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Mechanistic studies on long peptide based vaccins for the use in cancer therapy.

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Mechanistic studies on long peptide-based vaccines for the use in cancer therapy

Mechanistic studies on long peptide-based vaccines for the use in cancer therapy

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Cover: Top view of a MHC class I molecule with the OVA₂₅₇₋₂₆₄ peptide in the peptide binding groove. Picture has been made by Drs. Wouter Pos with the use of the program 'pymol' and data from Fremont et al., PNAS 1995 .

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In the long run
the pessimist
may be right,
but the optimist
has a better
time on the trip.

Daniel L. Reardon

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1

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) OVA₂₅₇₋₂₆₄ (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 Endoplasmic 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 TNF α , IL-1 β , 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 IFN α/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

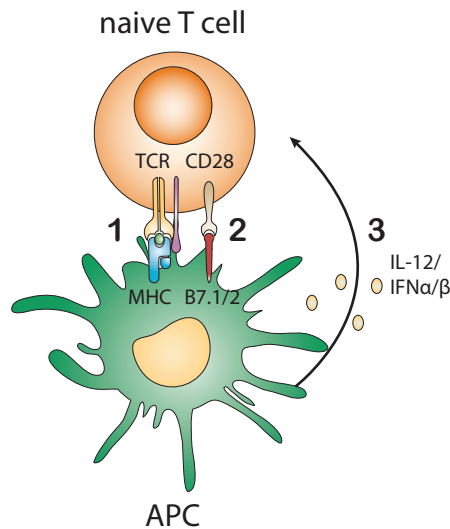


Figure 1. Three signal model for T cell activation. T cells require three signals for optimal activation: 1) TCR triggering on the T cell by MHC/peptide complex presented by the APC, 2) CD28 ligation on the T cell by the co-stimulatory molecules B7.1 and/or B7.2 on the APC, and 3) cytokines like IL-12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

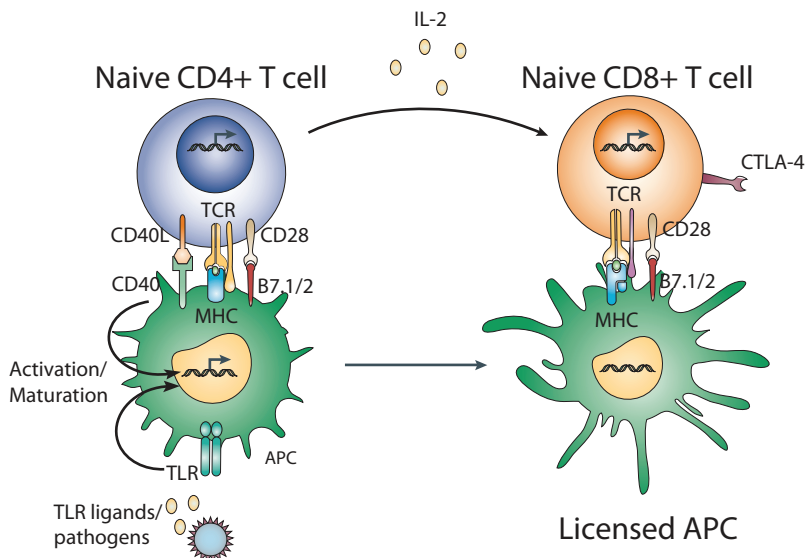


Figure 2. Licensing of the Antigen Presenting Cell. Immature APC can take up pathogens that will activate the APC via triggering of the TLR (left). Proteins derived from the pathogen are presented in the context of MHC class I and II. Ag-specific CD4⁺ T cells are activated by the APC and subsequently 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 described 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⁺ IFN γ ⁺ 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.

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CD8+ T cell tolerance

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CD8⁺ CTL priming by exact peptide-epitopes in Incomplete Freund's Adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity

Abstract. Therapeutic vaccination trials, in which patients with cancer were vaccinated with minimal CTL peptide in oil-in-water formulations, have met with limited success. Many of these studies were based on the promising data of mice studies, showing that vaccination with a short synthetic peptide in IFA results in protective CD8⁺ T-cell immunity. By use of the highly immunogenic Ovalbumin CTL peptide in IFA as a model peptide-based vaccine we investigated why minimal CTL peptide vaccines in IFA performed so inadequate in order to allow full optimization of peptide vaccination.

Injection of the minimal MHC class I-binding OVA₂₅₇₋₂₆₄ peptide in IFA transiently activated CD8⁺ effector T-cells, which eventually failed to undergo secondary expansion or to kill target cells, as a result of a sustained and systemic presentation of the CTL peptides gradually leaking out of the IFA depot without systemic danger signals. Complementation of this vaccine with the MHC class II-binding T-helper peptide (OVA₃₂₃₋₃₃₉) restored both secondary expansion and *in vivo* effector functions of CD8⁺ T-cells. Simply extending the CTL peptide to a length of 30 amino acids also preserved these CD8⁺ T-cell functions, independent of T-cell help, because the longer CTL peptide was predominantly presented in the locally inflamed draining lymph node. Importantly, these functional differences were reproduced in two additional model-antigen systems.

Our data clearly show why priming of CTL with minimal peptide-epitopes in IFA is suboptimal and demonstrates that the use of longer versions of these CTL peptide-epitopes ensures the induction of sustained effector CD8⁺ T-cell reactivity *in vivo*.

INTRODUCTION

Tumor cells express tumor associated antigens (TAA) on their cell surface in the context of MHC class I molecules. These TAA can be recognized by CD8⁺ T-cells, which are the essential players in the clearance of tumor cells *in vivo* (1,2). In recent years many epitopes have been identified (3,4), providing the option to apply these minimal MHC class I restricted CD8⁺ T-cell peptide-epitopes in vaccination strategies for the immunotherapy of solid tumors (3,4). Unfortunately, these minimal CTL peptide-based vaccines in oil-in-water formulations (e.g. IFA or Montanide) have in general met with limited immunological and clinical success (4,5).

The use of minimal CTL peptide-based vaccines was based on murine studies demonstrating that prophylactic vaccinations with minimal CTL peptides in IFA induced protective CD8⁺ T-cell (anti-tumor) immunity (6-9). However, other reports show that vaccination with the minimal CD8⁺ T-cell epitopes can also result in the induction of CD8⁺ T-cell tolerance (8,10-14). While in some of these studies tolerance was the result of repetitive vaccination with high dose of Ag (8,13,14), others reported that even after a single subcutaneous vaccination, tolerance could be induced (11,12). In these studies the injection of mice with low amounts of Adenovirus E1A (11) or E1B (12) minimal CTL peptide emulsified in IFA resulted in functional impairment of activated CD8⁺ T-cells, as was evident from the enhanced outgrowth of tumors (11,12). Intravenous co-injection of agonistic CD40 antibody (FGK), in order to activate and to mature dendritic cells (DC) (15,16), resulted in a transient adenovirus-specific CD8⁺ effector T-cell response, detectable 10 days after vaccination but not at 30 days after vaccination, and did not protect mice against a tumor challenge (17). These data indicate that an initial proper activation of the CD8⁺ T-cell response by minimal CTL peptide vaccines in IFA does not ensure long-term effectiveness of these CD8⁺ T-cells. Such long-term effectiveness is particularly important to control chronic diseases such as cancer.

So far it is unclear what the common long-term result is with respect to immunological outcome of injection with minimal CTL peptides. Therefore, we have thoroughly studied different peptide vaccinations strategies using the highly immunogenic model Ag Ovalbumin (OVA) (18-22), containing the MHC class I restricted CD8⁺ T-cell epitope OVA₂₅₇₋₂₆₄ (OVA8) and the CD4⁺ T helper (Th) cell epitope OVA₃₂₃₋₃₃₉ (ThOVA17). Peptides were mixed in IFA, as oil-in-water formulations are standard vehicles for peptide vaccination in clinical trials. The efficacy of the different peptide vaccine formulations was tested by the analysis of the following three important parameters: i) the magnitude of the CD8⁺ T-cell response, ii) the ability of CD8⁺ T-cells to undergo secondary expansion upon antigen challenge *in vitro* (23), and iii) the *in vivo* killing capacity of the CD8⁺ T-cells. These parameters were tested at

either day 10 (peak of the response) or at day 30 after vaccination - the time point where adenovirus-specific CD8⁺ T-cells were tolerized after injection with E1A-peptide in IFA (17). Furthermore, we investigated how to formulate a peptide-based vaccine that triggers CTL immunity without the risk for exhaustion/tolerance.

Here, we report that vaccination with immunogenic OVA8 peptide in IFA also results in the activation of effector CD8⁺ T-cells which eventually ceased to expand or to kill target cells. This cessation of T-cell function was associated with long-term systemic presentation of the minimal CTL peptide *in vivo* but could be prevented by either complementation with a minimal T helper peptide or by extension of the minimal CTL peptide to a 30 amino acid long peptide. These data clearly shows the potential hazard of using minimal CTL peptide vaccines, and the benefits of long peptide vaccines for the induction of an effective CD8⁺ T-cell response.

MATERIALS AND METHODS

Mice. C57BL/6 (B6; H-2^b) and MHC Class II knock out mice (class II^{-/-}) mice were purchased from Charles River (St. Germain sur l'Arbresle, France). CD90.1 mice were bred at TNO-PG (Leiden, the Netherlands). CD45.1 OT-1 (24) and OT-2 (25) mice are CD8⁺ T-cell and CD4⁺ T-cell TCR transgenic (Tg) mice expressing the TCR α -chain and β -chain recognizing OVA₂₅₇₋₂₆₄ in H2-K^b and OVA₃₂₃₋₃₃₉ in I-A^b, respectively and were bred at the Leiden University Medical Centre animal facility. All mice were kept at the Leiden University Medical Centre animal facility and used at 8–14 weeks of age in accordance with national legislation and under supervision of the animal experimental committee of the University of Leiden.

Cell lines. EG7 (EL4-OVA) (26) tumor cells were cultured in IMDM (Invitrogen Life Technologies, Rockville, MD) supplemented with 8% (v/v) FCS (Greiner), 50 μ M 2-mercaptoethanol, 2 mM glutamine, 100 IU/ml penicillin (complete medium) and 400 μ g/ml Geneticin (Gibco).

Peptides and peptide vaccination. Peptides were generated as described before (6). The following dominant minimal CTL peptides were used: OVA₂₅₇₋₂₆₄ SIINFEKL (OVA8), HPV16E7 peptide E7₄₉₋₅₇ RAHYNIVTF (HPV9) (6) and the Adenovirus protein E1A₂₃₄₋₂₄₃ SGPSNTPPEI (E1A10) (11). The minimal Th peptide sequence of OVA was the following; OVA₃₂₃₋₃₃₉ ISQAVHAAHAEINEAGR (ThOVA17). In addition the following long peptides deduced from the natural sequence of each protein were used: CTL peptide OVA₂₄₁₋₂₇₀ SMLVLLPDEVSGLEQLESSIINFEKLTEWTS (OVA30). Note that this peptide does not contain

the C-terminal Th epitope OVA₂₆₅₋₂₈₀ (27). Th peptide OVA₃₁₇₋₃₄₇ SSAESLKISOAVHAAH AEINEAGREVVGSAE (ThOVA31), CTL peptide of HPV protein E7₄₃₋₇₇ GQAEPDRAH YNIVTECCCKCDSTLRLCVQSTHVDIR (HPV35) (28), CTL peptide of protein E1A₂₂₃₋₂₅₂ RECNSSTDSCDSGPSNTPPEIHPVVRLCKK (E1A30). Mice were s.c. vaccinated with 40 nmol of (each) peptide admixed in PBS or in PBS and IFA (Difco Laboratories, Detroit, USA) (50% v/v) in a total volume of 200 µl.

Antibody treatment. Agonistic CD40 Ab (FGK45) (50 ug) was provided intravenously (i.v.) in the tail vein on day 0, 1 and 2 in 200 µl of PBS. Complete CD4⁺ T-cell depletion was obtained by i.p. injection of 25 µg of anti-CD4 (clone GK1.5) in PBS three days and one day before vaccination and once a week throughout the experiment. CD4 depletion was regularly checked by FACS analysis and showed that the mean percentage of CD4⁺ T-cells was $\leq 0.003\% \pm 0.005\%$ in the mice receiving GK1.5, while naïve mice displayed $13.8\% \pm 2\%$ of CD4⁺ T-cells.

CFSE labeling and adoptive transfer of Tg T-cells. Single cell suspension was made from spleen and peripheral lymph nodes of CD45.1 OT-1 mice or OT-2 mice. Erythrocytes were lysed by ammonium chloride treatment and 10×10^6 cells/ml were incubated at 5 µM CFSE end concentration in PBS 0.1%/BSA at 37° C for 15 minutes. The reaction was blocked with 10% v.v. of pure FCS. The cells were washed 2 times with PBS and $1-2 \times 10^6$ Tg T cells were injected into the tail vein in 200 µl of PBS.

Ex vivo detection of Ag. Mice were vaccinated with either OVA8, OVA30, ThOVA17 or ThOVA31 mixed in IFA. Two days later the dLN (inguinal and axillary) and the ndLN (mesenteric) were isolated. A single cell suspension was made using a 70µm cell strainer. To detect the CTL epitope *ex vivo* 0.5×10^6 LN cells were plated in a 96 wells plate and to detect the Th epitope, 1×10^6 LN cells were plated in a 96 wells plate. Purified and CFSE-labeled OT-1 (1×10^5) or OT-2 (2×10^5) Tg cells were added to these wells, respectively. Three days later division of OT-1 Tg T cells was measured by flow cytometry by gating on CD45.1⁺ and CD8⁺ lymphocytes. In the case of OT-2 cells, four days later division was determined by gating on Va2⁺ and CD4⁺ lymphocytes. Ag presentation was determined by the dilution of the CFSE of the Tg T cells.

In vivo cytotoxicity assay. Erythrocytes of B6/CD45.2 splenocytes were lysed and the splenocytes were split into two equal fractions. Cells were differentially labeled with CFSE to either 5 (target) or 0.5 (control) µM end concentration (see CFSE labeling). The target

cell population was pulsed with 1.0 ug/ml of OVA₂₅₇₋₂₆₄ and the control population with 1.0 ug/ml of p53 peptide (H2-K^b, p53₁₅₈₋₁₆₆) at 37° C in complete medium for 60 minutes. The cells were washed four times with PBS before the two populations were mixed in a 1:1 ratio and a total of 8*10⁶ cells was injected i.v. Either 1 or 2 days after injection of the target cells (day 10 or day 30 after vaccination, respectively) spleens were removed and analyzed for specific killing. The ratio of CFSElo/CFSEhi cells was determined by flow cytometry by gating on CD45.1⁺ lymphocytes. Specific killing of OVA₂₅₇₋₂₆₄ pulsed (CFSEhi) target cells was calculated as follows: $(1 - [(CFSEhi/CFSElo)_{vaccinated} / (CFSElo/CFSEhi)_{naive}]) * 100\%$.

In vitro stimulation. EG7 cells were incubated with 50 ug/ml of mitomycin C (Kyowa) in complete medium at 37° C for 1 hour. Subsequently, the cells were washed 4 times and then irradiated (4000 RAD). 1*10⁶ EG7 cells were incubated with 10*10⁶ splenocytes (V_{tot}=2 ml). After seven days viable splenocytes were isolated over a ficoll gradient and stained for H-2K^b Tetramer (TM)-OVA₂₅₇₋₂₆₄, CD8b2, and Propidium Iodide (to exclude dead cells).

Overnight Intracellular Cytokine Staining. Splenocytes were incubated for 1 hour with 1 μM of ThOVA17 or ThOVA31 peptide or no peptide (background) before 1 μg/ml Golgiplug (containing Brefeldin A; BD Pharmingen) was added. The next day cells were permeabilized and stained using the Cytofix/Cytoperm Plus kit (BD Pharmingen), according to manufacturer's instructions and stained for CD4 and intracellular IFNγ.

Flow cytometry. Single cell suspensions of spleens or lymph nodes were stained in PBS 0.1% BSA. The Abs that were used were the following: directly allophycocyanin-conjugated; TM-OVA₂₅₇₋₂₆₄; CD45.1 (A20, eBioscience); IFNγ (XMG1.2, Pharmingen); CD90.2 (53-2,1, Pharmingen); CD8a (53-6.7, Pharmingen) and PE-conjugated; CD8b2 (53-5,8, Pharmingen), CD4 (RM4-5, Pharmingen). Data acquisition and analysis was done on a BD Biosciences FACScan (San Jose, CA, USA) with CellQuest software.

Statistical analysis. Statistical analysis was done using GraphPad InStat software (version 3.0) and GraphPad Prism 4 (GraphPad Software, San Diego, CA). A two-tailed t test with Welch correction was applied for the statistical analysis of the samples, except for Fig. 1A OVA8 (IFA) d=10 vs. d=30 (non-parametric 2-tailed Mann-Whitney test) and Fig. 7 and 8 (Kruskal Wallis test non-parametric ANOVA).

RESULTS

Vaccination with the minimal CTL peptide OVA₂₅₇₋₂₆₄ in IFA induces a transient and then functionally impaired CTL response.

To explore the short and long-term outcome of the CD8⁺ T-cell response after peptide vaccination with the minimal MHC class I binding peptides, mice were vaccinated with OVA8 in IFA and subsequently the secondary expansion potential of the CD8⁺ T-cells *in vitro* as well as the *in vivo* effector function were determined, either 10 days (short-term) or 30 days after vaccination (long-term). In addition a strong DC stimulus was provided by i.v. injection of FGK in order to systemically activate APC *in vivo* (15,16,29).

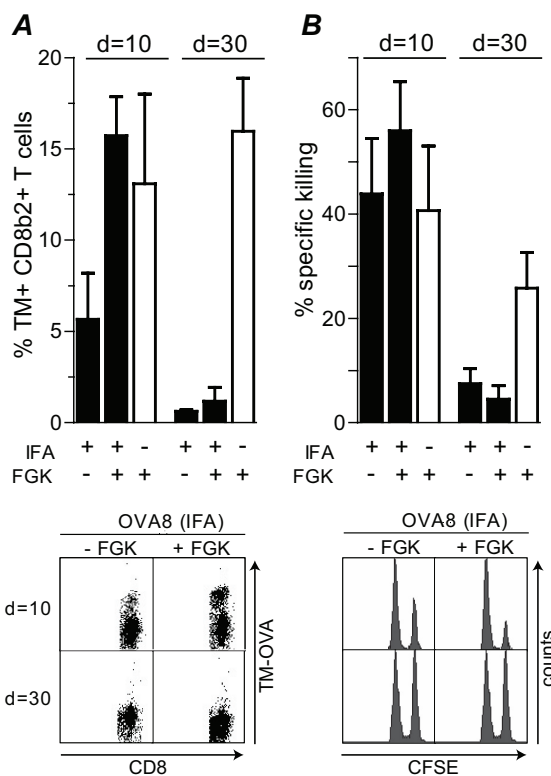


Figure 1. Minimal CTL peptide in IFA induces only transient CTL immunity. Mice were vaccinated s.c. with the minimal OVA8 CTL peptide mixed in IFA (black bars) or in PBS (white bars) in the presence or absence of 50 µg of FGK administered i.v. on day 0, 1 and 2. A, Enumeration of TM-OVA⁺ CD8⁺ T-cells by flowcytometry. Ten or 30 days after vaccination, splenocytes were harvested and stimulated *in vitro* with OVA-expressing tumor cells for seven days before analyzing the presence of TM-OVA⁺ CD8⁺ T cells (OVA8 (IFA) d=10 vs. d=30, $p < 0.01$; OVA8+FGK (IFA) d=10 vs. d=30, $p < 0.001$). The data are depicted as mean $\pm$ SEM ($n=5-7$). B, *In vivo* cytotoxicity assay. Nine or 28 days after vaccination, CFSE-labeled CD45.1⁺ target cells were injected and cytotoxicity was measured in the spleen at day 10 or day 30, respectively by FACS analysis (OVA8 (IFA) d10 vs. d30, $p < 0.01$; OVA8+ FGK (IFA) d10 vs. d30, $p < 0.01$). Cells were gated on CD45.1⁺ lymphocytes. The data are depicted as mean $\pm$ SEM ($n=6-10$). Representative Facs plots of the experiment are shown below in the figure.

Splenocytes were collected 10 days after vaccination and stimulated with OVA-expressing APC *in vitro*. The OVA-specific CD8⁺ T-cells in these cultures were capable of undergoing secondary expansion *in vitro*, as measured by high numbers of TM-OVA⁺ CD8⁺ T-cells (Fig. 1A, left). In order to determine whether vaccination resulted in effector CD8⁺ T-cells, an *in vivo* cytotoxicity assay was performed (30). Both groups of mice vaccinated with OVA8 peptide in IFA (+/- FGK) displayed a similar capacity to kill target cells *in vivo* 10 days after vaccination (Fig. 1B, left).

When splenocytes were isolated 30 days after vaccination, OVA-specific CD8⁺ T-cells displayed a strongly decreased capacity to undergo secondary expansion since only low numbers of OVA8-specific (TM-OVA⁺) CD8⁺ T-cells could be observed after stimulation *in vitro* (Fig. 1A). Accordingly, mice showed a markedly reduced capacity to kill target cells *in vivo* at day 30 (Fig. 1B), indicating that the ability to undergo secondary expansion *in vitro* correlated with the ability to lyse target cells *in vivo*. Provision of a DC stimulatory signal by FGK, although enhancing CTL levels at day 10, was unable to preserve the CTL response at day 30 (Figs 1A+B). Interestingly, when the OVA8 peptide was applied in PBS in combination with systemic administration of FGK, the CD8⁺ T-cell response was not lost over 30 days as indicated by their ability to expand after *in vitro* stimulation (Fig. 1A) and their capacity, albeit at lower levels, to kill target cells *in vivo* (Fig. 1B). Thus, vaccination with the minimal OVA8 CTL peptide in IFA induces a transient effector CD8⁺ T-cell response, followed by functional impairment of these activated CD8⁺ T-cells.

Peptide vaccination in IFA induces long-term presentation of the minimal CTL peptide.

The major difference between PBS and IFA is the capacity of the latter formulation to function as a depot for the peptides. To test whether the OVA8 peptide in IFA was presented for a long term *in vivo*, mice were vaccinated with the OVA8 peptide in IFA and after either 30 or 60 days, CFSE-labeled OT-1 CD8⁺ T-cells were adoptively transferred into C57BL/6 mice as probes to detect antigen presentation *in vivo*. Extensive proliferation of OT-1 T-cells was observed both at day 30 and day 60 (Fig. 2A) in the dLN of vaccinated mice, indicating that the minimal OVA8 CTL peptide was still presented 60 days after vaccination. In contrast, when the peptide was applied in PBS, no proliferation of OT-1 CD8⁺ T-cells was observed at day 30 (Fig. 2A). This indicated that vaccination with the OVA8 peptide in PBS was associated with relatively short duration of Ag presentation, while vaccination in IFA induced long-term Ag presentation *in vivo*.

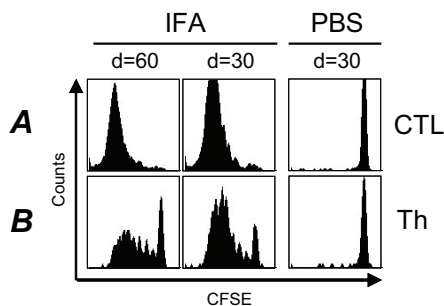


Figure 2. IFA promotes extended duration of minimal CTL and minimal Th peptide presentation *in vivo*. Mice were vaccinated s.c. with the minimal OVA8 CTL (A) or minimal ThOVA17 (B) peptide mixed in IFA. A, Either 30 or 60 days later, 1×10^6 CFSE-labeled CD8⁺ CD45.1⁺ OT-1 T-cells were i.v. injected into C57BL/6 mice. Three days later the dLN (inguinal) was harvested and proliferation was determined by flow cytometry analysis. Cells were gated on CD45.1⁺ CD8⁺ cells. B, Either at day 30 or 60, 1×10^6 CFSE-labeled CD4⁺ CD90.2⁺ OT-2 T-cells were i.v. injected into CD90.1⁺ recipient mice. Four days later the dLN (inguinal) was harvested and proliferation was determined by FACS analysis. Cells were gated on CD90.2⁺ CD4⁺ T cells. As a control, peptides were administered in PBS and antigen presentation was analyzed on day 30. The data are representative of 4 mice per time point and per vaccine.

Addition of Th OVA₃₂₃₋₃₃₉ peptide to the CTL/IFA vaccine retains CD8⁺ T-cell function.

Provision of FGK for APC activation (15,16), was unable to prevent CD8⁺ T-cell tolerance induction after vaccination with OVA8 peptide in IFA. As a dose of monoclonal Ab is generally rapidly cleared from the system (31,32), long-term presentation of OVA8 peptide beyond this period (Fig. 2) occurs in the absence of a potent APC activating agent. Since CD4⁺ T-cells can also activate APC via CD40-CD40L interaction (33), the minimal Th peptide ThOVA17 was mixed into the same IFA depot as the OVA8 peptide. Adoptively transferred CFSE-labeled OT-2 cells into ThOVA17 vaccinated mice, showed that also the ThOVA17 peptide in IFA is presented for at least day 60 *in vivo* in the dLN (Fig. 2B).

We therefore tested if vaccination with ThOVA17 peptide in IFA was able to induce long-lasting OVA₃₂₃₋₃₃₉-specific CD4⁺ T-cell responses in C57BL/6 mice. Indeed, OVA₃₂₃₋₃₃₉ specific IFN γ ⁺ CD4⁺ T-cells could be detected in all mice when tested directly *ex vivo*, 30 days after vaccination with the ThOVA17 peptide in IFA (Fig. 3A). Subsequently, we examined if addition of the ThOVA17 peptide to the IFA depot could rescue the function of OVA8 induced CD8⁺ T-cells. As shown in figure 3B, s.c. vaccination of mice with a combination of OVA8 and ThOVA17 peptide in IFA resulted in the detection of similar percentages of TM-OVA⁺ CD8⁺ T-cells following *in vitro* stimulation, both at day 10 and day 30 after vaccination. Moreover, the combination with the ThOVA17 peptide retained the cytolytic capacity of these CD8⁺ T-cells at day 30 (compare Fig 3C and Fig 1B, respectively). The addition of an extra DC-stimulatory signal, FGK, slightly enhanced the expansion of the OVA-specific CD8⁺ T-cell response (Fig.3B+C).

Injection of OVA8 and ThOVA17 in PBS, in combination with systemic injection of FGK, resulted in an OVA8-specific CD8⁺ T-cell response with a strong capacity to undergo secondary expansion but with only a moderate to kill target cells *in vivo* (Fig. 3B+C). Direct *ex vivo* analysis shows that at this time point the CD8⁺ T-cell response has contracted (% TM⁺CD8⁺ cells; 0.13 ± 0.03 vs. naïve 0.12 ± 0.04 , data not shown), with too low numbers of circulating T-cells to exert a direct strong *in vivo* measurable cytotoxic response. In contrast to the T-cells that were formed after vaccination with OVA8 in IFA, delivery of OVA8 + FGK ± ThOVA17 in PBS did not affect their potential to expand *in vitro* following one round of stimulation (Fig. 1+3).

In conclusion, simultaneous activation of T-helper cells for the sustained deliverance of license to kill signals (34), prevented premature termination of the CD8⁺ T-cell response that is observed otherwise after vaccination with OVA8 in IFA.

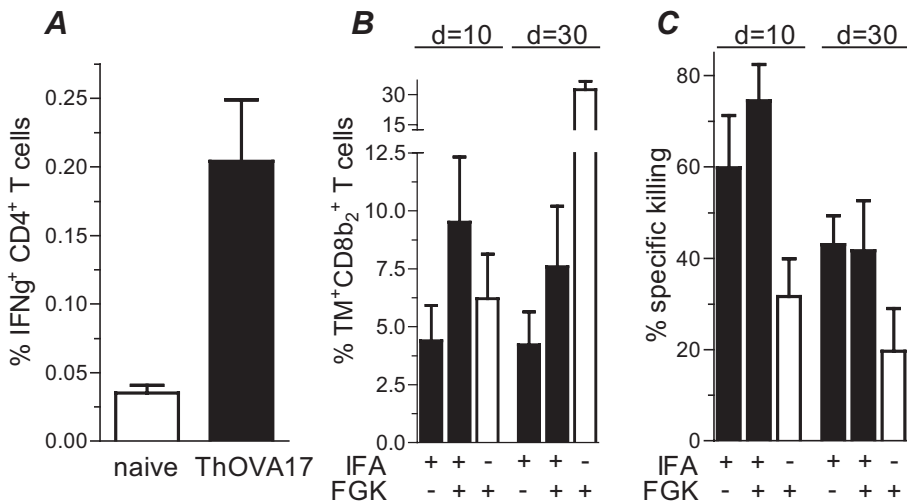


Figure 3. Long-term immunity after minimal CTL peptide plus minimal Th peptide vaccination in IFA. Mice were vaccinated s.c. with the minimal OVA8 CTL peptide and minimal ThOVA17 peptide mixed in IFA (black bars) or in PBS (white bars) in the presence or absence of 50 μ g of FGK (i.v. day 0, 1 and 2). A, Enumeration of CD4⁺ IFN γ ⁺ T-cells by intracellular cytokine staining. Thirty days after vaccination mice were sacrificed and the splenocytes were stimulated overnight in the presence or absence of the minimal ThOVA17 peptide and golgiplug. The next day the cells were stained for the surface marker CD4 and for intracellular IFN γ (n=5; naïve vs. vaccinated mice $p < 0.01$). B, Enumeration of TM-OVA⁺ CD8⁺ T-cells by FACS. Ten or 30 days after vaccination, splenocytes were harvested and stimulated *in vitro* with OVA-expressing tumor cells (EL4-OVA) for seven days and then analyzed for the presence of CD8⁺ T-cells capable of binding TM-OVA (n=5-8). C, *In vivo* cytotoxicity assay. Nine or 28 days after vaccination, target cells were injected and cytotoxicity was measured in the spleen at day 10 or day 30, respectively. Cells were gated on CD45.1⁺ lymphocytes. The data are depicted as mean $\pm$ SEM (n=6).

Long CTL peptide induces long-lasting CD8⁺ T-cell immunity independent of CD4⁺ T-cell help.

We have recently shown that long peptide-vaccination (35 a.a. long peptide) was superior to a minimal CTL peptide, with respect to the induction of the magnitude and functionality of the CD8⁺ T-cell response when vaccinated in PBS in combination with CpG-ODN (28). To study whether the functional impairment of the CD8⁺ T-cell response induced by the minimal OVA8 CTL peptide in IFA can be prevented by the use of a longer peptide in IFA, a long CTL peptide OVA30 and the long ThOVA31 peptide were designed, using the natural OVA protein flanking residues to extend the minimal CTL and Th peptides. By use of TCR transgenic detector T-cells OT-1 (CD8) and OT-2 (CD4), we confirmed that also the extended CTL (OVA30) and Th (ThOVA31) peptides were presented at least for 60 days *in vivo* in the dLN, when administered in IFA (Fig. 4A+B).

To ensure that vaccination with the ThOVA31 peptide also resulted in the induction of OVA-specific CD4⁺ T-cells, we enumerated the number of IFN γ producing CD4⁺ T-cells 30 days after vaccination by FACS analysis. The magnitude of the OVA-specific CD4⁺ T-cell response induced by the ThOVA31 peptide was comparable to what was observed after vaccination with the ThOVA17 peptide (Fig. 5A). To assess whether the use of long peptides in IFA prevented the induction of CD8⁺ T-cell tolerance at day 30, mice were vaccinated with the OVA30 peptide in the absence or the presence of the ThOVA31 peptide with or without i.v. injection of FGK (Fig. 5). In contrast to what we observed with OVA8, all the vaccine combinations with OVA30 resulted in high numbers of TM-OVA⁺ CD8⁺ T-cells after

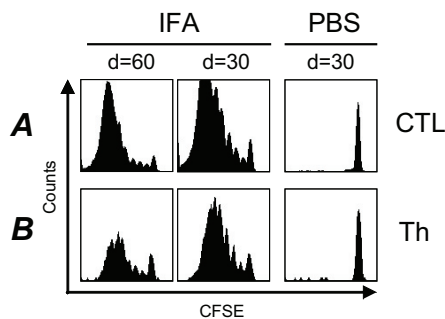


Figure 4. IFA promotes extended duration of long CTL peptide and long Th peptide presentation *in vivo*.

Mice were vaccinated s.c. with either the long CTL peptide OVA30 or with the long Th peptide ThOVA31 mixed in either IFA or PBS. A. Either 30 or 60 days later, 1.2×10^6 CFSE labeled CD8⁺CD45.1⁺ OT-1 T-cells were i.v. injected into C57BL/6 recipient mice. Three days later the dLN (inguinal) was harvested and proliferation was determined by FACS analysis gating on CD45.1⁺ and CD8a⁺ lymphocytes. B. Either 30 or 60 days later, 1×10^6 CFSE labeled CD4⁺CD90.2⁺ OT-2 T-cells were injected i.v. into CD90.1⁺ recipient mice. Four days later the dLN (inguinal) was harvested and proliferation was determined by FACS analysis. Cells were gated on CD90.2⁺ and CD4⁺ lymphocytes. As a control, peptides were administered in PBS and antigen presentation was analyzed on day 30. Representative figure is shown of 4 mice per time point and per vaccine.

stimulation *in vitro* at day 30, indicating that the CD8⁺ T-cells were capable of undergoing extensive secondary expansion *in vitro* (compare Fig. 5B with Fig. 1A, right hand side). Addition of the ThOVA31 peptide to the OVA30 peptide/IFA bolus enhanced the CD8⁺ T-cell response (Fig. 5B) compared to OVA30 peptide alone (mean TM-OVA⁺ 28% vs. 13%, respectively). An additional, albeit small increase in the number of CD8⁺ T-cells was observed in mice that received the OVA30, ThOVA31 peptide in IFA in combination with systemic FGK (Figure 5B).

Subsequently, the effector function of the CD8⁺ T-cells was tested in an *in vivo* cytotoxicity assay at day 30. Vaccination with OVA30 in IFA resulted in strong *in vivo* lytic activity (Fig. 5C). To warrant that the effect seen by OVA30 does not rely on CD4⁺ T-cell help, by unknown cryptic helper sequences that may have been present in the long OVA30 peptide, the mice were depleted for CD4⁺ T-cells prior to vaccination and once every week throughout the experiment to maintain complete CD4⁺ T-cell depletion (≤ 0.01 % CD4⁺ T-cells) or MHC class II^{-/-} mice were used. The absence of CD4⁺ T-cells during the priming and effector phase did not demonstrably affect the OVA30 induced CD8⁺ T-cell killing of target cells. Nor did the absence of MHC class II strongly influence the killing capacity of OVA30 induced CTL (Fig. 5C). Although the numbers of OVA-specific CD8⁺ T-cells were higher when the OVA30 peptide was complemented with either systemic FGK, the ThOVA31 peptide, or both, it was not possible to measure a potential increase in effector function since the percentage of *in vivo* killed target cells in mice vaccinated with OVA30 in IFA was high already (Fig. 5C). The cytolytic activity of these CD8⁺ T cells was preserved over time, as a comparable killing capacity was observed at day 90 (Fig. 5D).

Similar to what was observed with the injection of OVA8 and ThOVA17 in PBS, the injection of the long peptide(s) in PBS in combination with systemic FGK resulted in an OVA-specific CD8⁺ T-cell response which was contracted at day 30 (%TM⁺ CD8⁺ cells; OVA30+FGK 0.13 ± 0.05 , OVA30+ThOVA31+FGK 0.13 ± 0.03 , vs. naïve 0.12 ± 0.04 , data not shown) and of which the number of circulating T-cells was too low to measure direct *in vivo* effector function (Fig. 5C). However, these vaccine-induced T-cells were still capable of undergoing secondary expansion *in vitro* (Fig. 5B) to a similar extend as seen with OVA30 in IFA. In contrast, higher numbers of circulating OVA-specific cytotoxic CD8⁺ T-cells were detected in mice vaccinated with the long OVA30 CTL peptide in IFA (%TM⁺CD8⁺ cells; 0.83 ± 0.61 , data not shown).

In conclusion, despite the fact that vaccination with the OVA8 peptide or the OVA30 peptide in IFA resulted in a comparable long Ag presentation *in vivo*, extension of the minimal OVA8 peptide to the OVA30 peptide prevented the functional impairment of activated CD8⁺ T-cells and instead induced a CTL response that was sustained over a long period.

