants was agonistic CD40 antibody (Chapter 3-7). Agonistic antibodies bind their target and induce downstream signaling, often independent of the ligand-binding domain of the receptor[17]. Triggering CD40 on dendritic cells leads to upregulation of maturation markers, antigen presentation, cytokine production (in particular IL12) and activation of T cells[18–24]. Activating CD40 on DCs has therefore been used in vaccination strategies to boost T cell immunity[21,25–27]. CD40 is expressed by DCs, in particular monocyte-derived DCs[28]. Conventional and plasmacytoid DCs (cDCs and pDCs) express only low levels of CD40 and need a microbial stimulus to upregulate CD40 for synergistic activation[29,30]. We have found that the oil-in-water emulsion AddaVax (MF59) recruits monocytes that partially differentiate into moDCs (Chapter 4 and 6) and find that AddaVax synergistically boosts the adjuvanticity of agonistic CD40 antibody (Chapter 3, 6 and 7). AddaVax in itself does not have compounds that activate PRRs and needs to be completely formulated (Span 85, Tween 80 and Squalene), since the individual compounds do not show similar adjuvanticity[31]. Instead, AddaVax has been shown to mediate the cellular components needed for adaptive immune responses[32–34]. Indeed, AddaVax injection may recruit the CD40-expressing moDC susceptible to CD40 agonism and mediator of adaptive immunity. In fact, by disconnecting the moDC recruitment and the antigen targeting by sequential AddaVax and moDC targeting injections, we showed increases in early antibody production at the expense of T cell activation (Chapter 6). However, agonistic CD40 has additional effects outside of DC activation by providing T cell help to $CD8^+$ T cells[35–37]. Also, agonistic CD40 antibody has clinically been restricted by dose-limiting toxicities[38], although antibody modification and slow-releasing compounds may reduce toxicities[39–43].
A systematic approach to identify the most potent adjuvants is lacking in this thesis and is a drawback when selecting the most effective vaccination strategy for clinical testing. The clinical efficacy of most successful adjuvants are tested empirically in the clinic and not presumptive or pre-clinically[44]. However, we do provide evidence for the inherent adjuvanticity of bacterial vaccine components and propose alternative adjuvants whenever necessary. Hence, rational adjuvant selection remains difficult to pre-clinically substantiate.

8.1.2 The specificity component

An adaptive immune response towards pathogens or tumors requires antigens specific for the pathogen, infected- or tumor cell and that do not cross-react with the hosts healthy cells. T cells recognize linear peptides bound by MHC class I/-II complexes, whereas B cells often recognize proteins or structure with a tertiary molecular structure (Chapter 1.2.3). As such, a vaccine aimed at generating high affinity antibodies requires intact pathogen/tumor-associated structures. Vaccines aimed at generating a T cell response, however, require DC-mediated presentation of degraded linear peptides. Whereas peptides can be easily chemically synthesized (Chapter 7), proteins are much more difficult to produce chemically and require biological production systems, like bacteria, yeast, plants or insect cells[45–48]. Since we have mostly focused on generating T cell responses, most products reported in this thesis contain peptides and were evaluated on their capacity to induce T cell responses, not B cell responses. However, in the case of antibody targeting to DC-$SIGN^+$ moDCs, the model antigen ovalbumin (OVA) was covalently attached to the antibody, allowing the *in vivo* induction of OVA-directed antibodies (Chapter 4 and 6).

Linear antigens in the form of peptides are easier to produce, although short minimal forms of the antigen are not preferred since these directly replace peptides presented by MHC class I or –II on the surface of any cell. It has been shown that minimal epitopes accumulate on non-APCs in the injection sites, creating a depot for activated $CD8^+$ T cells, leading to a defective adaptive immune response[49].

Instead, longer peptides are preferred, which require intracellular proteolytic degradation by APCs for presentation to T cells[49]. This directly poses several challenges in vaccine design: 1) exogenous peptides need to localize to the cytosol of APCs before degradation in lysosomes for presentation of MHC class I, 2) the amino acid sequence should be optimized to allow N-terminal trimming events for MHC class I loading, 3) peptides should have a high enough affinity for MHC class I complexes and 4) MHCI and –II-binding peptides require different intracellular routing.

Importantly, all products were empirically tested on all these capacities by testing antigen-specific DC-mediated T cell activation *in vitro*. However, we have optimized certain aspects and made several predictions to maximize successful antigen presentation. First, flanking sequences that facilitates proper proteolytic degradation (by the proteasome and in dendritic cells was experimentally tested in our lab, resulting in the choice for cys-glu-glu-lys (CEEK) N-terminal sequences and lys-glu-glu-lys (KEEK) C-terminal sequences (Chapter 7 and data not shown). The N-terminal cysteine allows chemical modification like glycosylation or AMAX construction using sulfhydryl-reactive cross linker chemistry. Biologically, C terminal flanking allows appropriate proteasome processing, which does not occur by cytosolic carboxypeptidases. N-terminal flanking is processed by the MHC class I peptide loading complex (PLC) after a fitting C-terminal peptide is captured by the MHC class I complex[50–52]. Solid phase peptide synthesis is limited by the length of the peptide (Chapter 7). The CD8- and CD4- epitope were synthesized adjacently, instead of flanked by the natural ovalbumin sequences as used in OMVs and mDC-SIGN targeting antibody. Using this sequence, both epitopes were successfully processed and presented to $CD8^+$ and $CD4^+$ T cells by BMDCs pulsed with peptide, lipopeptide or IBs (Chapter 3, 5 and 7).

However, for the generation of a vaccine strategy that allows the inclusion of any antigen, universal flanking sequences for different sequences would be ideal. In the IB study (Chapter 3), we have made attempts to predict ideal epitope flanking sequences based on proteolytic cleavage site preference of relevant proteases/peptidases. To optimize proteasomal processing, we included an alanine-alanine-tyrosine (AAY) sequence on the C terminal of the CD8 epitope sequence. AAY sequences act as proteasome cleavage sites and aids the processing of $CD8^+$ T cell reactive epitopes in DCs[53–56]. For antigens expressed by MHC class II molecules, processing occurs in the endo-lysosomal components of dendritic cells, where cysteine proteases cleave proteins into peptides for MHC class II loading[57]. Data from Choe et al., showed the preferred cleavage sites of a wide variety of cathepsins involved in MHC class II peptide processing[58]. Based on this we assumed His-Arg-Leu-Lys (HRLK) as highly preferred CD4-epitope flanking sequence (Chapter 3). *In vitro* and *in vivo* data using these flanking sequences with the ovalbumin sequences showed functional antigen processing and presentation to $CD4^+$ and $CD8^+$ T cells. However, when MC38 colorectal cancer neoantigens, B16 melanoma tumor-associated antigens or PDAC-associated antigens (all CD8-epitopes) were used in combination with the pan HLA-DR epitope (PADRE), only PADRE-reactive $CD4^+$ T cells were found in immunized mice (data not shown). Additionally, using a variety of adjuvants with ovalbumin-IBs, we found that the higher the $CD4^+$ T cell response, the lower the $CD8^+$ T cell response (or vice versa), suggesting at least some level of competition or trade off at this level. Since we did not observe this effect in other compounds, we believe this to be an IB-specific phenomenon that warrants further investigation.

