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General introduction
THE IMMUNE SYSTEM.
The immune system is an organization of cells and molecules that is specialized in defending
the organism against pathogens such as bacteria, viruses, worms, fungi, and yeasts. In addition,
the immune system is also capable of eradicating malignant cells. To fight these pathogens
and malignant cells, two fundamentally different immune responses work intimately together:
the innate (natural) immune response, and the acquired (adaptive) immune response.
The innate immune response is the first line defense that readily takes action after infection
and causes an inflammatory reaction. The innate immune system consist of all the immune
cells that lack immunological memory such as: phagocytic cells (neutrophils, macrophages,
and monocytes); cells that release inflammatory cytokines (basophils, mast cells, and
eosinophils); and natural killer cells. The innate immune response is mediated by germ-line
encoded receptors and is therefore genetically predetermined. These receptors have defined
specificity and recognize conserved structures, so called pathogen-associated molecular
patterns that are shared between large groups of micro-organism (1). The best known
pattern-recognition receptors that can bind these conserved structures and subsequently
activate the immune system are the Toll-Like Receptors (2,3) of which already 11 receptors
have been characterized in mice (4,5).
The adaptive immune response possesses immunological memory and consists of
B- and T cells. By means of unique recombination processes, it has been estimated that
B- and T cells are capable of producing about $10^{15}$ different unique receptors from only 400
different genes, each. With this extremely diverse repertoire, the acquired immune response
ensures specific recognition of any foreign antigen. The main action of B cells is to excrete
neutralizing antibodies to clear viruses and bacteria (humoral response). T cells recognize
small protein fragments (peptide) from extracellular or intracellular proteins and T cells can
either provide help in the induction of an immune response or can kill infected or malignant
cells (cellular response). The ligand for T cells is peptides that are presented as a complex
with the major histocompatibility complex molecules on the cell surface.
Upon activation, B- and T cells divide, rapidly increase in numbers and acquire effector
mechanisms that endow them to clear infections or malignant cells. When the infection is
cleared, immunological memory is established that will ensure rapid activation of the immune
system upon re-challenge.
WHAT DO T CELLS RECOGNIZE?

CD4+ and CD8+ T cells.

Naive T cells can be subdivided into two classes: CD4+ and CD8+ T cells. CD4+ T cells can be further subdivided into T helper 1 (Th1), Th2 and T regulatory (Treg) T cells. Th cells recognize predominantly extracellular Antigen (Ag) in Major Histocompatibility Complex (MHC) class II molecules presented by professional Antigen Presenting Cells (APC), such as Dendritic Cells (DC) and B lymphocytes. Th2 cells can activate B cells to produce antibodies (Ab), while Th1 cells can have several functions for instance: activation of DC via CD40-CD40Ligand (CD40L) ligation; they are great sources of inflammatory mediators (cytokines); or activation of macrophages to clear intracellular pathogens. The function of Treg cells is to dampen the immune response to prevent over activation and to avoid auto-immunity.

CD8+ Cytotoxic T Lymphocytes (CTL) are killer T cells that recognize small peptides presented in MHC class I molecules that are expressed on all nucleated cells of the body. These peptides are derived from intracellular proteins and the CD8+ T cells can thereby recognize infected or malignant cells. Killing of these target cells is mediated by targeted release of granzymes, perforins or activation of a death receptor on the target cells (Fas) that subsequently induces apoptosis (6).

Cytotoxic T lymphocytes recognize short linear peptide sequences presented on the cell surface.

Until the publication of Townsend et al. 1984 (7) it was generally thought that the CD8+ T cells recognized Ag in their native conformation on the cell surface of target cells. In an elegant system - in which they transfected fibroblast cells with either the Hemagglutinin or the Nucleoprotein (NP) DNA of influenza virus - they showed that cells that expressed native glycoprotein Hemagglutinin on the cell surface were not recognized and lysed by CTLs. Only cells that expressed the intracellular NP were susceptible for CTL lysis. Most importantly, the NP positive cells that were recognized by the CTL did not express any surface native NP protein at all. Townsend et al. suggested that proteins were proteolytically degraded in the cytosol and that these fragments were presented on the cell surface that could then be recognized by CTLs (7,8). In 1986 they actually demonstrated that CTLs recognized short linear peptide sequences instead of the 3-dimensional protein structure (9) and by fine mapping of the protein they revealed that the CTL sequence was about 16 amino acid (a.a.) in length (9).
T cell epitope prediction.