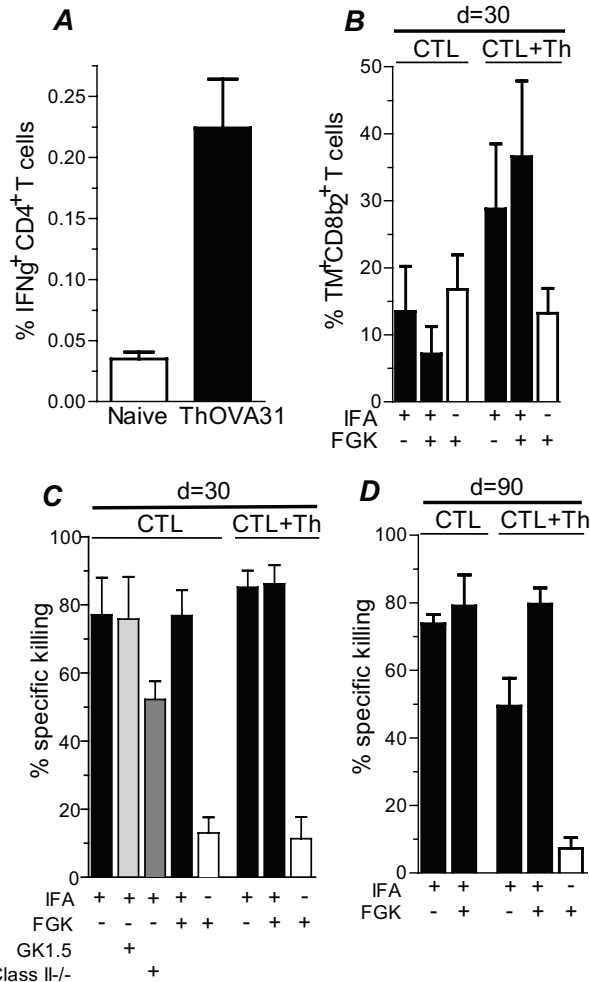


Figure 5. Long CTL peptide induces long-lasting CTL immunity independent of CD4⁺ T-cell help. Mice were vaccinated s.c. with the long CTL peptide OVA30 with or without the long Th peptide ThOVA31 mixed in IFA (black bars) or in PBS (white bars) in the presence or absence of 50 μ g of FGK (i.v. on day 0, 1 and 2). A, Enumeration of CD4⁺ IFN γ ⁺ T-cells by intracellular cytokine staining. Thirty days after vaccination mice were sacrificed and the splenocytes were stimulated overnight in the presence or absence of the ThOVA31 peptide and golgiplug. The next day the cells were stained for the surface marker CD4 and for intracellular IFN γ (n=5; naïve vs. vaccinated mice p<0.001). B, After 30 days the spleen was harvested and cells were stimulated *in vitro* with OVA-expressing tumor cells (EL4-OVA) for seven days and then analyzed for the presence of CD8⁺ T-cells capable of binding TM-OVA (n=4; OVA30 (IFA) vs. OVA30+ThOVA31 (IFA) p=0.06; OVA30+FGK (IFA) vs. OVA30+ThOVA31+FGK (IFA), p=0.09]. *In vivo* cytotoxicity assay C, day 30 and D, d90. Target cells were injected at day 28 (C) or day 88 (D) and two days later cytotoxicity was measured in the spleen by FACS analysis. Cells were gated on CD45.1⁺ lymphocytes. The data are depicted as mean $\pm$ SEM (n=5-10; C+D: IFA vaccination vs. PBS vaccination, p<0.01). Mice that were depleted for CD4⁺ T helper cells (GK1.5, light grey bar) received 25 μ g i.p. of depleting α CD4 at 3 days and one day before vaccination and once every week during the experiment. The dark grey bar indicates MHC class II knock-out mice (Class II^{-/-}; n=6).

Dominant negative effect of the OVA8 CTL peptide on induction of effector CD8⁺ T-cells by an immunogenic vaccine.

In addition to the vaccine draining lymph nodes, also the non-draining lymph nodes of mice injected with either OVA8, OVA30, ThOVA17 or ThOVA31 were analyzed for the presentation of these peptides. In contrast to the long peptides, which were presented predominantly in the draining lymph node, presentation of the OVA8 peptide was also observed in non-draining lymph nodes indicating that this peptide spreads systemically (Fig. 6). Despite the fact that sustained presentation of the ThOVA17 peptide is readily detected *in vivo* (Fig. 2B), we were not able to detect presentation of the ThOVA17 peptide *ex vivo* (Fig. 6B) suggesting that the MHC class II off-rate of this peptide is too high to allow direct *ex vivo* detection. This must be related to the affinity of this peptide for MHC class II I-A^b, which is probably lower than for the MHC class II molecule I-A^d which was first found to present this peptide (35).

Importantly, systemic presentation of OVA8 CTL peptide is associated with the functional impairment of the CD8⁺ T-cell response (Fig. 1B) while the predominant local presentation of the OVA30 peptide results in immunity. Therefore, we wondered what effect of OVA8 vaccination has on a concomitant immunogenic vaccination (OVA30, Fig. 5C). To test this, mice were vaccinated with the OVA8 peptide, the OVA30 peptide or both peptides mixed in IFA and the *in vivo* killing capacity was determined at day 30. As shown in figure 7, vaccination with OVA8 was unable to induce effector CD8⁺ T-cells at day 30, while

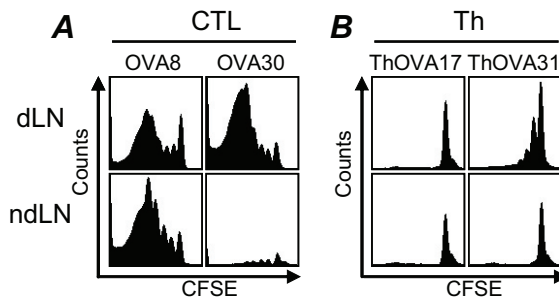


Figure 6. Location of Ag presentation. Mice were vaccinated with the indicated peptide mixed in IFA. Two days later the dLN (inguinal and axillary) and a ndLN (mesenteric) were isolated. A, *Ex vivo* detection of the OVA8 epitope was performed by incubation of 0.5×10^6 LN cells with 100,000 OT-1 CFSE-labeled Tg CD8⁺ T cells for 3 days. B, *Ex vivo* detection of the Th epitope was performed by incubation of 1×10^6 LN cells together with 200,000 CFSE-labeled OT-2 cells for 4 days. Dilution of CFSE was measured by gating on CD45.1⁺CD8⁺ lymphocytes for OT-1 cells, or Vα2⁺CD4⁺ lymphocytes for OT-2 cells.

vaccination with the OVA30 peptide resulted in a CTL response. When the OVA8 peptide was mixed with the OVA30 peptide in the same IFA depot, a significant reduction in the *in vivo* effector function of OVA-specific CD8⁺ T-cell reactivity was observed (Fig 7). These data show that vaccination with the minimal OVA8 peptide has a dominant negative effect on the effector functions of CD8⁺ T-cell induced by an immunogenic peptide vaccine such as OVA30 CTL peptide.

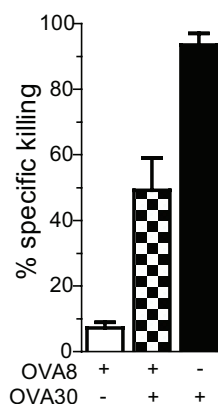


Figure 7. Dominant negative effect of the minimal OVA CTL peptide on immunogenic vaccination with the long OVA30 CTL peptide. Mice were vaccinated s.c. with the minimal CTL peptide (white bars), the long CTL peptide (black bars), or both peptides (blocked bars) mixed in IFA. Thirty days later mice were subjected to an *in vivo* cytotoxicity assay (n=4-6). (p<0.01, Kruskal Wallis test non-parametric ANOVA).

Failure to induce long-lasting CTL immunity is a more general characteristic of vaccination with minimal CTL peptide epitopes but can be overcome by the use of long peptides.

Because injection of the dominant minimal CTL peptide OVA8 of the model Ag OVA results in the functional impairment of CD8⁺ T cells when injected s.c. at a low dose in IFA, and as such acts similar to the earlier described adenoviral E1A (E1A10) (11,17) and E1B (12) minimal CTL peptides, the impact of minimal and extended CTL epitope vaccines was compared in two additional antigenic systems.

First we tested the HPV16 E7 minimal CTL peptide (HPV9) and the extended CTL peptide (HPV35) (28). As shown in Fig. 8A, vaccination with the HPV9 peptide resulted in marginal killing (8%) of HPV peptide loaded target cells *in vivo* 30 days after vaccination whereas the use of the HPV35 peptide resulted in 45% specific killing of target cells (Fig. 8A). The enhanced effector response induced by HPV35 in IFA was independent of CD4⁺ T-cell help (Fig. 8A).

Similarly, vaccination with the minimal CTL peptide of the Adenovirus E1A protein E1A10, known to tolerize the CD8⁺ T-cell response (11,17), failed to induce CD8⁺ T-cell effector function 30 days after vaccination (3% killing) (Fig. 8B). In contrast, when the extended CTL peptide E1A30 was used 52% of the target cells were lysed 30 days after vaccination, independently of the presence of CD4⁺ T-cell help (Fig. 8B). These results indicated that the use of extended CTL peptide vaccines may prevent T cell tolerance (E1A10 peptide) and, in general, induce a sustained *in vivo* CD8⁺ effector T-cell response, when compared to their minimal CTL peptide counterparts.

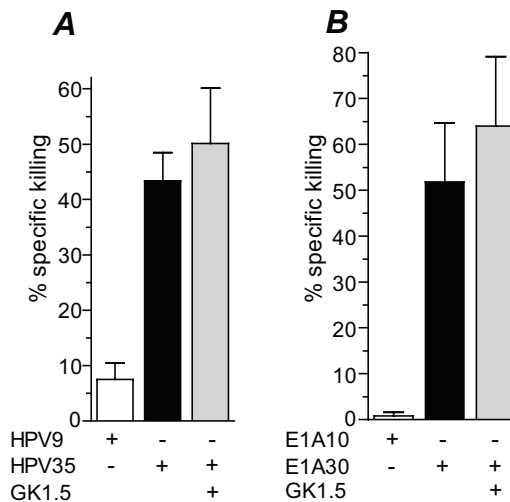


Figure 8. Failure to induce long-lasting CTL immunity is a general feature for minimal CTL peptides. Mice were vaccinated s.c. with peptides mixed in IFA with either the minimal CTL peptide (white bars), the long CTL peptide (black bars), or the long CTL peptide in which mice were depleted for CD4⁺ T-cells with GK1.5 Ab (light grey bars). Mice that were depleted for CD4⁺ T-cells received three and one day prior to vaccination, 25 μ g of CD4 depleting Ab GK1.5 (i.p.) and thereafter once every week 25 μ g to maintain complete CD4⁺ T-cell depletion. Thirty days later mice were subjected to an *in vivo* cytotoxicity assay. A, HPV peptides (n=10) (HPV9 vs. HPV35 with or w/o GK1.5, $p < 0.001$, Kruskal Wallis test non-parametric ANOVA) B, E1A peptides (n=5) (E1A10 vs. E1A30 with or w/o GK1.5, $p < 0.001$, Kruskal Wallis test non-parametric ANOVA). The data are depicted as mean $\pm$ SEM.

DISCUSSION

In this study we showed that vaccination with a low dose of a highly immunogenic exact MHC class I binding peptide (OVA₂₅₇₋₂₆₄) can result in the activation of CD8⁺ effector T-cells which eventually cease to expand or to kill target cells, provided that this peptide is administered in IFA. The injection of a minimal CTL peptide in IFA did not immediately impair the responding CD8⁺ T-cells but was preceded by induction of a complete functional

CD8⁺ T-cell response that was still present at day 10 but absent at 30 days after vaccination. APC activation by co-administration of FGK was unable to rescue the responding CD8⁺ T-cells from their fate. The basis for this dominant negative effect lies in the duration of antigen presentation *in vivo*, the location where these peptides are presented and the absence of sustained systemic danger signals.

When the minimal CTL peptide is administered in PBS, this antigen is presented to CD8⁺ T-cells for less than 30 days. In contrast, when the peptide is mixed with IFA, the CTL peptide was still presented at 60 days after vaccination. FGK is likely to be cleared rapidly from the system, similar to other Ab (31,32), and as such can only temporarily activate APC. When systemic presentation of the minimal CTL peptide ensues in the absence of a continuous helper/danger signal (36), it is likely that this will result in CD8⁺ T-cell tolerance (Fig. 1, Fig. 2 and (17)). In order to provide continuous helper signals we included the minimal ThOVA17 peptide in the CTL/IFA bolus to activate APC via CD40-CD40L interactions (15,16,33), and showed that also the Th peptide was presented for more than 60 days (Fig. 2). As both peptides are continuously leaking out of the IFA depot, activation of APC, CD4⁺ and CD8⁺ T-cells will take place simultaneously. The induction of CD4⁺ T-cells not only enhanced the CD8⁺ T-cell response (Fig. 3) as was previously reported (37,38), but our experiments also show that it prevented the impairment of CD8⁺ T-cell reactivity, resulting in the preservation of long-term CD8⁺ T-cell immunity (Fig. 3).

Recently, we found that the activation of CD8⁺ T-cells by minimal CTL peptide vaccines strongly depended on the presence of non-professional APC (Bijker, manuscript in preparation). Indeed, minimal MHC class I binding peptides are known to bind directly to their restriction elements at the cell surface of MHC class I expressing cells, including non-professional APC. However, our experiments teach us that CD8⁺ T-cells responding to minimal CTL peptides injected in PBS (and FGK) can become fully activated and as well form memory CD8⁺ T cells that are able to expand upon secondary antigen stimulation *in vitro*, to a similar extent as when the longer peptide is injected (compare figures 1A and 5B, day 30, open bars). Thus, even while suboptimal presentation may occur, this not necessarily leads to the activation of CTL that subsequently fail to expand or survive. This implies that at least other factors, such as the long term systemic presentation of the OVA8 peptide, may play a role in the detrimental outcome of minimal CTL peptide vaccines in IFA.

Despite the fact that both the long OVA30 CTL peptide and the minimal OVA8 CTL peptide were presented for at least 60 days *in vivo* when applied in IFA (Fig. 4A and 2A, respectively), vaccination with the OVA30 peptide did not result in an eventual impairment of CD8⁺ T-cell reactivity and did not require CD4⁺ T-cell help to preserve the CD8⁺ T-cell response (Fig. 5). This was not only the case for OVA, but could be generalized for 3 Ag systems (Figs 1,5,8).

Extension of the minimal CTL peptide of either the OVA, HPV or E1A protein induced CD8⁺ T-cells with potent *in vivo* killing function until at least 30 days after vaccination. Furthermore, increasing the length of the minimal E1A10 CTL peptide to a 30 a.a. long peptide, prevented the induction of CD8⁺ T-cell tolerance that was previously observed when the minimal CTL peptide was used (11).

In contrast to the minimal CTL peptides - which spread systemically to non-draining and non-inflamed LNs (17,29,39) where antigen probably is presented in a tolerizing fashion (40) - the longer peptides are predominantly presented by APC in the vaccine dLN (Fig. 6). As IFA induces a local inflammatory response (41,42) it will thereby provide the necessary danger signals (36,43) that are needed to activate the local APC that have ingested the long peptide vaccine. Together, this can explain why vaccination with long peptides in IFA does not result in a functionally impaired CD8⁺ T-cell response and why CD4⁺ T-cell help is not required.

So, why did many papers report that minimal peptide vaccination in IFA induces an effective CD8⁺ T-cell response (6-9) instead of CD8⁺ T-cell tolerance? At one hand the outcome of vaccination was studied shortly after peptide vaccination when the CD8⁺ T-cells are not expected to be tolerized as shown here (Fig. 1A+B). On the other, many of the peptides used were much longer than the minimal CTL sequence, reaching up to 27 a.a. in length (2,6,7,44-48), and/or contained a Th sequence (45,49). In view of the current results, this might explain why these so called “short synthetic peptides” performed so well compared to the exact minimal CTL peptides used here, or in clinical trials (4).

In addition, it should be noted that vaccination with minimal CTL peptide represses the induction of CD8⁺ effector T-cells generated in response to concomitant proper antigen presentation (Fig. 7). One can envisage that such an effect is highly undesirable in cancer patients that receive minimal CTL peptide vaccination after chemotherapy or radio therapy. In these cases, large amounts of Ag are released and cross presented by DC (50), which may result in the activation of tumor-specific CD8⁺ T cells that recognize the same sequences also present in the minimal CTL peptide vaccines in IFA. As a result such T-cell responses may be functionally impaired following vaccination.

In conclusion, our data clearly answers the question why vaccines consisting of minimal CTL peptides in oil-in-water emulsions (e.g. IFA) may have only limited immunological and clinical success in cancer patients. These vaccines need to be complemented at least by T-helper epitopes in order to sustain effective CD8⁺ T-cell reactivity. In case that specific CD4⁺ T-cell help is not readily available, extension of CTL peptides to longer variants may form an excellent alternative. Vaccine trials should be performed in order to confirm our results in a human setting.

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3

Pharmacokinetics of short and
long peptide-based vaccines

Submitted

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Superior induction of anti-tumor CTL immunity by extended peptide vaccines involves prolonged, DC-focused antigen presentation

3

Abstract. Anti-tumor vaccines consisting of extended CTL peptides in combination with CpG are superior to those comprising minimal CTL epitopes and CpG, in that they elicit stronger effector CTL responses with greater tumoricidal potential. This superiority is primarily due to the focusing of CTL epitope presentation to activated DC in the inflamed lymph nodes draining the vaccination site. In the case of vaccination with minimal peptides, additional APC including T- and B-cells are also loaded with CTL epitopes and, once loaded, can circulate through the lymphoid system. Consequently, epitopes are presented in non-inflamed lymphoid organs distal from the vaccination site, in the absence of potent costimulatory signals required for efficient CTL priming. The resulting blend of pro-immunogenic and tolerogenic signals, which results in suboptimal activation of the CTL response, is avoided by vaccinating with extended CTL peptides. An additional advantage of extended CTL peptide vaccines is an increased duration of *in vivo* epitope presentation, which is particularly apparent for vaccines comprising epitopes with weaker MHC class I binding.

INTRODUCTION

The immune system has evolved to fight a diversity of invading pathogens, but is not optimally equipped to combat most cancer types (1). An important reason for this is that tumors arise slowly from single cell precursors and thereby fail to efficiently activate the immune system, due to the lack of strong pro-inflammatory signals (2,3). The immune system can be forced to respond against tumor-associated antigens (TAAs) by means of vaccination (reviewed in (4)). Peptide-based vaccines are intensively studied as a means for enhancing anti-cancer immunity, in particular T-cell responses. Their development was sparked by the identification of MHC-bound peptides, derived from various TAA, that are presented at the surface of murine and human tumor cells (5-7). Peptide-based vaccines constitute an attractive platform for immune intervention against cancer, because synthetic peptides with defined sequences can be readily produced in clinical-grade quality (4). Furthermore, synthetic peptides are molecularly defined, in that they do not contain antigenic components other than the epitopes of interest that could divert the attention of the immune response, or potentially pathogenic remnants of micro-organisms that could compromise the safety of their use in patients. Moreover, synthetic peptides constitute ‘off the shelf’ components that can be used to compose multi-peptide vaccines tailored to the antigen-content of the tumor and the HLA-type of the patient. Finally, the known antigen-content of peptide-based vaccines facilitates evaluation of antigen-specific immunity pre- and post-vaccination and, thereby, systematic analysis of vaccine-induced T-cell immunity in relation to clinical efficacy.

The vast majority of pre-clinical and clinical vaccination studies involving peptide-based vaccines have employed formulations comprising short peptides that match the exact, minimal sequences of MHC class I-binding CD8⁺ T-cell epitopes (8-14). Their apparent efficacy in eliciting protective anti-tumor T-cell immunity in mouse tumor models (9,14) has resulted in the testing of this concept in a considerable number of clinical studies involving cancer patients. However, the clinical and immunological impact of these vaccines can be considered disappointing (reviewed in (15,16)). In hindsight, this lack of therapeutic efficacy was to be expected. Although studies in multiple mouse models have shown that prior immunization of mice with vaccines comprising minimal CTL epitopes can protect against outgrowth of subsequently transplanted tumors, such vaccines were rarely found to elicit truly therapeutic T-cell immunity capable of clearing pre-existing tumors (reviewed in (4)). In our previous work, we have demonstrated this feature with a peptide vaccine comprising a CD8⁺ T cell epitope derived from the human papillomavirus type 16 E7 oncoprotein that is presented in the context of MHC class I (H-2Db) of HPV16-transformed murine tumor cells.

Even though administration of a vaccine comprising this minimal peptide epitope can protect mice against a subsequent challenge with HPV16-positive tumor cells (9), this vaccine fails to elicit therapeutic T-cell immunity in mice with pre-existing tumors (17). Importantly, we have found that the therapeutic efficacy this HPV16E7 peptide can be significantly increased by either combining it with a systemic dose of agonistic anti-CD40 antibody (18), or by extending the length of the peptide with natural flanking sequences (17). In the former case, *in vivo* ligation of CD40 was shown to replace the need for CD4⁺ T-cell help in mediating efficient CTL priming through the activation of antigen-presenting dendritic cells (DC) (18). In the latter case, the superior anti-tumor immunity induced by the extended peptide vaccine was shown to relate, at least in part, to the presence of a CD4 T-helper epitope in this peptide (17). This resulted in the induction of HPV16E7-specific T-helper responses and enhanced CTL-responses, where the enhancement of CTL immunity by these T-helper responses depended on CD40-mediated interactions with professional APC. Importantly, the extended HPV16E7 peptide vaccine also resulted in superior induction of CTL immunity in settings where the contribution of CD4⁺ T-cell help was excluded. If the minimal and extended peptides were administered in combination with the potent DC-activating agent CpG ODN, the extended peptide elicited much higher CTL responses, in a manner that did not depend on CD40-mediated interactions with professional APC.

In view of the potential of more effective, long-peptide based vaccines for immunotherapeutic use, we further investigated the mode of action of these vaccines, in particular the importance of peptide length on vaccine performance. The use of the chicken Ovalbumin (OVA) antigen and the corresponding OVA₂₅₇₋₂₆₄-specific OT-1 TCR transgenic CD8⁺ T-cell system allowed detailed analysis of the impact of peptide length on epitope presentation and CTL activation. We compared the minimal CTL peptide OVA₂₅₇₋₂₆₄ (OVA8) to a peptide that was extended at the N and C terminus with the natural flanking residues of the OVA protein OVA₂₄₁₋₂₇₀ (OVA30). Because the OVA epitope is highly immunogenic (19,20), while many epitopes derived from TAA may have less optimal characteristics (20), we also compared minimal and extended OVA peptides that, due to a point mutation in an anchor residue, displayed weaker MHC binding (21). The results of our studies showed that the induction of therapeutic anti-tumor CTL immunity critically depends on the use of extended peptide vaccines, in particular when the target epitope concerned is suboptimal. Our data furthermore demonstrate that the superiority of vaccines comprising extended peptides relies on an increased duration of antigen presentation that is focused on CD11c⁺ dendritic cells in the lymph node draining the vaccination site.

MATERIALS AND METHODS

Mice. C57BL/6 (B6; H-2b) mice were purchased from IFFA Credo. B-cell-deficient uMT H-2Kb mice were purchased from Charles River (St. Germain sur l'Arbresle, France). OT 1, CD45.1 mice, expressing the OVA-specific, H2-Kb -restricted T-cell receptor (22) were bred at the Leiden University Medical Centre animal facility. All mice were used at 8–12 wk of age in accordance with national legislation and under supervision of the animal experimental committee of the University of Leiden.

Peptides and vaccination. Peptides were generated, purified, dissolved and stored as described previously (9). We have used the following peptides; OVA8 (OVA₂₅₇₋₂₆₄, SIINF EK L), OVA30 (OVA₂₄₁₋₂₇₀ (SMLVLLPDEVSGLEQL ES IINF EK L TEW TS), OVA8LI (SIINF EK I) and OVA30LI (SMLVLLPDEVSGLEQL ES IINF EK I TEW TS). The extended OVA peptides do not contain the known T-helper epitope OVA₂₆₅₋₂₈₀ (23), nor any cryptic helper epitopes (24). Mice were vaccinated s.c. in the flank with 40 nmol of peptide (40 ug for 8-mer peptides; 140 ug for 30-mer peptides) admixed with 20 ug of CpG-ODN (25) in a total volume of 200 ul PBS.

Tumor cells and their use for *in vitro* stimulation and tumor challenge experiments. EG7 tumor cells expressing the full length OVA antigen (26) were cultured in IMDM (Invitrogen Life Technologies, Rockville, MD) supplemented with 8% (v/v) FCS (Greiner), 50 μ M 2-ME, 2 mM glutamine, 100 IU/ml penicillin (complete medium) supplemented with 400 ug/ml G418 (Gibco). EG7 cells were used for *in vitro* stimulation of splenocytes from immunized animals as follows. Three weeks after vaccination, spleens were removed and single spleen cell suspension of 10^6 cells/ml were made. EG7 cells were incubated with 50 ug/ml of mitomycin C (Kyowa) in complete medium at 37° C for 1 hour, washed 4 times with medium, irradiated (4000 RAD) and resuspended and used as stimulator cells. Splenocytes were incubated in a 10:1 ratio with EG7 cells. Seven days later, viable cells were isolated over a ficoll gradient and were stained with H-2Kb Tetramer (TM)-OVA₂₅₇₋₂₆₄ complexes and CD8. Propidium Iodine was used to exclude dead cells.

B16-OVA cells, which express the OVA antigen (27) were cultured in complete medium supplemented with Non essential amino acids (1:100) (Gibco), 1mM Sodium Pyruvate (Gibco), 60 ug/ml Hygromycin B (Invitrogen) and 1000 ug/ml G418 (Gibco). For therapeutic vaccination experiments, mice were challenged with 5×10^4 B16-OVA tumor cells in the left flank. The following day, mice were vaccinated on the right flank with peptide vaccine and boosted two weeks later. Tumor size was measured with a capillary in 3 dimensions three

times a week. Mice were sacrificed when the tumor exceeded 1000 mm³ in accordance with national legislation. The group size was n=15 for the naïve groups and n=10 for the vaccinated groups.

In vivo cytotoxicity assay. *In vivo* cytolytic activity was determined with peptide-loaded B6/CD45.1 splenocytes as target cells that were differentially labeled with the fluorescent dye CFSE (Molecular Probes). First erythrocytes were lysed by ammonium chloride treatment (3 min at room temperature). Splenocytes were then split into two fractions: i) target population and ii) internal control population. The target cell population was pulsed with 1.0 ug/ml of OVA₂₅₇₋₂₆₄ peptide and the internal control population with 1.0 ug/ml of p53 peptide (H2-Kb, p53₁₅₈₋₁₆₆) in complete medium at 37° C for 60 minutes. Subsequently, the cells were washed three times with PBS 0.1% BSA to remove excess of free peptide. The target population was labeled with 5 µM (CFSEhi) and the internal control population with 0.5 µM (CFSElo) of CFSE for 20 minutes. The cells were washed two times with PBS before the populations were mixed in a 1:1 ratio and a total of 8*10⁶ cells in 200 µl PBS was injected i.v. The next day spleens were removed and single cell suspension was made. The ratio of CFSElo/CFSEhi cells was determined by flow cytometry by gating on CD45.1+ lymphocytes. Specific killing of OVA₂₅₇₋₂₆₄ pulsed (CFSEhi) target cells was calculated as follows: $(1 [(CFSEhi/CFSElo)_{vaccinated} * (CFSElo/CFSEhi)_{naive}]) * 100\%$.

CFSE labeling of Tg T cells and adoptive T cell transfer. Single cell suspension was made from spleen and peripheral lymph nodes of OT-1 CD45.1+ mice. Erythrocytes were lysed by ammonium chloride treatment (3 min at room temperature). Cells were labeled with CFSE (28) as described above (5µM). OT-1 CD8+ T cells (2 x 10⁶ in 200 ul PBS) were injected into the tail vein or used for *in vitro* experiments (in complete medium).

Ex vivo Ag detection. Lymph nodes were isolated and incubated for 30 minutes with Collagenase IV (250 U/ml; Sigma-Aldrich, St. Louis, MO) and DNase (50 ug/ml; Sigma-Aldrich) at 37° C. Single cell suspension was made and the cells were incubated with Abs against CD11c (DC), CD19 (B cells) and CD3 (T cells) and sorted on a FACSort. These sorted cells were used as stimulator cells in co-cultures with naïve CFSE-labeled OT-1 CD8+ T cells for three days, after which proliferation of the OT-1 cells was evaluated by flow cytometry on basis of CFSE-dilution. Co-cultures contained 1*10⁵ OT-1 cells and one of the following stimulator cells: CD11c+ 5*10⁴; CD19+ cells 4*10⁵, CD3+ cells 8*10⁵, We have used 5*10⁴ CD11c+ cells, because only limited amounts of these cells could be isolated

from the draining lymph nodes (dLN). The numbers of CD19+ and CD3+ cells used for this experiment, were based on their ratio to the CD11c+ cells found in the dLN; 8 fold more CD19+ cells, and 16 fold more CD3+ cells.

Flow cytometry. Single cell suspensions of spleens were stained in PBS 0.1% BSA. The Abs used, were the following: directly allophycocyanin-conjugated; H-2Kb Tetramer OVA₂₅₇₋₂₆₄; CD45.1 (A20, eBioscience), CD19 (1D3, Pharmingen); PE conjugated; CD8b2 (53-5,8, Pharmingen), CD11c (HL3, Pharmingen) and, Va2 (clone B20.1, Pharmingen); FITC-conjugated CD3 (145-2C11, Pharmingen). Data acquisition and analysis was done on a BD Biosciences FACScan (San Jose, CA, USA) with CellQuest software.

Statistical analysis. Statistical analysis was done using GraphPad InStat software (version 3.0) and GraphPad Prism 4 (GraphPad Software, San Diego, CA). A two-tailed t test with Welch correction was used for Fig. 1,4 and 5. For the comparison of survival curves in Fig. 2, the Logrank test was applied.

RESULTS

Magnitude and anti-tumor efficacy of the CD8+ T cell response is determined by peptide affinity and length.

In order to validate whether the OVA epitope constitutes a suitable model for evaluating the mode of action of peptide vaccines containing extended CTL epitopes (17), mice were vaccinated with the minimal peptide OVA8 (OVA₂₅₇₋₂₆₄) or the extended peptide OVA30 (OVA₂₄₁₋₂₇₀). Because our study focused on the impact of peptide length on vaccine performance, both peptides were administered in combination with CpG. In this setting, CTL priming is independent of CD4+ T-cell help (17). Analysis of the number of OVA-specific CTLs by means of flow cytometry revealed only a modest difference between mice immunized with the minimal and extended peptides (Fig. 1). The lack of correspondence of these results with our previously published data concerning minimal and extended peptides containing the HPV16 E7 CTL epitope (17) could be explained by the fact that the OVA8 epitope is a highly immunogenic epitope that strongly binds to MHC class I, while the HPV16E7 epitope is a subdominant epitope that displays intermediate MHC binding (8,20). In view of this, we repeated our analyses with minimal and extended peptides comprising a modified OVA epitope, in which the secondary anchor residue Leucine at position 264 was substituted for an Isoleucine (OVA8LI and OVA30LI). This substitution was reported to result in decreased MHC binding without altering its interaction with the T cell repertoire (21). Importantly,

comparison of the CTL numbers induced by minimal and extended peptides comprising this modified CTL epitope did reveal a clear superiority of the extended peptide vaccine (Fig. 1), in agreement with our previous results for peptides comprising the HPV16 E7 epitope (17). To test if the capacity of the OVA peptide vaccines to increase CTL numbers corresponded with their capacity to elicit therapeutic anti-tumor immunity, groups of mice were challenged with tumorigenic doses of B16-OVA cells and subsequently vaccinated with the peptide vaccines under examination. Interestingly, vaccination with extended peptides did not only result in superior anti-tumor immunity in the case of the wild type OVA epitope, but also in the case of its modified counterpart (Fig 2A,B). In line with these findings, the *in vivo* cytolytic activity against peptide-loaded splenocytes was significantly stronger in mice immunized with extended peptides as compared to mice immunized with the corresponding minimal peptides (Fig. 2C, D). For vaccines comprising the wild type OVA peptide, this difference was already apparent after a single vaccine dose (Fig. 2C), while induction of detectable cytolytic activity with vaccines comprising the modified OVA peptides required two subsequent doses of vaccines (Fig 2D). Overall, the levels of *in vivo* cytolytic activity induced by prime-boost

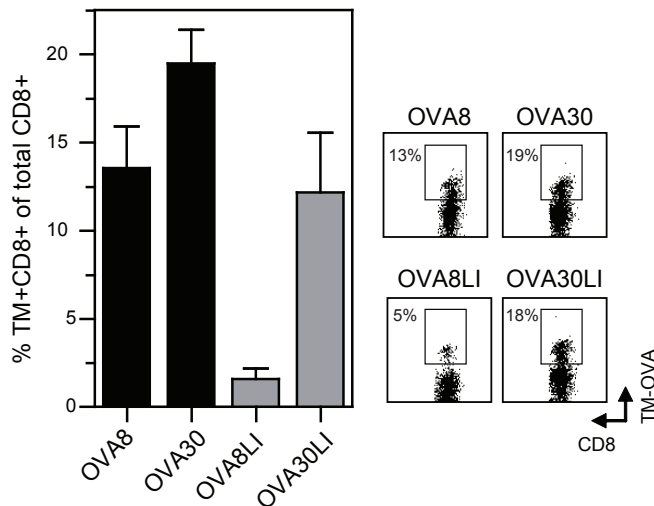


Figure 1. The magnitude of the CD8+ T cell response is determined by peptide affinity and length. Mice (>15/group) were vaccinated s.c. with either OVA8, OVA30, OVA8LI, or OVA30LI peptide (40 nmol) in combination with CpG (20ug). Three weeks afterwards, splenocytes were isolated and stimulated *in vitro* with OVA-expressing tumor cells for 7 days before analyzing the frequency of TM-OVA+ CD8+ T cells. The data are depicted as mean values $\pm$ SEM. Results with OVA and OVALI peptides are shown with black and grey bars respectively (OVA8 vs. OVA30, $p=0.058$; OVA8LI vs. OVA30LI, $p<0.01$). In addition, representative FACS plots are shown.

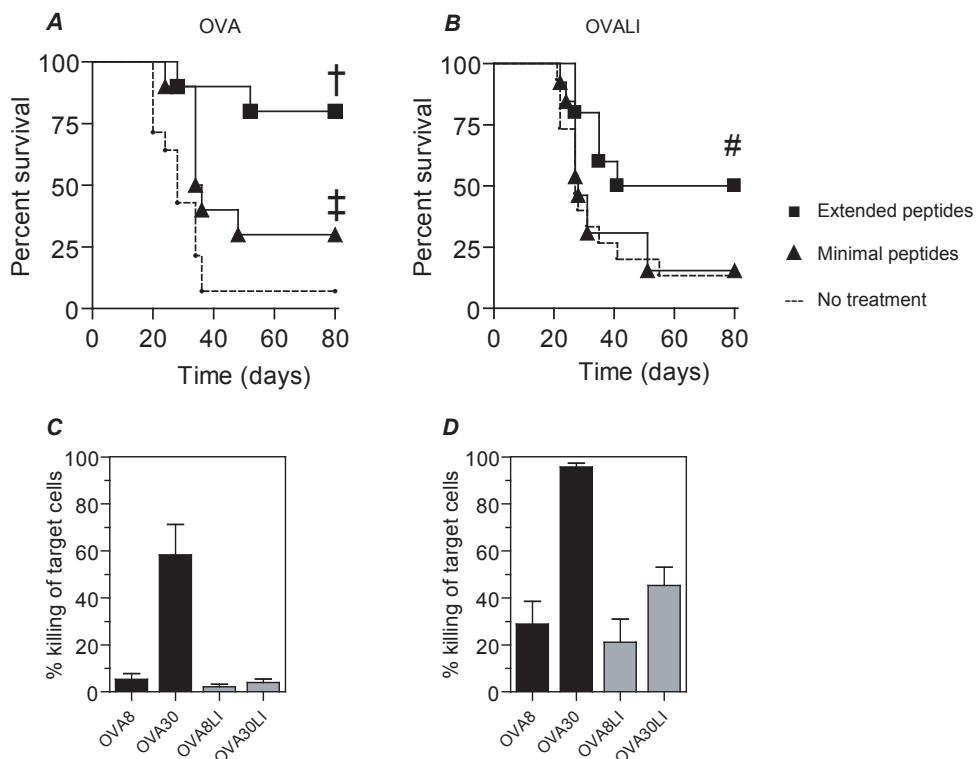


Figure 2. Improved therapeutic efficacy of anti-tumor vaccines comprising extended CTL peptides.

A. Mice were challenged s.c. with 5×10^4 B16-OVA tumor cells in the left flank (day -1). The following day (d0), the mice (n=15) were left untreated or vaccinated in the right flank with either OVA8 or OVA30 peptides (40 nmol) in combination with CpG (20 ug) and boosted 2 weeks later (10 mice/group). Tumor size was measured 3 times per week and mice were sacrificed when the tumors exceeded 1000 mm^3 . B. Mice were challenged with B16-OVA tumor cells as in A, and left untreated or vaccinated with either OVA8LI, or OVA30LI peptide in combination with CpG and boosted 2 weeks later (10 mice/group). The graphs depict the percentages of tumor free mice (significant improvement of tumor free mice as compared to untreated mice: †, $p < 0.0002$; ‡, $p < 0.03$; #, $p < 0.03$). C, D: Analysis of antigen-specific in vivo cytolytic activity in mice vaccinated with one (C; day 0) or two (D; days 0 and 14) doses of vaccines containing the indicated peptides. Mice (6/group) received a 1:1 mixture of OVA-peptide loaded and control peptide-loaded, CFSE-labeled splenocytes as target cells at day 9 (C) or day 19 (D). The relative amounts of OVA- and control peptide-loaded target cells in the spleen were evaluated by flow cytometry. Data are depicted as mean percentage of OVA-specific target cell killing $\pm$ SEM.

regimens (Fig 2D) correspond well with the protective effect observed in tumor challenge experiments (Fig 2A, B), in accordance with the fact that also the latter experiments involved prime-boost vaccination.

Taken together, our data indicate that the capacity of CTL peptide vaccines to induce CTL effector responses is greatly enhanced by the use of extended peptide sequences. Even though this is particularly critical for vaccines comprising suboptimal CTL epitopes, this concept also applies to vaccines that comprise highly immunogenic CTL epitopes. Notably, such differences in vaccine efficacy may be overlooked when enumeration of antigen-specific CTL by flow cytometry is used as a read out (Fig 1).

Increased duration of antigen presentation after vaccination with extended CTL peptides.