The delivery of antigens in the right intracellular compartment for efficient antigen presentation remains a challenge. One strategy is targeting an antigen to a DC-expressed endocytic receptor known to cross-present bound antigen, like DC-SIGN[59–61]. In chapter 7 we have shown that the AMAX platform can be modified to accommodate DC-SIGN targeting ligands, like Lewis Y, increasing $CD8^+$ T cell responses in hDC-SIGN transgenic mice (Chapter 7). In chapter 4 we have shown that the mouse variant of DC-SIGN, CD209a/mDC-SIGN, can also be targeted using a specific antibody coupled to ovalbumin. This resulted in potent $CD8^+$ and $CD4^+$ T cell responses *in vitro* and *in vivo*[62]. Targeting of antigens conjugated to specific antibodies may be an interesting approach, since synthesized peptides can also be used as payload instead of difficult to synthesize proteins[63]. A downside of antibody-

mediated antigen delivery is that the affinity of the antibody may be too high to release antigens or allow receptor recycling, as has been shown for DC-SIGN[64].

8.1.3 Adaptive immune effector components

The effector components of the adaptive immune system are in part defined by the mode of innate activation and antigen presentation in the lymph node. DC-mediated T cell activation after antigen uptake, processing and presentation on MHC class I and –II can be investigated *in vitro*. Benefits of *in vitro* systems is the direct investigation of DCs in their interaction with the vaccine compounds and the correct presentation to antigen-specific transgenic T cells (ie. Ovalbumin-reactive OTI $CD8^+$ and OTII $CD4^+$ T cells). Designing the composition of antigen sequences and flanking regions that allows correct proteolytic processing in DCs is crucial before experiments can be performed *in vivo*. Additionally, *in vitro* antigen presentation systems allow the investigation of cross-presentation and innate signaling needed for optimal cross-presentation. For example, we have shown that bacteria-derived products like OMVs and IBs require MyD88 signaling in DCs for optimal cross-presentation to $CD8^+$ T cells (Chapter 2 and 3). These data are in line with previous reports showing that TLR4-MyD88 signaling reduces phago-lysosomal fusion and acidification, resulting in reduced protein degradation and increased cross-presentation to the cytosol[65]. Still, it remains to be defined how antigens derived from OMVS or IBs are routed or retained in the dendritic cell for antigen presentation to T cells. OMVs have shown to translocate to the cytosol (either whole or in part) and a similar translocation process may facilitate cross-presentation, especially in TLR4-stimulated conditions where OMVs are more likely to survive proteolytic degradation for longer periods. Also, the location of the antigen on the outer membrane (coupled to the Hbp transporter protein) is inherent to the design of the OMV and may allow quick release in the endosomes compared to antigens localized to the lumen of the OMV. Similarly to OMVs, the propensity for cross-presentation of IB-derived antigens was in part MyD88-dependent (Chapter 3). How IBs are retained in DCs remains to be defined, although $CD8^+$ T cell activation is intact even a day after DCs were loaded with IBs. It therefore seems likely that IBs provide a strong intracellular depot providing persistent antigen release. However, whether this happens in endosomes, lysosomes or even the cytosol is unknown.

DC activation, antigen presentation and T cell activation *in vitro* is not a guarantee for a T cell response *in vivo*. The number of confounding factors affecting both innate and adaptive immunity *in vivo* is difficult to estimate from *in vitro* experiments. DCs may be the main inducer of T cell immunity, but the vaccine is often not delivered to DCs exclusively *in vivo*. For example, OMVs are efficiently cross-presented to $CD8^+$ T cells by DCs *in vitro*, but no T cell response was found in pilot immunization experiments (data not shown). One explanation may be the relatively dose of OMVs. However, OMVs injected subcutaneously were poorly tolerated and induced skin lesions, preventing any continued experimentation. Most cells in the skin can take up OMVs, resulting in extensive off target effects like cell death that may blunt the DC-mediated T cell response[4,66.] Indeed, engineered OMVs may benefit from a DC-targeting that allows reduction in concentration and off-target pathology. In contrast, IBs are very well tolerated but do not induce robust T cell responses *in vivo* without additional adjuvants (Chapter 3 and data not shown). IBs show inherent innate/DC activation capacity in a MyD88-dependent manner *in vitro*, but IBs alone are apparently not sufficient to drive T cell immunity. The large particulate nature of IBs prevents drainage to skin-draining lymph nodes after subcutaneous injection and therefore requires local uptake by dendritic cells that migrate to the lymph node for presentation to T cells. Perhaps highly phagocytic macrophages are the main interacting cell in the skin upon subcutaneous injection, which do not induce *de novo* T cell responses. However, the combination of IBs with agonistic CD40 induced robust $CD8^+$ T cell responses, opposing the hypothesis that IBs are not delivered to DCs because of off-target effects. Indeed, a variety of innate stimuli induced T cell responses when combined with IBs (Chapter 3). It therefore seems that the capacity of IBs to activate DCs (ie. adjuvanticity) *in vitro* is less well translated to the *in vivo* setting and warrants further investigation.

Off target effects of vaccine components can be reduced by a DC-targeting component[67–69]. Targeting allows tailored responses by choosing DCs and the endocytic routing of the antigen. Because the human endocytic receptor DC-SIGN has proven to be an excellent target for cross-presentation to CD8$^+$ T cells, targeting human DC-SIGN with an antibody or glycan has shown promising results. However, recent years the expression of DC-SIGN on professional APCs (DCs, capable of kickstarting adaptive immunity) has been debated[70–72]. DC-SIGN is described mostly as an moDC-associated receptor in the context of binding pathogen-associated glycans like HIV or endogenous proteins like LFA1[73,74]. Focusing on the skin, DC-SIGN is expressed by CD14$^+$ DCs in steady state conditions and their function as DCs or tissue-resident APCs remains unclear. However, under inflammatory conditions, infiltrating monocyte-derived DCs express high levels of DC-SIGN providing a target for antigen loading. Moreover, monocyte-derived DC have shown to be crucial for the adaptive immune response generated by several clinically successful adjuvants[75,76]. In the case of mouse DC-SIGN/CD209a, antigen targeting is successful because it is an internalizing receptor like human DC-SIGN and supports cross-presentation to CD8$^+$ T cells (Chapter 4). However, receptors like DC-SIGN are not expressed by all types of DC and the receptor expression should fit the DC and its downstream effector activation signals. In an effort to exploit mDC-SIGN$^+$ cells, we show that local recruitment of moDCs can be targeted by an antigen-conjugated antibody, resulting in increased humoral responses. Indeed, timed vaccination after sterile recruitment om DC-SIGN$^+$ moDCs, further boosts B cell responses instead of CD8$^+$ T cell responses (Chapter 6). An explanation for the increased B cell responses could be the local activation by skin-derived moDCs, blood-derived LN-infiltrating moDCs or through antibody:receptor recycling of mDC-SIGN resulting in intact presentation to B cells[77–79]. Although DC-SIGN$^+$ moDCs recruited by AddaVax injection express CCR7 and take up the antigen-conjugated antibody, it remains to be defined whether these cells actively migrate to the draining lymph node for T- and B cell activation.