In order to determine the exact peptide length and peptide sequence, the group of Rammensee used acid-elution to elute peptides from the MHC class I molecules (10-12). They showed that the minimal sequence of MHC class I was not 11-16 a.a., as was published by Townsend et al. (8), but was much shorter - 8 a.a in length (11). They found that, despite different genetic background of the cells, similar peptides of a given protein are presented if these cells share a MHC class I molecule. This provided evidence that the MHC class I molecule is the main factor that determines which peptide of a given protein is presented on the cell surface. Sequencing of all the eluted peptides revealed that there are MHC allele-specific motifs that determine which peptides will (strongly) bind to the MHC class I molecules (13).

The knowledge of the restriction patterns of peptides for MHC class I binding resulted in the rapid identification of CTL epitopes. Straightforward scanning of the protein sequences revealed: i) the first bacterial CTL epitope from Listeria Monocytogenes (14), ii) the dominant epitope of the chicken egg Ovalbumin (OVA) OVA_{257-264} (15), and iii) epitopes for the hen egg lysozyme protein (16). However, identification of the primary a.a. sequence turned out to be not sufficient per se, as different peptides were found that did not meet the criteria (16) or predicted CTL epitope sequences that did not induce any CTL responses (17).

ANTIGEN PRESENTATION BY DENDRITIC CELLS TO T CELLS

Antigen scavenging and antigen presentation by Dendritic Cells.

Immature DC are the gatekeepers of the body and are therefore lined in the periphery at the barriers of the body – such as skin and mucosa (in the lungs and gut) – places that are easily accessed by invading pathogens. Immature DC are specialized to bind and engulf all kinds of pathogens and Ag formulations (18). Upon endocytosis of pathogens, the immature DC will be activated and migrates to the draining LN (dLN) where it enters the paracortical area where the T cells reside (19). During that migration the ability of the DC to take up Ag is decreased while on the other hand the machinery to process Ag is enhanced (18). Proteins from ingested pathogens, dying virally infected cells, or tumor cells are degraded by the DC’s endosomal and lysosomal proteases and results in the production of small peptides (20). When the endocytic compartments fuse with the lysosomal compartments, the peptides can associate with newly synthesized MHC class II molecules, which are subsequently transported to the cell surface for presentation to CD4+ Th cells.

CD8+ T cells recognize peptides that are bound to MHC class I molecules. Because most viruses do not infect DC and most of the exogenous Ag from dying cells is presented in the MHC class II route, activation of CD8+ T cells requires a different mechanism called
cross-presentation (21). Ag that is taken up by the DC via endocytosis can leave the endosomal compartment and enters the cytosol. In the cytosol the proteins are proteolitically cleaved by the proteasome into small peptide fragments that are subsequently transported via the Transporters of Ag Processing to the Endoplasmatic Reticulum (ER). In the ER these peptides are further trimmed to allow exact binding into the groove of the MHC class I molecule after which the peptide:MHC class I complex is transported to the cell surface. This route ensures presentation of Ag from the periphery such as self proteins, proteins derived from pathogens (e.g. viruses), and aberrantly expressed proteins or neo-proteins from tumor cells, to CD8+ T cells in the dLN (22).

Dendritic Cell activation and maturation.

DC can be activated in several distinct ways. The commonly shared structures of pathogens (pathogen-associated molecular patterns) can interact with its cognate receptor and thereby activate the DC. These pathogen-associated molecular patterns can consist for instance of DNA (23), RNA (24-26), lipoproteins and cell wall proteins of the pathogen (27-30). Additionally, uric acid is a major endogenous danger signal that is released from injured or dying (cancer) cells and can also induce DC activation (31). Moreover, activation via CD40 receptor ligation by CD40L on Ag-specific Th cells in the dLN (32,33) or by agonistic CD40 Ab to activate DC (34-36) will endow the DC with the ability to cross-present exogenous Ag to CD8+ T cells (37). Finally, DC can be activated by soluble inflammatory mediators such as TNFα, IL-1β, and PGE-2 whose secretion is triggered by invading pathogens (18,38). Following activation, DC enhance the Ag processing pathways, increase MHC class I and II presentation and induce co-stimulatory molecules on the surface of the DC to facilitate optimal T cell activation and T cell expansion (38).

The requirements for T cell activation.