Several studies have demonstrated that duration of antigen presentation is an important parameter in determining the magnitude of the CTL response (29-31). We therefore tested the impact of peptide length on longevity of antigen presentation after vaccination, as detected by naïve CFSE-labeled OT-1 CD8⁺ T cells (32). OT-1 T cells can be used to detect the presentation of both wild type and modified OVA peptides, because the CTLs respond to these peptides *in vitro* and *in vivo* with comparable efficiency (Fig. 3A, B).

To test the duration of antigen presentation *in vivo*, naïve CFSE-labeled OT-1 T cells were infused 2, 6, or 10 days after vaccination with the minimal and extended OVA peptides (Fig. 3C). For peptides comprising the wild type OVA epitope, peptide length had only a modest impact on duration of antigen presentation. Even though OVA peptide was presented up to 10 days after vaccination with either OVA8 or OVA30, antigen presentation at day 10 was still maximal in mice immunized with OVA30, while being in decline in mice immunized with OVA8. The impact of peptide length was much more profound in case of the modified OVA peptides, in that most antigen presentation was already lost within 2 days after vaccination with the short OVA8LI peptide, while being constant for at least 10 days in case of the extended OVA30LI peptide.

Our data on longevity of antigen presentation are in striking correspondence with those on the induction of *in vivo* effector CTL responses, in that the use of extended peptides is critical for good performance of vaccines comprising the suboptimal OVA epitope, while being less essential (but still important) for vaccines comprising the optimal OVA epitope.

The short *in vivo* duration of antigen presentation of the minimal CTL peptide OVA8LI (Fig.3C) correlated with its poor capacity to elicit CTL responses (Fig. 1&2). We therefore analyzed whether increasing the duration of antigen presentation by repeated administration of the OVA8LI peptide would improve its capacity to elicit CTL immunity. Indeed, such

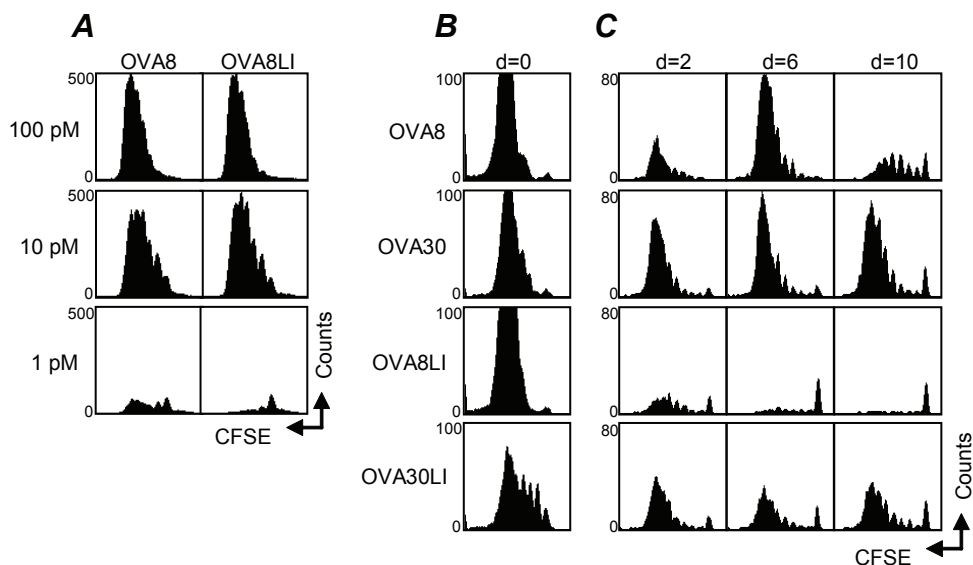


Figure 3. Increased duration of antigen presentation after vaccination with extended CTL peptides. A. Ex vivo recognition of OVA8 and OVA8LI peptides by OT-1 T-cells. Naïve CFSE-labeled OT-1 T-cells (1×10^5) were incubated in vitro with the indicated amounts of OVA8 or OVA8LI peptide. Three days afterwards, proliferation was determined by measuring CFSE dilution. Representative histograms of duplicate experiments are shown. B. In vivo recognition peptide antigens by OT-1 T-cells. Mice were infused with naïve CFSE-labeled OT-1 T cells (2×10^6) and were vaccinated s.c. on the same day with the indicated peptides in combination with CpG. Three days later, the extent of T-cell proliferation in the dLN (inguinal) was analyzed by flow cytometry. C. In vivo longevity of antigen presentation. Mice were vaccinated s.c. with the indicated peptides in combination with CPG. Two, 6, or 10 days afterwards, naïve CFSE-labeled OT-1 T cells (2×10^6) were infused. Three days later, proliferation of OT-1 T cells in the dLN was evaluated by flow cytometry. Each histogram depicts data representative of 4 mice.

repeated administration resulted in significantly increased numbers of OVA-specific CD8⁺ T cells as detected by MHC-tetramers (Fig. 4A). However, testing of this vaccine regimen for its potential to clear B16-OVA tumors revealed that, in spite the high levels of CD8⁺ T cells induced by this vaccine (Fig. 4A), no enhanced tumor clearance was observed compared to the naïve mice (Fig. 4B). These data suggest that the therapeutic impact of vaccines comprising extended CTL peptides is not merely due to prolonged antigen presentation.

Antigen presentation after vaccination with extended peptides is predominantly focused to DC in the draining lymph node.

Efficient induction of CTL immunity requires strong costimulatory signals in addition to the antigenic signal. Of all APC types, DC are best equipped to provide naïve CTLs with both types of signals, provided that they are activated by CD4 T-helper cells through CD40 and/

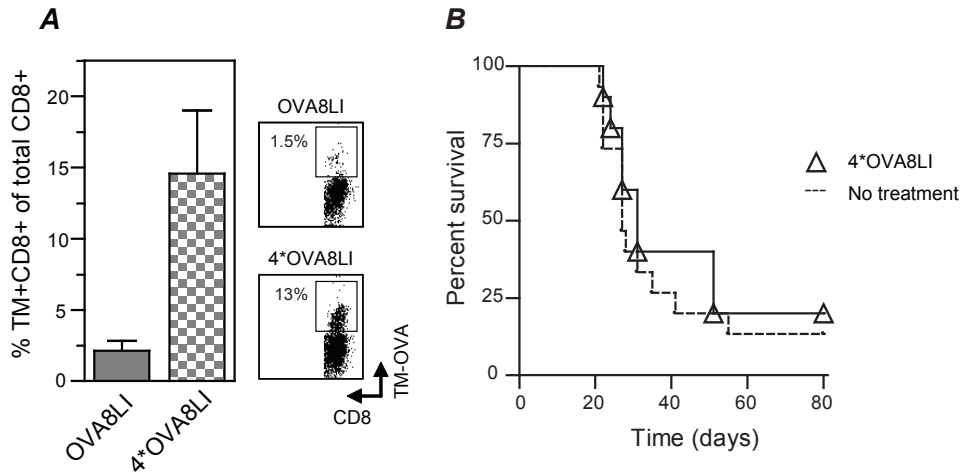


Figure 4. Extending in vivo antigen presentation by repeated injection of minimal peptide does not enhance effector CTL responses. A. Mice (13/group) were vaccinated s.c. with a single dose (day 0, grey bars) of OVA8LI (40 nmol) and CpG (20 ug) or were subsequently boosted with OVA8LI without CpG on days 3, 6, 9 (checked bars). Three weeks afterwards, splenocytes were stimulated in vitro with OVA-expressing tumor cells for seven days, after which the frequencies of TM-OVA+ CD8+ T cells were analyzed by flow cytometry. Histograms show mean values $\pm$ SEM (OVA8LI vs. 4*OVA8LI, $p < 0.013$). In addition, representative FACS plots are shown. B. Mice were challenged s.c. with 5×10^4 B16-OVA tumor cells on the left flank (day -1). The next day (d0), mice were either left untreated ($n=15$) or were vaccinated ($n=13$) with 4 consecutive doses of OVA8LI at days 0, 3, 6 and 9 (see above), and boosted at day 14. Tumor size was measured 3 times per week and mice were sacrificed when the tumors exceeded 1000 mm³. The untreated group is the same as shown in Fig. 2B.

or by pathogen-associated molecular patterns (PAMPs) through Toll-like receptors (TLRs). Therefore, one would expect to achieve optimal antigen presentation after vaccination if this presentation is restricted to DC in the inflamed lymph node(s) draining the vaccination site. To evaluate the localization of - and APC-types involved in - epitope presentation after vaccination, mice were vaccinated in the flank with minimal versus extended OVA peptides in combination with CpG and two days afterwards, the draining (inguinal and axillary) and non-draining (mesenteric) lymph nodes were isolated. The cells from the dLN were separated into CD11c+ DC, CD19+ B cells, and CD3+ T-cells, while the cells of the ndLN were separated into CD11c+ DC and CD11c- cells. The resulting cell populations were subsequently tested for their capacity to stimulate proliferation of naive CFSE-labeled OT-1 T-cells. As shown in figure 5A, vaccination with the minimal OVA8 peptide resulted in antigen presentation by CD11c+ DC, B-cells and T-cells in the dLN, as well as by the CD11c-negative cells in the



ndLN. In contrast, clearly detectable antigen presentation after vaccination with the extended OVA30 peptide was focused to the CD11c⁺ DC in the dLN, while no antigen presentation could be detected by cells isolated from the ndLN. These results are in line with the notion that uptake of exogenous antigen, such as extended peptides, and processing of the CTL epitopes contained into MHC class I is restricted to DC (33), while minimal peptide epitopes can be exogenously loaded into MHC class I molecules at the surface of various types of professional and non-professional APC. The finding that DC-mediated antigen presentation is limited to the dLN is in accordance with the expectation that activation of DC at the vaccination site, by the adjuvant CpG, will result in DC migration to the dLN and not in DC circulation throughout the body. Systemic migration after antigen uptake can, however, be envisioned for lymphoid APC, such as B and T-cells. We therefore tentatively conclude that the antigen presentation in ndLN by CD11c-negative cells, as detected after vaccination with the minimal OVA8 peptide, is mediated by circulating lymphocytes that have been exogenously loaded with peptide epitope in the dLN.

The analyses of antigen presentation by the different APC populations in dLN versus ndLN were also performed after vaccination with the minimal OVA8LI and extended OVA30LI peptides (Fig 5B). The levels of antigen presentation detected in these experiments were much lower. This is most likely the result of the weaker binding of the modified OVA CTL epitope to H-2Kb, which decreases the on-rate and increases the off-rate and thereby the level at which peptide/MHC complexes accumulate at the cell surface (21,34). Furthermore, the higher off-rate is expected to result in loss of MHC/peptide complexes over time after isolation from the lymph nodes, and thereby to negatively affect the detection of such MHC/peptide complexes in *in vitro* assays. In spite of the latter, also the experiment with the modified OVA peptides, clearly show that epitope presentation after immunization with the extended peptide is focused to the CD11c⁺ DC in the dLN (Fig. 5B).

Taken together, our data argue that vaccination with extended peptides plus CpG results in high-quality CTL epitope presentation that is focused to activated DC in the inflamed, draining lymph node. In comparison, vaccination with minimal peptides plus CpG results in CTL epitope presentation by DC and other APC including B- and T-cells. Epitope presentation by the latter APC is expected to be tolerogenic, because it is not accompanied by sufficient costimulatory signals (33,35,36). Notably, this tolerogenic antigen presentation is not limited to the inflamed draining lymph node, but also takes place in the absence of pro-inflammatory signals in non-inflamed lymph nodes distal to the vaccination site.

In view of the magnitude of CTL epitope presentation by B-cells isolated from mice immunized with the minimal OVA8 peptide (Fig 5A), and the abundance of this cell type in the hematopoietic system, we examined the importance of B-cell mediated epitope presentation

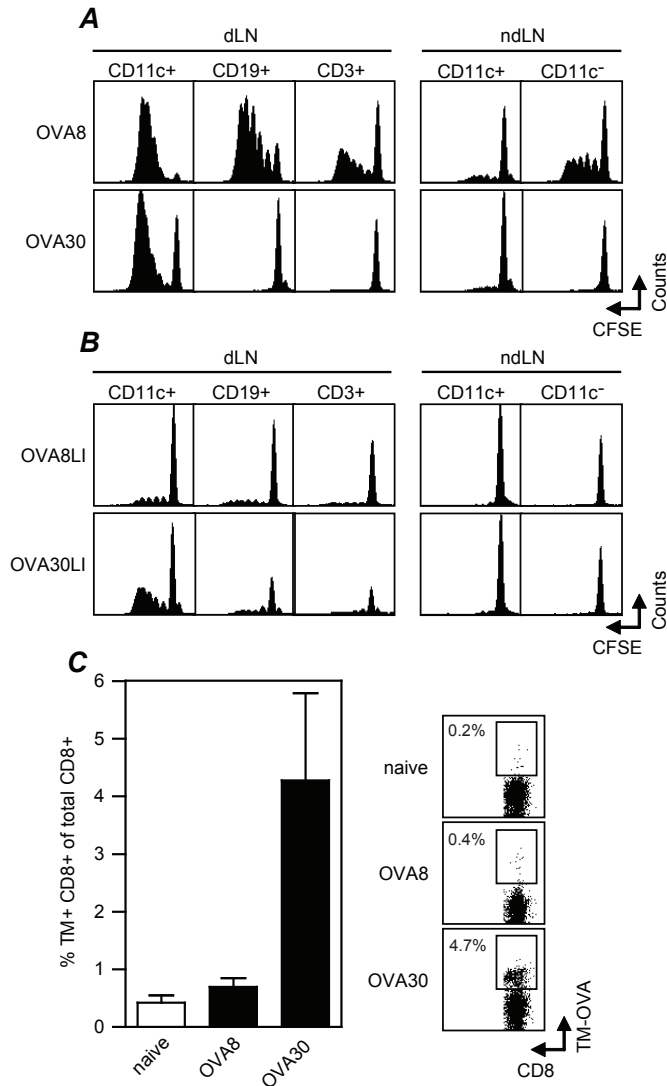


Figure 5. Antigen presentation after vaccination with extended peptides is predominantly focused to DC in the draining lymph node. A,B. Mice were vaccinated s.c. with the indicated peptides in combination with CpG. Two days afterwards, the draining (inguinal and axillary) and non-draining LN (mesenteric) were isolated. The dLN cells were sorted into CD11c+ DC, CD19+ B cells and CD3+ T-cells while the ndLN cells were sorted into CD11c+ DC and CD11c- cells. The resulting cell fractions CD11c+ cells 5×10^4 ; CD19+ cells 4×10^5 ; CD3+ cells 8×10^5 ; CD11c- cells 8×10^5 were used as stimulator cells in a 3-day co-cultures with 1×10^5 naïve CFSE-labeled OT-1 T cells. Proliferation of OT-1 T cells was measured as dilution of CFSE by flow cytometry. C. Antigen presentation on B-cells is important for CTL-priming after minimal CTL peptide vaccination. B cell deficient mice ($n=7$) were vaccinated s.c. with either OVA8 or OVA30 peptide in combination with CpG. Three weeks later, splenocytes were stimulated in vitro with OVA-expressing tumor cells for seven days, after which the frequencies of TM OVA+ CD8+ T cells were evaluated. Histograms show mean values $\pm$ SEM (OVA30 vs. naïve, $p<0.03$). In addition, representative FACS plots are shown.

for CTL priming in mice immunized with minimal versus extended OVA peptides. Mice deficient for B cells were vaccinated with either of these peptides plus CpG and stimulated *in vitro*, after which the frequencies of OVA-specific CD8⁺ T cells were determined by flow cytometry. This experiment showed that B-cell deficiency profoundly affected CTL priming after minimal OVA8 peptide vaccination, while leaving CTL priming by vaccination with the extended OVA30 peptide largely intact (Fig. 5C). These findings support our notion that antigen presentation by B-cells plays a prominent role in T-cell priming by minimal CTL epitope vaccines.

In conclusion, the focused antigen presentation on activated DC in inflamed, draining lymph nodes (Fig 5), together with the increased longevity of antigen presentation (Fig 3) can readily explain why vaccination with extended CTL peptides results in superior CTL effector responses as compared to vaccination with minimal CTL peptides.

DISCUSSION

In the present study, we addressed the question why peptide-based vaccines comprising extended CTL peptides, in particular the HPV16E7-specific anti-tumor vaccine on which we reported previously (17), elicit superior T-cell immune responses as compared to vaccines consisting of minimal peptide epitopes. Our experiments, in which we used the chicken Ovalbumin (OVA) CTL epitope as a model antigen, showed that vaccination with extended CTL peptides resulted in increased duration of antigen presentation, especially in the case of a peptide vaccine that comprises a modified OVA epitope with suboptimal MHC-binding characteristics. Although this correlation between duration of *in vivo* antigen presentation and vaccine performance is in line with previous observations by others (29,30), it cannot fully account for the different capacities of minimal and extended peptide vaccines to elicit CTL immunity. Because maintenance of *in vivo* antigen presentation through repeated administration of short peptides, although resulting in the priming of higher CTL frequencies, failed to elicit tumoricidal CTL effector responses. A plausible explanation for this failure is offered by the outcome of further experiments, in which we evaluated the location of antigen presentation and the APC-types involved after vaccination with minimal versus extended peptides. These data revealed that antigen presentation after vaccination with extended CTL peptides is focused to the DC in the draining lymph nodes, while vaccination with minimal peptides can result in antigen presentation on multiple APC types in both the draining lymph nodes and in distal lymphoid organs. As a consequence, vaccination with extended peptides primarily results in pro-immunogenic presentation of CTL epitopes by DC, in the context of strong costimulatory signals and in an inflamed lymph node. In contrast, vaccination with minimal CTL peptides, although resulting in this pro-immunogenic mode of antigen

presentation, also leads to presentation of CTL epitopes by cell types that cannot provide T-cells with the costimulatory signals required for optimal T-cell activation, such as T- and B-cells. Importantly, the latter antigen presentation takes place in non-inflamed lymph nodes, distal from the source of adjuvant, and thereby in the absence of a pro-inflammatory context. It is conceivable that the resulting blend of T-cell activating and tolerizing signals does not constitute an optimal setting for mobilization of potent effector CTL responses. This notion is supported by our finding that vaccination with minimal peptide vaccines, although increasing the numbers of antigen-specific CTLs, fails to launch potent effector CTL immunity capable of therapeutic efficacy against tumors.

Our recent data do not only provide insight into the mode of action of minimal and extended peptide vaccines in pre-clinical models, but may also explain the lack of therapeutic efficacy of minimal CTL epitope vaccines in the clinic, in particular with respect to two recent studies in which melanoma patients were vaccinated with minimal CTL peptides derived from gp100 and MART-1 (12,13). In both cases, peptide vaccination resulted in significant levels of circulating, antigen-specific CTLs that exhibited clear antigen-specific reactivity *in vitro*. However, there was no correlation between the CTL frequencies detected in peripheral blood and the clinical impact of vaccination. In fact, clinical impact was similarly poor as that of previous peptide vaccination studies (15,16). As such, the results of these clinical vaccination studies bear a strong resemblance to those of our mouse studies with minimal CTL peptide vaccines. Interestingly, one of these studies tested whether complementation of the minimal peptide vaccine with CpG would increase the potency of the vaccine, and concluded that addition of this adjuvant enhanced CTL frequencies but not the anti-tumor efficacy (13). In view of our data, this lack of true improvement can be explained by the fact that CpG, although activating the DC in the draining lymph node, does not empower the other antigen-loaded APC, such as B-cells and T-cells, in draining and non-draining lymphoid organs to efficiently activate CTLs, and therefore does not improve the overall quality of the antigen presentation.

Our observation that extended peptide length is especially important for greater duration of *in vivo* antigen presentation in case of the modified OVA epitope (Fig. 3), which displays weaker MHC-binding, argues that vaccination with extended peptides creates an antigen depot that compensates for loss of surface expressed epitope. Although the nature of this depot awaits further investigation, it is likely to constitute an extracellular depot rather than storage of antigen within the DC. This is suggested by our observation that *ex vivo* detection of antigen presentation on DC isolated from draining lymph nodes of OVA30LI vaccinated mice is rather difficult. If the DC are used in co-cultures with OT-1 T-cells, a modest level of T-cell proliferation reflecting antigen presentation can be observed (Fig. 5A, B). However,



this antigen presentation is lost if the DC are not rapidly isolated and immediately used for the co-cultures (data not shown). Nevertheless, *in vivo* antigen presentation as detected by proliferation of OT-1 T-cells can be observed for at least 10 days after vaccination with the OVA30LI8 peptide (Fig 3C). We have previously shown that such a depot can also be created for minimal peptide vaccines by administering these peptides with oil-in-water formulations, such as an emulsion with IFA. Importantly, the resulting prolonged presentation fails to induce an effective CD8⁺ T cell response and eventually result in T-cell tolerance through exhaustion of the antigen-specific T-cells (24,37). Our cumulative data indicate that the tolerance induced by this peptide in IFA vaccine results from prolonged systemic presentation of antigen in the absence of sufficient costimulation, because it can be overcome, by addition of a CD4⁺ T-helper epitope to the vaccine, or by extending the length of the peptide (24). In case of the CD4⁺ T-helper epitope, the helper signal to the DC improves the net quality of antigen presentation and thereby the immunogenic potential of the vaccine (38,39), while the use of extended peptides focuses antigen presentation to the DC in the inflamed lymph nodes draining the vaccination site.

In conclusion, our studies strongly support the use of vaccines comprising extended instead of minimal CTL peptides, as this results in a prolonged *in vivo* antigen presentation as well as in a better quality of the antigenic signal that is confined to activated DC in the inflamed lymph nodes.

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4

Dendritic Cell activating agonists

Vaccine

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Multiple CD4 and CD8 T-cell activation parameters predict vaccine efficacy *in vivo* mediated by individual DC-activating agonists.

Abstract. A systematic comparison of the immunostimulatory capacity of TLR 2, 3, 4, 5, 7 and 9 agonists and an agonistic CD40-specific antibody was performed in a single long peptide vaccination model. All adjuvants activated DC *in vitro* but not all induced a strong functional T-cell response *in vivo*. Optimal clonal CD8(+) T-cell expansion depended on the capacity of agonists to mature pro-inflammatory DC and the duration of their *in vivo* stimulatory effect. Strong agonists promoted the induction of both antigen-specific IFNgamma-producing CD4(+) T-helper cells and high numbers of IFNgamma producing CD8(+) effector T-cells that killed target cells *in vivo*. Importantly, the capacity of an agonist to function as an adjuvant depended on the vaccine strategy used. Collectively, the multi-parameter system presented here can be used as a general road map to develop therapeutic vaccines.

INTRODUCTION

Dendritic cell (DC) activation is key to the induction of an effective cytotoxic T-cell (CTL) response (1). In effective natural immune responses CD4⁺ T-helper cells can fully activate DC through the CD40/CD40L signaling pathway (2, 3). Other DC activation signals that can support induction of powerful CTL responses involve the innate immune receptors and include the molecularly defined agonists for Toll-like receptors (TLR), which is a family of pattern recognition receptors that recognize structural components of bacteria, viruses and fungi (4-6). The use of these TLR agonists in vaccine formulations may permit the development of effective therapeutic vaccine strategies for the immunotherapy of cancer. Several reports show an improved efficiency of vaccines in mice, when antigen delivery is combined with a TLR 9 agonist. In a C57BL/6 mouse model, the combined injection of the TLR 9 agonist CpG-ODN1628 and an HPV16-specific long peptide comprising CD4⁺ and CD8⁺ T-cell epitopes resulted in a strong expansion of HPV16-specific CD8⁺ T-cells and the subsequent eradication of established HPV16⁺ TC-1 tumors (7). However, the distribution of TLR 9 differs substantially between human beings and mice. While in C57BL/6 mice TLR 9 is broadly expressed on all types of DC (8, 9), the expression of TLR 9 in humans is restricted to the plasmacytoid DC (9-11). Importantly, the expression pattern of the other TLRs is similar in both humans and mice. TLR 2, 3, 4, 5 and 7 are broadly expressed on several DC types of both species (9, 10, 12). However, only few studies have reported the use of TLR agonists other than TLR 9 as adjuvants for vaccines. In some mouse studies TLR 3, 4 and 7 agonists showed a weak capacity to activate antigen-specific CD8⁺ T-cell responses whereas in other studies activation of these TLR resulted in a strong CD8⁺ T-cell response (13-18). Combinations of immunostimulatory molecules are expected to enhance the therapeutic potential of anti-tumor vaccines as it was shown that agonists for TLRs synergized with CD40 triggering and stimulated a 10-20 fold greater expansion of antigen-specific CD8⁺ T-cells than either agonist alone (17).

Because the above-mentioned studies were all carried out in different mouse models, we performed a systematic comparison in which the adjuvanticity of TLR 2, 3, 4, 5, 7 and 9 agonists as well as an agonistic CD40-specific antibody was tested in a single long peptide vaccination model. Our experiments show that profound differences exist between these agonists with respect to a) the efficacy to activate DC *in vitro*; b) the capacity to induce the production of inflammatory cytokines by DC; c) their capacity to sustain T-cell expansion *in vivo*; and d) their ability to trigger direct T-cell effector function as measured by IFN γ production, *in vivo* cytotoxicity, T-cell migration and protection against the outgrowth of established tumors. Collectively, these data provide key information regarding the expected features and design of potent therapeutic vaccines.

MATERIAL AND METHODS

Mice. Female C57BL/6 (B6, H-2b) mice were purchased from Charles River (Paris, France). Mice were maintained under specific pathogen-free conditions and used at 6-10 weeks of age. OT-1 Rag-/- CD45.1 mice were bred at the LUMC animal facility under specific pathogen-free conditions. These mice were used as a source of OVA-specific CD8+ T-cells for adoptive transfer studies.

Cells and cell lines. The tumor cell line 13.2 was derived from B6 mouse embryo cells (B6 MECs) transformed with adenovirus type 5-derived E1 protein in which the H-2D^b E1A epitope was replaced with the HPV16-E7₄₉₋₅₇ CTL epitope (19). Notably, no HPV16-specific T-helper epitopes are present in this cell line. TC-1 was derived from primary epithelial cells of C57BL/6 mice co-transformed with HPV-16 E6 and E7 and c-Ha-ras oncogenes (a kind gift of dr. T.C. Wu). Both tumor cell lines were cultured in IMDM (BioWhittaker, Verviers, Belgium) and 10% FCS (Greiner, Alphen aan de Rijn, The Netherlands) (19). Primary bone-marrow-derived DC cultures (BM-DC) were generated as described previously (20). Both floating and adherent DC were used for the experiment and the purity of the cultured cells was determined by flow cytometry using the following antibodies: APC-conjugated anti-CD45R/B220 (clone RA3-6B2; BD Pharmingen, San Diego, CA, USA), FITC-conjugated anti-CD11b (clone M1/70; BD Pharmingen) and PE-conjugated anti-CD11c (clone HL3; BD Pharmingen).

Antigens and peptides. As human papillomavirus (HPV)-specific antigens the H-2D^b-restricted CTL epitope HPV16-E7₄₉₋₅₇ (RAHYNIVTF) and the HPV16 E7₄₃₋₇₇ 35-residue long peptide QAEPDRAHYNIVTFCCCKDSTLRLCVQSTHVDIR, covering both the CTL epitope (in bold) and the T-helper (Th) epitope (underlined), were used. In addition, we used a 32-mer long OVA peptide LPDEVSGLEQLESIINFEKLTEWTSSNVMEER that encodes the H-K^b-restricted CTL epitope SIINFEKL (in bold), which is recognized by the T-cell receptor transgenic OT-1 cells. The purity of the peptides was determined by RP-HPLC and was found to be routinely over 90%. Peptides were dissolved in 0.5% DMSO in PBS and, if not used immediately, stored at -20°C. The recombinant HPV16-E7 protein was produced in recombinant Escherichia coli transformed with Pet-19b-HPV16-E7 and purified as described previously (21).

Molecularly defined adjuvants. The following agonists for the different murine Toll-like receptor (TLR) were used: Palmitoyl-Cys(CRS)-2,3-di(palmitoyloxy)-propyl (PAM₃CSK₄; EMC Echaz Microcollections, Tübingen, Germany) for TLR 2. Poly I:C₁₂U (Ampligen;

Bioclones, Sandton, South Africa; kindly provided by dr. M. Adams, Cardiff, UK) for TLR 3. Monophosphoryl lipid A (MPL; detoxified LPS) was kindly provided by Corixa Corporations (Seattle, WA, USA) for TLR 4. Flagellin (FliC protein from *Salmonella enterica* serovar Typhirium, kindly provided by dr. JC Sirard, INSERM, Lille, France) for TLR 5. R848 (InvivoGen, San Diego, CA, USA) for TLR 7 and CpG (ODN1826: TCCATGACGTTCTGACGTT, kindly provided by dr. G. Lipford (Coley Pharmaceutical Group, Wellesley, MA, USA) for TLR 9. In addition, the agonistic CD40 antibody FGK-45 was used (22). As a standard we used incomplete Freund's adjuvant (IFA).

***In vitro* DC stimulating experiments.** Activated DC are characterized by cytokine production and the up-regulation of accessory and co-stimulatory molecules expressed at the cell surface, including CD40, CD80, CD86 and CD83 as well as MHC class II molecules (23). Therefore, 10-days cultured BM-DC were incubated at 40,000 cells/well in a flat-bottom 96-well plate for 48 h with a concentration range (in 2 to 5-fold stepwise dilutions from 50 µg/ml down to 0.5 ng/ml for all adjuvants except for flagellin which had to be tested in a broader range namely from 50 µg/ml down to 1 pg/ml) of the various adjuvants to activate these antigen-presenting cells. Supernatants of all the cultures were harvested after 24 h and 48 h to analyze the cytokine profile (IL-6, TNF- α , and IL-10) by cytometric bead array (CBA; BD Pharmingen). As an universal marker for activation of all DC subsets present in BM-DC (8) we determined the concentration of mouse IL-12p40 in the supernatants by a standard sandwich ELISA using rat anti-mouse IL-12p40 (clone C15.6; BD Pharmingen) for catching and biotinylated rat-anti mouse IL-12p40 (clone C17.8; BD Pharmingen) for the detection. Streptavidin-HRP and ABTS (Sigma-Aldrich, St. Louis, MO, USA) were used as enzyme and substrate, respectively. At 48 h of culturing the cells were harvested and stained for the activation markers with PE-labeled anti-CD40 (clone 3/23; BD Pharmingen), FITC-labeled anti-CD80/B7.1 (clone 16-10A1; BD Pharmingen) and PE-labeled anti-CD86/B7.2 (clone GL-1; BD Pharmingen) and analyzed by flow cytometry.

Vaccination of mice. Mice were vaccinated subcutaneously (s.c.) in the right flank with 200 µl of the vaccine, consisting of 150 µg of long HPV16 E7 peptide or 150 µg of long OVA peptide, respectively, admixed with or without 50 µg PAM₃CSK₄, 20 µg Poly I:C₁₂U (Ampligen), 10 µg MPL, 50 µg R848 or 50 µg CpG per mouse, respectively. In case of CD40 ligation 100 µg FGK-45 per mouse was subcutaneously injected the day after peptide vaccination. After 10 days mice were sacrificed and spleen cells were isolated.

The used doses of the various adjuvants were either advised by the provider but also demonstrated to be effective (PAM₃CSK₄, MPL, CpG (7, 24)) or deduced from a dose finding

pilot experiment (range 10–50 µg adjuvant/mouse). Although R848 was successfully used at a concentration of 17 µg per mouse (25) and Poly I:C₁₂U (Ampligen) at 25–50 µg/mouse (17, 26, 27) we first tested these components in the dose finding experiment. The dose with the relatively highest number of tetramer positive CD8⁺ T-cells was used for further vaccination experiments. The amount of FGK-45 used was based on previous work of our group (22).

Tumor challenge experiments. Mice were injected with 25,000 TC-1 tumor cells in the left flank (the tumor take was 100%). At day 9–10, when the tumors were palpable, mice were vaccinated at the right flank with indicated vaccines. Fourteen days later, these mice received a booster injection with the vaccine. Tumor growth was monitored every 2–3 days and followed for approximately 80 days.

***In vivo* cytotoxicity analysis.** Mice were vaccinated as described above. After 9 days peptide-loaded Thy 1.1+ (CD90.1) target cells differentially labeled with CFSE were injected intravenously (i.v.) into Thy 1.2+ (CD90.2) mice. At day 10 mice were sacrificed to obtain both draining lymph node cells and spleen cells. For target cells the spleen and lymph nodes were obtained from a naïve mouse (28). After a red blood cell lysis the cells were purified for T-cells by nylon wool column and resuspended at 10×10^6 cells per ml in medium. Then, the obtained number of cells were split into two equal amounts and incubated for 90 minutes at 37°C either with 0.5 µg/ml of the HPV16 E7_{49–57} peptide (RAHYNIVTF as specific target peptide) or OVA_{257–264} peptide (SIINFEKL as non-specific target or control peptide). After 4 wash steps the specific target cells were labeled with a final concentration of 5 µM CFSE and the non-specific target cells with 0.5 µM CFSE for 10 minutes at 37°C. Both target cells were resuspended at 50×10^6 cells/ml, mixed at a 1:1 ratio, and injected i.v. at a volume of 200 µl per mouse. By flow cytometry the amount of remaining/non-killed specific (CFSE^{high}) and non-specific (CFSE^{low}) target cells were established by gating on Thy1.1+ cells (clone H1551; BD Biosciences). The percentage of specific killing was calculated according to the following formula: $(1 - (\% \text{ of specific peptide-loaded target cells} / \% \text{ of control peptide-loaded target cells})) \times 100\%$.

Analysis of HPV16-E7-specific T-cells. The spleen cells were either analyzed directly by tetramer staining as described below or after expansion of the T-cells by culturing spleen cells (4×10^6 cells/well of a 24-wells plate) in complete medium in the presence of 5×10^5 HPV16 E7_{49–57}-expressing cells (tumor cell line 13.2). Cultures were maintained at 37°C in humidified air containing 5% CO₂. No exogenous IL-2 was added thereby enabling the *in vivo* generated memory T-cells to undergo a secondary expansion *in vitro*, which is a well

known hallmark of a proper immune response (29). On day 6, dead cells were removed from the culture by centrifugation over a Ficoll density gradient and remaining viable cells were seeded in 24-wells plates at 1.5×10^6 cells/well. On day 7, tetramer staining or intracellular cytokine staining was performed.

PE-labeled H-2D^b epitope E7₄₉₋₅₇ (RAHYNIVTF)-containing tetramers were constructed and used for the analysis of peptide-specific CTL-immunity as described earlier (19). FITC-labeled anti-CD8b.2 (Ly-3.2) antibody (clone 53-5.8; BD Pharmingen), APC-labeled anti-CD4 (clone RM4-5; BD Pharmingen) and PE-labeled anti-IFN γ (clone XMG1.2; BD Pharmingen) were used for the analysis of antigen-specific IFN γ production of HPV16 E7-specific CD8⁺ and CD4⁺ T-cells as described previously (19, 30).

Adoptive transfer of T-cell receptor transgenic CD8⁺ T-cells. To determine the fate of T-cells primed by antigen-presenting DC, which have been stimulated by the various adjuvants, mice were vaccinated s.c. with 150 μ g long OVA peptide with or without the DC stimulating agent in a total volume of 200 μ l. After 4 days, T-cell receptor transgenic, SIINFEKL-specific CD8⁺ T-cells (OT-1 cells) were isolated from spleen and lymph nodes of naïve OT-1 mice and after lysis of red blood cells, labeled with 5 μ M CFSE for 10' at 37 degrees Celsius, and injected i.v. at a dose of $1-2 \times 10^6$. After 5 days mice were sacrificed and the CFSE dilution of the labeled OT-1 cells was analyzed by flow cytometry. Gates were set based on CD45.1 (clone A20; BD Pharmingen) and CD8 α (clone 53-6.7; BD Pharmingen) expression.

RESULTS

In vitro activation of antigen presenting cells by adjuvants.

The induction of potent tumor-specific T-cell immunity by anti-tumor vaccination critically depends on the proper conditioning and activation of DC that take up and process the delivered antigens. Fully activated DC that display a Th1 polarizing profile exert optimal CTL-inducing activity (31). Therefore, the efficacy to activate and polarize DC of the TLR-agonists PAM₃CSK₄ (TLR 2), Poly I:C₁₂U (TLR 3), MPL (TLR 4), Flagellin (TLR 5), R848 (TLR 7) and CpG (TLR 9), as well as that of the agonistic CD40 antibody FGK-45, was systematically compared *in vitro*. As a source of DC bone-marrow derived DC (BM-DC) were used as they are considered to be a proper source for physiological relevant DC (32) and they behave functionally very similar to DC *in vivo* (33). The minimum required concentration to activate $\geq 50\%$ of BM-DC, as indicated by the increased expression of the cell surface markers CD40 and CD86 (23) differed per adjuvant (Table 1). On a molar basis Flagellin

Table 1: *In vitro* activation of bone marrow derived dendritic cells and their cytokine profile

Receptor	Compound	TLR	≥50% maturation		Cytokine profile			
			μg/ml	M	IL-12 p40	IL-6	TNFα	IL-10
-	None	-	-	-	32	<20	30	<20
TLR2	PAM ₃ CSK ₄	2	0.05	3.10 ⁻⁸	774	2790	>5000	<20
TLR3	poly I:C ₁₂ U	3	10	1.10 ⁻⁶	176	46	<20	<20
TLR4	MPL	4	0.1	6.10 ⁻⁸	252	>5000	717	<20
TLR5	Flagellin	5	0.0001	2.10 ⁻¹²	30	<20	<20	61
TLR7	R848	7	1	3.10 ⁻⁶	840	1340	880	<20
TLR9	CpG1628	9	0.5	8.10 ⁻⁸	850	>5000	1456	<20
CD40	FGK-45	-	2.5	2.10 ⁻⁸	392	79	<20	<20

BM-DC were cultured in the presence of a range of 2-5 fold diluted concentrations (0.5 ng/ml – 50 μg/ml, except for flagellin which was tested in a range of 1 pg/ml – 50 μg/ml) of the indicated DC agonists for 48 hours, after which the cells were harvested and analyzed for cell surface expression of CD40 and CD86 by flow cytometry. On the basis of these analyses the minimal required concentration of each agonist (indicated in μg/ml and Molar) that resulted in a strong increase in the expression of these markers in at least 50% of the DC was determined for each compound. Supernatants of the DC's incubated with the minimal required concentration of each agonist to activate ≥50% DC were analyzed for the presence of the cytokines (pg/ml) listed. The <20 pg/ml indicates that the values were lower than the detection limit of the assays used.