Perhaps the most straightforward strategy explored in this thesis for both CD8$^+$ and CD4$^+$ T cell responses is the peptide modified with a single palmitic acid, lipopeptide. Since hypothetically half of the lipopeptide is quickly localized to the cytosol for cross-presentation on MHC class I, the other half remains in the outer leaflet, which upon endocytosis is localized to the endo-lysosomal pathway where MHC class II loading occurs (see also Chapter 8.1.4 below). As a result the lipopeptide is excellent for both CD8$^+$ and CD4$^+$ responses (Chapter 5). The lipopeptide may also be interesting for antigen presentation to B cells, since B cells need to recognize high densities of membrane-bound antigen[80]. Direct incorporation of palmitoylated antigen in the membrane of follicular DCs (fDCs) would provide a stable antigen presentation platform for affinity maturation. However, the unselective nature of lipopeptide membrane targeting hampers a simple *in vivo* fDC loading platform and would need to be used in *ex vivo* fDC loading therapy[81,82]. While antibodies are most often directed at proteins with tertiary molecular structures, antibodies directed against linear peptides exist and may provide some level of protection against viral infection or opsonize antigens[83–86].

8.1.4 Form factor

How the immune system perceives vaccine components is largely dependent on the (macro)molecular structure, the form factor. Examples of different form factors investigated in this thesis are vesicular (OMVs, liposomes), particulate (IBs), soluble (AMAX, lipopeptide) and targeted forms (AMAX:Lewis Y, antibody). Uptake of these components by DCs depends on several properties, including size, shape, surface charge, hydrophobicity and specific receptor interactions[87–89]. For example, particles with hydrophobic surfaces activate the complement pathway, resulting in opsonization, increased phagocytosis by DCs and macrophages, and antigen-specific adaptive immunity[90,91]. These type of antigen modification are essential to successful immunization, since soluble peptides are generally poorly immunogenic and are inefficiently taken up through macropinocytosis by dendritic cells[82].

OMVs are vesicular particles that effectively bud off bacteria (in research derived from a hypervesiculating strain[92]), containing proteins derived from the outer membrane as well as proteins

from the luminal side of the outer membrane[93]. Because bacteria have two membranes, targeting cytosolic proteins to the outer membrane of the bacteria requires specific shuttling proteins, termed auto-transporters (ATs)[94]. Genetically engineering a recombinant protein to the shuttling partner, allows the expression of heterologous antigen on the outside of OMVs[95,96]. However, they can be difficult to produce in high concentrations, to isolate and to purify[97]. Still, engineered OMVs include ample antigen and are presented to the dendritic cell in a multivalent manner, with several receptors engaged, leading to receptor-mediated endocytosis and innate signaling[4,98]. This makes OMVs an attractive alternative to attenuated forms of the host bacteria. The downside of vesicular particles like OMVs as antigen platform is the stability[99], which requires at least 4 °C for short-term storage[5,100] or even -70 °C for long-term storage[101]. Innovative ways of stabilizing OMVs and other vesicular vaccine components like drying are now being explored[101,102]. Preferably complete vaccines should be stable for storage at room temperature or even higher temperatures for delivery in developmental countries where a proper cold-chain is difficult to provide[103,104]. Alternatively, bacterial IBs provide an antigen source with excellent stability profiles[105,106]. IBs are large and highly concentrated protein aggregates sized around 100-800 nm, the ideal size for phagocytosis by DCs and macrophages[82]. The highly concentrated and folded nature of the IB may allow for slow enzymatic proteolysis and therefore long-term continuous internal antigen release. IBs are known to slowly release the misfolded antigen either passively or by extracellular proteases[8,107,108]. Indeed, subcutaneously injected IBs may have a depot function, with slow antigenic release, engaging the lymphatics. More research is required on the *in vivo* stability and behavior of injected IBs in terms of their interaction with the immune system. An interesting form of vaccine component tested is the mono-palmitoylated peptide (lipopeptide). The chemical modification of a single C16 palmitic acid facilitates the association of the lipid tail to the outer leaflet of any lipid bilayers (Chapter 5). While mono-palmitoylated peptides have been used in several studies for vaccination[16], its mode of action is still underexplored. We observed binding and internalization of lipopeptide to dendritic cells *in vitro*, within 15 minute of exposure at 4 °C. The localization within the cytosol as determined by electron microscopy (EM) under these conditions, internalization experiments and the fact lipopeptide bound synthetic liposomes suggested a passive receptor/endocytosis-independent mode of membrane binding and internalization to the cytosol (Chapter 5). We hypothesize mono-palmitoylated peptides can flip the cellular membrane and are released to the cytosol. What is the evidence that such a unique mechanism may exist? First, palmitoylation is a post-translational modification used by cells to shuttle proteins between intracellular membranes[109]. The thioesther linkage of fatty acyl moieties (ie. 16C palmitate) to cysteine by palmitoyl transferases in the Golgi, tags proteins for shuttling to the plasma membrane[110,111], preferably in lipid raft domains[112–114]. This would explain the propensity of exogenous palmitoylated peptide to associate with lipid bilayers like the plasma membrane. Second, the association of exogenous lipopeptide to the outer leaflet of the cellular membrane creates asymmetry between the inner and outer leaflet of the bilayer; there are more lipids in the outer leaflet compared to the inner leaflet. To re-establish lipid homeostasis, we hypothesize that ATP-independent scramblases “flip” the appropriate amount of lipopeptide from the outer leaflet to the inner leaflet. Similar trans-membrane equilibration mechanisms have been described for other exogenous lipids, like detergents, phospholipids and ceramide[115–118]. Third, once the lipopeptide would be flipped to the inner leaflet, the synthetic thioester bond of the lipopeptide would have to be cleaved in order for the peptide to localize to the cytosol. The inner leaflet of the plasma membrane of cells inhabits acyl protein thioestarases (APTs), specifically APT1 and APT2[109,119,120]. The molecular rules to which APTs abide to cleave a palmitoylated bond at the inner leaflet are now being studied[121–123], but the depalmitoylation machinery has been shown to only require the target cysteine side-chain[109]. In fact, peptides with non-natural D-configured cysteine side chains or changes in peptide sequences had no effect on the de-/palmitoylation machinery[109]. Interestingly, APT1s themselves are palmitoylated, resulting in localization to lipid rafts, where other palmitoylated proteins (including flipped lipopeptides) are abundant[124]. Hence, we hypothesize that the palmitic acid tail of the lipopeptide incorporates in the outer leaflet of the plasma membrane of DCs (or any other cell), creating lipid bilayer asymmetry, which is resolved by ATP-independent scramblase activity. On the inner leaflet, APTs cleave the palmitic acid bond and releasing peptide

into the cytosol for cross-presentation on MHC class I. This would explain 1) the ubiquitous and continuous binding of lipopeptide to lipid bilayers 2) the continuous loading and cytosolic localization in a endocytosis-independent manner and 3) the superior cross-presentation capacity. Also, since only part of the externally loaded lipopeptide flips, a substantial amount would remain localized on the outer leaflet which is localized inside endosomes upon endocytosis, facilitating MHC class II loading and $CD4^+$ T cell activation. We initially started several lines of investigation into APTs, scramblases and ubiquitous cross-presentation by conventional cells, but due to time limitations, these data have not been conclusive and require follow up.