Naïve T cells that enter the LN from the blood and subsequently migrate to the paracortical area to scan DC for their cognate Ag, require three signals from the DC for optimal priming (Fig. 1 and reviewed in (39)): i) peptide:MHC complexes on the APC that interact with the TCR on the T cell, ii) co-stimulatory molecules on the APC such as B7 that ligate with the CD28 receptor on the T cell, and iii) additional cytokines such as IL-12 and IFNα/β that function as a third signal to enhance T cell survival (40,41). Although efficient T cell activation can occur in the absence of co-stimulation, it will require high concentrations of Ag to overcome the lack of co-stimulation (42,43). Co-stimulation, however, reduces the need for high amount of peptide/MHC complexes in order to activate naïve T cells and in addition, it results in the stabilization of IL-2 mRNA to allow transcription into IL-2 protein
General introduction

Figure 1. Three signal model for T cell activation. T cells require three signals for optimal activation: 1) TCR triggering on the T cell by MHC/peptide complex presented by the APC, 2) CD28 ligation on the T cell by the co-stimulatory molecules B7.1 and/or B7.2 on the APC, and 3) cytokines like IL-12 and IFNα/β secreted by the APC to enhance T cell survival.

(44-46) - an important growth hormone required by CD8+ T cells (47,48).

In the absence of strong inflammatory responses the DC will not upregulate co-stimulatory molecules, nor produce the necessary inflammatory cytokines. Ag presentation in the absence of co-stimulatory molecules will therefore result in the incomplete activation of the T cell and will lead to either a state of specific unresponsiveness termed anergy (associated with impaired intracellular signaling) or will lead to apoptosis (programmed cell death) (49); collectively called peripheral tolerance (50).

THE IMMUNE SYSTEM, CANCER, AND PEPTIDE VACCINATION

The role of the immune system in tumor clearance.

The first indication that the immune system could be induced to fight cancerous tissue was seen in the 18th century. Spontaneous tumor regression was sporadically observed in patients that had high fever. Occasionally complete remission could be induced by deliberately infecting cancer patients with the bacteria Streptococcus pyogenes, also know as Coley’s toxin (51). In the 1950s the role of the immune system in cancer was further explored. It was shown that unknown items of the tumor - tumor associated Ag (TAA) - were recognized by T cells. Mice could be rendered immune against syngenetic transplanted carcinogenic tumors when the primary (carcinogenic) tumor was excised and the mice were challenged
with the parental tumor (52-55). By the use of depleting Antibodies (Ab), it was shown that several immune cell type such as: natural killer cells (56), neutrophils (56), macrophages (56), eosinophils (57), CD4+ Th2 cells (57), or CD8+ T cells (58,59) have the ability to clear malignant cells. Eventually, in the 1990s the first immunogenic human and mouse TAA were identified (60-64). This knowledge about what the T cells actually recognize, resulted in a gain in momentum in the applicability of tumor specific immunological therapies, of which peptide vaccinations is one of the best explored so far.

**Synthetic peptide vaccines.**

The first synthetic peptide vaccine that induced virus-specific CD8+ T cells *in vivo* was a peptide derived from lymphocytic choriomeningitis virus (LCMV) (65). Soon two papers followed that showed that peptide vaccination protected mice against a lethal virus challenge of either Sendai virus (66) or LCMV (67). In addition, peptide vaccination also protected mice against a lethal tumor challenge of a Human Papilloma Virus (HPV) type 16 positive tumor (68), or enhanced the survival of mice in a spontaneous metastasis model (69).

The immunogenicity of a peptide is determined by the affinity of the peptide for its MHC molecule (70-73) and its dissociation constant (74). Together these parameters influence the duration of Ag presentation on the APC and thereby the time of TCR triggering. Most TAA have relatively low affinity and higher dissociation constants for the MHC class I molecule compared to most viral Ag and are therefore less immunogenic. Using site directed substitution of specific a.a. positions in the peptide, the affinity of the peptide can be enhanced and the dissociation can be reduced for the MHC class I molecule, resulting in higher half-life of the peptide:MHC complex. Vaccination with these enhanced binding affinity peptides were shown to give protection against a subsequent lethal tumor challenge in two different Ag systems in mice (75) and were reported to increase T-cell immunity in melanoma patients vaccinated with a modified gp100 peptide (76-80).