(<1 picomolar) was the most potent activator of BM-DC, followed by PAM₃CSK₄, MPL, CpG and FGK-45 of which concentrations in the 20-80 nanomolar range were sufficient to mature DC. Poly I:C₁₂U and R848 were less effective in that 2-3 micromolar of these TLR agonists was needed to mature DC.

Supernatants from DC-cultures incubated with the minimal required concentration of each agonist to activate ≥ 50% of DC were collected and the amount of cytokine produced was measured in order to analyze the polarization of matured DC. BM-DC produced the pro-inflammatory cytokines IL-12p40, IL-6 and TNFα when incubated with the compounds PAM₃CSK₄, MPL, R848 and CpG. Poly I:C₁₂U and the agonistic CD40 antibody FGK-45 displayed only a moderate capacity to trigger the production of such cytokines. In contrast, BM-DC incubated with Flagellin were not able to develop a T-helper type 1 (Th1) polarizing profile because they did not produce any of the pro-inflammatory cytokines. However, flagellin-triggered DC produced the regulatory cytokine IL-10 instead (Table 1), which is

well known to be non-supportive for induction of cytotoxic T-cell (CTL) responses.

In conclusion, all the tested adjuvants were able to activate C57BL/6 mouse-derived BM-DC in accordance with the presence of TLR on these cells (9, 10, 12). Striking differences were found with respect to the amount of each compound required to mature DC and activate the production of pro-inflammatory cytokines. PAM₃CSK₄, MPL, CpG and FGK-45, are expected to function as potent adjuvants in vaccines that aim at the induction of effective Th1 and CTL responses, as nanomolar concentrations of these compounds are able to stimulate DC to produce pro-inflammatory cytokines.

The efficacy of DC activator agents to induce HPV-specific T-cell responses *in vivo*.

The marked differences between the concentrations of DC-activating agents that were needed to mature DC *in vitro* prompted us to evaluate these compounds, and in particular those agents that promoted the development of DC secreting pro-inflammatory cytokines, for their efficacy to stimulate the induction of HPV16-specific T-cell responses *in vivo*. The HPV16 E7₄₃₋₇₇ peptide that contains both a CTL epitope and a Th epitope (7) was used as vaccine. Mice received a single dose of this peptide-antigen in combination with one of the indicated DC agonists (see M&M). Splenocytes were isolated 10 days after vaccination, stimulated *in vitro* for 7 days with 13.2 tumor cells, and the secondary expansion capacity of memory T-cells, as a hallmark for good immunity (29) was measured by flow cytometric analysis of the number of CD8⁺ T-cells stained with H-2D^b/E7₄₉₋₅₇-specific tetramer (Fig. 1a).

Very few tetramer positive (TM⁺) CD8⁺ T-cells were detectable when mice were injected with HPV16 E7₄₃₋₇₇ in the absence of any stimulatory agent (PBS). A minor increase in the number of TM⁺ CD8⁺ T-cells was found when mice were vaccinated with peptide emulsified in IFA or its clinical grade counterpart Montanide ISA 51. In concordance with the limited capacity of poly I:C₁₂U and R848 to trigger DC activation *in vitro*, no increase in the number of HPV16-specific CD8⁺ T-cells was found when these agonists were used in the vaccine (Fig. 1a). Additional experiments in which these adjuvants were administered either in an IFA depot or every 2 days, did not dramatically improve the outcome of vaccination (less than 3% TM⁺ CD8⁺ T-cells). This suggests that predominantly their insufficient capacity to properly mature DC is responsible for their failure to induce strong HPV16-specific CD8⁺ T-cell immunity.

The most potent adjuvants were MPL, CpG and agonistic CD40 antibody FGK-45 (Fig. 1a). In all three cases approximately 20% of the CD8⁺ T-cells stained with the H-2D^b-E7₄₉₋₅₇ tetramer, which is significantly higher than responses induced by peptide in PBS (mean=0.93%), IFA (mean=5.6%) or Montanide ISA 51 (mean=1.5%). Unexpectedly, PAM₃CSK₄ was not able to support the induction of a strong CD8⁺ T-cell response, despite its capacity to activate a

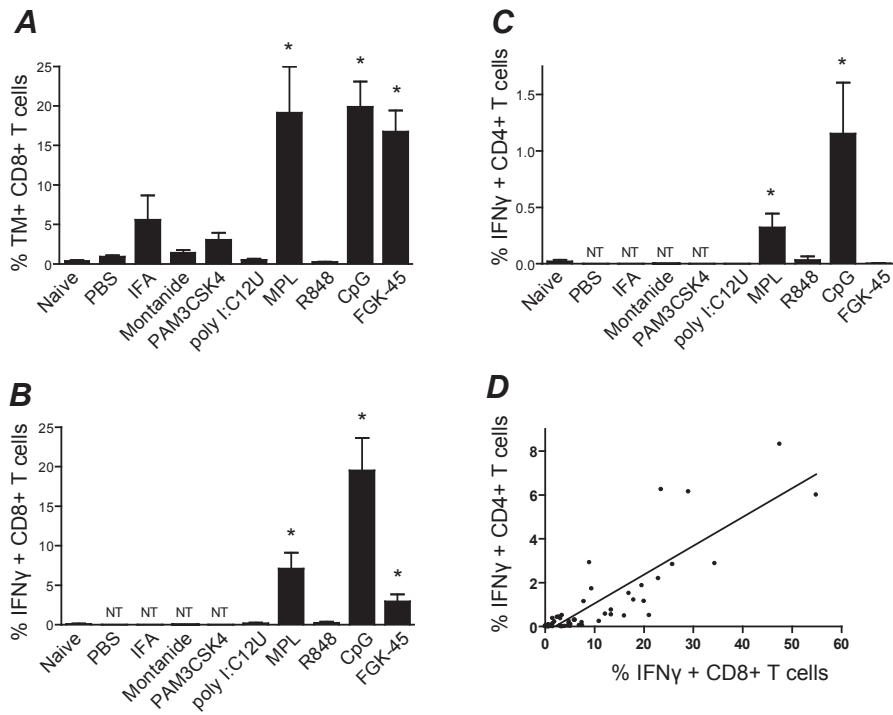


Figure 1. Efficacy of DC agonists to induce HPV16-specific T-cell immunity. Groups of 6-16 C57BL/6 mice were vaccinated s.c. in the right flank with a 35-mer HPV peptide, comprising an overlapping T-helper epitope and a CTL epitope, in combination with the indicated DC agonists. The used dose of the adjuvants were 50 µg PAM₃CSK₄, 20 µg Poly I:C₁₂U (Ampligen), 10 µg MPL, 50 µg R848, 50 µg CpG or 100 µg FGK-45 per mouse (See M&M). After ten days the mice were sacrificed and the spleens harvested. (A) The number of HPV16 E7-specific CD8+ T-cells by H-2D^b-E7₄₉₋₅₇ tetramer analysis was determined after a 7-day incubation period of the splenocytes with E7₄₉₋₅₇-expressing cells (tumor cell line 13.2). Mice vaccinated with the TLR agonists MPL and CpG as well as the agonistic CD40-specific antibody FGK-45 display a significantly increased number of HPV16-specific CD8+ T-cells compared to mice vaccinated with peptide in IFA, Montanide or PBS (*, $p < 0.05$). The functionality of the (B) HPV16-specific CD8+ T-cells and (C) HPV16-specific CD4+ T-cells was determined by intracellular cytokine staining for IFN γ . Significant higher T-cell responses compared to the T-cell immunity of naive mice are indicated with an asterisk ($p < 0.05$). (D) Linear regression analysis showed a strong correlation between the percentage of HPV16-specific IFN γ -producing CD8+ T-cells and HPV16-specific IFN γ -producing CD4+ T-cells ($r^2 = 0.73$; $p < 0.0001$).

pro-inflammatory DC response *in vitro*.

The differences between the potency of the adjuvants to stimulate HPV16-specific CD8+ T-cells became even more pronounced when the functionality of these CD8+ T-cells was analyzed by intracellular staining of antigen-specific IFN γ production (Fig. 1b). We focused on the three adjuvants (MPL, CpG and agonistic CD40 antibody FGK-45) that stimulated the largest expansion of CD8+ H-2D^b-E7₄₉₋₅₇ tetramer positive T-cells. The percentage of HPV16-specific IFN γ -producing CD8+ T-cells in the splenocyte cultures of CpG treated

mice (mean =19.5%) was similar to that found by the tetramer analysis. Importantly, even though the induction of TM+ CD8+ T-cells was comparable to CpG treatment, the percentage of HPV16-specific IFN γ -producing CD8+ T-cells in the groups of mice vaccinated with MPL (mean=7.1%) or the agonistic CD40 antibody FGK-45 (mean=3.0%) was significantly lower ($p<0.01$). HPV16 E7-specific IFN γ -producing CD4+ T-cells were only detected in mice vaccinated with peptide in combination with CpG or MPL (Fig. 1c).

Overall, the comparative analysis of DC agonists revealed a good correlation between the numbers of IFN γ -producing CD8+ and CD4+ T-cells induced (Fig. 1d), which is in line with the prevailing views on the importance of CD4+ Th1 immunity for efficient activation of CD8+ T-cell responses (reviewed in Ref. 4). The agonistic CD40 antibody FGK-45, although also very potent in the induction of high numbers of antigen-specific CD8+ T-cells, failed to trigger strong IFN γ production by E7-specific CD8+ T-cells (Fig. 1c). This failure is paralleled by the absence of an E7-specific CD4+ Th1 response (Fig. 1c), the induction of which may have been prohibited as a result of interference by the agonistic CD40 antibody with the CD40L signaling pathway.

In conclusion, the TLR 9 ligand CpG clearly acts as the most potent adjuvant for the peptide vaccine tested, in that it induces the strongest E7-specific Th1 and CTL responses. The peptide vaccine supplemented with TLR 4 ligand MPL similarly triggers such T-cell responses, albeit of a lower magnitude. This correlates with the *in vitro* capacity of these adjuvants to induce pro-inflammatory cytokine production and DC maturation at low doses. However, this latter capacity per se is not sufficient for strong *in vivo* adjuvanticity because PAM₃CSK₄, which is also very potent in the activation of pro-inflammatory DC, did not trigger a strong HPV16-specific T-cell response *in vivo*.

Potent DC agonists have a sustained capacity to induce expansion of antigen-specific T-cells.

In order to study the apparent discrepancy between the capacity of some of the agonists to properly activate DC while failing to induce a strong HPV16-specific CD8+ T-cell response, we studied the proliferation and expansion of T-cells that are activated *in vivo* by peptide and the different adjuvants. For this we made use of a T-cell receptor transgenic OT-1 system (SIINFEKL-specific CD8+ T-cells). Since the OT-1 cells will recognize this injected cognate SIINFEKL peptide with the same T-cell receptor affinity and will respond all exactly the same upon triggering, differences in the OT-1 response will reflect the efficacy of the different DC activating agents co-injected with the peptide vaccine. By exploiting the CD45.1/CD45.2 polymorphism, these CD45.1+ CD8+ T-cells (OT-1 cells) can be easily tracked *in vivo* when adoptively transferred into congenic CD45.2+ C57BL/6 mice. CFSE-labeled naïve

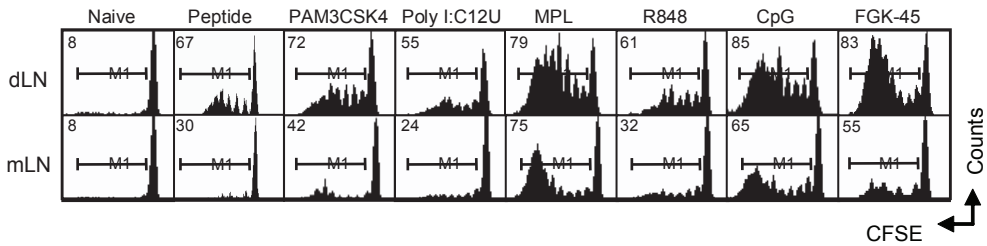


Figure 2. *In vivo* capacity of adjuvants to induce and sustain the expansion of specific T-cells. C57BL/6 mice were vaccinated s.c. in the right flank with long OVA peptide with or without the indicated adjuvants. Subsequently, these mice received $1\text{-}2 \times 10^6$ CFSE-labelled CD45.1+ OT-1 T-cells i.v. Then, five days later the draining lymph node (DLN: inguinal LN) and the non-draining lymph node (MLN: mesenteric LN) were harvested and the obtained cells were analysed by flow cytometry thereby acquiring 200,000 life cells. Histograms were plotted by gating on CD45.1+ and CD8a+ cells. Numbers in the graph indicate the percentage of cells that have divided at least once, as gated in M2. Data are representative for two mice per group in two different experiments.

OT-1 cells were adoptively transferred into mice vaccinated with a 32-mer OVA peptide comprising the CTL epitope SIINFEKL. Subsequently, the proliferative response of OT-1 cells in the vaccine-draining lymph node (DLN) and in the non-draining (mesenteric) lymph node (MLN) was analyzed after 5 days by flow cytometry (Fig. 2).

The vaccines containing MPL, CpG or FGK-45 were able to trigger a strong and sustained proliferative response of OT-1 cells, as is indicated by the increase in height of the peaks of the divided T-cells (compare to peptide only). In contrast, OT-1 cells primed by either peptide alone or with peptide in combination with PAM₃CSK₄, poly I:C₁₂U or R848 did divide in the DLN, however, we did not observe accumulation of T-cells in higher dividing cell populations (i.e. the typical cell expansion peaks). Actually, after each division the height of the peak decreased, pointing at either migration of the divided cells to other parts of the lymphoid system or increased cell death. Analysis of the non-draining lymph node (i.e. MLN) revealed that the OT-1 cells primed by peptide and MPL or CpG sequester and migrate from the DLN to other lymphoid organs. In contrast, this was not observed when PAM₃CSK₄, poly I:C₁₂U or R848 were used (Fig. 2), suggesting that under these circumstances OT-1 cells divided but did not migrate or survive after antigenic triggering. A failure of PAM₃CSK₄, poly I:C₁₂U and R848 to support the sustained expansion of antigen-specific T-cells may explain why it failed to induce vigorous HPV16-specific T-cell responses *in vivo* (Fig. 1a). Moreover, the results using the 32-mer OVA peptide (without a T-helper epitope present) reinforces the observations made with the 35-mer HPV16 E7 peptide (containing a CTL and T-helper epitope), in that the observation of abortively expanded OT-1 cells (Fig. 2) was correlated with the disability to induce a strong CD8+ T-cell response as measured by tetramers (Fig. 1a) in mice treated with the long HPV peptide together with PAM₃CSK₄, poly I:C₁₂U or R848.

MPL and CpG, but not agonistic CD40 antibody FGK-45 mediated immune activation, confer *in vivo* killing capacity to vaccine-induced CD8+ T-cells.

The three most potent adjuvants (CpG, MPL and agonistic antibody FGK) were tested for their capacity to enhance the *in vivo* cytolytic activity of vaccine-induced HPV16 E7-specific CD8+ T-cells, as this is the key effector mechanism for destroying virus infected cells as well as for tumor eradication in many solid tumor settings. HPV16 E7₄₉₋₅₇ or control peptide loaded target cells, labeled with CFSE at different fluorescent intensities to allow differential analysis, were simultaneously injected in a one to one ratio into immunized mice at day 9. After 24 hours, the recovery of these labeled target cells in the spleen was analyzed by flow cytometry and the percentage of *in vivo* killed target cells was determined (28). Whereas the spleens of naïve mice contained equal numbers of both target cell types, only the CFSE^{high} E7 peptide loaded cells were clearly killed in spleens of mice immunized with peptide vaccine containing CpG or MPL (Fig. 3). The highest percentages of HPV16 E7-specific cytotoxicity were found in mice vaccinated with peptide and CpG followed by mice vaccinated with peptide and MPL. The efficacy of vaccination with peptide and agonistic CD40 antibody FGK-45 was disappointing because there was no significant *in vivo* killing of specific target

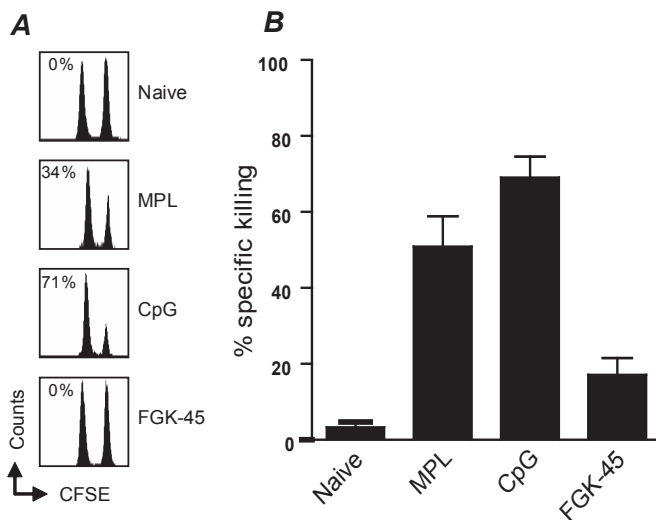


Figure 3. *In vivo* cytolytic function of vaccine-induced CD8+ T-cells. C57BL/6 mice were vaccinated with E7 peptide supplemented with MPL, CpG or agonistic CD40-specific antibody FGK-45. After 9 days, the mice received i.v. 2×10^6 CFSE^{high} splenocytes pulsed with the target peptide (E7₄₉₋₅₇; RAHYNIVTF) along with 2×10^6 CFSE^{low} splenocytes pulsed with a control peptide (OVA; SIINFEKL). Spleens of these mice were harvested 24 hrs. later and the relative numbers of CFSE^{high} and CFSE^{low} target cells were determined by flow cytometry. Two independent experiments with 5 mice per group were performed. (A) Histograms of representative examples. (B) Percentage of specific *in vivo* killing of E7-labeled target cells.

cells observed in mice vaccinated with peptide and FGK-45. Overall, the magnitude of the E7-specific *in vivo* cytolytic response corresponded with that of the number of IFN γ -positive CD8 $^{+}$ T-cells detected by flow cytometry (Fig. 1b).

The CD8 $^{+}$ T-cell effector function in an *in vivo* cytolytic assay does not depend on T-cell homing, because the target cells were injected intravenously. Therefore, the effector function of the vaccine-induced T-cell response was also tested against a subcutaneous tumor. C57BL/6 mice bearing palpable tumors of syngenic HPV16E7-expressing TC-1 tumor cells were vaccinated twice, with a 14-day interval and in the flank contra-lateral to the tumor site, with the aforementioned formulations. The tumors in non-vaccinated mice grew out rapidly, whereas the outgrowth of tumors in mice treated with peptide vaccine containing MPL or FGK was slightly delayed (Fig. 4). Despite the fact that MPL conferred *in vivo* killing of peptide-pulsed target cells in half of the vaccinated mice (Fig. 3), it did not work as well in the more demanding setting of an experimental tumor model since tumor eradication was only occasionally (2/19 mice) observed. Importantly, treatment with the peptide vaccine supplemented with CpG resulted in a significant therapeutic efficacy, in that half of the mice (11/23) showed complete remission, whereas the other mice showed delayed tumor growth (Fig. 4). Thus, strong cytolytic activity was induced by the combination of the long HPV peptide and CpG or MPL, but not by agonistic CD40 antibody FGK-45, and this is in line with the fact that these two compounds were the most potent inducers of both a HPV16-specific CD8 $^{+}$ and CD4 $^{+}$ type 1 T-cell response (Fig. 1-3).

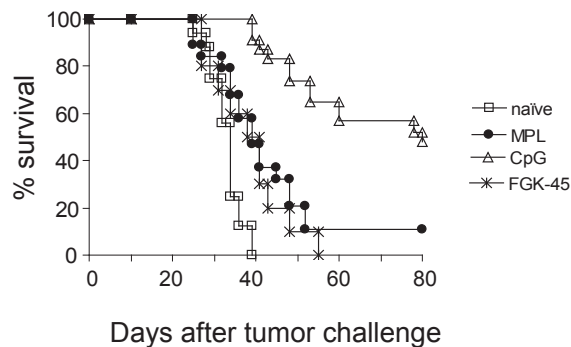


Figure 4. Therapeutic efficacy of vaccine-induced T-cell immunity in the TC-1 tumor model. C57BL/6 mice were given 25,000 TC-1 tumor cells s.c. in the right flank. At day ten, when tumors were palpable, mice were either left untreated (naïve) or received the vaccine in combination with one of the indicated DC activating adjuvants, s.c. in the left flank. Mice received a second vaccine dose in the left flank 14 days later. Tumor size was determined 2-3 times per week, and mice with tumors exceeding 1000mm ^{3} were sacrificed. The percentage of mice surviving tumor challenge is shown for each of the groups. The graph consists of the combined data ($n \geq 10$ per experiment) of two different tumor experiments. Treatment with peptide and CpG resulted in a significant therapeutic efficacy when compared to no treatment ($p < 0.01$), FGK ($p < 0.01$) and MPL ($p = 0.02$) as reflected by a complete remission in 11 out of 23 treated mice, and a clear delay of tumor growth in the other mice.

Distinct effects on CD8⁺ T-cell responses of local versus systemic immunization with combinations of strong adjuvants.

The finding that vaccination with the best formulation, peptide and CpG, resulted in tumor eradication in 50% of the mice argues that further optimization of this vaccine formulation is desirable. We explored combinations of the three most potent agonists because these adjuvants trigger different immunostimulatory molecules present on DC and may act synergistically. Strikingly, our data show that the numbers of HPV16 E7 peptide-specific CD8⁺ T-cells induced by vaccines containing a given combination of DC agonists were at best comparable, but in general lower, than those induced by vaccines containing only one of these DC agonists (Fig. 5: e.g. compare MPL, FGK45 and MPL + FGK45). The strongest significant decrease in immunostimulatory capacity of the vaccine was observed when CpG was combined with agonistic CD40 antibody FGK-45 (Fig. 5).

These experiments show that peptide vaccines containing both a TLR ligand and agonistic CD40 antibody FGK-45 were suboptimal with respect to the induction of strong, systemic CTL immunity. The difference between this outcome and that of previously reported analyses by Ahonen et al., (17) may be related to differences in the experimental set up. Our experiments involved local co-injection of peptide and DC agonists, and thereby targeting of antigen and

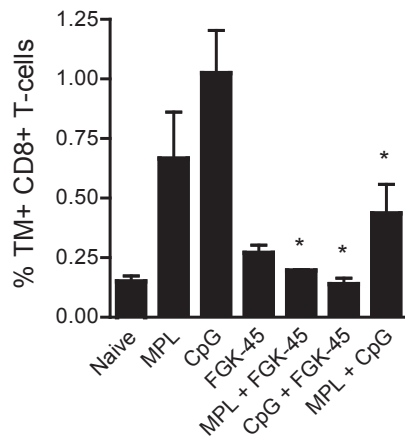


Figure 5. Combinations of strong adjuvants result in decreased CD8⁺ T-cell immunity. C57BL/6 mice were vaccinated s.c. with the HPV16 E7 peptide vaccine and indicated (combinations of) DC agonists. After 10 days, the spleens were harvested and the percentage of H-2D^b-RAHYNIVTF tetramer⁺ CD8⁺ T-cells was determined by flow cytometry, directly ex-vivo. The results of two independent experiments with 5 mice per group are depicted. (*) A significant decrease in the number of tetramer⁺ CD8⁺ T-cells was observed when CpG was combined with MPL (CpG vs. combination, $p=0.014$) or FGK-45 (CpG vs. combination, $p<0.01$) and when MPL was combined with FGK-45 (MPL vs. combination, $p=0.04$).

DC agonist to the DC in the DLN. In the experiments by Ahonen and co-workers, peptide antigen and DC agonists were administered through systemic routes. As such, DC in all secondary lymphoid organs will become activated and present the cognate peptide-epitope to antigen-specific T-cells and this may explain why they observe such a strong T-cell response in the spleen. In view of these essential differences, we immunized mice with our HPV16 E7 peptide and (combinations of) DC agonists through the systemic route, and analyzed the E7-specific CTL numbers induced in the spleen. When using this method of vaccine delivery, we also observed strong synergy between CpG and the agonistic CD40 antibody FGK-45 (Fig. 6), exceeding that of what can be found after s.c. delivery by injection with either single compound (see Fig. 5). Our data clearly illustrate that the impact of DC agonists on vaccination efficiency depends on the manner in which the vaccine is administered. It is important to realize, that systemic administration of agonist CD40 antibodies, although acting synergistically with CpG, also results in splenomegaly as a consequence of polyclonal B-cell activation and expansion. As this is a side effect one would try to avoid in human beings, it is important to take into account that local administration of agonistic CD40 antibodies can negatively affect the outcome of vaccination.

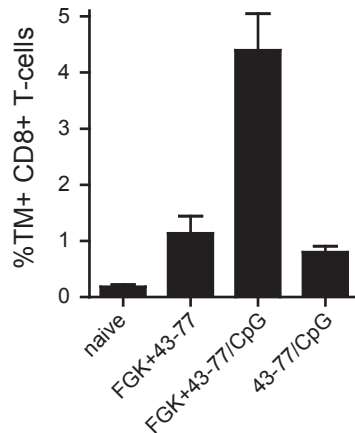


Figure 6. Increased numbers of antigen-specific T-cells in the spleen after systemic vaccination with 2 different DC activating adjuvants. C57BL/6 mice were vaccinated either with FGK-45 i.p. followed by i.v. administration of the peptide with or without the adjuvant CpG after 4 hours. Six days later the spleens were harvested and subjected to H-2D^b-RAHYNIVTF tetramer analysis, directly ex-vivo. The percentages of tetramer-positive CD8+ T-cells measured for 10 mice per vaccination regime (in 2 independent experiments) are plotted. The combination of CpG and antagonistic CD40 antibody FGK-45 when administered systemically with the peptide vaccine resulted in a significant increase (4-fold; $p \leq 0.0005$) in circulating TM+ CD8+ T-cells compared to the response of either adjuvant alone.

DISCUSSION

Full mobilization of CD8⁺ lytic effector cells crucially depends on proper activation of DC either by innate immunity triggers such as microbial ligands of TLR or by adaptive immunity triggers such as CD40 ligand (CD40L) on activated CD4⁺ Th cells. In infections, DC will be activated through both pathways whereas in tumor immunity DC activation relies on the interaction with CD4⁺ T-cells (reviewed in Ref. 4). We have tested 6 different commonly used ligands that are known to trigger TLR on antigen presenting cells as well as an agonistic CD40-specific antibody for their capacity to enhance the T-cell responses induced by subcutaneous vaccination. Our experiments showed that, although all these agonists were able to activate DC *in vitro*, several agonists failed to induce an effective T-cell response *in vivo*. This failure, such as it was observed for Poly I:C₁₂U (Fig. 1), is presumably not due to differences in antigen uptake by activated DC since West et al., (34) described enhanced antigen uptake in several DC types activated by PAM₃CSK₄, Poly I:C₁₂U, LPS and CpG. In addition, Datta et al., (32) showed that the ligands for TLR 3 and TLR 9 were able to boost cross presentation, while this was not the case for TLR 2, TLR 4 and TLR 5 ligands. Since Poly I:C₁₂U is a rather weak stimulant in our model and MPL a strong stimulatory adjuvant it seems that other factors are responsible for the *in vivo* outcome. Moreover, the differential responsiveness of BM-DC to TLR ligands does not correlated with the TLR mRNA (measured by RT-PCR) expression by murine DC (8). The limiting factors of DC-activating agonists to elicit an effective T-cell response *in vivo* included high concentrations (e.g. Poly I:C₁₂U and R848) required to mature DC, disappointing T-cell expansions, poor systemic migration of activated T-cells, a failure to trigger an antigen-specific CD4⁺ Th1 cell response as well as failure to endow the expanded CD8⁺ T-cell population with full effector function. Of all the agonists tested, the use of CpG in the subcutaneously administered long peptide vaccine consistently resulted in a high number of antigen-specific fully activated CD8⁺ T-cells that are easily detected by tetramers, directly ex-vivo within 10 days after priming. This rapid induction of high numbers of CD8⁺ effector T-cells may be advantageous for situations where a quick and strong T-cell response is mandatory such as lethal viral infections but also for cytopathic virus infections with a high economical impact, for instance infections with new variants of influenza and the immunotherapy of cancer.

CpG, MPL and the agonistic CD40 antibody FGK-45 triggered the production of pro-inflammatory cytokines by DC *in vitro* (Table 1) and supported a vigorous clonal expansion of adoptively transferred transgenic CD8⁺ T-cells *in vivo* (Fig. 2). A major difference between FGK-45 on one hand and CpG or MPL on the other hand was the failure of FGK-45 to endow vaccine-induced HPV16-specific CD8⁺ T-cells with full effector function. Mice vaccinated with peptide plus FGK-45 did not display the capacity to directly kill target cells present in

the spleen. In addition, no HPV16-specific CD4⁺ T-cells were detected in these spleens and the numbers of HPV16-specific IFN γ -producing CD8⁺ T-cells were lower when compared to peptide vaccination with CpG or MPL. However, the T-cells that did migrate to the spleen displayed a well preserved capacity to undergo secondary expansion as indicated by the high numbers of HPV16-specific TM⁺ CD8⁺ T-cells detected after one round of *in vitro* stimulation of spleen cells (Fig. 1a). But this does not explain the lack of effector function of these in lymph node resident CD8⁺ T-cells. Recently, Bachmann et al. reported that the presence of Th cells, but not CD40/CD40L interaction, was key to an effective antiviral cytotoxic response by CD8⁺ T-cells upon viral challenge (35), implying that CD4⁺ Th cells play a role that is beyond CD40/CD40L mediated DC-activation and involves the regulation of CD8⁺ T-cell effector function. Indeed, in our model the presence of HPV16-specific IFN γ -producing CD4⁺ T-cells, which were detected in MPL and CpG vaccinated mice but not in FGK-45 treated mice, is associated with high numbers of HPV16-specific cytotoxic CD8⁺ T-cells (Fig. 1 and 3). Previously, antibodies reacting with CD40L on T-cells were shown to block the interaction between DC and CD4⁺ T-cells (36). The failure of the CD40 binding antibody FGK-45 to facilitate the induction and/or expansion of HPV16-specific CD4⁺ T-cells probably is the result of a similar interference by the agonistic CD40 antibody with the CD40-CD40L signaling pathway.

Analysis of vaccine-induced expansion of OT-1 CD8⁺ T-cells *in vivo*, showed that the subcutaneous administration of peptide mixed with a TLR 2, 3, or 7/8 agonist failed to induce a continued expansion and accumulation of OT-1 cells in the DLN (Fig. 2). In contrast, the TLR 4 and 9 ligands as well as the agonistic CD40 antibody FGK-45 were able to drive the expansion of T-cells and this was reflected in the numbers of HPV16-specific CD8⁺ T-cells detected in mice vaccinated with the HPV16 vaccine in combination with either of these adjuvants (Fig. 1 and 2). Similar observations have been made by Lefrancois et al. who showed that short-lived optimal stimulations resulted in rapid but abortive proliferative T-cell responses whereas a sustained response initiation drives optimal clonal T-cell expansion (37). In some cases the failure of a TLR agonist to sustain the activation of T-cells may be overcome. For instance, application of the TLR 7 agonist imiquimod twice a day for 2 days at a significantly higher dose (50-fold per application) than in our experiments can also trigger functional CTL responses (38). Similarly, intravenous (systemic) administration of a 10 fold higher dose of the TLR 3 agonist Ampligen induces an enhanced expansion of antigen-specific CD8⁺ T-cells, albeit that the magnitude of the response as well as the functional capacity of these CD8⁺ T-cells was significantly lower when compared to a low dose of subcutaneously administered CpG (39). In addition, we observed that, compared to the subcutaneous injection of the agonistic CD40 antibody FGK-45 (Fig. 5), the systemic

administration results in a stronger CD8+ T-cell response (Fig. 6). Furthermore, the systemic injection of CpG in combination with FGK-45 resulted in a HPV-specific CD8+ T-cell response that significantly exceeded the response of either compound alone ($p \leq 0.0005$, Fig. 6). These data indicate that a change in the dose and/or route of administration of the DC-activating agent can improve the priming of antigen-specific T-cells and this may have far-reaching consequences. For instance, in human beings the intravenous injection of the TLR 3 ligand poly(I:C) is associated with intolerable side effects at the doses that are effective in mice (40, 41). Ampligen, which has a reduced toxicity compared to its parent compound poly(I:C), also has a reduced bioactive half-life and this is reflected by a reduced stimulating capacity *in vivo*. Application of high and/or frequent doses of the TLR 7 and 8 agonists, imiquimod or resiquimod, gives rise to stronger T-cell responses but also to severe local reactions including ulceration and excoriation in patients (42, 43). Similarly, repeated intravenous injections of agonistic CD40 antibody or CpG can cause splenomegaly and lymphoid follicle destruction (reviewed in Refs. 44 and 45). These apparent side effects may restrict the use of such compounds for direct injection into patients, although they may still be very useful for the *in vitro* activation of DC for adoptive transfer protocols (46).

Last but not least we observed that there was no difference in T-cell reactivity when peptide plus CpG was delivered either subcutaneously or systemically (Fig. 5 and 6). When CpG was combined with FGK-45 the systemic administration of this vaccine resulted in strong synergy as indicated by a 4-fold increase in circulating TM+ CD8+ T-cells (Fig. 6), which is in accordance with Ahonen et al. (17). Interestingly, s.c. injection of both compounds did not result in increased numbers of HPV16 E7 specific T-cells in the spleen but rather lowered this number, suggesting that s.c. delivery of two strong agonists might result in a lymph node shut down as observed for other DC activating agents (47Y, thereby precluding the migration to secondary lymph nodes within the optimal test period for each of the single compounds (10 days). Potentially, a synergistic effect of these s.c. injected compounds may become evident at later time points than currently studied.

In conclusion, we have performed a head-to-head comparison of several DC agonists within a single long peptide vaccination model, using a multiple parameter analysis. The parameters used unerringly indicate to what extent these adjuvants are capable to induce a strong T-cell immunity within a chosen setting. A failure to pass one of these check-points does not automatically label an agonist as a useless adjuvant. As illustrated by the differences in T-cell reactivity observed when CpG and FGK-45 are both locally or systemically injected (Figures 5 and 6), it merely indicates that an alternative strategy should be sought to overcome the problem. The multiple parameter system presented here can be used as a general road map to develop therapeutic vaccines.

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5

Toll-like receptor ligands
conjugated to peptides

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Distinct uptake mechanisms but similar intracellular processing of two different TLR-peptide conjugates in Dendritic Cells

Abstract. Covalent conjugation of Toll-like receptor ligands (TLR-L) to synthetic antigenic peptides strongly improves antigen presentation *in vitro* and T lymphocyte priming *in vivo*. These molecularly well defined TLR-L-peptide conjugates, constitute an attractive vaccination modality, sharing the peptide antigen and a defined adjuvant in one single molecule. We have analyzed the intracellular trafficking and processing of two TLR-L-conjugates in dendritic cells (DCs). Long synthetic peptides containing an Ovalbumin cytotoxic T-cell epitope were chemically conjugated to two different TLR-Ls the TLR2 ligand, Pam(3)CysSK(4) (Pam) or the TLR9 ligand CpG. Rapid and enhanced uptake of both types of TLR-L-conjugated peptide occurred in DCs. Moreover, TLR-L conjugation greatly enhanced antigen presentation, a process that was dependent on endosomal acidification, proteasomal cleavage, and TAP translocation. The uptake of the CpG approximately conjugate was independent of endosomally-expressed TLR9 as reported previously. Unexpectedly, we found that Pam approximately conjugated peptides were likewise internalized independently of the expression of cell surface-expressed TLR2. Further characterization of the uptake mechanisms revealed that TLR2-L employed a different uptake route than TLR9-L. Inhibition of clathrin- or caveolin-dependent endocytosis greatly reduced uptake and antigen presentation of the Pam-conjugate. In contrast, internalization and antigen presentation of CpG approximately conjugates was independent of clathrin-coated pits but partly dependent on caveolae formation. Importantly, in contrast to the TLR-independent uptake of the conjugates, TLR expression and downstream TLR signaling was required for dendritic cell maturation and for priming of naïve CD8(+) T-cells. Together, our data show that targeting to two distinct TLRs requires distinct uptake mechanism but follows similar trafficking and intracellular processing pathways leading to optimal antigen presentation and T-cell priming.

INTRODUCTION

Toll-like receptors (TLR) are germ-line encoded receptors expressed mainly on cells of the innate immune system, such as granulocytes, macrophages and dendritic cells (DCs). These receptors are important in sensing infectious agents through recognition of pathogen-associated molecules, and act as a communicator between innate and adaptive immune responses. The receptors are expressed either on the cell surface or in the endosomal organelles. This compartmentalization of the TLR correlates with the type of ligands with which they interact. The TLRs expressed on the cell surface bind to extracellular components of the microorganisms (such as bacterial LPS to TLR4, bacterial lipopeptide to TLR2). In contrast, the TLRs found in the endosomes bind to ligands derived from intracellular molecules of the pathogen, such as unmethylated CpG DNA sequences to TLR9, and ssRNA to TLR7 (1). Studies have shown that ligands interacting with the latter type of TLRs are internalized independently of the TLRs (2). Upon engagement of the ligand to its receptor, a cascade of intracellular signaling events is initiated, which involves docking of different adaptor molecules such as MyD88, and TRAM to the TLR receptors and recruitment of proteins belonging to the IRAK-family, that ultimately culminate in the activation of the NF- κ B transcription factor and gene transcription leading to production of pro-inflammatory cytokines (3).