The effect of size and tertiary structure in the vaccine form factor was shown in Chapter 7. Here, we showed the effect of constructing synthetic linear peptides into trimeric structures (antigen matrix or AMAX) with increased *in vivo* immunogenicity. Initially studied as an excellent DC-SIGN targeting platform (see also data from colleagues[125]), we found consistently higher T cell responses when the peptides were oriented in a trimeric structure compared to the individual building blocks (ie. synthetic long peptides). It is known that the geometry of nanoparticles affects endocytosis by phagocytes[87,126]. However, there was little difference when peptides or AMAX were compared *in vitro*, suggesting that different properties enhances the immunogenicity of AMAX compared to peptide. Both soluble antigens of low molecular weight (beween 3-4kD for synthetic long peptide and 10/15kD for AMAX), the access to the lymph node (LN) through the afferent lymphatics into the conduit system (where LN-resident DCs have access to the draining antigen) is assumed to be similar[127]. We have not investigated the advantage of AMAX over single peptides in a mechanistic manner and remains to be clarified.

In terms of form factor, the single palmitic acid is relatively easy to conjugate to any peptide of choice and can be stored in lyophilized form. The hydrophobic tail prevents the long term storage in aqueous solutions and the concentration cannot exceed the critical micelle concentration (CMC), since that would render the membrane-targeting capacity void. There are obvious downsides to the ubiquitous loading of cellular membranes upon subcutaneous vaccination. In fact, it has been shown that the local presentation of antigen is detrimental to the T cell response, because local cells present the antigen to which effector T cells are generated[49]. The vaccination site could become a depot for defective $CD8^+$ T cells. Additional, assuming the "flipping-hypothesis" is correct, the lipopeptide is limited in the length, since flipping would involve the hydrophilic peptide passing the hydrophobic domains of the lipid bilayer. We can assume this dynamic is hindered by the length of the peptide. We have tested several synthetic long peptides containing a CD8 and CD4 sequence (or other tags of similar size), but nothing longer. It would be interesting to investigate what the maximum size of the peptide sequence is for flipping.

Antibodies targeted against DC-specific endocytic receptors can function as highly specific carriers of antigenic loads that can bind locally to target DCs or drain through the lymphatics to target lymph node-resident DCs[2,67,128,129]. However, injected in tissue like the skin, antibodies that are not directly bound to their target are cleared by Fc receptor-expressing cells like macrophages, severely limiting the long term availability of the compound. Since the availability of the targeting antibody may be limited, injection should be timed with the presence of the cell expressing the targeted receptor. We have attempted to optimize antigen delivery by synchronizing the presence of the DC in the tissue with the injection of the payload (ie. antigen-coupled DC-targeting antibody; Chapter 6). However, there seems to be a trade off in terms of cellular versus humoral response when targeting DC-$SIGN^+$ DCs in the skin at different time points. In order to maximize translational value of our "vaccine-site pre-conditioning" approach, we have obtained mice that express the human DC-SIGN molecule driven by its endogenous human promoter[130]. Because expression of mouse DC-SIGN does not completely mimic the expression of human DC-SIGN in humans[62,131] or in CD11c-driven hSIGN mice[132], this novel model would validate many DC-SIGN targeting vaccination approaches. A downside of the antibody targeting approach, at least concerning DC-SIGN, is the affinity prevents the release of the antigen in endosomes and re-expression of the receptor[64,133]. An solution would be to use the natural ligands

for DC-SIGN, like Lewis antigens, to target DC-SIGN[134–137]. The AMAX platform allows the synthesis of high order structures by direct conjugation of pre-synthesized peptides. Since peptides can be easily modified to accommodate terminal glycans like Lewis Y, a glycosylated AMAX can function as DC-SIGN targeting platform (Chapter 7). The tertiary structure, however, is unpredictable and dependent on the exact sequence of amino acids. For example, while the OTI-OTII products are predicted to have relatively straight single-peptide-arms, the OTI-OTIII product has a distinct nick in the peptide structure because of a predicted α-helix. As a result, the orientation of the terminal glycan moiety is different, likely affecting the binding capacity to DC-SIGN. It is unclear how promiscuous the cell membrane-bound DC-SIGN tetramer truly is in terms of molecular weight, spacing and glycan orientation.

In summary, several novel vaccine components were tested with each their own benefits and drawbacks. This begs the question: Do we know enough about the highly complex immune system to construct a vaccine that does your bidding?

8.2 Beyond the three signal paradigm

Recent vaccinology has been trying to build vaccines based on our fundamental knowledge of immune responses, with increasing help of systems biology (Chapter 1.3). However, proper T cell-mediated protection is coordinated far beyond the principles of antigen-TCR engagement, co-stimulation, and priming cytokine production (ie. the three-signal paradigm). Indeed, many cells are involved in the DC-mediated T- and B cell response in lymph nodes and this needs to occur in the correct space and time[138–140]. While we are starting to understand the steps involved in adaptive immune activation, using this knowledge to tailor immunity in space and time can be notoriously difficult.

8.2.1 Spatiotemporal dynamics of infection and vaccination

Systemic tissue alert | An elegant study using two poxvirus strains with either vaccinating (modified Vaccinia Ankara (MVA)) or pathogenic (Western Reserve (WR)) properties investigated the immune response in an organism-wide manner[141]. In both mouse models, 17 tissues were collected at 11 different time-points after infection/injection for organism-wide gene expression profiling. Pathogenic intranasal WR infection showed increased viral load in many organs across the organism within 2 days with death after 8 days. However, when mice were subcutaneously immunized with the highly similar MVA a week before, mice survived intranasal WR infection, even though the same organs were initially infected two days after intranasal WR challenge[141]. The subcutaneous MVA attenuated infection caused an organism-wide upregulation of type I interferon genes, suggestive of an immediate "alert" response to prevent systemic infection. Other studies have shown similar systemic effects that "prime" the host to subsequent infections[142,143]. Interestingly, temporally-controlled parabiont studies showed that upon MVA injection tissue-resident memory (Trm) $CD8^+$ T cells spread across tissues to confer host protection. Hence, the attenuated MVA strain conferred organ-wide protection through the upregulation of type I interferon systemically and generation of long-lived tissue-resident effector cells, which can prevent future infection. Once infection retracts, several factors, including local antigen competition makes sure the resident memory niche is retained[144]. The infection itself causes inflammation, which drives antigen sensitivity in memory $CD8^+$ T cells irrespective of antigen-specificity, thus preparing these cells for cytotoxicity in the case of low amount of cognate antigen expressed[145]. Therefore, the type and timing of inflammation is key in the recall response that provides host protection. This is especially important since the recall response to viral infection seems to heavily rely on $CD8^+$ Trm cells[143,146]. The role and induction of $CD8^+$ and $CD4^+$ Trms in vaccination is only recently being explored and will undoubtedly affect rational vaccine design[147–152].

Microbiome | Another example of how distant sites may influence inflammation and vaccine efficacy is demonstrated by changes in the microbiota. For example, mice that lack a microbiota show major defects in activated and memory $CD8^+$ T cells because of impaired metabolism. Microbiota-derived

short chain fatty acids like butyrate enhances a metabolic switch needed for proper activation and memory development, which is absent in germ-free mice[153]. Indeed, the role of the gut microbiome is increasingly gaining interest in the field of vaccinology[154–156].