**Tolerance induction after synthetic peptide vaccination.**

Although minimal CTL peptide vaccines were reported to induce CTL responses and give protection against lethal tumor challenges, there were also publications about the lack of CTL responses and even data showing the induction of CD8+ T cell tolerance. For instance, vaccination with the minimal CTL peptide of the Murine leukemia virus (MuLV) did not protect mice against a subsequent MuLV+ tumor challenge (81). Moreover, multiple high dose of minimal CTL (GP33-41) peptide vaccination of the GP33 protein of LCMV resulted in the induction of CD8+ T cell tolerance (82) or the deletion of GP33-41 specific Transgenic CD8+ T cells (83). Although multiple vaccinations with high doses of GP33-41 peptide seemed
to be required in the LCMV system to induce tolerance (82,83), Toes et al. demonstrated that in the Adenovirus system, already a single injection of low Ag dose with the minimal Adenovirus CTL peptide was sufficient to induce CD8+ T cell tolerance or to enhance tumor outgrowth (84,85). Despite these potential dangerous effects of minimal CTL peptide vaccination, many clinical trials have been initiated, so far with limited immunological and clinical responses (86).

Help for the Cytotoxic T Lymphocyte.

The low magnitude of CD8+ T cell responses can be enhanced by inclusion of a Th epitope in the same vaccine. This helper mechanism is versatile, CD4+ T cells; i) can secrete the growth hormone IL-2 (87), ii) can activate the APC via CD40-CD40L interactions (34-36) that can subsequently license (88) the CD8+ T cell to kill its target cell (Fig. 2) and, iii) are required for the induction of CD8+ T cell memory (89). The first synthetic peptide vaccine that protected against a lethal challenge with LCMV (65) not only contained a CD8+ T cell epitope, but also a CD4+ T cell epitope (90). Deletion of the CD4+ T cells almost completely abrogated this protective effect, showing the importance of the concomitant activation of CD4+ and CD8+ T cells. Accordingly, addition of a Th peptide to a minimal CTL peptide enhanced the CTL

![Figure 2. Licensing of the Antigen Presenting Cell.](image)

Immature APC can take up pathogens that will activate the APC via triggering of the TLR (left). Proteins derived from the pathogen are presented in the context of MHC class I and II. Ag-specific CD4+ T cells are activated by the APC and subsequently activate the DC via CD40-CD40L interactions (left). This "licensed" APC can activate naive CD8+ T cells to kill their target cells (right).
response in a malaria vaccination system (91). Similarly, the addition of the MuLV Th peptide in combination with the minimal MuLV CTL peptide protected mice against a subsequent MuLV+ tumor challenge, while mice vaccinated with just the minimal CTL peptide died from the tumor (81). Although it was claimed that the T helper response had to be tumor Ag-specific (81), this tumor specific Th-dependence theory has been challenged by others (92). Most likely the differences in peptide binding affinity of these specific or unspecific Th peptides were the cause of the enhanced tumor clearance; resulting in enhanced CD4+ T cell responses and cytokine secretion, rather than that the Th peptides had to be derived from the tumor Ag.

Help can also be provided in the form of an agonistic CD40 Ab that triggers the CD40 receptor on the DC (34-36). Using CD40 agonistic Ab, a prophylactic vaccine - HPV16 E7 minimal CTL peptide - was altered into a vaccine with therapeutic tumor potency (93). Also for the minimal CTL peptide of the E1A protein of Adenovirus (84,85) addition of CD40 agonistic Ab enhanced the Ag-specific CD8+ T response (94).

**Increased peptide length.**

Zwaveling et al. have published that the use of a long peptide based vaccine of the HPV16 E7 protein, induced a more robust CTL response capable of eradicating a pre existing tumor compared to the minimal CTL peptide (95). In this paper it was hypothesized that the additional advantage of the use of long peptide-based vaccines compared to minimal CTL peptide-based vaccines might be the differences in dependency of Ag processing. While minimal CTL peptide can exogenously bind to any cell that contains MHC class I molecules, it was suggested that long peptides are taken up and processed by professional APC in order to be presented in the context of MHC class I molecules. In addition, the T cell epitopes in long peptides are protected by the N- and C-terminal flanking a.a. from direct proteolytically degradation and thereby prevent destruction of the epitope. Finally, the enhanced CTL response in the long HPV16 E7 peptide could have been due to the existence of a Th epitope in the peptide (95), as co-delivery of the Th and CTL epitope to the same APC results in APC activation (36) and has been shown to be more effective than vaccination with the minimal Th and CTL epitopes as a mix (96,97).