DCs are both initiators and regulators of T cell responses (4). Dendritic cells constantly screen the environment for potential foreign antigens by a variety of mechanisms such as phagocytosis, macropinocytosis, caveolin-mediated or clathrin-dependent endocytosis. The manner of uptake dependent on the size and nature of material to be internalized (5, 6). As specialized antigen presenting cells, DCs have the capacity to efficiently process exogenous proteins and present the peptides in major histocompatibility complex (MHC) class I molecules, a process known as cross-presentation. In this scenario, exogenously derived antigens are internalized and translocated from the endosomal route into the cytosol, where the proteasome complex processes the antigen. The generated peptides are transported from the cytosol into the endoplasmic reticulum via the peptide transporter TAP (7), after which the peptides undergo further trimming and are finally loaded onto MHC class I molecules, which translocate to the cell surface, where the peptide is presented to CD8⁺ T-cells.

The ability of DCs to cross-present peptides on MHC class I to CD8⁺ T-cells together with the capacity of TLR ligands to deliver maturation signals, have inspired efforts to explore the use of DCs as a vaccine vehicle in the fight against infectious diseases and cancer (8, 9). Covalent linkage of immunogenic peptides to the TLR9 ligand, CpG DNA or TLR2 ligands, like Pam₃CysSS and Pam₃CysSK₄ induces a more prominent T-cell response than administration of free TLR2-L or TLR9-L mixed with protein (2, 10-16).

To explore the mode of action of TLR-L antigen conjugates, we have designed well-defined synthetic vaccines, composed of peptides containing the model antigen Ovalbumin CD8⁺ cytotoxic T-lymphocyte (CTL) epitope (SIINFEKL) chemically linked to either the TLR2-ligand, Pam₃CysSK₄, or the TLR9 ligand, CpG. These conjugates were used to study the uptake, intracellular routing and processing. We show that not only TLR9-L conjugates but also the TLR2-L conjugates are taken up independently of TLR expression, albeit through two distinct internalization mechanisms. Down-stream processing route for MHC class I antigen presentation, however, were similar and requires endosomal acidification, TAP translocation, and proteasomal processing. Importantly, whereas the uptake of both types of TLR-L conjugates was independent of TLR expression, priming of specific CD8⁺ T-cell response required TLR signaling in dendritic cells.

MATERIAL AND METHODS

Mouse strains and chemicals. C57BL/6 (B6; H-2^b) were obtained from Charles River Laboratories. TLR2-deficient mice were purchased from Jackson Laboratories, whereas the TLR9-deficient mice were obtained from S. Akira Osaka University, Osaka, Japan. Bone marrow from TAP-deficient mice and TAP/β2m-deficient mice strains were kindly provided by Prof. H.G. Ljunggren, Karolinska Institutet, Sweden. LPS of *Escherichia coli* (serotype 026:B6), Monodansylcadaverine (MDC) and filipin were purchased from Sigma-Aldrich (St. Louis, MO). Epoxomicin and chlorophenol red-β-D-galactopyranoside (CPRG) were from Calbiochem.

Cell lines. Freshly isolated DCs were cultured from mouse bone-marrow cells as described elsewhere (17). D1 cell line, a long term growth factor-dependent immature splenic DCs line derived from B6 (H-2^b) mice, was cultured as described (18). B3Z is a T-cell hybridoma specific for the H-2K^b CTL epitope SIINFEKL, which expresses a beta-galactosidase construct under the regulation of the NF-AT element from the IL2 promoter (19). EG7 (EL4-OVA) (20) was cultured in complete medium with 400 μg G418 (Gibco).

Generation of Pam₃CysSK₄- or CpG-conjugated peptides and labeling.

Table I shows the conjugates and peptides used in this study.

Chemicals. HCTU was purchased from IRIS Biotech GmbH (Germany) and Pam₃Cys-OH was from Bachem. PyBOP (Benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate) was purchased from MultiSynTech GmbH. Reactive fluorescent dyes BODIPY-FL N-(2-aminoethyl) maleimide, Alexa Fluor 488 C₅ maleimide

and Alexa Fluor 488 carboxylic acid succinimidyl ester were purchased from Invitrogen. Fmoc-amino acids were from SENN Chemicals or from MultiSynTech GmbH. Tentagel based resins were bought at Rapp Polymere GmbH (Germany). All chemicals and solvents used in the solid phase peptide synthesis were from Biosolve. Chemicals, resins and solvents used in the solid phase DNA synthesis, except of Beaucage reagent and Control Pore Glass (CPG) support used to introduce 3'-thiol modification were from Proligo and used as received. 3'-thiol modifier C3 S-S CPG and Beaucage reagent were purchased at Glen Research. All chemicals were used as received.

General methods. Mass spectra were recorded on a PE/SCIEX API 165 (Perkin-Elmer) mass spectrometer. Analytical LC/MS was conducted on a JASCO system using an Alltima C₁₈ analytical column (4.6 x 150 mm, 5μ particle size, flow: 1.0 ml/min) detecting at 214 and 254 nm. Solvent system: A: 100% water, B: 100% acetonitrile, C: 1% TFA. Gradients of B in A were applied over 15 minutes, keeping C isocratic at 10%. Purifications of the synthetic peptides were conducted on a BioCAD "Vision" automated HPLC system (PerSeptive Biosystems, inc.), supplied with a Alltima C₁₈ column (10 x 250 mm, 5μ particle size, running at 4ml/min). Solvent system: A: 100% water, B: 100% acetonitrile, C: 1% TFA unless stated otherwise. A Varian DMS 200 UV VIS spectrophotometer was used to measure UV absorption MALDI-TOF spectra were recorded on a Voyager-DE PRO mass spectrometer (PerSeptive Biosystems, inc.).

Peptide synthesis. Fmoc based solid-phase peptide synthesis was performed on a CS Bio 336 automated instrument (CS Bio, California, USA) starting from either preloaded Fmoc-Leu-PHB-Tentagel resin or from Tentagel –RAM resin. The synthesis was performed on a 50 or 250 μmol scale according to established methods (21). HCTU was used as coupling reagent. All peptides (see Table I) were cleaved from the resin using trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H₂O (95/2.5/2.5) for 2h at room temperature (RT), precipitated from diethyl ether, redissolved in 20% aqueous 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and purified by RP HPLC and characterized using LC-MS and MALDI MS (see General methods).

Oligonucleotide synthesis. DMT based solid-phase phosphorothioate oligonucleotide (ODN) synthesis was performed on an Expedite automated instrument (PerSeptive Biosystems) starting from Control Pore Glass support with the 3'-thiol modifier. The syntheses were performed on a 10 μmol scale according to established methods (22). Elongation was performed using DMT protected DNA monomers 5'-DMT-A(TAC)-OH, 5'-DMT-C(TAC)-OH, 5'-DMT-G(TAC)-

OH and 5'-DMT-T-OH under the agency of dicyanoimidazole (DCI). After each coupling, remaining free 5'-hydroxyls were blocked using a capping solution (t-butylphenoxyacetic anhydride (T_{ac}O)/1-methylimidazole in THF/pyridine), followed by sulphurization of the phosphite linkage to the phosphorothioate linkage using the Beaucage reagent. Next the 5'-DMT protecting group was removed by trichloroacetic acid (TCA) after which elongation was continued. After final DMT removal the DNA oligomer was cleaved from the resin by 25% ammonium hydroxide solution to give a 3'-disulfide modified ODN (ODN-SS-propyl-OH). ODNs were purified on a Q-Sepharose column pre-equilibrated with 50mM NaOAc applying a gradient of 2M NaCl in 50mM NaOAc. Fractions containing the pure product were combined and dialysed three times with millipore water using dialysis tubing with 1 kD cut-off (Spectrum). Quantification was performed by UV absorbance at 260 nm. Sequences of the ODN prepared in this study were CpG: 5'-TCCATGACGTTCTGACGTT-3'; GpC: 5'-TCCATGAGCTTCCTGATG-3'.

Maleimidopropionyl peptides. Maleimidopropionyl-OVA₂₄₇₋₂₆₄ (Mal-OVA₂₄₇₋₂₆₄) and Maleimidopropionyl-OVA_{247-264A5K} (Mal-OVA_{247-264A5K}). Fmoc-deprotected peptide resin (200 mg, 30 μmol) was suspended in 2 ml NMP, 3-maleimidopropionic acid (5 eq., 250 μmol, 42.2 mg), HCTU (5 eq, 250 μmol, 103.2 mg) and DiPEA (5 eq., 250 μmol, 42 μl) were added subsequently. The mixture was shaken for 2 hours after which the resin was filtered, washed with NMP, DCM and dried. TNBS test indicated complete coupling. The products were processed as described in Peptide synthesis section.

ODN-peptide conjugates (CpG-OVA₂₄₇₋₂₆₄). 3'- disulfide modified CpG- SS-propyl-OH (266 nmol) was converted to 3'-SH modified CpG-SH overnight with dithiothreitol (DTT) containing buffer (35 mg DTT, 26 mg NaOAc. 3H₂O, 1 mL water). DTT was removed from the mixture using a PD-10 desalting column (Amersham) that was pre-equilibrated with 25 ml of a 50mM phosphate buffer (25mM Na₂HPO₄, 25mM NaH₂PO₄, 1mM EDTA in water, continuously degassed with helium. Filtrate (3.25 ml) was directly transferred to a tube containing 5 mg of maleimidopropionyl peptide Mal-OVA₂₄₇₋₂₆₄. The resulting solution was sonicated and placed under blanket of argon. The tube was sealed and shaken for 2 days at RT. The mixture was purified over a Superdex 75 gel filtration column using isocratic elution with 0.15 M triethylammonium acetate. Fractions containing the product were collected and lyophilised. Excess triethylammonium acetate was removed by lyophilization from water (3 times). Quantification was performed by UV-absorbance (260 nm).

CpG-OVA_{247-264A5K} and GpC-OVA₂₄₇₋₂₆₄ were prepared as described for CpG-OVA₂₄₇₋₂₆₄ starting from the corresponding ODNs and maleimidopropionyl peptides.

Lipopeptides Pam₃CysSK₄-OVA₂₄₇₋₂₆₄. Fmoc-protected peptide resin (300 mg, 45 μ mol) was suspended in 1.4 ml, 1/1 NMP/DCM. Pam₃Cys-OH, (91 mg, 2 eq., 100 μ mol) and PyBOP (80 mg, 3 eq., 150 μ mol) were added. DiPEA (30 μ l, 175 μ mol) was added in two portions of 15 μ l with an interval of 15 minutes and the mixture was shaken for 4 hours.⁵ The resin was washed with NMP, DCM and dried. TNBS test indicated complete coupling. The product was cleaved from the resin as described in Peptide synthesis section, dissolved in ^tBuOH/ACN/H₂O 1/1/1 and purified on a Alltima CN column (10 x 250 mm, 5 μ particle size) with gradient of B in A; C kept isocratic at 10% (A; 1/1 MeOH/H₂O, B; ACN, C; 1% TFA in MeOH/H₂O 9/1). Pam₃CysSK₄-OVA₂₄₇₋₂₆₄ and Pam₃CysSK₄-C-OVA₂₄₇₋₂₆₄ were prepared and purified as described above for Pam₃CysSK₄-OVA₂₄₇₋₂₆₄ starting from the corresponding peptide resins.

Fluorescently labeled peptides and conjugates [Alexa488]OVA₂₄₇₋₂₆₄. Fmoc-protected peptide resin (100 mg, 15 μ mol) was treated twice with 2 ml of a capping reagent (0.5M Ac₂O, 0.125M DiPEA in NMP). TNBS test indicated complete acetylation. The cleaved and purified peptide (1 mg) was dissolved into 50 μ l buffer (300mM NaHCO₃ in 30% acetonitrile/water) and Alexa Fluor 488 carboxylic acid succinimidyl ester (0.3mg) was added. Another 50 μ l of buffer was added and the mixture was let to shake overnight. The product was purified by RP HPLC (see General methods).

CpG-[Alexa488]OVA₂₄₇₋₂₆₄ and GpC-[Alexa488]OVA₂₄₇₋₂₆₄ - Conjugate CpG-OVA₂₄₇₋₂₆₄ (296 nmol) or GpC-[Alexa488]OVA₂₄₇₋₂₆₄ (296 nmol) was dissolved in 50 μ l buffer (300mM NaHCO₃, 30% ACN in H₂O) and Alexa Fluor 488 carboxylic acid succinimidyl ester (1.0 mg) was added. The bright green mixture was let to shake overnight. The mixture was diluted 5 times with H₂O before subjection to RP-HPLC purification. (Alltima C₁₈, gradient of B in A; C kept isocratic at 10%; A:H₂O, B: ACN, C: 100 mM aq. NH₄OAc).

Pam₃CysSK₄-C[BDP-FL]OVA₂₄₇₋₂₆₄ -Lipopeptide Pam₃CysSK₄-C-OVA₂₄₇₋₂₆₄ (0.46 μ mol, 1.69 mg) and BODIPY-FL N-(2-aminoethyl)maleimide were transferred to a vial containing 0.5ml of 50mM phosphate buffer (25mM NaH₂PO₄/25 mM Na₂HPO₄, 1mM EDTA in 2/1/1, H₂O/MeOH/ACN). The mixture was sonicated and let to shake for 60 hours under argon. The mixture was diluted 5 times with 40/30/30 H₂O/ACN/^tBuOH, 1% TFA before subjection to RP-HPLC purification as described for Pam₃CysSK₄-C-OVA₂₄₇₋₂₆₄.

SK₄-C[BDP-FL]-OVA₂₄₇₋₂₆₄ - Synthesized as described for Pam₃CysSK₄-C[BDP-FL]OVA₂₄₇₋₂₆₄ using 50 mM phosphate in 3/2 H₂O/ACN as a ligation buffer and SK₄-C-OVA₂₄₇₋₂₆₄ as the peptide substrate.

Pam₃CysSK₄-C[Alexa488]OVA₂₄₇₋₂₆₄ was synthesized as described for Pam₃CysSK₄-C[BDP-FL]OVA₂₄₇₋₂₆₄ using Alexa Fluor 488 C₅ maleimide as the reactive dye.

IL-12p40 ELISA. DCs (4×10^4) were plated into 96-well round bottom plate, and incubated for 24-48h with the compounds indicated in the figure legends. Supernatants were harvested, and tested for IL-12 p40/p70 content using a standards sandwich ELISA, as previous described (23).

Confocal microscopy. DCs were plated out into glass-bottom petrish dishes (MatTek) two days before the experiment. Cells were incubated either with the fluorescence labeled TLR-L peptide conjugate or the fluorescence labeled peptide at 37°C for different time-periods, at the concentrations indicated in the figure legends. In some experiments, as indicated, cells were coincubated with 1 μ M LysoTracker for 5 min to stain endosomal/lysosomal compartments. In experiments with inhibitors, cells were pre-incubated for 30min either with 50 μ M MDC, or 10 μ g/ml filipin, followed by extensively washing before incubation with the TLR-L conjugate either alone (pretreatment) or in the presence of inhibitors (coincubation). Cells were then washed and imaged using an inverted Leica SP2 confocal microscope. Dual color images were acquired by sequential scanning, with only one laser line per scan to avoid cross-excitation. The images were processed using the software program ImageJ.

Flow cytometry. For analyzing the effect of the different compounds on dendritic cell phenotypic profile, DCs were incubated with the different compounds at a final concentration of 1 μ M for 48 h. Subsequently, cells were harvested and resuspended in FACS buffer (PBS / 0.1% BSA) and incubated for 20 min with the following panel of monoclonal antibodies FITC-anti-CD86 (clone GL-1), PE-anti-I-A^b (clone M5/114 15.2), PE-anti-CD40 (clone 3/23), APC-anti-K^b (24). Cells were washed twice and fixed with 0.5% PFA before being subjected to flow cytometry measurements.

MHC-class I-restricted antigen presentation assay. DCs were incubated for 2 h, (unless stated otherwise in the figure legends), with 1) the parent peptide (DEVSGLEQLESINF EKLA²⁴⁷⁻²⁶⁴AAAK, OVA₂₄₇₋₂₆₄ASK^{*} or DEVSGLEQLESINF EKL, OVA₂₄₇₋₂₆₄), 2) the peptide-conjugate, or 3) the mixture of the parent peptide and the Pam₃CySK₄ or CpG, at the indicated concentrations. Cells were washed five times with medium before the T-cell hybridoma B3Z cells were added and incubated for 16h at 37°C.

Antigen presentation of the Ovalbumin cytotoxic T-cell epitope, SIINF EKL (OVA₂₅₇₋₂₆₄) in H-2K^b was detected by activation of B3Z cells measured by a colorimetric assay using chlorophenol red- -D-galactopyranoside as substrate to detect lacZ activity in B3Z lysates, as described (23).

In some experiments cells were preincubated for 30 min with titrated amounts of epoxomicin ranging from 0.01-10 μ M epoxomicin or with 3mM NH₄Cl, or preincubated for 60min with titrated amounts of monodansylcadaverine (25-50 μ M), or with filipin (10-20 μ g/ml) before adding the peptide compound still in the presence of the inhibitors. Cell viability was confirmed by tryphan blue exclusion at the indicated concentration range of inhibitors.

Priming of endogenous naïve CD8⁺ T-cells. To determine the endogenous CTL response, five nmol of the different compounds was injected s.c. into naïve C57BL/6 mice. After 10 days spleen cells were stimulated *in vitro* by plating 10x10⁶ splenocytes with 1x10⁶ mytomyacin C (Kyowa) treated (50ug/ml 1 hour at 37 degrees) and irradiated (4000 rad) EG7 cell line (EL4-OVA), in the absence of additional cytokines. After seven days viable splenocytes were isolated over a ficoll gradient and stained for H-2K^b Tetramer (TM)-OVA₂₅₇₋₂₆₄ and CD8b2 (clone 53-5.8), and propidium iodide to exclude dead cells as described previous (23).

Intracellular cytokine staining. An aliquot of spleen cells after re-stimulation and ficoll purification (see above) were subjected to stimulation *in vitro* with or without 1 μ g/ml OVA₂₅₇₋₂₆₄ peptide (H2-K^b restricted SIINFEKL) overnight in the presence of GolgiPlug (BD Pharmingen, San Diego, CA, USA). Cells were then washed twice with FACS buffer and stained with PE-conjugated monoclonal rat anti-mouse CD8b2 antibody. Cells were subjected to intracellular cytokine staining using the Cytotfix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). Intracellular IFN- γ was stained with APC-conjugated rat anti-mouse IFN- γ . All antibodies were purchased from BD Pharmingen. Flow cytometry analysis was performed using FACSCalibur with CELLQuest software (BD Biosciences, Mountain View, CA, USA). Splenocytes without peptide stimulation were used as a negative control.

***In vivo* uptake studies.** To monitor the uptake of the TLR-L conjugated peptides and the free peptide *in vivo*, mice were injected s.c. either with 5nmol of Alexa 488 Fluor labeled CpG~conjugated peptide, 5nmol of BODIPY-FL Pam~conjugated peptide, CpG mixed with Alexa 488 Fluor labeled peptide or Pam mixed with BODIPY-FL labeled peptide. Three days later, mice were sacrificed and a single cell suspension of the draining lymph nodes was stained for CD11c (clone HL3) before being subjected to flow cytometry analysis.

RESULTS

To study the uptake, trafficking and processing of two distinct TLR-ligands peptide conjugates in dendritic cells for MHC class I presentation to CD8⁺ cytotoxic T-lymphocytes, we selected the TLR9 ligand CpG, and the TLR2 ligand Pam₃CysSK₄ (Pam), based on the fact that these two ligands interact with two distinct receptors located either in the endosomal compartment (TLR9) or on the plasma membrane (TLR2). As a model antigen we made use of peptides containing the CTL epitope (SIINFEKL, designated OVA₂₅₇₋₂₆₄) derived from the Ovalbumin protein. Two different peptide length variants were synthesized, an extended peptide that required proteasome-dependent processing on both the N- and C-terminus to release the CTL epitope (DEVSGLEQLESIINFEKLAAAAAK) designated OVA_{247-264A5K}, and a shorter peptide, of which C-terminal processing by the proteasome is not required (DEVSGLEQLESIINFEKL), designated OVA₂₄₇₋₂₆₄. For example, peptide OVA_{247-264A5K} conjugated to Pam₃CysSK₄ is designated as Pam~OVA_{247-264A5K}.

Crucially, all of our compounds (listed in Table I) are produced synthetically, therefore chemically well defined, of high purity and of constant quality, avoiding potential contamination with other TLR-ligands such as LPS, which commonly occur in purified TLR ligand preparations.

Table I. List of compounds generated

Abbreviation	TLR ligand	Peptide	Fluorophore
OVA ₂₅₇₋₂₆₄		SIINFEKL	
OVA ₂₄₇₋₂₆₄		DEVSGLEQLESIINFEKL	
OVA _{247-264A5K}		DEVSGLEQLESIINFEKLAAAAAK	
CpG-OVA ₂₄₇₋₂₆₄	CpG	DEVSGLEQLESIINFEKL	
CpG-OVA _{247-264A5K}	CpG	DEVSGLEQLESIINFEKLAAAAAK	
GpC-OVA ₂₄₇₋₂₆₄	GpC	DEVSGLEQLESIINFEKL	
Pam ₃ CysSK ₄ -OVA ₂₄₇₋₂₆₄	Pam ₃ CysSK ₄	DEVSGLEQLESIINFEKL	
Pam ₃ CysSK ₄ -OVA _{247-264A5K}	Pam ₃ CysSK ₄	DEVSGLEQLESIINFEKLAAAAAK	
[Alexa488]OVA ₂₄₇₋₂₆₄		Ac-DEVSGLEQLESIINFEKL	Alexa488
SK ₄ -C[BDP-FL]-OVA ₂₄₇₋₂₆₄		SKKKKDEVSGLEQLESIINFEKL	Bodipy-FL
CpG-[Alexa488]OVA ₂₄₇₋₂₆₄	CpG	DEVSGLEQLESIINFEKL	Alexa488
GpC-[Alexa488]OVA ₂₄₇₋₂₆₄	GpC	DEVSGLEQLESIINFEKL	Alexa488
Pam ₃ CysSK ₄ -C[Alexa488]OVA ₂₄₇₋₂₆₄	Pam ₃ CysSK ₄	DEVSGLEQLESIINFEKL	Alexa488
Pam ₃ CysSK ₄ -C[BDP-FL]-OVA ₂₄₇₋₂₆₄	Pam ₃ CysSK ₄	DEVSGLEQLESIINFEKL	Bodipy-FL

Robust induction of naïve CD8 specific T-cells mediated by the conjugates.

To establish the quality of our generated TLR-L-peptide conjugates, we first investigated the induction of an endogenous T-cell response, following s.c. injection of either TLR-L-conjugated peptide or free peptide into naïve C57BL/6 mice. After 10 days, the induction of OVA₂₄₇₋₂₆₄-specific CD8⁺ T-cells was analyzed. As is shown in Fig. 1A, the magnitude of the OVA₂₅₇₋₂₆₄-specific T-cell response induction by either CpG~OVA_{247-264A5K} or Pam~OVA_{247-264A5K} was significantly higher than that in mice injected with non-conjugated OVA_{247-264A5K}.

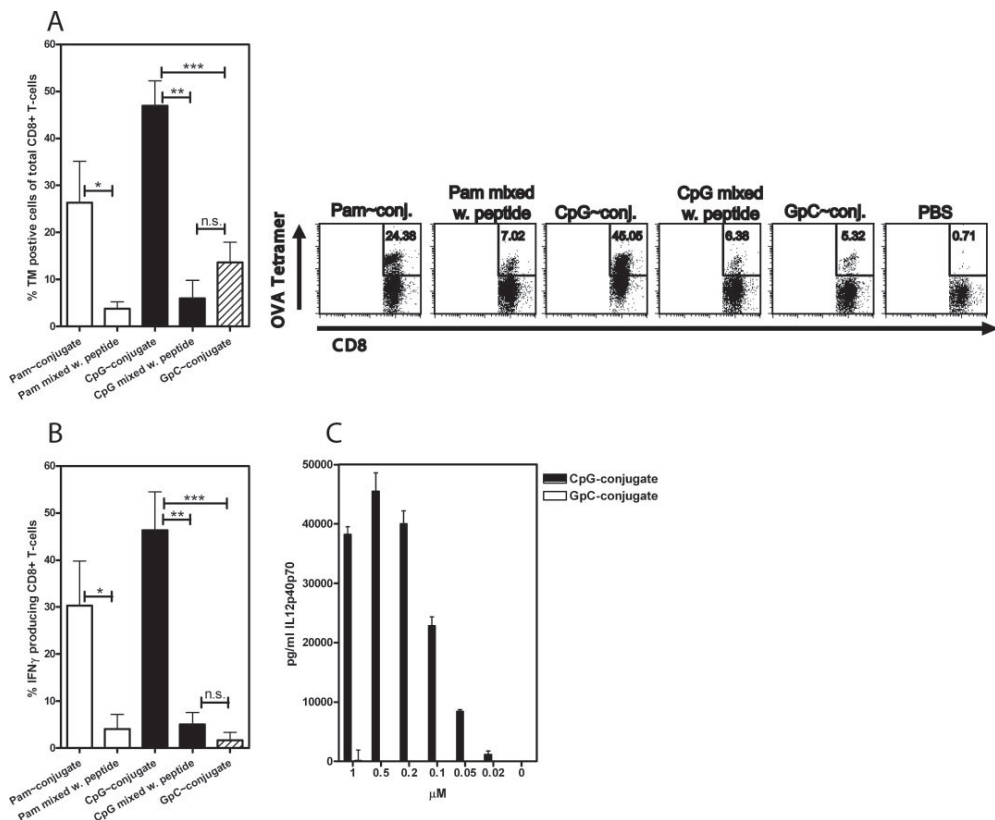


Figure 1. Robust induction of naïve CD8+ specific T-cells mediated by the TLR L-conjugates. A, Naïve C57/B6 mice were injected with either Pam mixed with OVA_{247-264A5K} (Pam mixed w. peptide), Pam~OVA_{247-264A5K} (Pam~conjugate), CpG mixed with OVA_{247-264A5K} (CpG mixed w. peptide), CpG~OVA_{247-264A5K} (CpG~conjugate), or GpC~OVA₂₄₇₋₂₆₄ (GpC~conjugate). After stimulation *in vitro*, cells were analyzed for the presence of CD8b2 cells capable of interacting with H-2K^b-OVA₂₅₇₋₂₆₄ tetrameric complexes. The y-axis displays the percentages of tetrameric positive cells out of total CD8b2+ cells. The % of tetrameric CD8b2+ cells (<1%) of the naïve mice (PBS injection only) has been subtracted from all the values. Right hand panel shows a representative FACS plot, cells were gated on CD8+ and the percentage given in the top of right quadrant are the percentages of tetramer positive cells of total CD8+ T cells. Unpaired Student's t-test, *: P=0.04; **: P=0.0006; ***: P=0.001; n.s.: not significant. B, IFN- γ production in specific T-cells was measured as described in material and methods. Shown are results gated on CD8+ events. Pam mixed with OVA_{247-264A5K} (Pam mixed w. peptide), Pam~OVA_{247-264A5K} (Pam~conjugate), CpG mixed with OVA_{247-264A5K} (CpG mixed w. peptide), CpG~OVA_{247-264A5K} (CpG~conjugate), or GpC~OVA₂₄₇₋₂₆₄ (GpC~conjugate). Unpaired Student's t-test, *: P=0.05; **: P=0.001; ***: P=0.0007; n.s.: not significant. Experiments were conducted with five mice per group. C, BMDCs were incubated either with CpG~OVA_{247-264A5K} (black bars) or GpC~OVA₂₄₇₋₂₆₄ (white bars) for 48h. Supernatant was harvested and the concentration of IL-12p40 was determined as out-lined in material and methods. Results are means of triplicates $\pm$ SEM. Data are representative of three independent experiments.

peptide mixed together with either free CpG or free Pam. To address whether the induction of specific T-cells depended on activation of the DCs, we injected GpC~OVA₂₄₇₋₂₆₄ conjugate, which is a non-stimulatory oligonucleotide as shown by its lack of capacity to induce IL-12 production by DCs (Fig. 1C). Injection of GpC~peptide conjugate into naïve mice led to a significantly lower induction of specific CD8⁺ T-cells than of CpG~conjugated peptide, but was still as high as the response obtained after mixing of peptide with the CpG (Fig. 1A). Importantly, only when a stimulatory TLR-L-conjugate was given the majority of CD8⁺ T-cells were able to produce interferon- γ (Fig. 1B), indicating that signaling via the TLR is essential for the generation of large numbers of functional T-cells *in vivo*.

These results suggest that the enhanced induction of specific T-cells is primarily the result of efficient delivery of the TLR-L-conjugated peptide into the antigen-presenting cell.

TLR-conjugates activate dendritic cells.

Next we analyzed the ability of the different conjugates to induce maturation of DCs. As evident from Table II, increased surface marker expression of CD40, CD86, MHC I, and MHC II, was observed in dendritic cells after treatment with the different TLR-L-conjugates to a similar extent as the free TLR-Ls. To confirm the involvement of the TLRs in DCs activation, we isolated bone-marrow derived dendritic cells (BMDCs) from WT mice, TLR2-deficient mice and TLR9-deficient mice and stimulated these cells with the different conjugates, followed by phenotypic characterization by staining for different surface markers associated with DCs maturation (25). No up-regulation of the cell surface markers CD86 and MHC class II was detected when BMDCs from TLR2-deficient mice or TLR9-

Table II. FACS analysis of cell surface expression of markers after TLR-L-conjugate induced DCs maturation.

Treatment	Mean fluorescence			
	CD40	CD86	MHC I	MHC II
Untreated	8	21	923	277
Peptide	8	22	ND	271
CpG	59	136	3421	512
CpG mixed with peptide	48	134	ND	903
CpG~conjugate	71	132	3229	725
Pam	16	38	ND	327
Pam mixed with peptide	18	40	ND	346
Pam~conjugate	14	35	1348	329
LPS	46	137	3572	576

Dendritic cells were incubated for 48 h in the presence of 1 μ M of either OVA₂₄₇₋₂₆₄ (peptide), CpG, CpG mixed OVA₂₄₇₋₂₆₄ (CpG mixed with peptide), CpG~OVA₂₄₇₋₂₆₄ (CpG~conjugate), Pam, Pam mixed with OVA₂₄₇₋₂₆₄ (Pam mixed with peptide), Pam~OVA₂₄₇₋₂₆₄ (Pam~conjugate) or with E.coli LPS (10 μ g/ml). Cells were stained with specific antibodies as described in material and methods, and subjected to flow cytometry analysis. Indicated are the mean fluorescence intensities of positive cells. The results were obtained from a single experiment, and are representative of four similar experiments. N.D indicates not done.

deficient mice were stimulated with Pam~conjugated peptide or CpG~conjugated peptide, respectively (Fig. 2). This impaired up-regulation was not due to a general defect in the maturation signaling pathway, as stimulation with LPS could induce up-regulation of CD86 and MHC II in BMDCs derived from both TLR2- and TLR9-deficient mice to a similar extent as observed for BMDCs derived from wild type mice. Taken together, these results demonstrate that the conjugates are as effective as free TLR ligand in activating the DCs, and show that the expression of the cognate TLR is required for activation of DCs by the TLR-L-peptide conjugates.

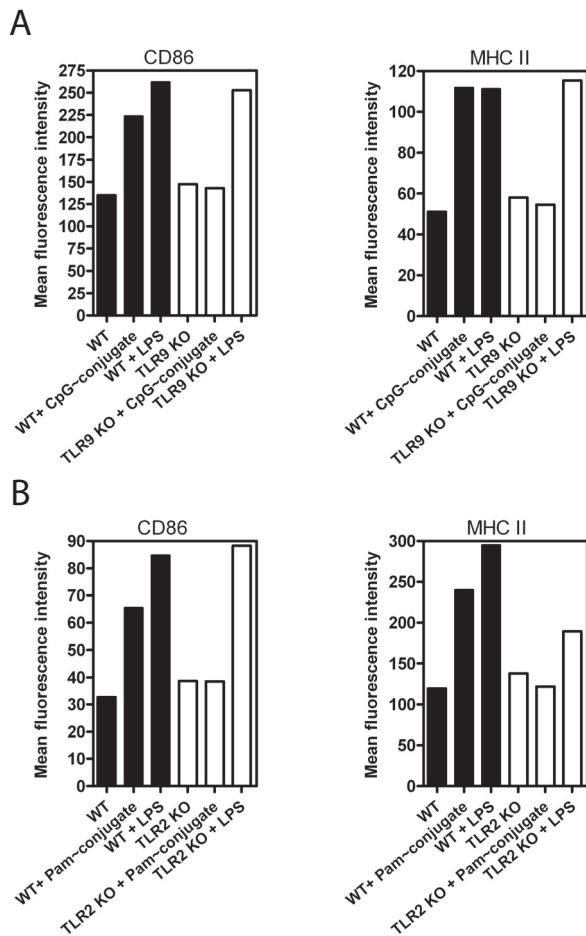


Figure 2. TLR-dependent DCs activation. A, BMDCs from either WT or TLR9-deficient mice (TLR9 KO) were incubated either with 1 μ M CpG~OVA_{247-264A5K} or E.coli LPS (10 μ g/ml) for 48 h. Cells were stained with CD86 or MHC II antibodies as described in material and methods, and subjected to flow cytometry analysis. B, BMDCs from either wild-type (WT) or TLR2-deficient mice (TLR2 KO) were incubated either with 1 μ M Pam~OVA_{247-264A5K} or E.coli LPS (10 μ g/ml) for 48 h, cells were treated as stated under (A).

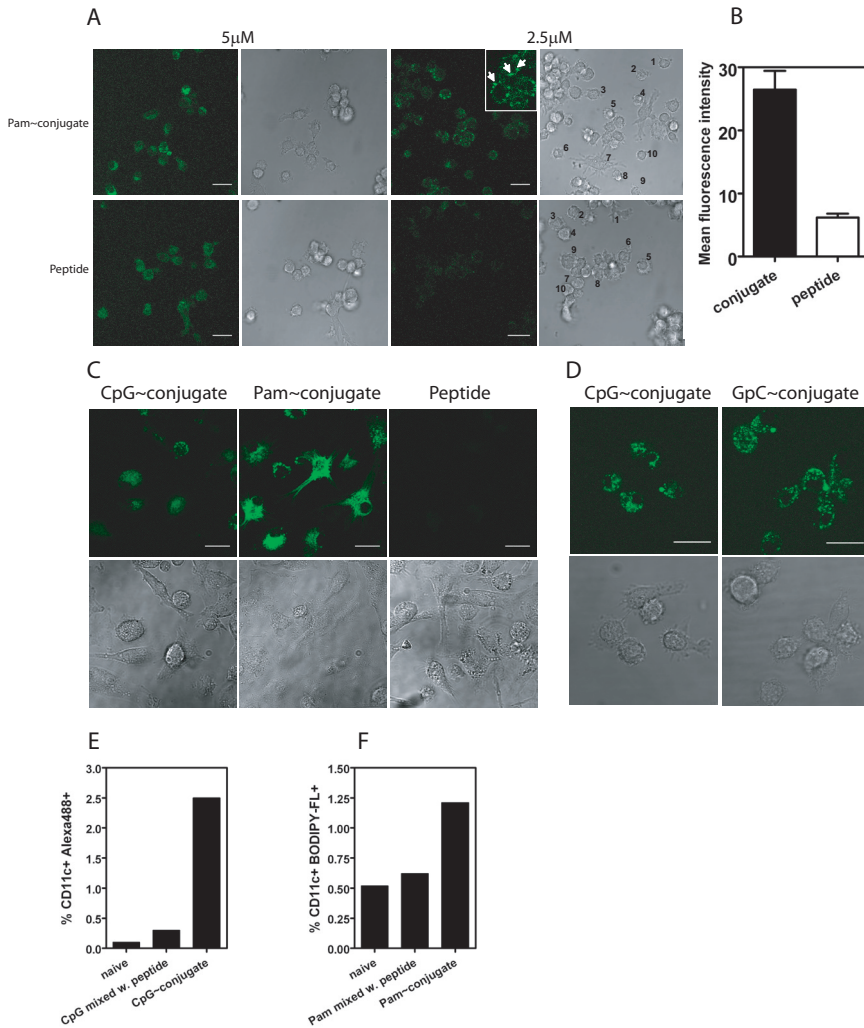


Figure 3. Efficient antigen uptake mediated by the TLR-L-conjugates. A, Confocal images of the dendritic cell line D1 incubated for 15 min with either BODIPY-FL labeled Pam~OVA₂₄₇₋₂₆₄ (Pam~conjugate) or BODIPY-FL labeled peptide OVA₂₄₇₋₂₆₄ (peptide) at different concentrations. Arrows indicate accumulation of Pam~conjugates. B, Quantification of mean intensity fluorescence (MFI) of fluorescence inside numbered cells selected from Fig. 3A. C, DCs were incubated with either Alexa 488 Fluoro labeled CpG~OVA₂₄₇₋₂₆₄ (CpG~conjugate), Pam~OVA₂₄₇₋₂₆₄ (Pam~conjugate), or OVA₂₄₇₋₂₆₄ peptide for 30 minutes, all compounds were used at 2.5 μM. D, DCs were incubated with either Alexa 488 Fluoro labeled CpG~OVA₂₄₇₋₂₆₄ (CpG~conjugate) or GpC~OVA₂₄₇₋₂₆₄ (GpC~conjugate) for 30min at a final concentration of 5μM. All scale bars in A, B, C and D 20 μm. E, Mice were injected s.c. with either Alexa 488 Fluoro labeled peptide OVA₂₄₇₋₂₆₄ mixed with CpG, or Alexa 488 Fluoro labeled CpG~OVA₂₄₇₋₂₆₄. 72 hr later mice were sacrifice and draining lymph node cells were stained for CD11c. The y-axis displays the percentages of Alexa488 positive cell out of total CD11c+ cells. F, Mice were injected s.c. with either BODIPY-FL labeled peptide OVA₂₄₇₋₂₆₄ mixed with Pam, or BODIPY-FL labeled Pam~OVA₂₄₇₋₂₆₄. 72 hr later mice were sacrifice and draining lymph node cells were stained for CD11c. The y-axis displays the percentages of BODIPY-FL positive cell out of total CD11c+ cells. Experiments were conducted twice with similar results.