Finding infection | Another dimension of control of infection can be illustrated in the host defense against brain infection caused by Toxoplama gondii. T. gondii is an intracellular protozoan that is primarily subclinical, but may cause life-threatening encephalitis in immunocompromised patients[157]. The resistance to encephalitis in immunocompetent humans and mice was shown to be dependent on IFNγ produced by $CD8^+$ T cells infiltrating the brain[158–162]. How $CD8^+$ T cells in the brain clear T. gondii was elegantly shown in a study by Harris and colleagues. Using *in vivo* imaging approaches and bioinformatics modelling, the search strategies of $CD8^+$ T cells patrolling the brain was investigated. $CD8^+$ T cells were found to rely on the cytokine CXCL10 for 1) maintenance during infection and 2) increasing the migration speed in search for infected cells. More importantly, CXCL10 changed the search strategy of $CD8^+$ T cells in the infected brain from Brownian (stochastic motion) to Lévy walks (long distance jumps) with interspersed Brownian motion[163,164]. This type of search strategy is assumed to be especially efficient when the target is sparse and distributed unpredictably. In fact, predators like sharks switch from Brownian hunting patterns to Lévy patterns when the availability of prey becomes sparse[165,166]. Hence, the immune system has adapted local control of search strategies of adaptive immune cells to most efficiently battle pathogens (expertly reviewed by Krummel and colleagues[167]).

Aging immunity | Lastly, both the resistance to infection and the efficacy of host protection through vaccination decreases as people age[168–170]. Especially the elderly are susceptible to influenza and Streptococcus pneumonia and would greatly benefit from preventative vaccines[171]. The risk of developing influenza after immunization was highest among the old subjects, which showed a lack of T- and B cell responses[172]. Also, vaccination to pneumococcal pneumonia showed significantly impaired IgA and IgM responses in older subjects[173]. Although immune aging affects most parts of the immune system[174], it has been suggested that particularly $CD4^+$ T cell functioning is responsible for the decline in responsiveness to vaccination[175]. Memory $CD4^+$ T cells accumulate during aging relative to naïve $CD4^+$ T cells[176,177], which is hypothesized to be in part due to thymic involution[178,179]. With age, the capacity of the thymus to produce naïve T cells, capable of responding to novel infections, reduces[179,180]. However, thymic involution has been shown to be partially reversible by treatment with thymostimulatory cytokines (like keratinocyte growth factor, interleukin 7 and growth hormone) and androgen blockade, restoring thymic T cell output[179]. Perhaps unsurprisingly, it has been suggested that aging of the immune system is not a passive process, but is instead influenced by lifestyle[181]. As people are getting older and the number of elderly increases, the impact on society will increase and vaccine design will need to take age into account[182,183].
In summary, protective immunity and therefore vaccination is highly coordinated in space and time. This presents new challenges for future approaches to vaccine design. However, as the number of critical variables increase, the paradigm of reverse vaccinology or rational vaccine design may become more challenging to justify.

8.2.2 Protective immunity is a matter of life and death

Not every infection leads to a full-blown systemic immune response with all parts of the adaptive immune system activated. Immune responses are costly processes in terms of energy and nutrient expenditure, as well as risk of auto-immunity and collateral tissue damage. Therefore, the magnitude and duration of the effector phase of the immune response has evolved to minimize cost and optimize protection[184]. These factors are dependent on the initial size and the persistence of the infection and its virulence factors[185]. In this sense, several factors have been identified to scale the microbial threat and tailor the appropriate response[185]. In fact, the viability of the pathogen determines a major part of the immune response, via viability-associated PAMPs (vita-PAMPs)[186]. Not only does the immune system discriminate between live and dead microbial matter, it also affects vaccine efficacy[186].

Indeed, the success of live vaccines compared to killed counterparts is now thought to arise from the presence of these vita-PAMPs[187,188]. Mechanistically, prokaryotic RNA from viable bacteria (which was absent in dead bacteria) leaked from phagosomes of innate immune cells to the cytosol where it activated the NLRP3 inflammasome and induced the production of IL1β[187]. Importantly, dead bacteria induced antigen-specific class-switched IgG production after vaccination only when supplemented with prokaryotic RNA[187]. This response was dependent on the detection of bacterial RNA by TLR8-expressing DCs, resulting in follicular helper T cell differentiation and high affinity B cell responses[189]. Another vita-PAMP has since been identified. STING can sense cyclic-di-adenosine monophosphate (c-di-AMP), a bacterial second messenger protein[190]. C-di-AMP acts as a vita-PAMP in phagocytes by binding STING, resulting in endoplasmic reticulum (ER) stress, inactivation of mTOR and induction of autophagy[191]. Additionally, a type I IFN response was initiated, which was blunted whenever any step of c-di-AMP availability to STING expression, ER stress, mTORC1 activity, or autophagy was modulated[191]. Since even fish seem to distinguish between live and dead bacteria in their immune response, the vita-PAMPs may very well be an evolutionary adaptation to ensure sensing of real danger[192]. However, it should be mentioned that in the case of innate immune activation by prokaryotic RNA, pathogenic and non-pathogenic bacteria induced a similar response[187], suggesting that so-called "danger" signals are not defined at this stage and probably needs additional PRR stimuli. Nonetheless, the need for a viability signal for a mature adaptive immune response is an important finding for vaccine design[188].

8.2.3 Evolution and protective immunity, a place for rational vaccine design?

The immune system does not distinguish between pathogen or commensal, evolution does

Vaccines and adjuvants have mostly been tested empirically with a minimum of a priori assumptions about the precise workings of the desired adaptive immune response[14]. However, empirical testing of undefined vaccines is becoming increasingly difficult or expensive and side effects of vaccination are increasingly less tolerated by the public (Chapter 8.2.4). Instead, recent efforts in vaccine design have used a different approach; reverse vaccinology, rational vaccine design or vaccinomics. Indeed, the technological and bioinformatics advances of the last decade have changed the way antigens and vaccines are viewed and used[193,194]. Now, the whole genome of a pathogen can be sequenced and bioinformatics can make reliable predictions on the immunogenicity of antigens expressed within the organism for vaccination purposes. However, as we have argued, there is more to the initiation of the adaptive immune response to a pathogen or vaccine than just the antigen. Rational vaccine design would require complete knowledge on the rules that govern successful host protection, which are only just being understood.

Firstly, most successful vaccines are based on the generation of antigen-specific antibodies and therapeutic efficacy during vaccination programs is measured as antibody titers to the antigen. Predicting immunogenicity of non-linear B cell epitopes based on amino acid sequences (similar to T cell epitopes) is still very difficult, with most algorithms marginally performing above random[195–197]. This can in part be explained by the lack of known rules that define the tertiary structure of proteins, the dimensions to which most antibodies respond. Indeed, the tertiary molecular structure of an antigen is defined by protein folding, a process that is difficult to predict from amino acid sequence[198]. While beyond the scope of this thesis, current bioinformatic methods that incorporate deep machine learning algorithms might shed light on this process in the near future[199–202]. Nonetheless, once potentially immunogenic B cell epitopes could be predicted after whole exome sequencing, T cell-help and affinity maturation still needs to take place for B cells to class-switch and become able antibody-producing cells (Chapter 1.2.3).

Secondly, our knowledge on dendritic cells as initiators of immune responses suggests that monocyte-derived DCs (moDCs) are not the main driver of adaptive immune responses[203–205]. Paradoxically, moDCs are thought to be crucial for successful vaccination, via their recruitment to the vaccination

site or lymph node and presentation of antigen to B- or T cells[75]. As such, there is discrepancy between the role of DCs upon vaccination or infection and initiating protective immunity that allows rational decisions in vaccine design beyond empirical testing.