**Toll Like Receptor ligands as adjuvant.**

Besides APC activation by CD4+ T cell via CD40-CD40L interactions, pathogens possess strong immune stimulatory molecules that can trigger Toll Like Receptors (TLR). These TLR are widely expressed on immune cells, especially on APC. Except for TLR9, which is in humans restricted to plasmacytoid DC and B cells, the expression of TLR3, 4, 7, and
8 is broadly expressed on myeloid DC in both human and mouse DC (98-100). These TLR ligands can be made synthetically and can be used in various vaccination forms to enhance the (vaccine induced) immune response by ways of: mixing a TLR ligand with the Ag of interest (101,102), using TLR ligand-cream mixed with peptides (103), or covalently linking the TLR ligands to the Ag of interest (104-107). Besides that covalently linking the TLR ligand to the Ag results in enhanced uptake of the Ag-TLR complex (104-107), another great advantage of covalently linkage is the simultaneous delivery of Ag and the activation signal to the same APC, comparable to physically linking of the Th and the CTL epitopes (96,97,108). Therefore, these chemically designed and well defined vaccines hold great promises for a variety of vaccine formulations against multiple diseases.

SCOPE OF THIS THESIS

In this thesis we have explored the mechanisms of minimal and extended CTL peptide-based vaccines to induce an optimal immune response, without the risk for tolerance induction. Minimal CTL peptide vaccinations are widely used to induce a CD8+ T cell response in cancer patients, however, without the expected success.

In chapter 2 the work is described on the difference between long and short-peptide based vaccinations. We have used the Ovalbumin (OVA) antigenic system to investigate the long-term effects of minimal CTL peptide vaccination after application in oil-in-water formulation; Incomplete Freund’s Adjuvant (IFA). The reason we chose for this type of adjuvant is that it is the most standard vehicle used in clinical vaccinations strategies. Vaccination with the minimal (8 a.a.) CTL peptide-based vaccine in IFA induced a transient CD8+ T cell response that is readily tolerized at day 30. Tolerance induction could, however, be prevented by the addition of a minimal Th peptide or using an extended (30 a.a.) CTL peptide that was CD4+ T cell independent.

In Chapter 3 the mechanisms is discussed of the enhanced immunogenicity of extended peptide-based vaccines compared to their minimal counterparts in the OVA system after vaccination in combination with the TLR9 ligand CpG (in saline). The use of an extended peptide resulted in an increased duration of in vivo Ag presentation and induced a greater CTL response compared to the minimal CTL peptide. In addition, the extended peptide was presented predominantly by DC in the local draining LN, whereas the minimal CTL peptide was presented by DC, B and, T cells in the dLN and was transported by B and T cells to non-draining LN. Furthermore, B cells turned out to be important APC in the induction of CD8+ T cells after minimal CTL peptide-based vaccination.

In chapter 4, a head-to-head comparison of different TLR compounds and a CD40 agonistic Ab is discribed using the HPV16 E7 extended peptide-based vaccination model (95). We
have compared the differences in potency of inducing CD8+ T cell responses with enhanced in vivo effector function using these adjuvants. Only the addition of MPL, CpG, or CD40 agonistic Ab to the vaccine (in saline) was able to induce comparable high magnitudes of the CD8+ T cell response. However, CpG was the only adjuvant that induced fully functional CTL that correlated with the simultaneous induction of Ag-specific CD4+ IFNγ+ T cells.

In the 5th chapter the work is described on delineating the underlying molecular and cellular mechanisms of the superior working mechanism of a TLR2 or TLR9 ligand coupled to an extended peptide, compared to the uncoupled TLR ligands and peptide. We have shown that Ag coupling to a TLR ligand enhanced immune activation and showed that this required endosomal acidification and proteasomal cleavage. Surprisingly, the enhanced uptake of the TLR-ligand peptide conjugate was independent of expression of the cognate TLR. Together, these data show that simultaneous entry of antigen and delivery of a maturation signal to the same DC is responsible for the improved action of the TLR-ligand peptide conjugates.

In chapter 6 we have described the anti-cancer potential of a mouse line deficient for Cbl-b. Cbl-b is a member of the mammalian family of Cbl E3 ubiquitin ligases and functions as a negative regulator of antigen-specific T cell activation. Cbl-b is consequently upregulated in T cells that are stimulated in the absence of co-stimulation and Cbl-b is therefore a critical mediator of T cell anergy. Cbl-b deficient mice spontaneously reject a lethal dose of HPV16 E7 positive tumors and CD8+ T cells were identified as the key players. Loss of Cbl-b not only enhanced the anti-tumor reactivity of CD8+ T cells but also uncoupled in vivo anti-tumor immunity from CD4+ T cell help. Importantly, therapeutic transfer of naïve Cbl-b-/- CD8+ T cells was sufficient to mediate rejection of established tumors.

Finally, an overall view of the design and development of synthetic peptide vaccines is discussed in chapter 7.

REFERENCE LIST

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