Efficient uptake of CpG – and Pam-conjugated antigen peptides by dendritic cells.

Having demonstrated that TLR signaling was important for DC activation and priming of T-cells, we decided to compare the down-stream cellular mechanism used by the two types of TLR-L with respect to uptake, routing, and cellular processing. Therefore the efficiency of antigen uptake of conjugated versus non-conjugated peptide by DCs was determined. DCs were incubated with either Pam~conjugated peptide, or free peptide. Both compounds were labeled with a fluorophore (attached on the peptide backbone), which allowed us to monitor the internalization of these compounds by confocal microscopy analysis. Introduction of the fluorophore (either Alexa 488 or BODIPY-FL) into the conjugates did not alter the ability of the conjugates to activate DCs as comparable level of the IL-12 cytokine was produced by the fluorescent conjugates and the dark conjugates (unpublished data). As indicated by the increased intensity of fluorescence inside the cells, the Pam~conjugated peptides were taken up far more efficient than the non-conjugated peptide (Fig 3A). Interestingly, the Pam~conjugated peptide was found to accumulate in hot spots (indicated by arrows in Fig. 3A), whereas a more diffuse pattern was observed in DCs incubated with the peptide alone. Quantification of the mean fluorescence intensity (MFI) revealed a more than 4-fold higher fluorescence in DCs incubated with the Pam~conjugate compared to DCs incubated with the peptide (Fig. 3B). Similarly, CpG~conjugated peptide was internalized more efficiently than the unconjugated peptide by DCs (Fig. 3C). In addition, the non-stimulatory GpC~conjugate (MFI 63 ± 7.3) was internalized to a similar extent as the stimulatory CpG~conjugate (MFI 72 ± 9.1) (Fig. 3D).

These comparisons indicate that the fluorescent TLR-L conjugates are taken up much more efficiently by DCs than unconjugated peptides *in vitro*. To examine the uptake efficiency *in vivo*, mice were injected with either Alexa 488 Fluor labeled CpG~conjugate or peptide labeled with Alexa 488 Fluor mixed with dark CpG. Three days later draining lymph node cells were stained for the DCs surface marker CD11c. In line with the *in vitro* results, a significantly higher proportion of CD11c+ cells had taken up the CpG~conjugated peptide (2.5%), when compared to the population of DCs that ingested unconjugated peptide (0.3%), or non-injected mice (0.1%; Fig. 3E). A similar tendency was observed upon comparison of BOPIPY-FL labeled Pam~conjugate with peptide labeled with BODIPY-FL mixed with dark Pam, (Fig.3F). Collectively, these results indicate that it is the covalent linking of the peptide to the TLR-L that is responsible for the enhanced uptake by the DCs.

Conjugates translocate to endosomal/lysosomal compartment independently of TLR expression.

Our antigen uptake studies revealed that also the TLR9-L-conjugate was taken up more efficient despite that TLR9 is not surface expressed. Therefore to evaluate the relevance of TLR expression for the enhanced uptake, bone-marrow dendritic cells (BMDCs) purified from wild-type (WT), TLR2^{-/-}, and TLR9^{-/-} mice were incubated with Alexa 488 Fluor labeled TLR-L-conjugates. As shown in Fig. 4A and Fig. 4C BMDCs from TLR9^{-/-} mice internalized CpG~conjugates to a similar extent as BMDCs from wild type mice, as reported previously (2).

Surprisingly, similar experiment carried out with Pam-conjugate and BMDCs from WT mice and TLR2^{-/-} mice showed that also the Pam-conjugate, despite the fact that the receptor for Pam (TLR2) is located on the cell surface (1, 26), were internalized equally well by both types of DCs (Fig. 4B and Fig. 4D).

To monitor the intracellular localization of the conjugates, we performed a co-localization study between the conjugates (green) and the endosomes (red). As seen in Fig. 4E both the CpG~ and Pam~conjugates are co-localized partially with an endosomal tracker (lysotracker), in a pattern characteristic for the endosomal vehicles. Moreover, no overall difference in the uptake kinetic or in the trafficking of the compounds could be detected when comparing BMDCs from wild-type mice to mice deficient for either TLR9 or TLR2 expression (unpublished data). These results indicate that TLR expression is not required for uptake and that the conjugate relocates to the endosomal compartment.

Conjugation of peptide leads to pronounced enhancement in antigen presentation *in vitro*.

Having established that the conjugates were taken up much more efficiently than the free peptide harboring the OVA CTL epitope SIINFEKL, we next addressed the effect of conjugation of peptide to TLR-L on antigen presentation. DCs were loaded with either the CpG~conjugated peptide, Pam~conjugated peptide, the CpG or Pam mixed with the peptide, or the peptide alone (Fig. 5A and 5B) before incubation with the peptide-specific T-cell hybridoma B3Z cells that recognize the H-2K^b, SIINFEKL CTL epitope (27). As the concentration of the compounds decreased, antigen recognition was rapidly lost when DCs were incubated either with the peptide alone, or with the peptide mixed with CpG but not when the CpG~conjugated peptide was used. This indicates that the conjugation of peptides to TLR-L enhance antigen presentation (Fig. 5A). Likewise, an increased antigen presentation was observed for the Pam~conjugated peptide (Fig. 5B). In this case the difference in antigen presentation between conjugate and non-conjugate was even more prominent. Incubation

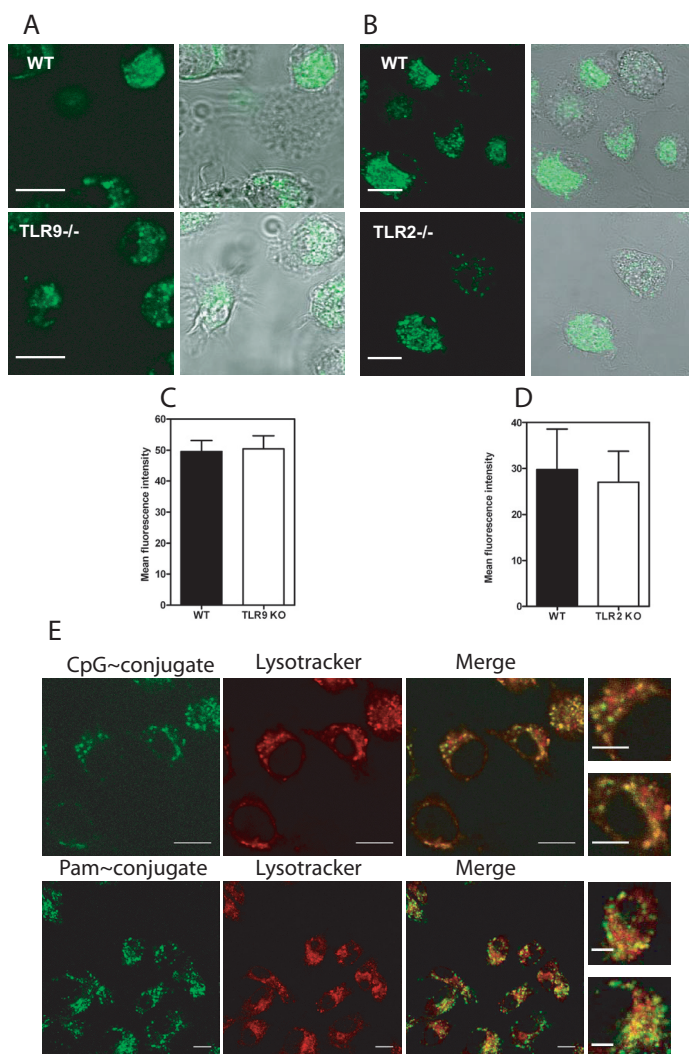


Figure 4. TLR-conjugates translocate to the endosomal compartment independently of TLR expression. A, BMDCs from wild type mice or TLR9 deficient mice were incubated for 30 min with Alexa 488 Fluor labeled CpG~OVA₂₄₇₋₂₆₄ (5 μ M). B, BMDCs from wild type mice or TLR2 deficient mice (TLR2 KO) were incubated for 30 min with Alexa 488 Fluor Pam~OVA₂₄₇₋₂₆₄ (2.5 μ M). Scale bars in A and B 15 μ m. C, Quantification of uptake of CpG~conjugate in BMDC from wild type (WT) and TLR9 deficient mice (TLR9 KO) was done on ten random cells selected from similar pictures as depicted in Fig.4A. D, Quantification of uptake of Pam~conjugate in BMDC from wild type (WT) and TLR2 deficient mice (TLR2 KO), was performed on ten random cells selected from similar pictures as depicted in Fig.4B. E, BMDCs were incubate with Alexa 488 Fluor labeled CpG~OVA₂₄₇₋₂₆₄ (5 μ M), or Pam~OVA₂₄₇₋₂₆₄ (2.5 μ M) in combination with 1 μ M Lysotracker-DND99, before imaging. Images were acquired by sequential scanning, with only one laser line per scan to avoid cross-excitation. Scale bars 10 μ m, and 5 μ m in enlarged images right panel.

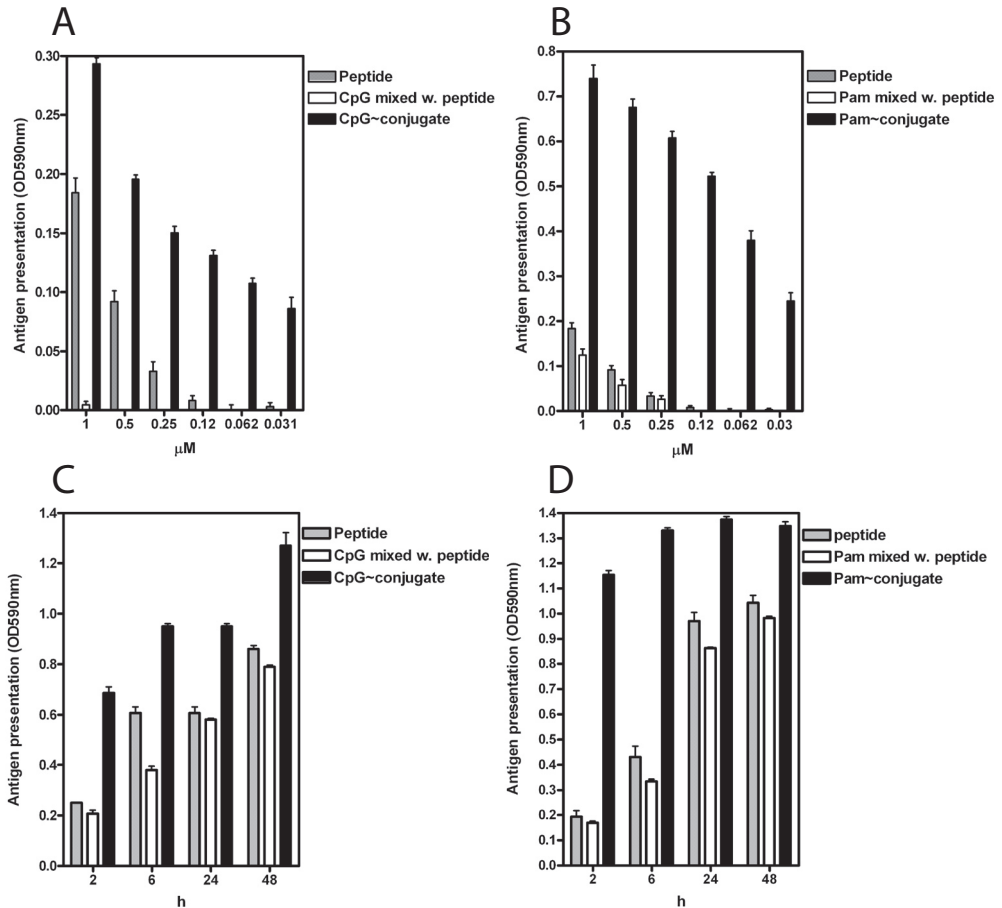


Figure 5. TLR-L conjugates strongly enhance antigen presentation. A, The dendritic cell line, D1 was incubated with either the OVA₂₄₇₋₂₆₄ peptide (gray bars), OVA₂₄₇₋₂₆₄ peptide mixed with CpG (white bars), or CpG~conjugated to OVA₂₄₇₋₂₆₄ (black bars). After 2h, cells were washed and B3Z cells were added and co-cultured for 24h, before their activation was measured. B, D1 cells were incubated under the same conditions as indicated under (A), but in the presence of either the OVA₂₄₇₋₂₆₄ peptide (gray bars), OVA₂₄₇₋₂₆₄ peptide mixed with Pam (white bars), or Pam-conjugated OVA₂₄₇₋₂₆₄ peptide (black bars). Results are means of triplicates $\pm$ SEM. C, BMDCs were incubated for various time period with either OVA_{247-264A5K} (gray bars) CpG mixed with OVA_{247-264A5K} peptide (white bars), or CpG~OVA_{247-264A5K} (black bars), or D, with OVA_{247-264A5K} peptide (gray bars), Pam mixed with OVA_{247-264A5K} (white bars), or Pam~OVA_{247-264A5K} (black bars) before co-culturing with B3Z T-cell hybridoma as outlined in material and methods.

with a mixture of free TLR-L (Pam or CpG) and the peptides resulted in a decreased antigen presentation by DCs, when compared to loading with peptide alone or with conjugated peptide. This might be related to decreased uptake, since it has previously been reported that the endocytotic capacity of DCs declines upon encountering maturation signals (28, 29).

To gain insight in the kinetics of antigen presentation, DCs were incubated for various time periods with either TLR-L-conjugated peptide, peptide mixed with TLR-L, or peptide alone. As shown in Fig. 5C and 5D, it required 24h-48h of continuous presence of peptide mixed with CpG or Pam, or of peptide alone to reach the level of antigen presentation acquired already after 2h of incubation with the conjugated peptide, as measured by an equal ability to stimulate the peptide-specific B3Z hybridoma T-cells. Thus, conjugation greatly improves the swiftness of presentation of antigen by DCs for stimulation of T-cells.

The confocal microscopy results indicated that uptake of the conjugates occurred independently from the expression of the respective TLRs. Therefore we next evaluated the impact of TLR expression upon antigen presentation. To this end BMDCs from WT, TLR2^{-/-}, and TLR9^{-/-} mice loaded with the conjugates were incubated *in vitro* together, and subsequently incubated with the peptide-specific T-cell hybridoma B3Z cell line. In line with the confocal uptake studies, BMDCs derived from WT, the TLR2- or TLR9-deficient mice strains were recognized to the same extent (Fig 6A and 6B).

Disruption of clathrin formation and caveolea clustering blocks antigen presentation of TLR-L-conjugates.

DCs can take up exogenous antigens via different mechanisms such as clathrin-mediated endocytosis, fluid phase endocytosis, and macropinocytosis (30). To define the pathways by which the TLR-L-peptide conjugates were internalized, DCs were pre-treated with different inhibitors before loading with the TLR-L conjugates. Macropinocytosis inhibitor 5-(N,N-dimethyl) amiloride (29) had no effect on antigen presentation (unpublished data). On the other hand pretreatment with filipin, a sterol-binding agent that disrupt caveolea structures (31) and thereby lipid-raft formation, reduced antigen presentation of both Pam~conjugates as well as CpG~conjugates in a dose dependent manner, whereas antigen presentation of the CTL epitope OVA₂₅₇₋₂₆₄ was only marginal affected (Fig. 6C). As lipid raft formation is involved both in clathrin-dependent endocytosis as well as caveolae dependent internalization (32), we analyzed the impact of monodansylcadaverine (MDC), a specific inhibitor of clathrin formation (33, 34) upon antigen presentation. Interestingly, whereas antigen presentation of CpG~conjugated peptides was not affected by MDC, antigen presentation of Pam~conjugated peptides was abrogated in a dose dependent fashion (Fig. 6D). To further explore the distinct uptake mechanisms used by the two types of conjugates, confocal microscopy was performed

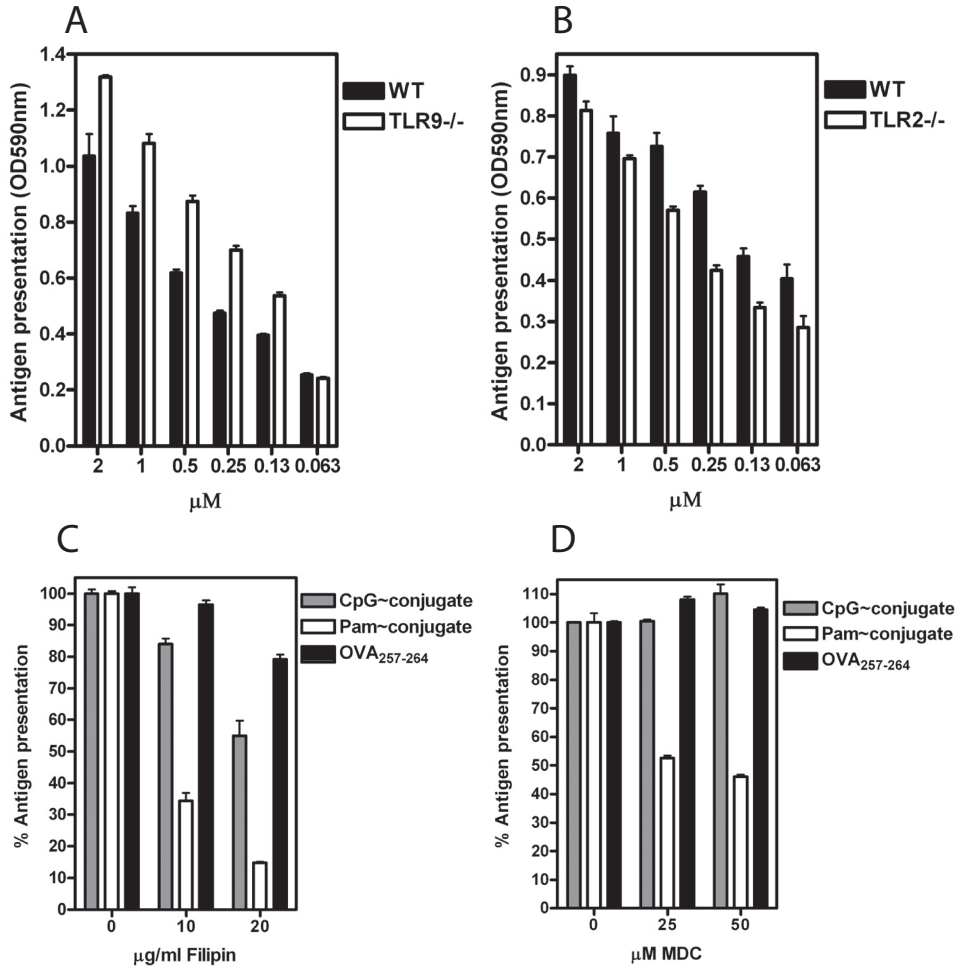


Figure 6. Antigen presentation of TLR-L-conjugates does not require TLR expression, but is dependent on receptor-mediated endocytosis. A, BMDCs from wild type mice (black bars) or TLR9 deficient mice (white bars) were incubated for 2 h with CpG~OVA₂₄₇₋₂₆₄ and processed for antigen presentation as described in material and methods. B, BMDCs from wild type mice (black bars) or TLR2 deficient mice (white bars) were incubated for 2h with Pam~OVA₂₄₇₋₂₆₄ and processed for antigen presentation. C, DCs were left untreated, or pre-treated for 60min with various concentrations of filipin, or with various concentration of MDC D, before addition of 0.5 μM CpG~OVA_{247-264A5K} (gray bars), 0.5 μM Pam~OVA_{247-264A5K} (white bars), or 0.01 μM OVA₂₅₇₋₂₆₄ (black). Cells were incubated with the peptides for 3hr in the presence of inhibitors, before processed as outlined in material and methods section. Actual OD-values in Fig. 6C in the absence of filipin was: CpG~conjugate=0.5; Pam~conjugate=1.4; OVA₂₅₇₋₂₆₄=2.0. Actual OD-values in Fig. 6D in the absence of MDC was: CpG~conjugate=0.5; Pam~conjugate=1.0; OVA₂₅₇₋₂₆₄=1.4. Background OD-level of cells incubated without peptides were below 0.1 in both experiments.

on DCs treated with the two inhibitors. As evident from Fig. 7 both MDC and filipin abolished the internalization of the Pam-conjugate in terms of mean fluorescence per cell, whereas inhibition of clathrin-formation had a less pronounced effect on internalization of the CpG-conjugate (Fig. 7C). On the other hand, inhibition of caveolin-formation by filipin reduced the mean fluorescence of cells incubated with the CpG-conjugate. The selective effect of the inhibitors was not due to a direct effect on one conjugate, as preincubation of cells with the inhibitor, followed by extensive washing before incubation with the conjugates (indicated as preincubation in Fig. 7) in the absence of inhibitors, led to similar results. Thus these results indicate that the two TLR-L-conjugates are internalized by distinct uptake receptors.

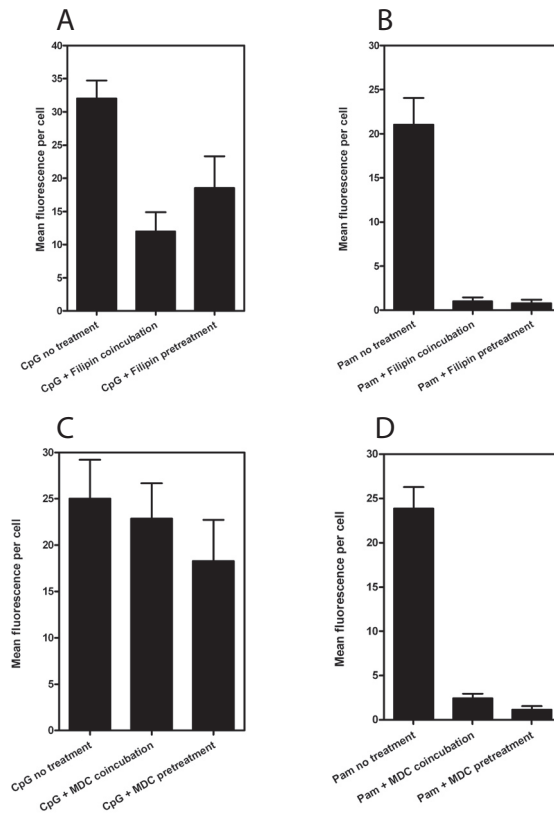


Figure 7. Effect of Filipin and MDC on internalization of the TLR-L conjugates. DCs were left untreated, or pretreated for 30min with 10 μ g/ml filipin, before adding Alexa488 labeled CpG-conjugate A or BODIPY-FL labeled Pam-conjugate B either in the presence of filipin (coincubation) or in the absence of filipin (pretreatment) for 30min at 37°C before being subjected to confocal microscopy analysis. DCs were left untreated, or pretreated for 30min with 50 μ M MDC, before adding Alexa488 labeled CpG-conjugate C or BODIPY-FL labeled Pam-conjugate D either in the presence of MDC (coincubation) or in the absence of MDC (pretreatment) for 30min at 37°C, before being subjected to confocal microscopy analysis. Shown is the mean fluorescence intensity per cell based on quantification of ten random selected cells.

Antigen presentation depends upon endosomal acidification, proteasome activity, and TAP translocation.

Next, we examined the impact of different proteases upon antigen presentation. To address this issue, we made use of the C-terminal extended peptides (OVA_{247-264A5K}), which require both N- and C-terminal processing to release the SIINFEKL CTL peptide-epitope. DCs were pre-treated either with epoxomicin, which inhibits the proteasome or NH₄Cl that increases the pH in the acidic endosome/lysosome environment and thereby inhibiting the proteases that depend on acidification (35-38), before incubation with either of the conjugates. As evident from Fig 8A and Fig.8B when DCs were pretreated with the lysosomotropic agent, NH₄Cl, a decrease in antigen presentation was seen ranging from 45% inhibition for the Pam~conjugates to 70% for the CpG~conjugated peptide. Similar, inhibition of the proteasome activity resulted in an overall decrease in antigen presentation of both the CpG~conjugated and Pam~conjugated peptide (up to 50% inhibition) in a dose dependent manner (Fig. 8C). To ascertain that the inhibitory effect observed was not due to an overall decrease in the surface expression of MHC class I, DCs that had been pretreated with either epoxomicin or NH₄Cl were incubated with the minimal CTL epitope OVA₂₅₇₋₂₆₄. As expected, the inhibitors did not cause major affect upon antigen presentation of exogenous loaded OVA₂₅₇₋₂₆₄ peptide (Fig. 8B and Fig. 8C).

Following proteasomal processing, CTL peptide-epitopes need to be translocated into the luminal side of endoplasmic reticulum via the transporter complex TAP, in order to be loaded onto MHC class I molecules. To address the involvement of TAP for cross-presentation of the TLR-L-conjugates, BMDC from either wild-type mice, or TAP-deficient mice were loaded with the TLR-L conjugates or the minimal CTL epitope OVA₂₅₇₋₂₆₄. As evident in Fig. 8D, antigen presentation of both the TLR2L- and TLR9L-conjugates depended upon TAP activity, as the presentation was abrogated in TAP-deficient mice. Importantly, antigen presentation of the minimal CTL epitope OVA₂₅₇₋₂₆₄ was not affected in the TAP-deficient DCs, showing that the observed TAP-dependence of the conjugates was not due to an overall reduced surface expression of MHC class I in the TAP-deficient DCs. In contrast, when using BMDC from mice deficient in both TAP and β 2-microglobulin expression (TAP^{-/-} β 2m^{-/-}), completely lacking MHC class I surface expression (39), antigen presentation was completely lost for all peptides (Fig. 8D).

Collectively, these results indicate that endosomal acidification, proteasomal activity and TAP translocation are required for antigen presentation of the TLR-L peptide-conjugates, and suggest that the peptide (conjugate) translocate via the endosomal compartment into the cytosol where the peptide undergoes proteasomal processing before being loaded onto MHC class I molecules in the endoplasmic reticulum.

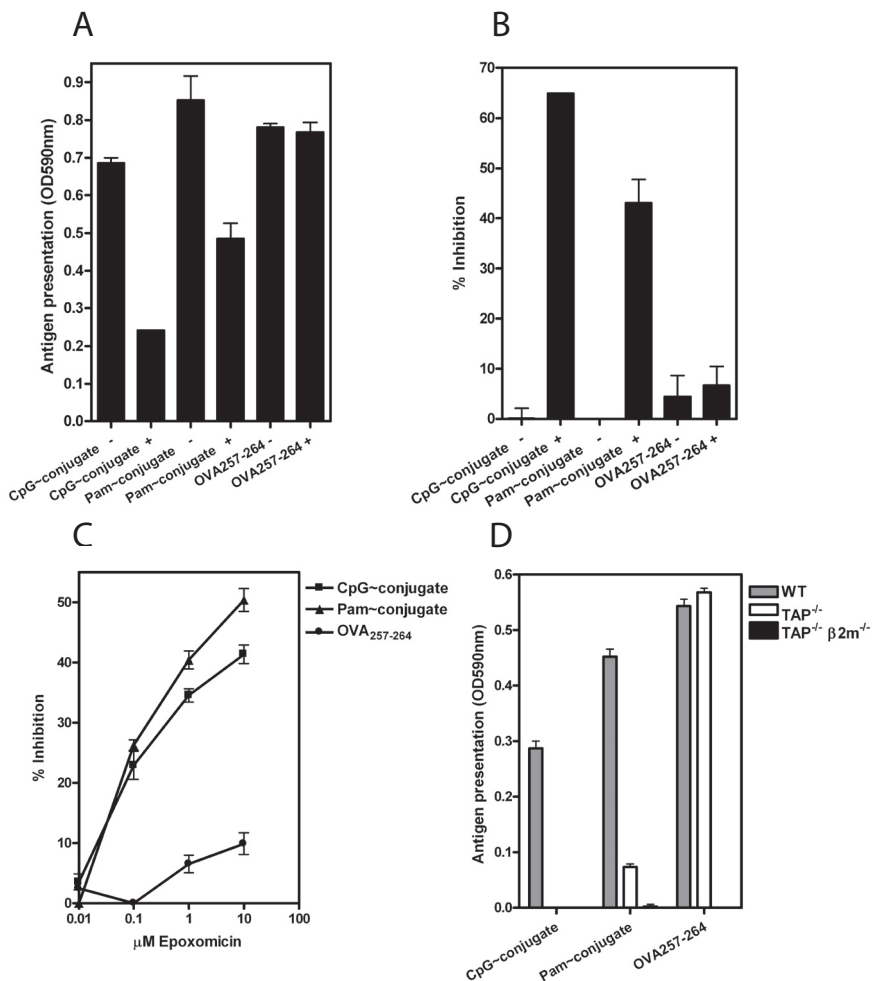


Figure 8. Antigen Presentation depends upon endosomal acidification, proteasomal activity and TAP translocation. DCs were left untreated, or pre-treated for 60min with various concentrations of 3mM NH_4Cl A, or epoxomicin B, before addition of 0.5 μ M CpG~OVA_{247-264A5K} (squares), 0.5 μ M Pam~OVA_{247-264A5K} (triangle), or 0.01 μ M OVA₂₅₇₋₂₆₄ (circle). Cells were incubated with the peptides for 3hr in the presence of inhibitors, before processed as outlined under material and methods. (-) indicate untreated; (+) indicate with NH_4Cl . Results are means of triplicates $\pm$ SEM. C, BMDC from wild-type (WT) mice, TAP-deficient mice (TAP^{-/-}), or mice deficient in TAP and β 2m (TAP^{-/-} β 2m^{-/-}) were pre-loaded either with CpG-conjugates, Pam-conjugate, or OVA₂₅₇₋₂₆₄, before being processed as outlined under material and methods.

DISCUSSION

In this study we analyzed the cellular uptake and trafficking of two distinct TLR ligand-antigen conjugates that ultimately lead to the induction of an efficient CTL response. Strikingly, one single s.c. immunization with conjugate in saline induced an impressive systemic expansion of antigen-specific CD8 T-cells (Fig. 1). Thus, conjugation resulted in a stronger systemic response than what was observed for the mixed vaccines.

Our immunofluorescence analysis revealed that conjugates of both types of TLR-ligands were taken up very efficiently compared to unconjugated peptides (Fig. 3). Internalization was a very rapid process since uptake studies showed that already within 15-30 min, the major part of the conjugates could be found in endosome/lysosome compartments (Fig. 4, and unpublished data). For this we have used fluorescent conjugates, these may have slightly different properties from that of the unmodified conjugates, which could influence the uptake and function. However, the fluorescent conjugates induced DCs maturation to a similar extent as the unmodified conjugates (unpublished data). In line with our findings, CpG linked to FITC labeled Ovalbumin protein was recently shown to translocate to LAMP-1 positive endosomal-lysosomal compartments (10). Further support of enhanced uptake mediated by the conjugates was provided from our *in vivo* uptake analysis, which revealed a 6-8-fold increase in uptake of the CpG-conjugated peptide by CD11c⁺ cells, and a 2-fold increase in uptake of the Pam-conjugated peptide by CD11c⁺ cells compared to uptake of non-conjugated peptide (Fig. 3E and Fig. 3F). We found that CpG-conjugated peptides were internalized independently from the expression of TLR9 and could also support antigen presentation *in vitro* independently of TLR9 expression (Fig. 4). Accordingly, Wagner and co-workers (2) showed that cross-presentation of OVA linked CpG occurred independently from TLR9 expression, but that TLR9 expression nevertheless was essential for activation of the DCs. At first sight these findings might seem paradoxical, however, TLR9 are mainly expressed in the endoplasmic reticulum, followed by recruitment to the endosomes upon dendritic cell maturation (40).

Unexpectedly, considering the cell surface expression of TLR2, we found that internalization of the Pam-conjugate was taken up independently from the expression of TLR2. BMDCs isolated from TLR2 deficient mice internalized the Pam-conjugate to a comparable level as BMDCs from wild-type mice, and antigen recognition was unaffected in BMDCs from TLR2-deficient mice. This could not be attributed to a side effect mediated by the peptide part, since TLR2-independent internalization was also observed when incubating the cells with free Pam (unpublished data). Importantly, inhibition of clathrin-dependent endocytosis or caveola formation abrogated both uptake and antigen presentation of Pam-conjugates (Fig. 6 and Fig. 7), whereas internalization of CpG-conjugates was independent of clathrin-

coated pits, but dependent on caveolae formation. These results indicate that other (distinct) receptors than the TLRs are involved in the uptake of the TLR-conjugates, although the exact nature of these receptors remains to be established. Other TLR2 ligands have been reported to be internalized independently from the expression of TLR2. Outer membrane protein A, a conserved major component of the outer membrane of Enterobacteriaceae family that triggers cytokine production in macrophages and DCs (41), was recently shown to be internalized by the scavenger receptor LOX-1, independently from the expression of TLR2 (42). Moreover, lipoteichoic acid (LTA) has also been reported to be internalized independently from TLR2 expression (43), although the receptor involved in the uptake of LTA still remains to be identified. Therefore the contribution of TLR2 and other receptors expressed on the cell surface, to the uptake of Pam and other TLR ligands remains to be established.

Optimal presentation of the peptide antigen cargo in the conjugates required endosomal acidification (Fig.8). Although it can not be ruled out that the fusion of early endosomal vesicles with late endosomal vesicles is hampered by the lysosomotropic agent NH_4Cl (37, 44-46). These results imply that endosomal proteases, such as cathepsins (45, 47), might be involved in the processing of the TLR-L-peptide conjugate. Furthermore, proteasomal cleavage was required since proteasome inhibition greatly decreased antigen presentation of the TLR-L-conjugates (Fig.8C). As proteasomes are mainly located in the cytosol (48, 49), our results indicate that the peptide/conjugate, after being released in the endosomal compartment, translocate to the cytosol to undergo proteasomal processing (45). In this regard, it was recently reported that the translocon complex SEC61 could be involved in facilitating the translocation of peptide from the endosomes to the cytosol (50). Moreover, abrogation of translocation of peptides from the cytosol to the endoplasmic reticulum completely abrogated antigen presentation of both types of TLR-L-peptide conjugates (Fig 8D). Aside from being internalized efficiently, we found that all of the TLR-L conjugates retained their capacity to activate DCs to a comparable level as the free TLR-L, both in terms of production of the Th1-favoring cytokine, IL-12 (unpublished data) and up-regulation of DCs maturation surface markers (Table II). Importantly, TLR expression was required for DCs activation, since BMDCs lacking TLR2 or TLR9, were not able to up-regulate co-stimulatory molecules upon stimulation with either Pam~conjugate or CpG~conjugate, respectively (Fig. 2). In addition, conjugation of the non-stimulatory GpC oligonucleotide to the peptide antigen resulted in inefficient CTL priming (Fig.1) showing that the DCs activation of TLR-L peptide conjugates is essential. Therefore, intracellular signaling of TLR is crucially important for effective CTL priming by the conjugates.

In summary, we demonstrate that well-defined synthetic TLR-L-peptide conjugates induce a robust and systemic response of specific T-cells due to the combined action of enhanced

antigen uptake, improved MHC class I antigen presentation, and dendritic cell maturation. Our data show that targeting to two different TLRs requires distinct uptake mechanism, independent of TLR expression, but follows similar trafficking and intracellular processing pathways leading to optimal antigen presentation and T-cell priming. The chemical properties of these conjugates, which ensure that the same DCs that takes up the antigen receives simultaneously a proper maturation signal is likely to be the mechanism behind the superior activity of the peptide-conjugates. Accordingly, Medzhitov and co-workers recently reported that synchronous entrance of TLR-L and antigen enhanced MHC class II presentation, although in their system antigen and TLR-L was delivered on microspheres in a non-covalent manner (51). The collective features of our TLR-L peptide conjugates, together with their convenient manufacture and handling, makes synthetic peptide-TLR-ligand conjugates an attractive novel vaccine modality.

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Cbl-b and anti-tumor immunity

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Spontaneous tumor rejection by cbl-b deficient CD8⁺ T cells.

Abstract. The concept of tumor surveillance implies that specific and non-specific components of the immune system eliminate tumors in the early phase of malignancy. Understanding the biochemical mechanisms of tumor immunosurveillance is of paramount significance because it might allow one to specifically modulate spontaneous anti-tumor activity. Here we report that inactivation of the E3 ligase Cbl-b confers spontaneous *in vivo* rejection of tumor cells that express Human Papilloma Virus antigens. Moreover, cbl-b^{-/-} mice develop significantly fewer UVB-induced skin malignancies and reject UVB-induced skin tumors. CD8⁺ T cells were identified as key players in the spontaneous tumor rejection response. Loss of Cbl-b not only enhances anti-tumor reactivity of CD8⁺ T cells but also occurs in the absence of CD4⁺ T cells. Mechanistically, cbl-b^{-/-} CD8⁺ T cells are resistant to Treg mediated suppression and exhibit enhanced activation and rapid tumor infiltration. Importantly, therapeutic transfer of naïve cbl-b^{-/-} CD8⁺ T cells is sufficient to mediate rejection of established tumors. Even up to one year after the first encounter with the tumor cells, cbl-b^{-/-} mice carry an “anti-cancer memory”. These data identify Cbl-b as a key signaling molecule that controls spontaneous anti-tumor activity of cytotoxic T cells in different cancer models. Inhibition of Cbl-b is a novel approach to stimulate long-lasting immunity against cancer.