Thirdly, the boundary between pathogen and commensal is still largely undefined, apart from the definition of whether a microbe causes harm. In fact, both pathogens and commensals initiate immune activation in a flexible continuum that defy clear distinctions in terms of adaptive immune response[206–209]. This fits with the hypothesis that the immune system did not evolve as a strict set of immunological rules, but as a "survival of the fittest" where host-microbiota interactions have shaped humans as an holobiont[210–212]: assemblies of different species that form one ecological unit[213]. The human as holobiont is already shaping the way pharmacological research is designed[214]. Vaccines will need to catch up and abandon the oversimplified and misleading "danger" hypothesis that proposes an "on or off" switch of innate immunity and control of adaptive immunity.

Until the rules for the initiation of protective immunity are clear, rational vaccine design will be destined to repeat itself to the point of empirical testing. Then why rational vaccine design or reverse vaccinology and not sensible empirical evidence? Society expects protection without side effects driven by vaccines containing highly defined components.

8.2.4 Vaccines in the public eye

Refusal of vaccines by parents due to social media, Dunning-Kruger effects and epistemic self-reliance; a new kind of cheater?

Modern day vaccine design faces more challenges than just scientific ones[215]. It is clear that prevention far outweighs treatment of the disease, both in terms of monetary costs and human suffering[216,217]. The economic burden of vaccine-preventable diseases in the US was estimated at $9 billion per year[218]. These are costs associated with diseases for which a vaccine is available, so almost 80 percent of these costs are due to unvaccinated invididuals[218]. The financial costs of epidemics for which there are currently no vaccines are enormous. For example, the financial losses of airline companies during the global SARS epidemic was over $7 billion, while the global reduction in GDP was $33 billion[219]. The SARS epidemic lasted a year and was fatal to 916 people, scaling it as a relatively small global health crisis. In comparison, the 1918-19 Spanish flu would (by estimation) reduce the world's current economic output by almost 5% and cost more than $3 trillion[219]. To put these costs in perspective, it was recently estimated that the costs for progressing at least one vaccine through to the end of phase 2a for each of the major 11 epidemic infectious diseases* would cost a minimum of $2,8-3,7 billion per disease[220]. Both people and pathogen vectors (like malaria mosquitoes) transverse national borders[221–224], making pandemics a world-wide problem. Indeed, global vaccine-development funds have been proposed, but remain difficult due to different risk assessments, governmental prioritization or a lack of incentive because the market is considered too small[225]. While these cost-benefit comparisons are difficult to completely objectify and weigh, it is clear prevention far outweighs the costs of treatment, even in the case human suffering does not offer sufficient justification.

Nonetheless, a growing group of parents refuse to vaccinate their children, a phenomenon known as "vaccine hesitancy". Since unvaccinated individuals put communities risk of disease[226], interventions for reducing parental vaccine hesitancy are critically needed[227]. In fact, understanding vaccine hesitancy has become an international priority, as the World Health Organization outlined in the global vaccine action plan 2011-2020[228]. Vaccine hesitancy is largely defined as the "delay in acceptance or refusal of vaccination despite availability of vaccination services"[229]. It is a highly complex phenomenon but was modeled by the "Three Cs"; Confidence, Complacency and Convenience[229]. Confidence is defined as the trust in the therapeutic efficacy of vaccines, the health system (physicians, pharmaceutical

*Crimean Congo hemorrhagic fever, chikungunya, Ebola, Lassa, Marburg, Middle East respiratory syndrome coronavirus, Nipah, Rift Valley fever, severe acute respiratory syndrome, severe fever with thrombocytopenia syndrome, and Zika.

companies etc) and the motivation of policy-makers on vaccination programs. Complacency is the lack of perceived risk of vaccine-preventable diseases and lack of personal and societal health responsibilities. Convenience is the physical/geographical availability, affordability, ability to understand and the appeal of vaccines. While the factors influencing the three Cs is far beyond the scope of this thesis[230–232], several notable influences are becoming increasingly important to "vaccine hesitancy" in the Western world.

Firstly, vaccine hesitancy is largely a decision making problem and can as such, at least at the individual level, be described by psychological and cognitive models[233]. While decision making on vaccination generally would require the consideration and incorporation of information concerning vaccination, the availability, sources and reliability of such information is diverse and rapidly changing[234]. In particular, people are increasingly moving from "controlled" forms of mass media (for example, newspapers) towards uncontrolled social media like Facebook and Twitter[235,236]. On social media, so-called "opinion leaders" or "influencers" with a significant following affect the way information (even incidental) is consumed and incorporated[237]. Moreover, highly active users of social media are more likely to consider themselves opinion leaders and show increased efforts to try and persuade the opinions of others[238]. Also, social media has immense influence when certain posts are going "viral", an exponential sharing of a single article/post among social media users that reaches millions within a timespan of hours. For example, a parent intended to travel with their child posted on social media that certain airline companies secretly vaccinate passengers through the air conditioning system, which was widely shared by others[239]. While the sharing may not necessarily be in agreement with the content of the post, fictitious information is allowed to thrive and likely affects the opinion of many. One study in Italy found an inverse correlation between MMR vaccine coverage and internet search activity, Facebook posts and tweets (posts of Twitter)[240]. In the US, negative representation of vaccines by over 250.000 tweets sent over two years concerning HPV vaccine-related information affected the acceptance and coverage of vaccines[241]. Finally, an analysis of 153 YouTube videos on immunization showed that videos disapproving vaccines were more viewed, liked and shared[242]. Negative vaccine sentiment on social media is framed around skepticism and distrust of government organizations that communicate scientific evidence supporting positive vaccine benefits[243,244]. These studies show that social media can act as a breeding ground and echo chamber for misinformation and conspiracy theory, but also provides health professionals with a unique platform to reduce vaccine hesitancy[245–248]. Indeed, traditional educational tools like information pamphlets appear to have little or no effect on vaccine hesitancy, or in some instances even reinforced vaccine hesitancy[249]. For example, pregnant women presented with a website containing vaccine information and interactive social media components positively influenced vaccination rates of their infants, compared to usual care[250]. Pro-vaccination messaging, however, needs to be carefully approached, as a study reported that parents receiving images of sick children increased expressed belief in a vaccine/autism link and a dramatic narrative about an infant in danger increased self-reported belief in serious vaccine side effects[251]. As societies are becoming increasingly connected and influenced by social media, vaccination sentiments need to be carefully monitored and addressed[252,253], in order to rightfully gain public credibility and trust[254,255].