INTRODUCTION

More than 100 years ago, it was discovered that tumors regress in patients injected with bacterial extracts (1), suggesting that immune cells might be capable to eliminate cancer cells in the early phase of malignancy (2, 3). In many cases, tumor growth and lack of anti-cancer immunity can be ascribed to the fact that tumor cells do not provide sufficient T cell stimulation or induce tolerance in the tumor reactive T cell population (4-7). Several attempts have been made to break such tumor tolerance and to specifically enhance anti-tumor immunity by modulating immune cells (8, 9). However, immunotherapy is still difficult because most therapies result in severe side-effects, require large amounts of immune cells, or depend on extensive genetic manipulations of effector cell populations. Thus, identification of a key dominant “tolerogenic” factor in T cells that directly controls activation of tumor-reactive cytotoxic T cells *in vivo* might circumvent these limitations of T cell immunotherapy.

The Casitas B-cell Lymphoma-b protein, Cbl-b, is a member of the mammalian family of Cbl E3 ubiquitin ligases (10). Proteins of this family contain an N-terminal tyrosine kinase binding domain, a RING finger, a C-terminal proline-rich sequence, and can thus function as both E3 ligases and molecular adaptors (10). Studies of Cbl-b-deficient mice have revealed an essential role for this molecule in T cell tolerance induction. Cbl-b^{-/-} T cells show effective activation in the absence of costimulation, resulting in spontaneous autoimmunity or enhanced susceptibility to autoantigens (11-14). Moreover, Cbl-b sets the threshold for T cell activation to “weak” antigens (11, 15, 16) and controls immunotolerance in multiple experimental systems *in vitro* and *in vivo* (13, 14, 17, 18). Thus, Cbl-b functions as a negative regulator of antigen-specific T cell activation and is a critical mediator of T cell anergy. Based on these findings, we hypothesized that Cbl-b-regulated T cell activation may hold the key to our understanding of induction and/or maintenance of T cell responses to cancer cells.

RESULTS

Cbl-b mutant mice spontaneously reject tumors.

To determine whether Cbl-b contributes to anti-cancer immunity *in vivo* we tested the TC-1 cancer model in cbl-b deficient mice. TC-1 cells are c-H-ras transformed C57BL6-syngeneic fibroblasts expressing the Human Papilloma Virus (HPV) 16 derived oncoproteins E6 and E7 as tumor relevant T cell antigens (19). High risk HPV infection is a major cause of cervical cancer in women, with a high mortality rate, and the HPV-16 E6 and E7 oncoproteins are almost invariably expressed in early cervical cancer (20). Importantly, HPV vaccinations can protect against cervical cancer, indicating that immunoreactivity against HPV antigens plays a key role in cancer prevention and therapy (21).

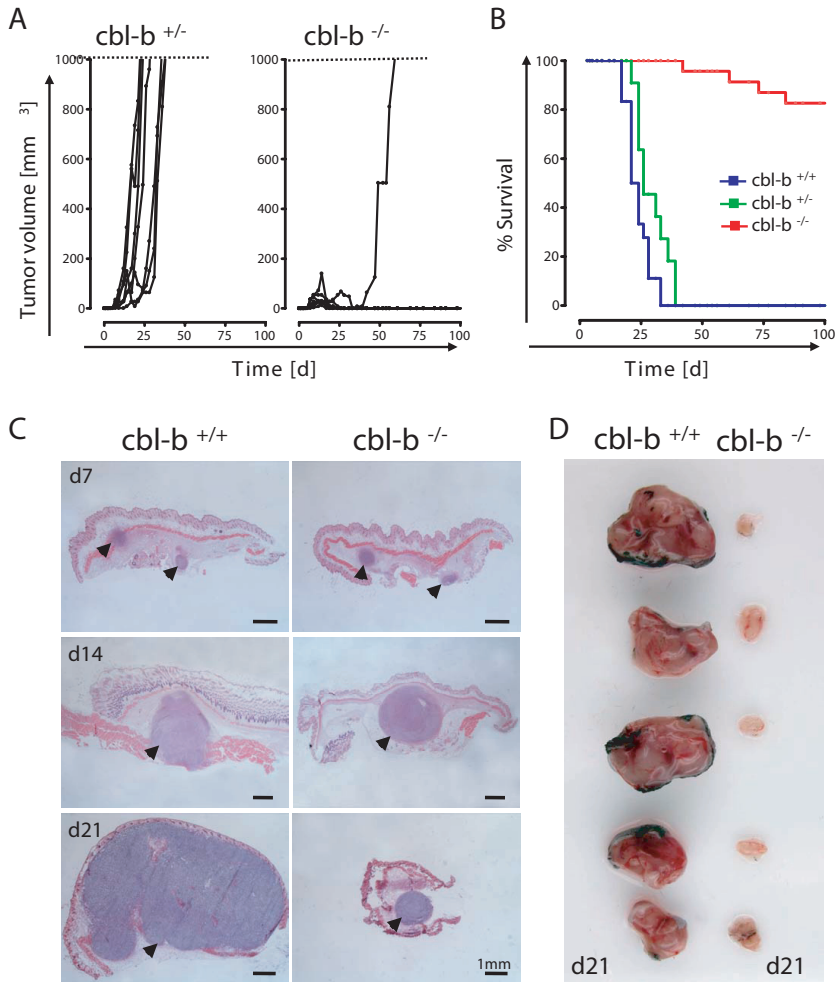


Figure 1. Spontaneous tumor rejection in *cbl-b*^{-/-} mice. A, Kinetics of TC-1 tumor cell growth in *cbl-b*^{+/-} (n=6) and *cbl-b*^{-/-} (n=7) mice. 2.5×10^5 TC-1 cells were injected into the flanks of 8-12 week old littermate mice and tumor volume was measured with a caliper [mm³] over time [days]. Of note, only mice that developed a palpable tumor were included into the experimental cohorts. B, Kaplan-Meier survival curves of *cbl-b*^{+/-} (n=18), *cbl-b*^{-/-} (n=11), and *cbl-b*^{-/-} (n=28) mice inoculated with 2.5×10^5 TC-1 tumor cells. Data are pooled from four different experiments. C, Representative histology of TC-1 tumors isolated on different days (d7, d14, and d21 after inoculation) from *cbl-b*^{+/-} and *cbl-b*^{-/-} mice. H&E staining. Arrows point at tumor mass. Bars indicate 1mm. D, Macroscopic appearance of TC-1 tumors in 5 different *cbl-b*^{+/-} and 5 different *cbl-b*^{-/-} mice on day 21 (d21) after inoculation.

In mice, injection of HPV16 E6 and E7 expressing TC-1 tumor cells into syngenic recipients results in rapid tumor growth that can be abrogated by vaccination with an E7-peptide-based vaccine (22, 23). We therefore injected TC-1 subcutaneously into the left flank of wild type (cbl-b^{+/+}), cbl-b heterozygous (cbl-b^{+/-}), and cbl-b deficient (cbl-b^{-/-}) mice. In all recipients, tumor growth was first macroscopically observed at approximately 3 to 5 days after inoculation of 2.5×10^5 tumor cells (Fig. 1A). As reported previously in naïve C57BL/6 mice (24), the tumors continued to grow progressively and with similar kinetics in all cbl-b^{+/+} and cbl-b^{+/-} mice analyzed (Fig. 1A,B). The histology of tumors, the kinetics of tumor growth, and tumor incidences were comparable between cbl-b^{-/-}, cbl-b^{+/-}, and cbl-b^{+/+} mice in the first two weeks after tumor inoculation (Fig. 1C and not shown). Intriguingly, around 2 weeks after TC-1 inoculation, naïve cbl-b^{-/-} mice spontaneously rejected the tumors (Fig. 1A). Tumor mass progressively reduced in cbl-b^{-/-} mice and became undetectable between days 25 and 35 after the initial inoculation of tumor cells (Fig. 1C). At 3 weeks post-inoculation, the average size of a wild type tumor was 835 mm³ (+/- 227,9 mm³ s.e.m.; n= 5) compared to an average size of 14 mm³ (+/-7,6 mm³ s.e.m.; n= 5, p < 0,003) in cbl-b^{-/-} recipients (Fig. 1D). Over 80% of cbl-b^{-/-} mice completely rejected the tumors and remained tumor free throughout the experimental observation period that in some cases was longer than one year (Fig. 1B and data not shown). Progressive tumor growth in cbl-b^{-/-} mice was observed in a few cases, but only after a longer latency period when compared to tumor growth in wild type mice (Fig. 1A,B). It should be noted that we injected a tumor cell number (2.5×10^5) into our experimental cohorts that is 10 times higher than the dose that is lethal for wild type mice. (24) These surprising data show that naïve cbl-b mutant mice can spontaneously reject a very high dose of aggressive TC-1 tumor cells.

Spontaneous tumor rejection in cbl-b mutant mice is mediated by CD8⁺ T cells.

To explore the underlying mechanisms of spontaneous tumor rejection in the cbl-b knockout mice, we assessed proliferation and cell death of tumor tissue. Tumor cell proliferation was comparable in both cbl-b^{+/+} and cbl-b^{-/-} mice on day 7, day 14 (Fig. 2A) and day 21 (not shown) suggesting that loss of Cbl-b expression in the host environment does not affect cell cycle progression of the tumor cells. By contrast, whereas cell death within early tumors (day 7 after inoculation) appeared comparable among the different cohorts, we observed markedly increased apoptosis in day 14 tumors taken from cbl-b^{-/-} mice (Fig. 2B). We next determined the numbers of lymphoid cells in tumor-bearing cbl-b^{+/+} and cbl-b^{-/-} mice. We did not observe alterations in CD11b⁺, CD11c⁺, NK1.1⁺ cells or Gr1⁺ granulocytes, nor in relative numbers of CD4⁺ or CD8⁺ T cells in the draining inguinal lymph nodes, non-draining contra-lateral inguinal lymph nodes, or the spleen (not shown). Immunohistochemistry (not shown) and

FACS analysis (Fig. 2C) of total tumor tissue revealed that CD4⁺ and CD8⁺ T cells infiltrate the tumors in both wild type and *cbl-b*^{-/-} mice. However we observed markedly increased ratios of CD8⁺ within the tumors of *cbl-b*^{-/-} mice compared to wild type mice (Fig. 2C). Of note, we observed tumor infiltration of CD8⁺ cells as early as day 8 after TC-1 inoculation in *cbl-b*^{-/-} mice (Fig. S1A,B). In line with increased CD8⁺ T cell infiltration of tumors, we also detected elevated levels of the CD8⁺ T cell chemokine RANTES (25) in tumors growing in *cbl-b*^{-/-} mice (not shown). These data show that loss of Cbl-b expression in mice results in increased tumor cell death, increased infiltration of CD8⁺ T cells into the tumor tissue, and, most importantly, spontaneous tumor rejection.

To investigate whether CD8⁺ T cells from wild type and *cbl-b*^{-/-} mice were reactive to the tumor specific antigens, we analyzed IFN γ production by CD8⁺ T cells isolated from the spleen (not shown) and lymph nodes (Fig. 3A) of tumor-bearing mice upon re-stimulation with the MHC class I (H2D^b) restricted E7 tumor-specific peptide antigen. In the draining lymph nodes of TC-1 challenged wild type mice, we consistently observed a low frequency of E7-reactive CD8⁺ IFN γ producing T cells. Importantly, in all tumor-bearing *cbl-b*^{-/-} mice analyzed, the frequency of IFN γ producing CD8⁺ T cells was markedly increased in response to stimulation with the E7 peptide (Fig. 3A). Of note, we also observed E7 tumor specific, IFN γ producing CD8⁺ T cells, albeit at lower numbers, in the non-draining contra-lateral inguinal lymph nodes of *cbl-b*^{-/-} mice. These data show that spontaneous tumor rejection in *cbl-b* mutant mice is associated with rapid CD8⁺ T cells infiltration into the tumor and hyper-activation of tumor-specific cytotoxic T cells.

To examine whether CD8⁺ T cells are indeed essential for the spontaneous rejection of TC-1 tumor cells in *cbl-b* mutant mice, we depleted CD8⁺ cells using specific antibodies prior to the tumor injection. Following confirmation of CD8⁺ T cell depletion (Fig. S2), *cbl-b*^{-/-} and *cbl-b*^{+/+} mice were injected with TC-1 tumors and tumor growth was monitored for up to one year. Tumors grew progressively in the *cbl-b*^{+/+} control mice and the kinetics of tumor expansion in the CD8⁺ T cell depleted group was comparable to the control cohort (Fig. 3B). In the *cbl-b*^{-/-} mice, tumors were spontaneously rejected in the control group with some cases of late-onset of tumor growth (Fig. 3B; see also Fig. 1A,B). Importantly, CD8⁺ T cell depleted *cbl-b*^{-/-} mice displayed progressive and lethal tumor growth (Fig. 3B).

In most experimental models of tumor rejection, CD4⁺ T cell help is required for effective anti-tumor immunity (26, 27). Similarly, it has been shown that the vaccination induced anti-TC-1 tumor response depends on CD4⁺ T cell help (22). To address the role of CD4⁺ T cells in spontaneous tumor rejection, we efficiently depleted CD4⁺ cells in *cbl-b*^{-/-} and *cbl-b*^{+/+} mice (Fig. S2) followed by TC-1 inoculation. As expected, depletion of CD4⁺ T cells in wild type mice did not change the kinetics or frequencies of tumor growth. Surprisingly,

ablation of CD4⁺ T cells in *cbl-b*^{-/-} mice did not affect their capacity to spontaneously reject the tumor (Fig. 3B) indicating that CD4⁺ T cells are not required for rejection of TC-1 tumors in our experimental system. These data show that CD8⁺ T cells play an essential role in the spontaneous rejection of TC-1 tumors in *cbl-b* mutant animals.

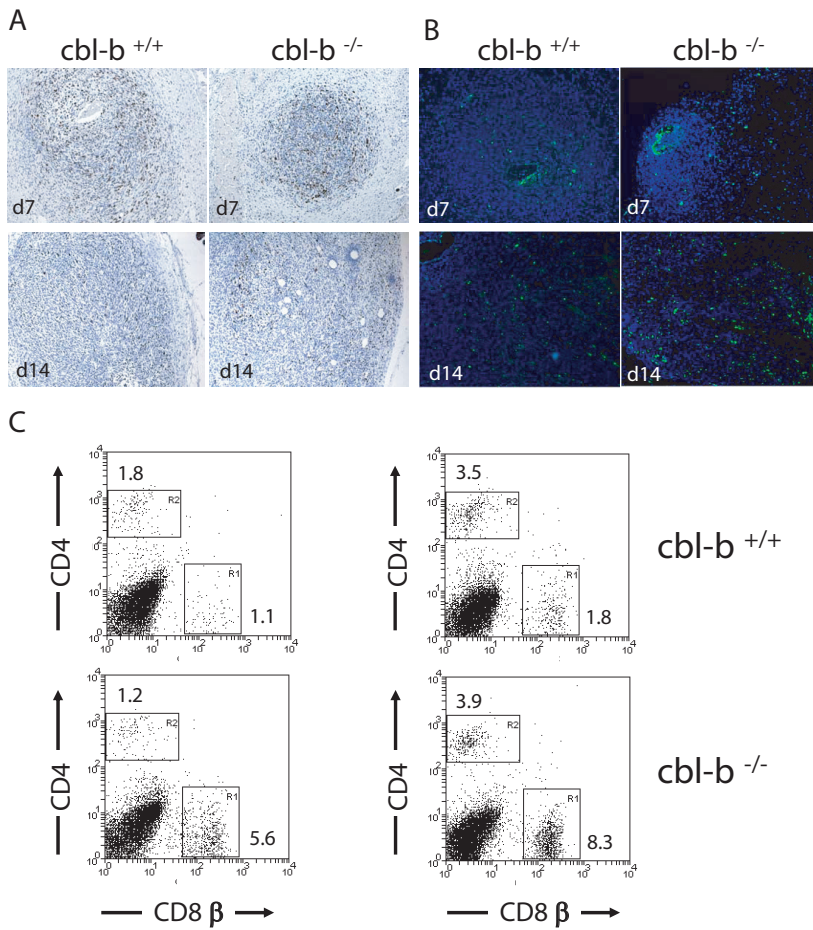


Figure 2. Infiltration of CD8⁺ T cells into tumors from *cbl-b*^{-/-} mice. A, Immunohistochemistry for proliferation marker Ki67 in TC-1 tumor samples from *cbl-b*^{+/+} and *cbl-b*^{-/-} mice at 7 and 14 days after tumor inoculation (2.5×10^5). Original magnifications X 50. Size bars represent 100 μ m. B, Increased cell death in tumor tissue from *cbl-b*^{-/-} mice 14 days after TC-1 tumor cell injection. Cell death was determined by TUNEL. Representative images of individual mice on day 7 and 14 are shown. Original magnifications X 50. Size bars represent 100 μ m. C, Analysis of tumor-infiltrating lymphocytes 17 days (left panels) and 21 days (right panels) after TC-1 inoculation into *cbl-b*^{+/+} and *cbl-b*^{-/-} mice. Single cell suspensions were analyzed by flow cytometry using antibodies reactive to CD8 and CD4. Numbers indicate percentages of cells within the R1 and R2 gates.

Therapeutic transfer of naïve *cbl-b*^{-/-} CD8⁺ T cells is sufficient to mediate spontaneous rejection of established tumors.

Our data show that *cbl-b* mutant mice spontaneously reject TC-1 tumors via CD8⁺ T cells. We then wanted to ask whether *cbl-b*^{-/-} CD8⁺ cells could also be used to treat a previously established cancer. To address whether *cbl-b*^{-/-} CD8⁺ cells function therapeutically, we set up an adoptive transfer model. In this model, T and B cell deficient *rag2* mutant mice were injected with 2.5×10^5 TC-1 tumor cells followed by infusion of 3×10^6 and 2×10^6 purified

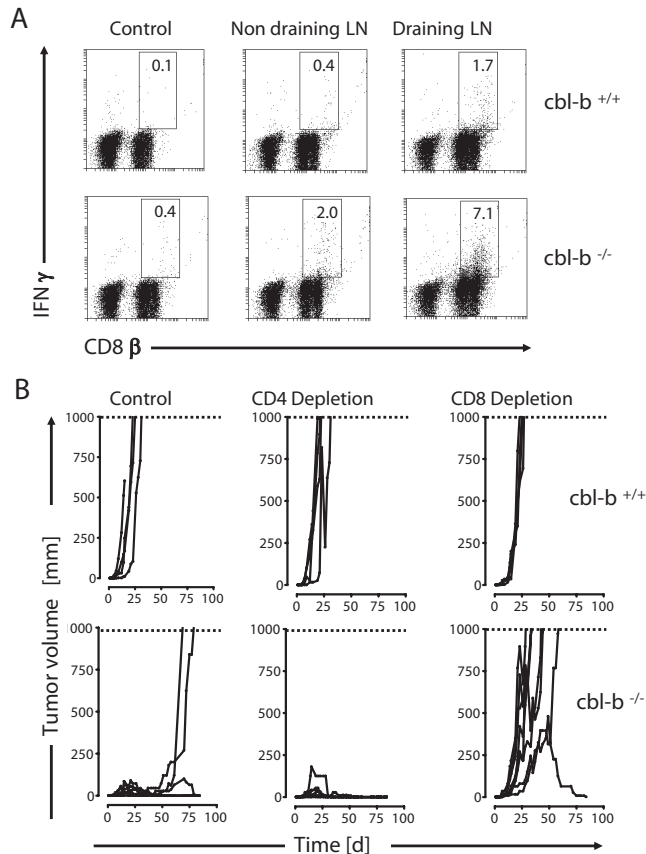


Figure 3. CD8⁺ cells mediate spontaneous tumor rejection independent of CD4⁺ T cell help. A, IFN γ production of CD8⁺ T cells isolated from draining and non-draining inguinal lymph nodes of tumor-bearing *cbl-b*^{+/+} and *cbl-b*^{-/-} mice (n= 5) and naïve control mice (n=3). Data are from day 21 after tumor inoculation. Purified CD8⁺ T cells were re-stimulated for 60h with the HPV16-derived peptide E7⁴⁹⁻⁵⁷, stained for intracellular IFN γ , and analyzed by flow cytometry. Numbers indicate percentages of IFN γ ⁺CD8 β ⁺ T cells. B, Kinetics of TC-1 tumor cell growth in *cbl-b*^{+/+} (top panels; n=6) and *cbl-b*^{-/-} (bottom panels; n=7) mice left untreated or following depletion of CD4⁺ or CD8⁺ T cell subsets. 2.5×10^5 TC-1 cells were injected into the flanks of 8-12 week old mice and tumor volume was measured with a caliper [mm³] over time [days]. Only mice that developed a palpable tumor were included into the experimental cohorts.

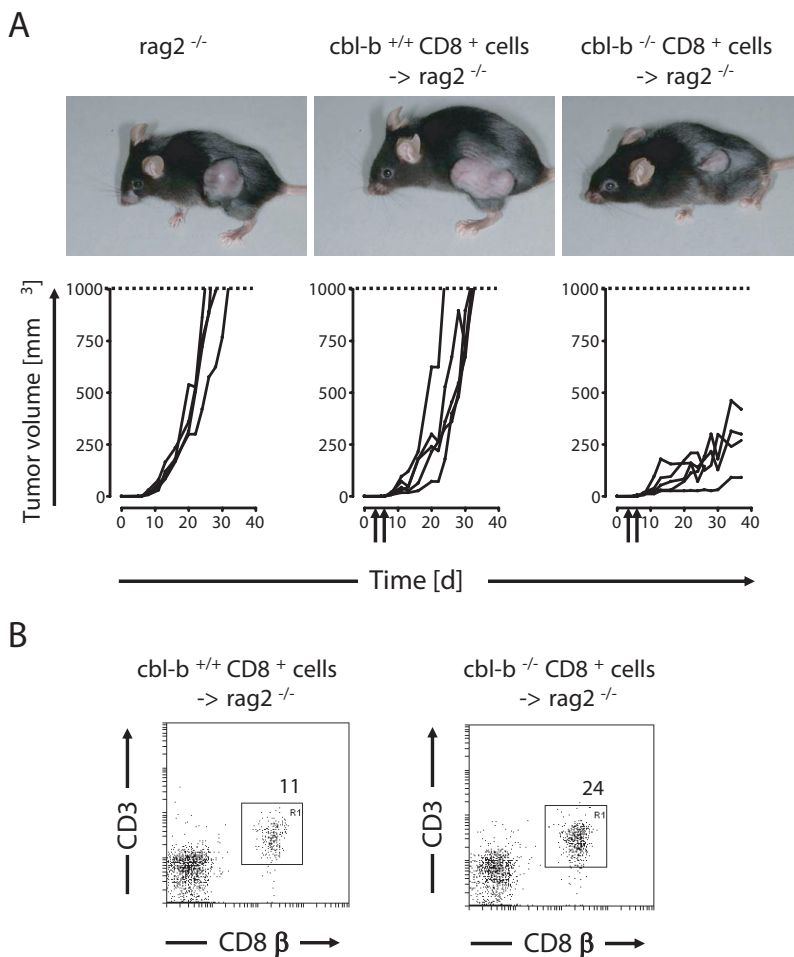


Figure 4. Therapeutic transfer of naïve $cbl-b^{-/-}$ CD8⁺ T cells is sufficient to mediate spontaneous rejection of established tumors. A, $rag2^{-/-}$ mice were subcutaneously injected with 2.5×10^5 TC-1 cells. At day 3 and day 6 post tumor cell injection, purified CD8⁺ cells from naïve $cbl-b^{-/-}$ and $cbl-b^{+/+}$ mice were adoptively transferred (i.v.) into the tumor bearing $rag2^{-/-}$ mice (arrows). $n=5$ per group. The $rag2^{-/-}$ control group ($n=4$) received tumor cells, but no donor T cells. Upper panels show representative tumor sizes at the end of the experiment. Lower panels indicate the kinetics of tumor growth. B, Relative percentages of CD3⁺CD8⁺ $cbl-b^{+/+}$ and CD3⁺CD8⁺ $cbl-b^{-/-}$ T cells in the blood of adoptively transferred $rag2^{-/-}$ mice carrying TC-1 tumors. Representative flow cytometry data show CD3⁺CD8⁺T cell populations on day 7 after the second T cell transfer (day 13 after the first TC-1 injection).

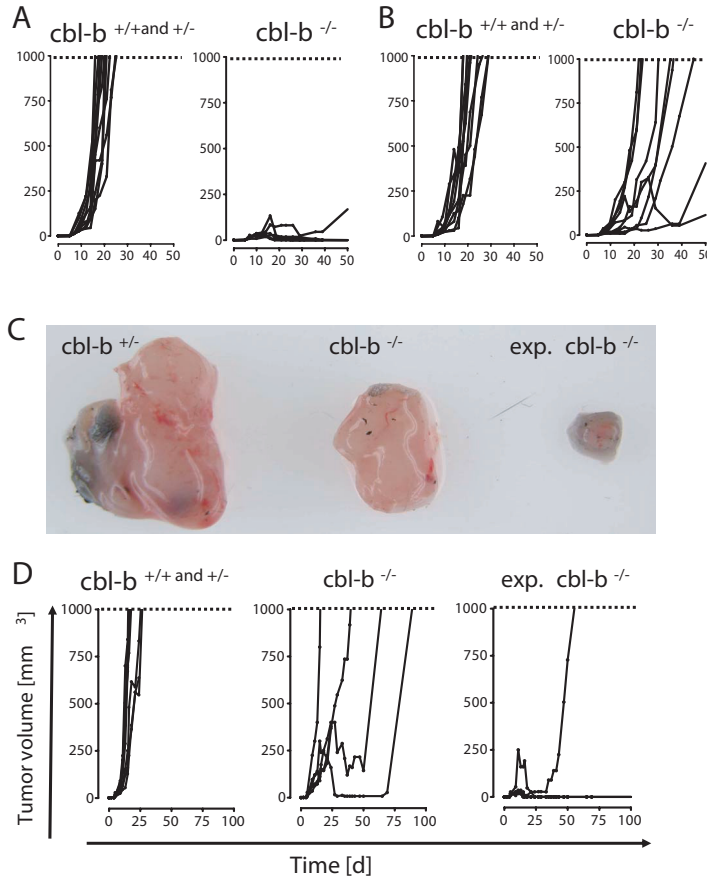


Figure 5. Tumor escape and long lasting anti-tumor memory in *cbl-b*^{-/-} mice. Escape of *cbl-b*^{-/-} mice-derived TC-1 tumors. Tumor cell lines were generated from A, TC-1 tumors growing in *cbl-b*^{+/-} mice or B, generated from late-onset TC-1 tumors growing in *cbl-b*^{-/-} mice. Tumor cells were injected (2.5×10^5) into 8-12 weeks old, naïve, *Cbl-b* expressing control mice and *cbl-b*^{-/-} recipients. Kinetics of tumor growth was analyzed over the indicated time period. Graphs represent data from two pooled experiment of *cbl-b*^{+/-} and *cbl-b*^{-/-} ($n=10$ for both cell lines) mice and *cbl-b*^{-/-} mice ($n=7$ for *cbl-b*^{+/-}-derived tumor cells and $n=10$ for *cbl-b*^{-/-}-derived tumor cells). Of note, the kinetics of tumor growth was comparable between *cbl-b*^{+/-} and *cbl-b*^{-/-} mice. C,D. Anti-tumor memory. *Cbl-b*^{-/-} mice, that had received 2.5×10^5 TC-1 cells at 8-12 weeks of age and stayed tumor free after rejection of the initial cancer, were kept under observation for 1 year post TC-1 injection. These experienced (exp) *cbl-b*^{-/-} mice together with age-matched naïve *cbl-b*^{-/-} and age-matched naïve *cbl-b*^{+/-} and *cbl-b*^{+/-} control mice were re-challenged with a 10 times higher dose of TC-1 cells (2.5×10^6). C, Appearance of representative tumors from each experimental group. Tumors were imaged 23 days after injection of 2.5×10^6 TC-1 cells. D, Kinetics of TC-1 tumor cell growth in age- matched naïve *cbl-b*^{+/-} and *cbl-b*^{-/-} control mice ($n=5$), naïve *cbl-b*^{-/-} mice ($n=6$), and experienced (exp) *cbl-b*^{-/-} mice ($n=6$). 2.5×10^6 TC-1 cells were injected into the flanks of 14 month old mice and tumor volume was measured with a caliper [mm^3] over time [days]. Of note, only mice that developed a palpable tumor were included into the experimental cohorts.

CD8⁺ T cells from naïve *cbl-b*^{+/-} and *cbl-b*^{-/-} donors on days 3 and 6, respectively, after initial tumor cell challenge. In this experimental system tumors grow progressively in a wild type environment followed by 2 therapeutic vaccinations with freshly purified, polyclonal, and naïve CD8⁺ T cells from syngenic donors. Tumor growth was monitored for a period of 6 weeks (Fig. 4). The kinetics and extent of TC-1 tumor growth was comparable between *rag2*^{-/-} control mice and *rag2*^{-/-} mice infused with wild type CD8⁺ T cells (Fig. 4A). In contrast, tumor growth was markedly reduced and delayed in *rag2*^{-/-} mice that received *cbl-b*^{-/-} CD8⁺ T cells as a therapeutic vaccine (Fig. 4A). Moreover, although the same numbers of cells were transferred from wild type and *cbl-b*^{-/-} donors, we observed a marked increase in the numbers of CD8⁺ *cbl-b*^{-/-} T cells in the blood of *rag2*^{-/-} hosts (Fig. 4B). These data show that therapeutic transfer of naïve *cbl-b*^{-/-} CD8⁺ T cells is sufficient to mediate spontaneous rejection of established tumors.

Tumor escape in *cbl-b*^{-/-} mice.

Recent results suggested that the dominant mechanism of spontaneous tumor growth is induction of immunotolerance rather than immuno-escape of the tumor cells (28). However, Cbl-b appears to be a critical regulator of antigen-specific T cell tolerance and it has been shown in multiple systems that *cbl-b*^{-/-} T cells cannot be anergized. (13, 17) Since some *cbl-b*^{-/-} mice developed a late onset tumor (Fig. 1A,B; Fig. 3B), we therefore addressed the mechanism by which Cbl-b can confer spontaneous tumor rejection. To test whether the late onset of tumor growth in *cbl-b*^{-/-} mice was the consequence of tumor-intrinsic “evasive” mechanisms rather than host-intrinsic “immunotolerance”, we established cell lines from TC-1 tumors that showed late onset growth in *cbl-b*^{-/-} mice. If tumor cells originating from *cbl-b*^{-/-} mice trigger immunotolerance in a particular host mouse, then these tumors should again be rejected in *cbl-b*^{-/-} mice that have survived a previous challenge of TC-1 cells (experienced *cbl-b*^{-/-} mice). If tumors developed due to an escape mechanism intrinsic to the cancer cell, then these tumors should also progress when transferred into experienced *cbl-b*^{-/-} mice. As a control we also established tumors that grew in *cbl-b*^{+/-} mice. As expected, tumors isolated from *cbl-b*^{+/-} mice rapidly formed large tumors in wild type mice, but were rejected when transferred into *cbl-b*^{-/-} hosts (Fig. 5A). Importantly, TC-1 tumors isolated from *cbl-b*^{-/-} mice grew progressively in both *cbl-b*^{+/-} and *cbl-b*^{-/-} recipients. These results indicate that tumor growth in *cbl-b*^{-/-} mice is a consequence of tumor escape rather than induction of immunotolerance.

Cbl-b^{-/-} mice carry long-lasting anti-cancer memory.

Our data so far showed that CD8⁺ T cells from cbl-b mutant mice can directly mediate and are sufficient for spontaneous tumor rejection. Moreover, mechanistically we failed to observe induction of immunotolerance in TC-1 challenged cbl-b^{-/-} mice. To expand these findings to additional therapeutic benefits of a potential T cell vaccine, we studied the anti-tumor memory response. To investigate whether aged cbl-b mutant mice, that rejected tumors and remained tumor free (“experienced cbl-b^{-/-} mice”), have a long lasting memory in response to the tumor, we re-challenged age matched naïve wild type, naïve cbl-b^{-/-}, and experienced cbl-b^{-/-} mice (1 year after the first challenge) with a hundred times the lethal dose of TC-1 cells. At such a tumor cell concentration, all age-matched wild type and cbl-b^{+/-} control mice rapidly developed tumors (Fig. 5C, D). Tumor development was abrogated in old naïve cbl-b^{-/-} mice, but all animals tested developed late onset tumors, indicating that the spontaneous anti-tumor response in cbl-b^{-/-} is dependent on the tumor load. Intriguingly, one year after the first challenge we observed that experienced cbl-b knockout mice were able to reject the tumors even at the 100 times lethal tumor dose (Fig. 5D). Moreover, three weeks after tumor challenge, tumors appeared significantly smaller in the re-challenged experienced cbl-b knockout mice compared to naïve knockout mice and naïve heterozygous control mice (Fig. 5C). It should be noted that incidence and severity of autoimmune organ infiltration were comparable between aged naïve and TC-1 challenged cbl-b mutant mice at one year after the first tumor challenge (not shown) suggesting that even such long-lasting anti-tumor reactivity did not enhance the incidence or severity of autoimmunity (11, 12). Thus, even up to one year after the first encounter with the tumor cells, cbl-b^{-/-} mice carry an anti-cancer memory.

Cbl-b^{-/-} CD8⁺ T cells are less sensitive to CD4⁺CD25⁺ regulatory T-cell suppression.

It has been reported that CD4⁺CD25⁺FoxP3⁺ T-regulatory (T-reg) cells suppress CD8⁺ effector cell immunity in cancer (29). Moreover, Tregs play a role in vaccination-mediated rejection of TC-1 tumors (30). Further it has been shown that cbl-b deficient CD4⁺ T-cells are resistant to CD4⁺CD25⁺ Treg suppression (17). However, whether loss of Cbl-b also confers such resistance to CD8⁺ T cells has never been established. We therefore first examined the numbers of Tregs in TC-1 tumors from cbl-b^{+/-} and cbl-b^{-/-} mice. At two weeks after TC-1 inoculation, the total numbers of CD4⁺CD25⁺ and CD8⁺ tumor infiltrating cells were similar in wild type mice (Fig. 6A). Tumors isolated from cbl-b^{-/-} mice contained slightly increased (~2 fold) numbers of infiltrating CD4⁺CD25⁺ cells. FoxP3 immunostaining of tumor-derived CD4⁺CD25⁺ cells showed that ~ 55% of these cells express FoxP3 both in cbl-b^{+/-} and cbl-b^{-/-} mice (Fig. 6A and Fig. S3A). The number of tumor infiltrating CD8⁺ cells was dramatically

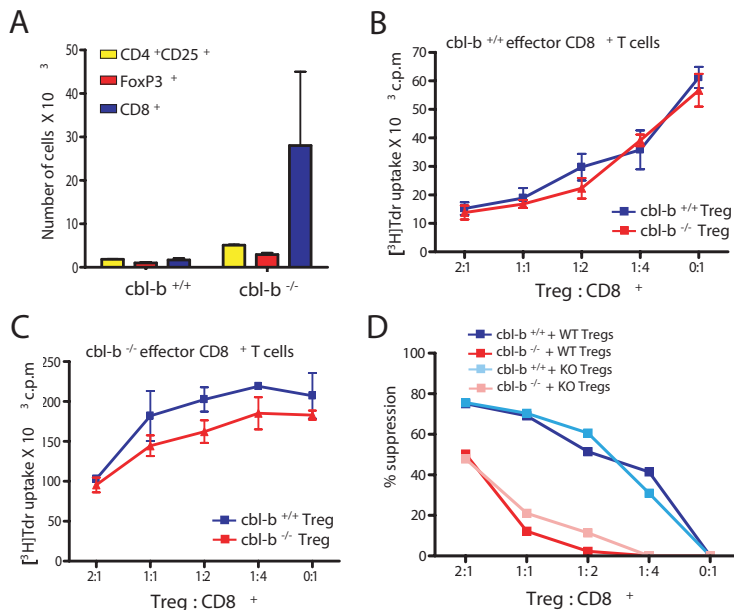


Figure 6. cbl-b^{-/-} effector CD8⁺ T cells are resistant to CD4⁺CD25⁺ Treg suppression. A, Numbers of tumor infiltration CD4⁺CD25⁺, FoxP3⁺, and CD8⁺ cells per 1 x 10⁶ tumor cells in cbl-b^{-/-} (n = 4 tumors) and cbl-b^{+/+} (n = 6 tumors) mice. Data are shown as mean $\pm$ s.e.m. B, Proliferation of wild type CD8⁺ effector T-cells and C, proliferation of cbl-b^{-/-} CD8⁺ effector T-cells in the presence of cbl-b^{+/+} and cbl-b^{-/-} CD4⁺CD25⁺ Tregs at various Treg:Teff concentrations. Proliferation in (B) and (C) was measured by [³H] Thymidine incorporation for the last 12 hours of a 72h stimulation with anti-CD3. Data (mean values of a triplicate culture $\pm$ s.e.m.) are from one out of three different experiments with similar results. D, Percent suppression of proliferation of CD8⁺ effector T-cells from cbl-b^{-/-} and cbl-b^{+/+} mice by cbl-b^{+/+} (WT) and cbl-b^{-/-} (KO) Tregs at various Treg : Teff concentrations.

increased in TC-1 bearing cbl-b^{-/-} mice as compared to wild type controls (Fig. 6A). Of note, the total number of tumor cells and tumor sizes were comparable in the cbl-b^{+/+} and cbl-b^{-/-} mice analyzed (Fig. S3B). These results show that Tregs infiltrate TC-1 tumors in both cbl-b^{+/+} and cbl-b^{-/-} mice; however, loss of Cbl-b dramatically changes the ratio of CD8⁺ T cells to Tregs within the tumors.