Secondly, educational levels of parents have also been implicated as contributing to vaccine hesitancy. A number of studies have reported that parents with less formal education have greater distrust in the medical community and their vaccines, and have less belief in the necessity and efficacy of vaccines[256–259]. For example, parents with less than a total of 12 years education were more likely to report a lack of information about vaccines, compared to parent with graduate education[259]. This allows the influence of less reliable sources on parents with limited education. Interestingly, anti-vaccine sentiments can partly be explained by the Dunning-Kruger effect[260]; being ignorant of one's own ignorance[261]. The psychologists Dunning and Kruger found that less intelligent/educated people overestimated their test performance and overall competence[262]. Importantly, these same subjects lacked the metacognitive capacity to recognize and judge their own lack of competence. Improving

their test performance helped them recognize the limitations of their abilities[262]. In the study from Motta et al., a third of participants responded to know as much or more on the cause of autism than doctors and scientists. This overconfidence is highest among those with low levels of knowledge about autism and is associated with increased support for the role that non-experts play in the policymaking process[260]. It seems a significant part of vaccine hesitant individuals are ignorant of their own ignorance. Of note, a similar phenomenon was observed in extreme opponents of genetically modified foods[263]. Anti-vaccinationists also tend towards low cognitive complexity in thinking patterns, flaws in deductive reasoning and a habit of substituting emotional anecdotes for data[264]. Still, it may not only be a lack of intelligence or education in "vaccine hesitant" parents, but also the lack of theoretical training to incorporate, assess and judge a larger amount of knowledge. Indeed, individuals are generally poor in distinguishing their own knowledge from the knowledge that resides in the community they belong to, resulting in overestimating their own knowledge[265]. However, vaccine hesitancy is also prevalent in parents with high levels of education[266,267]. Higher education cultivates epistemic self-reliance or intellectual autonomy; the belief in ones capacity to govern knowledge and constantly question the status quo. Perhaps, the refusal to adhere to a epistemic authority (a physician or scientist for example) by educated individuals is misplaced self-reliance concerning vaccines[268,269] *. This would argue for the lack of trust in authorities of knowledge and policymakers, a recurrent criticism on present-day vaccination programs.

Thirdly, the highly individualistic nature of Western culture may foster lower levels of collectivist behavior[270], like prosocial vaccination. For example, a large-scale experiment showed that participants with a collectivistic cultural background showed more prosocial vaccination motivation compared to participants with a individualistic cultural background[271]. Social aspects of vaccination are critical, since protection is only guaranteed when the frequency of vaccinated individuals reaches disease-specific herd immunity thresholds[272,273]. Interestingly, a Belgian study found that the social orientation of "vaccine hesitant" individuals is more tuned to individualistic inequality[274]. Nonetheless, in cultures that lack prosocial cultural background that motivate vaccination as a social decision, communicating the prosocial benefits of "herd immunity" results in improved willingness to vaccinate[275]. Herd immunity is a collective good and relies on the moral obligation of the individuals participating in that collective. However, problems arise when parents have to decide whether to vaccinate their children, since they have moral obligation to the safety of their children[276]. In this regard, herd immunity is a "public good" in the sense that it is not possible to exclude someone from benefitting from herd immunity once it is established. As a result, some parents argue their moral obligation to protect their children from risk of harm is high enough to free ride the benefits imposed by others through herd immunity[276]. This conflict has driven collectives to consider mandatory vaccination[277] with the reasoning that participation in the benefits of a society comes with the moral obligation and sacrifice for the greater good[278,279]. As Poland et al. put it: "Ultimately, society must recognize that science is not a democracy in which the side with the most votes or the loudest voices gets to decide what is right."[280] However, mandatory vaccination has previously been shown to increase polarization between government and vaccine-skeptics[277,281–284].
These arguments are only part of the debate surrounding "vaccine hesitance" and excludes important influences like religious, geographic and socioeconomic differences[229,232]. Also, vaccine hesitance is not a problem from the last decade per se[280], but with the advent of technology, widespread accessibility of information and increased individualism/entitlement, the paradigm has shifted and will require continuous attention. Vaccine design is ultimately futile if trust in the science is lost.

* The idea of the authority on knowledge and its role in intellectual self-reliance and the necessity of conscientious self-reflection is expertly argued by Linda Trinkaus Zagzebski, in "Epistemic Authority: A Theory of Trust, Authority, and Autonomy in Belief" 2012, ISBN-13: 9780199936472

8.3 The role of academia in vaccinology§

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them

W.L. Bragg

8.3.1 Academic and pharmaceutical operating framework in vaccine design

Vaccine designϕ is distinct from exploratory research in the fact that the outcome of the experiment has a predetermined quality. The goal is an increase in immunity of any form, or in other words; the outcome is positive only if the vaccine works. This is fundamentally different to exploratory research where the outcome of the experiment is of worth, regardless of the outcome, as long as the experimental approach is solid. For example, investigating whether amyloid β has a role in Alzheimer's disease is inherently worthwhile, because both outcomes of the research are of interest. It is the knowledge that counts (Figure 1). In contrast, if an "in house"-made synthetic vaccine does not perform according to the hypothesis, there is no interest or follow-up in "why it does not perform". Indeed, the number of unknown variables that generate the complexity of the immune system is poorly suited for empirical testing if the goal is to understand why a candidate vaccine does not perform; what would be the null-hypothesis? Whereas academic science is designed to increase any form of knowledge, vaccinology is designed to cure disease.

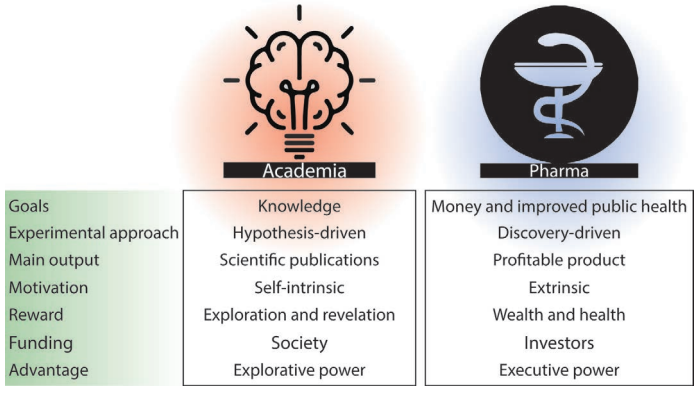

Figure 1 | Operating framework of academia and pharma The academic system and pharmaceutical companies (pharma) exhibit trivial (though not mutually exclusive) requirements to scientific conduct and success. Of note, in academia the requirements are mainly applicable on single scientists, whereas in pharma the requirements mainly apply to the overarching company.

The difference in scientific approach to vaccine design versus the search for knowledge can have a big impact on the motivation of the individual doing the experimentation. Motivation of scientists has been shown to be mostly self-intrinsic, the individual's desire to adhere to their own internal standards, competencies and values[285–287]. Similarly, both high school students and graduate students that score high in intrinsic traits like self-efficacy and self-determination perform better[288,289]. How does this relate to the difference in scientific approach to vaccine design and knowledge gathering? Perceived self-efficacy is defined as people's beliefs in their capabilities to produce desired effects by their actions[290]. Self-efficacy is developed by repeated experiences of perceived control, resulting in increased confidence[291–293]. For example, a scientist performing a typical vaccination experiment will perceive very little control over the success of the experimental outcome, since the variables of the complex immune system are unknown. In contrast, a scientist probing one variable of a complex and

§ I do not aim to extrapolate my viewpoint from a scientific subdiscipline or personal experience to the whole scientific community nor to make definitive *ex cathedra* statements about what research should be. However, befitting a discussion, interpretations, implications, limitations and recommendations are appropriate, may be freely challenged and enhance dialogue beyond a mere summary.
ϕ Defined as "product design and testing", not antigen discovery, which adheres more to the regular search for knowledge.

unknown immune system in an experiment may perceive control, because the goal is not success, but knowledge on the single testable variable. This difference is additionally illustrated by determining the sequence of experiments that are required to complete a line of research. Whereas the string of experiments for vaccine design is linear (chemistry, *in vitro*, *in vivo*, clinical) and irrespective of the outcome of intermediate experiments, the experiments for scientific knowledge are not predetermined and depend highly on intermediate results. Put differently, there is only one outcome for successful vaccine design, while the outcome of probing a scientific question is often unknown. As such, perceived control of scientists in the outcome of vaccine design is limited, resulting in decreased self-efficacy in the scientific method and potentially reduced motivation. In addition, the predefined experimental design in vaccine design abrogates the need for creativity. This is not trivial, since increases in creative self-efficacy corresponds with increases in creative performance[294], a critically important factor in problem solving and science[295,296]. In fact, increasing creativity in problem solving requires providing explicit strategies that promote cognitive flexibility, not constraint[297], as is the case for predetermined product testing. Especially in the case of young scientists, the bigger picture is less clear and short-term self-efficacy may be more important. Management styles of group leaders that promote independence and the ability to explore novel ideas increase perceived autonomy, which enhances self-efficacy and motivation[298].

Thus, is vaccine design without creativity? Absolutely not, vaccine conferences display a wealth of novel ideas. However, the testing is often linear and even regulated by legislation in terms of approval for clinical use. Also, the approach of pharmaceutical companies is more readily screening of expansive libraries of candidate compounds through rigorous empirical testing. Using a systematic approach through wide parallel screening of vaccine candidates is only feasible for pharmaceutical companies whose main drive is wealth (Figure 1). Of note, financial incentives for scientists decreases the level of research performance[286]. The end result is that pharmaceutical companies will vigorously test and only invest in candidates that are truly showing promise, since the monetary loss of pursuing non-viable leads increases exponentially with time[220]. An academic scientist, however, needs to publish a story, which will most likely be the most successful version of a compound that is difficult to compare to similar compounds, since it was never tested in the first place. Perhaps both the intrinsic motivation (driven by self-efficacy) and the publications as main output of academic science do not provide the proper conditions for vaccine design and testing. So what does the academic sciences provide and how is it able to do this?

8.3.2 Funding and expectation of applied research in academia; a charity case

Science provides a unique viewpoint to the world, build on empirical evidence that allows modelling of the truth so the world becomes predictable upon manipulation. However, academic science does not provide any direct or calculated societal gain and can therefore be viewed as a charity case. There is an historical "contract" between science and charities (or society) that science produces reliable knowledge, provided it communicates its findings back to society[299]. Academia in principle is provided with autonomy to engage in science and teaching, although this is based on the trust of the agreement[299]. Initially structured as a top-down structure, society has increasingly been involved in scientific conduct and its directions[299–301]. Science funding is becoming more defined as "science for the masses" and scientists are adapting by playing on the needs of the masses[302]. Unsurprisingly, a vaccine or cure of an untreatable infectious disease or cancer is among those needs and is increasingly incorporated in research plans as a goal. However, vaccine development takes an average of at least 10 years from pre-clinical discovery to market entry, with a 94% fail-rate[303]. With an average cost between $165-289 million, any academic scientist applying for grants promising the next vaccine is dreaming. In fact, the major source of cost inflation of vaccine development is the indirect costs associated with different levels of experience in the organizations developing the vaccine[220]. Therefore, the requirements of cost-effective development of vaccines is best done by experienced pharmaceutical companies within the pharma framework instead of creative academic scientists (Figure 1). Still, scientists get funding with initially big claims and likely cannot (or will not) live up to the overstated impact, ultimately

leading to loss of trust in science and a gap between society and academic scientists.
Academic science has a unique position as it is not tied by the capitalistic framework of pharmaceutical companies that require direct or credible return of investment. As such, there are some important counter arguments to be made for academic science when therapeutic vaccination is concerned, especially in the form of anti-tumor vaccines (see also Thesis Part II). Cancer patients diagnosed with immune-suppressed tumors can be treated through immune-(re)activating biologics, which find their origin of discovery in fundamental knowledge from academic research. For example, the understanding of the PD1 inhibitory receptor constitutively expressed by virally exhausted T cells led to the discovery of similarly exhausted tumor-specific T cells (Chapter 9). Blocking these molecular processes (that keep T cell suppressed) using antibodies (termed immune checkpoint blockade; ICB) reactivated exhausted T cells in mice and later in cancer patients, leading to tumor eradication and revolutionizing cancer therapy[304]. However, more than a decade later, we still do not fully understand the complete effect of these blocking antibodies in both mice and humans, regardless of the initial hypothesis that led to clinical testing. Similarly derived from academia, the expansion of tumor-derived T cells *ex vivo* for infusion back into the same patient (passive therapeutic vaccination) has led to impressive clinical responses[305,306]. In both cases, pharmaceutical companies made clinical trials possible that were initially based on academic research. Hence, where the operating frame work of academia may provide unique explorative power, pharma has the executive power to drive the translational value of academic discoveries (Figure 1).
There are several factors to consider these impressive academic discoveries in the light of vaccine design. First, cancer patients are a group of patients with an immediate medical need and new therapeutic approaches can often be tested as a last resort. This is distinctly different from prophylactic vaccine development, where threats to health are less visible or immediate, healthy volunteers are required and side effects are of higher concern. Second, through our fundamental understanding of a cascade of abnormalities underlying a certain disease, we can hypothesize that removing a part of the pathological cascade will reduce or disrupt the disease. By analogy, removing one fundamental building block of a tower (disease), the whole tower collapses. However, understanding the position of every building block of a tower to build one (rational vaccine design), requires significantly more knowledge and testing. Third, the high prevalence of cancer in Western countries drives immediacy in public opinion towards anti-cancer therapies and subsequently funding. Lastly, and perhaps the most telling example is that of prophylactic human papillomavirus (HPV) cancer vaccination. Cervical cancer is caused by the HPV in virtually all cases and is the second most common cause of cancer-related death among women worldwide[307]. HPV non-infectious subunit vaccines, containing virus-like particles from the L1 major capsid protein of the virus, have shown extraordinary clinical efficacy in preventing cervical cancer[308–310]. However, the coverage of national HPV immunization programs is inadequate due to low public acceptance[311,312], although the vaccination effects are clearly reducing the incidence of cervical cancer cases[313,314]. It is plausible the public is more tuned towards curing immediate illness instead of the prevention of the "chance" of disease, regardless of all the before mentioned downsides to curing compared to preventing. Academia seems suited to gain insight in understanding disease and pathology, which may lead to novel ways of interrupting existing disease processes and the development of novel cures. The realm of disease prevention (prophylactic vaccination) may be more difficult to leverage in a rational manner within the constraints of academia. Instead, academia may invest more in early detection of viable intellectual property (IP) that justifies additional funds and translational efforts. The translational value of their research may seem limited to academic researchers and requires alternative perspectives. In house IP scouts may not only generate additional funds from protected IP, but also provide the translational value that is expected by society, without devaluating the explorative power of academia.
In summary, there is clear merit for academics in testing novel therapeutic approaches based on a fundamental understanding of disease processes, but the goals should be realistic and overzealous claims should be frowned upon. Society will need to understand that science is not a grocery shopping list and that science can be unpredictable, regardless of the brilliance of the scientist or rigor of his/her experimental approach. In this regard, when academic scientists take their responsibility as teachers of

society, expectations can be managed and the gap between society and academia may decrease. The gap between the explorative power of academia and the executive power of pharma may be bridged by experienced IP scouts capable of evaluating fundamental research results. Hopefully, academic research may regain the explorative power that has enabled revolutions like genetic engineering, electricity or X-ray radiography. As Jean-Claude Petit put it: "Actual breakthroughs, true discoveries, unpredictable and radical changes of world views can only emerge from fundamental research"[315]

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