We next examined whether loss of Cbl-b might change the function of Tregs towards CD8⁺ effector cell proliferation. Suppression of wild type CD8⁺ effector cells was comparable between cbl-b^{+/+} and cbl-b^{-/-} Tregs (Fig. 6B,D and Fig. S4). In addition, similar to control CD4⁺CD25⁺ Tregs, cbl-b^{-/-} Tregs did not proliferate upon anti-CD3 stimulation *in vitro* (Fig. S4). Thus, cbl-b^{-/-} regulatory CD4⁺CD25⁺ T-cells are bona fide suppressors towards wild type and cbl-b^{-/-} responder CD8⁺ T-cells and loss of Cbl-b has no apparent effect on

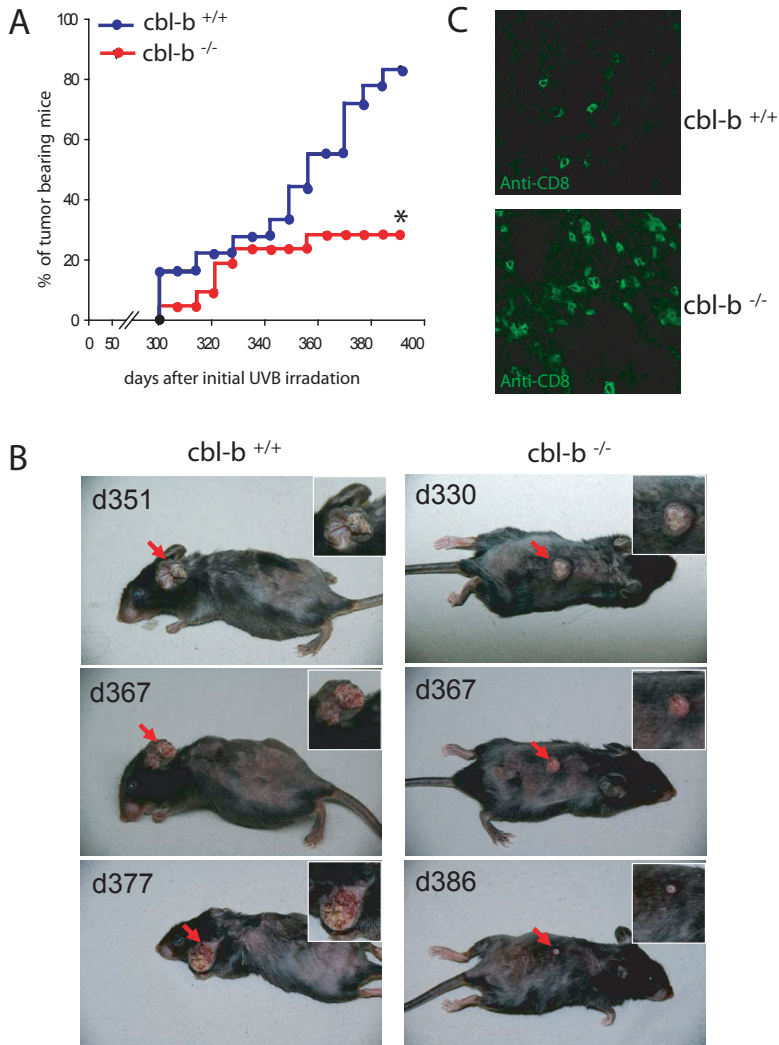


Figure 7. *cbl-b*^{-/-} mice show significantly decreased susceptibility to spontaneous UVB induced skin cancer. A, Kaplan Meier curves of tumor-bearing *cbl-b*^{+/+} (n=18) and *cbl-b*^{-/-} (n=21) mice during chronic UVB irradiation. Tumor incidence in the *cbl-b*^{-/-} cohort was significantly reduced as compared to *cbl-b*^{+/+} mice ($p < 0.05$ from day 363 and $p < 0.001$ from day 377 onwards; log-rank test). B, Representative UVB-induced tumor growth in one wild type (left panels) and one *cbl-b*^{-/-} mouse (right panels). Tumor growth is shown over time in the same two mice (days after first UVB irradiation is indicated) Note progressive reduction of tumor mass in the *cbl-b*^{-/-} mouse. Inserts are higher magnifications of tumors. Histology confirmed epithelial origin of the tumors in control and *cbl-b*^{-/-} mice. C, Confocal images of *cbl-b*^{-/-} and *cbl-b*^{+/+} skin tumor sections stained for CD8⁺ cells by immunofluorescence. Original magnifications x 400. Scale bars represent 25 μ m.

Treg functions. However, *cbl-b* deficient effector CD8⁺ T-cells were resistant to suppression by wild type as well as *cbl-b*^{-/-} Tregs at Treg: effector ratios that significantly suppressed proliferation of wild type CD8⁺ T cells. At a 2:1 Treg:CD8⁺ effector cell ratio we, however, still observed suppression, albeit at lower levels as in *cbl-b*^{-/-} effector CD8⁺ T cells (Fig. 6C,D and Fig. S4). These data show that *cbl-b*^{-/-} CD8⁺ effector cells are resistant to Treg suppression.

***cbl-b*^{-/-} mice show resistance to spontaneous UVB-induced skin cancer.**

Our results show that genetic ablation of *Cbl-b* confers spontaneous *in vivo* rejection of TC-1 tumor cells following subcutaneous inoculation. We therefore wanted to test whether *Cbl-b* also controls tumor resistance in a spontaneous tumor model relevant for human cancer, i.e., UVB triggered skin cancer in mice. UVB irradiation is the most important risk factor for the induction of non-melanoma skin cancer (31, 32). In addition it has been shown, that induction of immunosuppression by UVB is a skin cancer promoting factor (31). To determine the role of *Cbl-b* in the generation of UVB-induced cutaneous malignancies we chronically irradiated cohorts of *cbl-b*^{+/+} and *cbl-b*^{-/-} mice with UVB on their shaved backs. Tumor development was recorded over time (Fig. 7A and Fig. S5A,B). In both *cbl-b*^{+/+} and *cbl-b*^{-/-} mice the first visible progressively growing tumors appeared around day 300 after the initial UVB treatment suggesting that tumor onset is comparable between control and *cbl-b* mutant animals. Most UV-induced skin tumors were located on the ears and backs of the mice (Fig 7B). However, *cbl-b*^{-/-} mice exhibited markedly reduced susceptibility to photocarcinogenesis compared to wild type mice: whereas 15 of 18 wild type mice developed 2-3 visible tumors each, only 6 out of 21 *cbl-b*^{-/-} mice developed visible skin cancer and in only one case did we observe more than one tumor per *cbl-b*^{-/-} mouse (Fig. 7A).

Intriguingly, whereas in *cbl-b*^{+/+} mice skin tumors grew progressively in all cases observed (Fig. S5A), the initial phase of tumor growth in *cbl-b*^{-/-} mice was followed by a marked reduction in tumor mass (Fig. 7B and Fig. S5B). Note that one *cbl-b*^{-/-} mouse was euthanized for histology and therefore tumor progression or reduction could not be followed (Fig. S5B). In line with our TC-1 data, the number of tumor infiltrating CD8⁺ T cells was dramatically increased in the UVB-induced skin tumors of *cbl-b*^{-/-} mice as determined by immunofluorescence (Fig. 7C). Furthermore, using TUNEL staining, we detected increased numbers of apoptotic cells in skin tumors of *cbl-b*^{-/-} mice compared to *cbl-b*^{+/+} skin tumors (Fig. S6A). Numbers and ratios of T-cells as well as expression of surface markers (CD44, CD43, CD69, CD28, CTLA4, CD3, CD8, CD4, CD127, CD62L, CD25, FoxP3) in the draining lymph nodes were comparable among tumor-bearing *cbl-b*^{+/+} and *cbl-b*^{-/-} mice (not shown). To address whether, similar to our TC-1 tumor model, CD8⁺ T cells are also the

critical cell type involved in the surveillance of UVB-induced skin cancer, CD8⁺ cells were depleted in UVB treated *cbl-b*^{-/-} mice that had received UVB irradiation but never developed a tumor. Remarkably, only 10 day after starting the depletion by injection of the CD8 depleting antibody (Fig. S6B) 50% of UVB treated (and previously cancer free) *cbl-b*^{-/-} mice (n=4) developed rapidly growing tumors while all IgG isotype control-treated UVB treated *cbl-b*^{-/-} mice (n=4) remained tumor free (Fig. 8A, B, see page 138). In conclusion, our results show that *cbl-b* deficient mice exhibit reduced skin cancer and are able to reject spontaneous, UVB-induced skin tumors.

DISCUSSION

Various schemes for immunological treatment of tumors have been described including genetic alterations of tumors with cytokines or co-stimulatory molecules, or the generation of tumor specific cytotoxic T cells (33-35). However, immunotherapy is still difficult because most tumors are insufficiently recognized, do not elicit a robust immune response, or induce immunotolerance (4, 5, 36). Several attempts have been made to break tumor tolerance and enhance tumor immunity using transgenic models, transplantation of T cells or dendritic cells, or novel vaccination regimens against known tumor antigens (8, 9). Moreover, many immunotherapies are limited due to severe side-effects and the availability of tumor-reactive immune cells and combination therapies (7, 37).

Our results show that inactivation of a single negative regulator of T cell signaling confers anti-cancer activity *in vivo* using two distinct tumor models relevant for human cancers. This anti-tumor activity occurs spontaneously and tumor growth is completely eradicated in virtually all *cbl-b* mutant mice in the TC-1 tumor model as well as in our spontaneous UVB-induced skin cancer model. In the TC-1 model we could show that anti-tumor memory is maintained for more than one year in *cbl-b*^{-/-} mice. Thus, we have identified a dominant “tolerogenic” factor that actively represses activation of tumor-specific T cells *in vivo*. Although we explored the role of Cbl-b in two distinct tumor models, further studies are required to determine whether Cbl-b is indeed a key molecule that confers anti-tumor immunity in additional cancer types including tumor models with defined high or low immunogenicity. Moreover, it will be interesting to explore whether *cbl-b*^{-/-} CD8⁺ T cells cooperate with other cell types in tumor rejection.

Mechanistically, deletion of *cbl-b* might affect anti-cancer immunity at several levels. One rate limiting factor for successful anti-tumor immunity is the induction of Tregs in cancer. Interestingly, whereas loss of Cbl-b does not affect Treg mediated suppressor functions towards CD8⁺ T cells, *cbl-b*^{-/-} CD8⁺ effector T cells display resistance to proliferative suppression. Thus, similar to previous reports that Cbl-b may regulate suppression of CD4⁺ effector cells

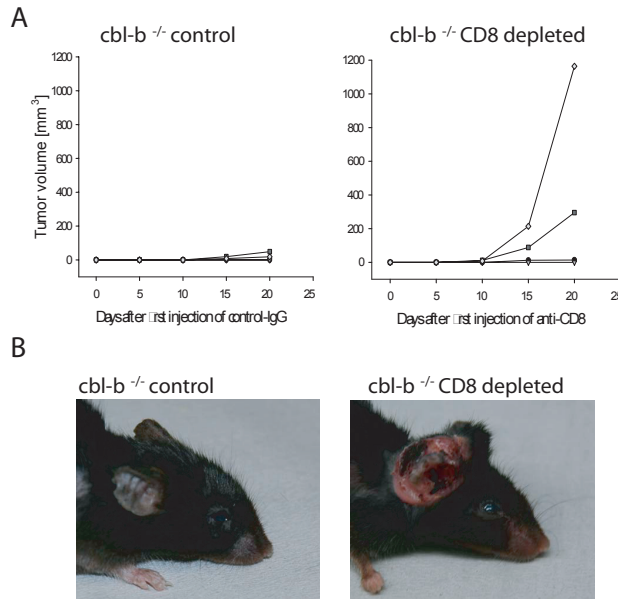


Figure 8. Depletion of CD8⁺ cells in UVB treated cbl-b^{-/-} mice leads to rapid tumor outgrowth. A, Kinetics of progressive tumor growth in individual CD8⁺ cell depleted UVB treated cbl-b^{-/-} (n=4) and IgG control-treated UVB treated cbl-b^{-/-} (n=4) mice. Depletion was performed 130 days after the last UVB treatment. Only mice that received UVB treatment for 9 month but did not develop skin cancer were included in this experiment. Tumor volume in [mm³] was measured over time [days]. B, Representative UVB-induced tumor growth in one IgG treated cbl-b^{-/-} mouse (left panel) and one CD8⁺ cell depleted cbl-b^{-/-} mouse (right panel) imaged on day 22 after initial depletion.

(17, 18), we have identified a novel function of Cbl-b in Treg mediated suppression of effector CD8⁺ T cells. Our results also indicate that Cbl-b must regulate additional mechanisms involved in tumor rejection by CD8⁺ T cells. For instance, the expansion and proliferation of CD8⁺ T cells is increased in cbl-b^{-/-} mice and we observed a rapid onset and elevated numbers of tumor infiltrating effector CD8⁺ T-cell in TC-1 tumors as well as our spontaneous skin cancer model. These results would be in line with enhanced penetration of T cells into the tumors possibly as a result of enhanced activation. Moreover, our preliminary data suggest that CD8⁺ T cells from cbl-b^{-/-} mice exhibit increased sensitivity to dendritic cells loaded with TC-1-derived tumor antigens. We propose that Cbl-b affects multiple regulatory circuits in anti-tumor immunity.

Importantly, established TC-1 tumors can be treated by the transfer of non-transgenic, “naïve” CD8⁺ cbl-b^{-/-} T cells that have previously never encounter the tumor antigen. Loss of Cbl-b in the CD8⁺ compartment alone is both necessary and sufficient to induce potent anti-tumor immunity, thereby perhaps providing a direct means of targeting tumors via CD8⁺ T-cell responses even in the context of ineffective co-stimulation, impaired CD4⁺ T cell help, or

Treg immunosuppression. Thus, inactivation of Cbl-b might be a potent new strategy for anti-cancer immunotherapy on multiple levels to augment the effectiveness of tumor specific CD8⁺ T cells in humans.

MATERIAL AND METHODS

Mice. cbl-b mutant mice have been previously described (11) and were crossed onto a C57BL/6 background for more than 10 generations. C57BL/6 wild type mice and rag2^{-/-} mice were obtained from our in house breeding stock. cbl-b^{+/-} littermates showed the same results as wild type mice. Only female mice were used in all experiments since TC-1 cells are derived from female mice. All mice were maintained under specific pathogen-free conditions and used in accordance with institutional guidelines (Permission from Magistrat 58 of the City of Vienna).

Tumor cells, dendritic cell culture, antibodies, and peptides. TC-1 cells have been previously reported and were generated by co-transformations of primary C57BL/6 mouse lung fibroblasts with an activated c-H-ras oncogene and the HPV-16 E6 and E7 oncoproteins (19). TC-1 cells were maintained in IMDM medium containing 10% FCS, 100 IU/ml penicillin, and 2 mM glutamine, supplemented with G418 (0.5mg/ml), Na pyruvate (1mM) and 30μM 2-ME. The HPV16-derived and H-2D^b restricted peptide E7⁴⁹⁻⁵⁷ (RAHYNIVTF) was used for all re-stimulation experiments (23). Antibodies against mouse CD3ε (clone 145-2C11), CD4 (RM4-5), CD8β.2 (53-5.8), CD8α (53-6.7), TCR-β (H57-597), CD11b (M1/70), CD11c (HL3), CD16/32 (2.4G2), GR1 (RB6-8C5), NK1.1 (PK136), IFNγ (XMG1.2), CD44 (IM7), CD62L (Mel-14), CD25 (PC61), CTLA-4 (UC10-4F10-11), CD43 (1B11), CD69 (H1.2F3), CD28 (37.51), and CD127 (clone SB/199) were purchased from BD Pharmingen. The anti-Ki67 antibody was purchased from Novacastra. The anti-mouse FoxP3 antibody (clone FJK-16s) was obtained from eBioscience. Hybridomas for production of the depleting antibodies to CD4 (clone GK1.5) and CD8 (clone 2.43) were grown in serum free medium and purified using a proteinA/proteinG column. Cells were analyzed by four-color flow cytometry on a FACScalibur™ cytometer (Becton Dickinson, BD) using the CELLQuest™ software.

In vivo TC-1 tumor cell growth. TC-1 cells were injected s.c. into the shaved left flank of 8-12 weeks old female mice. In all experiments, at day zero 2,5x10⁵ TC-1 tumor cells were injected s.c. whereas for tumor memory experiments, 2,5x10⁶ TC-1 tumor cells were s.c. injected. CD8⁺ cells or CD4⁺ cells were depleted by injection of 50 μg of depletion antibodies i.p. per mouse at day-4 and day-2 prior to tumor inoculation (day 0). At day-1 complete depletion of respective cell subsets was confirmed by FACS analysis. The depletion was repeated weekly throughout the experiment. In all experimental groups, tumor growth was

monitored three times per week by measuring tumor length, width and height with a caliper. Mice were euthanized when tumor volume reached 1 cm^3 . For tumor escape/immunotolerance experiments, tumors were isolated from $\text{cbl-b}^{+/-}$ or $\text{cbl-b}^{-/-}$ mice. Cells were passaged *in vitro* for one month and aliquots were kept. Prior to use for experiments cells were passaged 6 times. These newly established TC-1 tumor cell lines cells were then injected into 8-12 week old, 'naïve', $\text{cbl-b}^{+/+}$ or $\text{cbl-b}^{-/-}$ recipients and tumor growth was analyzed as described above. All experimental procedures performed on mice were in accordance with institutional guidelines.

UVB-induced photocarcinogenesis. Within the solar spectrum, the UV-B range (290–320 nm) is responsible for carcinogenesis and immunosuppression. Therefore, a bank of four Philips UV-B TL40W/12 sunlamps was used, which have an emission spectrum from 280 to 350 nm, with a peak at 306 nm. These lamps deliver an average dose of 8 W/m^2 , as measured with an IL-1700 UV detector and a SED 24 (no. 3124) filter (both from International Light). The mice were placed on a shelf 20 cm below the light bulbs for irradiation. The cage order was systematically rotated before each treatment to compensate for uneven lamp output along the shelf as described previously (38, 39). Female mice were shaved with electric clippers once per week on the entire dorsum. Beginning at 10 wks of age, mice were irradiated three times per week with 2.5 kJ/m^2 for the first 4 wks, followed by 5 kJ/m^2 for 4 wks, 7.5 kJ/m^2 for 4wks and finally for 6 month with 10 kJ/m^2 . All mice were monitored weekly for tumor development by two independent investigators for additional four months. The location and growth of each tumor exceeding 2 mm in diameter was recorded. The method of Kaplan and Meier was used to describe the probability of tumor development in the carcinogenesis study. Immunohistochemistry of UV-induced tumors was performed on cryostat sections ($3\text{ }\mu\text{m}$) fixed in acetone according to standard methods. Slides were incubated in the appropriate dilution of anti-CD8 (clone 53-6.7; Becton Dickinson) and an Alexa-488 labeled secondary antibody (Molecular Probes) and examined using a Zeiss 200M confocal microscope and the LSM510Meta software. Statistical differences for the development of tumors between the two strains of mice were determined using a log-rank test.

Therapeutic CD8⁺ T-cell transfer into $\text{rag2}^{-/-}$ mice. CD8⁺ cells were purified from spleens and lymph nodes of naïve $\text{cbl-b}^{-/-}$ and $\text{cbl-b}^{+/+}$ mice by positive selection using Magnetic beads against CD8 (Miltenyi Biotec) following the manufactures recommendations (purity was over 90% as determined by CD8 β and CD3 double staining). Three and six days after tumor cell injection 3×10^6 and 2×10^6 CD8⁺ cells were adoptively transferred into the tumor bearing $\text{rag2}^{-/-}$ mice, respectively. Tumor growth was monitored three times per week.

Re-stimulation and intracellular cytokine staining. Cells were isolated from spleens and lymph nodes of tumor bearing *cbl-b*^{-/-} and *cbl-b*^{+/+} mice as well as naïve controls of each genotype and CD8⁺ T cells were purified using positive magnetic bead sorting (Miltenyi Biotec). CD8⁺ cells (2x10⁵/well) were stimulated *in vitro* for 3 days with HPV16-E7⁴⁹⁻⁵⁷ peptide-loaded splenocytes (10µg/ml peptide; 20Gy irradiated; 2x10⁵ APCs per well) in complete medium. Golgi plug was added for the last 4h and the cells were stained for CD8β and CD3 surface expression. Following fixation, intracellular IFNγ levels were then determined using a cytoperm/cytofix kit from BD Pharmingen (according to the manufacturer's protocol). Cells were analyzed by FACS (BD).

Histology and immunohistochemistry. Tumors were dissected and frozen in OCT for cryosections or fixed with 3.7% formaldehyde prior to paraffin embedding. For histological analysis, 5 µm thick sections were cut and stained with hematoxylin and eosin (H&E) using standard protocols. TUNEL (Roche) and Ki67 stainings were performed on paraffin embedded sections. Tumor infiltrating CD8β⁺ and CD4⁺ were detected on frozen sections by immunofluorescence. FoxP3 staining was performed on frozen sections and on PolyPrepTM slides (sigma) coated with FACS-sorted CD4⁺CD25⁺ cells by immunocytochemistry. Confocal images were taken using a Zeiss LSM510 Microscope.

Treg suppressor assays. Different concentrations of sorted CD25⁺CD4⁺ Treg cells from naïve *cbl-b*^{+/+} and *cbl-b*^{-/-} mice were co-cultured with CD8⁺ effector T cells (5*10⁴/well) isolated from wild-type and *cbl-b*^{-/-} mice in the presence of 2*10⁵ irradiated (3,000 rads), T depleted splenocytes and 1 µg/ml of purified mouse anti-CD3ε in 200µl Iscove's modified Dulbecco's medium supplemented with 10% FCS in 96-well round-bottomed plates. Proliferation was measured by scintillation counting following a pulse with 1 µCi of [³H]thymidine per well for the last 12 h of a 72h -hour incubation period.

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Design and development of
synthetic peptide vaccines

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Design and development of synthetic peptide vaccines: past, present, and future.

Abstract. Synthetic peptide vaccines aiming at the induction of a protective CD8⁺ T cell response against infectious or malignant diseases are widely used in the clinic but despite their success in animal models they yet do not live up to their promise in humans. This review assesses the development of synthetic peptide vaccines, weighs it against the immunological concepts that meanwhile have emerged, and identifies the key issues that play a role in failure or success of a synthetic peptide vaccine. The current state-of-the-art peptide vaccine is a complete synthetic inflammatory product that is ingested by professional antigen presenting cells and stimulates both CD4⁺ and CD8⁺ T cells.

SYNTHETIC PEPTIDE VACCINES: IMMUNITY VERSUS TOLERANCE

In the 1950s the host's immune system was shown to reject tumors following the recognition of tumor-associated Ag (TAA) (1-4). Subsequently, Zinkernagel and Doherty discovered in 1974, that the recognition of target cells by CD8⁺ T cells was restricted by the Major Histocompatibility Class (MHC) I molecules (5). Townsend et al. then revealed that CD8⁺ T cells were able to sense short linear peptide sequences of 11-16 amino acid (a.a.) in length, generated by proteolytic degradation of the target antigens that were presented in the context of MHC class I molecules (6-8). Subsequently, the group of Rammensee and colleagues revealed that the exact MHC class I binding peptides (CD8⁺ T cell epitopes, Fig. 1A) recognized by CD8⁺ T cells were even smaller peptides - 8 amino acids (a.a.) (9-11) - and that these peptides contained specific amino acids that anchored the peptide in the pockets of the MHC class I molecule (12). This knowledge resulted in the rapid identification of CD8⁺ T cell epitopes in both mice (13-16) and human beings (12) and in exploration of the use of peptide vaccines against multiple diseases.

The first peptide vaccine able to induce a Cytotoxic T lymphocyte (CTL) response *in vivo*, was reported by Aichele et al. (17) who injected mice with a peptide encoded by lymphocytic choriomeningitis virus (LCMV) (Fig. 1B). Peptide vaccination was also shown to induce a protective CTL response against lethal challenges with live viruses, such as LCMV (18) and Sendai virus (19), against a lethal challenge with Human Papilloma Virus (HPV) type 16 positive tumor cells (20) or methylcholanthrene-induced sarcomas (21), and was shown to enhance the life-span of mice in a spontaneous lung carcinoma metastasis model (22) (Fig. 1B). However, not all peptide vaccines induced strong protective CTL responses. For instance, vaccination with peptides from the circumsporozoite protein from malaria (23), or GP100 (24) induced low levels of CTL responses while vaccination with Her2/neu peptides in patients induced only short-lived CD8⁺ T cell responses (25). In contrast, some peptide vaccines did not induce protective CTL responses (26-28) or even enhanced tumor cell growth due to the induction of CD8⁺ T cell tolerance (29,30). Despite these opposing outcomes, many synthetic peptide-based clinical trials have been initiated in cancer patients with disappointing immunological and clinical responses (reviewed in (31,32)). In this review we have explored the short-comings of these peptide-based vaccines and provide a basis for the rational design of improved peptide vaccines that are able to induce strong and sustained T cell responses. Since a complete overview of the results obtained by peptide vaccines in patients has recently been published by Mocellin et al. (31), we only refer to specific examples in this review.

HOW TO ENHANCE THE CD8⁺ T CELL RESPONSES

Increasing the peptide affinity.

Many (self) minimal CTL peptides have relatively low binding affinity for the MHC class I complex and, therefore, are not able to induce a strong CD8⁺ T cell response. The affinity of the peptide (33-35) and the dissociation constant of the peptide (36) determine the time before the peptide:MHC complex is displaced from the cell surface. Site directed substitution of specific amino acid positions in these peptides (Fig. 1C, I) can therefore induce more stable peptide:MHC class I complexes and thereby enhance the immunogenicity of these

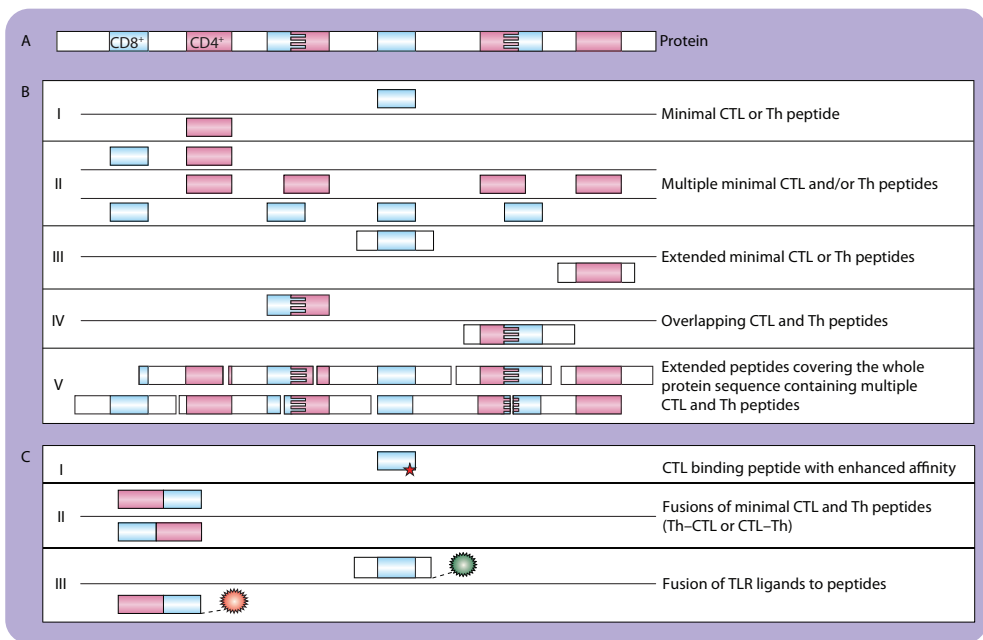


Figure 1. Design of peptide-based vaccines; choosing and designing the right peptide(s). A. Definition of CD8⁺ and CD4⁺ T cell epitopes. In a given protein sequence there can be multiple CD8⁺ (blue) and CD4⁺ (purple) T cell epitopes present that can bind to different MHC/HLA class I and II molecules, respectively. These CD8⁺ and CD4⁺ T cell epitopes can be located at distinct positions in the protein sequence, or could be (partially) overlapping; striped blue/purple bars. B. Peptide vaccines can come in many flavors, for instance: I) minimal CTL or Th peptides; II) multiple minimal CTL and/or Th peptides; III) minimal CTL and Th peptides that are N- and C-terminally extended; IV) single peptides that contain both a CTL and a Th epitope that (partially) overlap; or V) a set of overlapping peptides that cover the whole protein sequence, containing all possible CD8⁺ and CD4⁺ epitopes of the protein. C. Engineered peptide vaccines with enhanced immunogenicity. In order to enhance the immunogenicity of a peptide vaccine several strategies can be undertaken: I) the affinity of a MHC/HLA class I binding peptide can be altered/enhanced by changing certain amino acids (red star); II) minimal CTL and Th peptides can be physically linked to form long Th-CTL or CTL-Th fusion peptides; or III) different forms of peptides can be conjugated to TLR ligands.

peptides (33,37-39). As a result, injection of these modified peptides could confer protection against a subsequent lethal tumor challenge in mice (40,41) as well as increase the magnitude of the CD8⁺ T cell response in melanoma patients (42,43).

Another approach to enhance the immunogenicity of MHC class I restricted peptides is to design a super-agonist peptide able to interact more efficiently with the T cell receptor of CD8⁺ T cells (Fig. 1C, I), as demonstrated with the Melan-A/Mart-1₂₇₋₃₅ epitope (44). Site directed substitution of certain a.a. in the minimal CTL peptides could therefore be a genuine strategy to enhance the magnitude of peptide vaccine induced T cell responses. However, in some cases alteration of peptides resulted in the induction of T cell responses with TCRs not able to cross-react with the wild type peptide (45,46), indicating the limitations in applying such a strategy.

Inclusion of CD4⁺ T cell peptide-epitopes; a helping hand for the Cytotoxic T Lymphocyte.

Central to the induction of strong and sustained CTL immunity is the presentation of minimal CTL peptides by matured DC which endow the responding CD8⁺ T cells with the necessary signals that allow them to become functionally active (47). In addition, CD8⁺ CTL require the presence of cytokines such as IL-2 that support them to rapidly expand. Lack of DC maturation or supporting cytokines may result in a vaccine-induced CD8⁺ T cell response that is transient and weak, even when peptides with strong MHC binding affinity are used. A proper way to increase the magnitude and long-term efficacy of the CD8⁺ T cell response is through the provision of help by CD4⁺ T helper cells (Fig. 1B).

The helping role of CD4⁺ T cells is versatile. Upon activation, the CD4⁺ T cell can secrete the cytokine IL-2 (48), which is an important growth factor for (CD8⁺) T cells; CD4⁺ T cells can activate the DC via CD40-CD40L interactions (49-51) that can subsequently license the CD8⁺ T cell to kill its target cell and CD4⁺ T cells can program CD8⁺ T cells via the Antigen Presenting Cell (APC) to become memory cells (52).

This important role for the CD4⁺ T cell in the induction of a protective CD8⁺ T cell response by peptide vaccination against LCMV became apparent after it was realized that the peptide vaccine used by Aichele et al. (17) comprised both a CD8⁺ T cell epitope and a CD4⁺ T cell epitope (53) (Fig. 1B, IV). *In vivo* depletion of the CD4⁺ T cell population almost completely abrogated the vaccine-induced protective effect against LCMV (53). In addition, CD4⁺ T-cell help was shown to be indispensable for the induction of long lasting immunity against tumors. Eradication of MHC class II-negative FBL erythroleukemia cells was only observed when the mice were injected with a vaccine that only contained a tumor-specific Th peptide (54) (Fig. 1B, I). Indeed vaccination with an exclusive Th peptide protected mice against

MHC class II-negative virus induced tumors (Fig. 1B, I), but optimal protection was achieved by vaccination with both a minimal Th peptide and a dominant minimal CTL peptide (28) (Fig. 1B, II). This principle of providing help was endorsed in a phase I clinical trial in which patients were vaccinated with minimal CTL peptides of melanoma-associated tyrosinase antigen. Tyrosinase peptide specific IFN γ -producing T cells were detected only if the patients simultaneously received the non-specific helper protein keyhole limpet hemocyanin (KLH) in addition to the minimal tyrosinase CTL peptide vaccine (55).

Further improvements in peptide vaccines were achieved by directly linking the minimal Th peptide to the minimal CTL peptide to form a single linear hybrid peptide [PbCS(57-70)-(251260)] (Fig. 1C, II) showed enhanced CTL responses in a mouse model for malaria (23). Similar observations were reported for peptide vaccines aiming at the induction of Human Immunodeficiency Virus-specific CTL (56) and Herpes Simplex Virus-specific CTL (57). However, not all the combinations of minimal Th and CTL peptide linkage showed enhanced reactivity. For instance, in a phase I clinical trial with stage IV patients, a gp100 specific T cell response was only observed in patients that had received the minimal CTL peptide gp100 and not in patient vaccinated with the gp100 minimal peptide that was C-terminally extended with a Th peptide derived from tetanus (58). The reason for the absence of gp100 specific T cells could be the additional amino acid sequence located at the C-terminus of the CTL epitope. Changing of the natural occurring C-terminal amino acid sequence can change the processing by the proteasome and thus liberation of the CTL epitope (59,60).

Interestingly, a number of peptide vaccines able to induce strong CD8⁺ T cell responses, not only comprised the minimal CD8⁺ T-cell epitope but also a CD4⁺ T cell epitope, e.g. LCMV (53), HPV (61), and mutant p53 (21) (Fig. 1B, IV). Co-localization of CD4⁺ and CD8⁺ epitopes has also been described in proteins that are target antigens in human beings, e.g. NY-ESO (62), Synovial Sarcoma X-2 breakpoint protein (63), and Mart-1/Melan-A (64). An elegant approach was undertaken by Knutson et al. who identified Her2/neu CD4⁺ T cell epitopes that comprised a (HLA-A2⁺) CD8⁺ T cell epitope within the same peptide sequence (65) (Fig. 1B, IV). In contrast to their clinical trial in which only a CD8⁺ T-cell epitope was used (25), vaccination with the peptides, comprising both CD4⁺ and CD8⁺ T-cell epitopes, resulted in the induction of long-lived Her2/neu specific CD8⁺ T cell responses. Thus, a simple approach to induce strong and long-term CD8⁺ T cell reactivity is the inclusion of CD4⁺ T cell epitopes in peptide vaccines.

Antigen-related or non-related CD4⁺ T cell help.

Addition of a minimal Th peptide enhanced the capacity of peptide vaccines to induce strong malaria-specific CTL responses (23,66) and protected mice against a subsequent tumor challenge (28,67,68). As yet it is not clear whether the CD4⁺ T cell epitope that is incorporated in the vaccine should be derived from the same (tumor) Ag or pathogen as the CD8⁺ T cell epitope. It has been argued that for optimal results both epitopes should be encoded in the same pathogen or tumor (28) (Fig. 1B, II, IV, V), while others have shown that this is irrelevant (23,55,67,69). This issue can only be solved when the intrinsic properties of CD4⁺ T-cell epitopes (Fig. 1A) - i.e. binding affinity or antigenic origin - are taken into account. It can be envisaged that CD4⁺ (viral) T cell peptides with high binding affinity for the MHC class II molecule will be more helpful to a developing CD8⁺ T cell response than the use of CD4⁺ T cell peptides which have a low binding affinity for MHC class II, as was the case for the non-related MHC class II I-Ab-restricted epitope of Ovalbumin (28). Similarly, CD4⁺ T cell epitopes derived from proteins to which partial tolerance exist may be less powerful with respect to the induction of a strong helper T cell response (70).

Help can also be provided in the form of an agonistic CD40 Ab that triggers the CD40 receptor on APC (49). Using CD40 agonistic Ab, a prophylactic vaccine consisting of the minimal CTL peptide of HPV16 E7 protein could be converted in to a vaccine with therapeutic anti-tumor potency (71) (Fig. 2, I). Furthermore, the use of agonistic CD40 Ab allowed eradication CD40 negative tumors in tumor-bearing mice (72). This depended on the activation of tumor-antigen presenting CD11c⁺ APC in the draining Lymph Node (LN) (73). However, this type of “help” requires complete tumor or virus eradication, because it is associated with an acute wave of effector CTLs. In case of chronic disease, additional injections (of aCD40 Ab) are required.

This latter argument also applies to the use of non-related CD4⁺ T cell epitopes in peptide vaccines. Although such CD4⁺ T cell epitopes can effectively induce a CD4⁺ T cell response that helps the development of an acute wave of CTL, this CD4⁺ T cell response is rather useless in re-activating the CTL response upon secondary challenge or to sustain CTL responses in case of chronic infection/diseases, simply because the cognate Ag for these CD4⁺ T cells is lacking. Provision of both the minimal (non-related) Th and (related) CTL peptides in IFA - that induces long-term Ag presentation of both peptides simultaneously in the dLN (74) - might however, sustain APC activation and thereby CTL reactivity and might to some extent circumvent this problem. Note, however that the vaccination with Ag-related minimal Th peptides will ensure reactivation of these Ag-specific CD4⁺ T cells, that subsequently provide help to the Ag-specific CD8⁺ T-cell response upon re-challenge.

I	 APC- and T-cell-activating Abs (aCD40, OX-40, 4-1-BB, aCTLA-4, aPD-L1)	Abs that enhances T-cell activation, expansion and survival; or activate APCs in vivo
II	 Cytokines/T-cell growth hormones  Cytokine complexed to specific Abs  Cytokine complexed to soluble receptors	Enhances expansion, effector function, and survival of T cells. Complexation to specific Abs or soluble receptors reduces the in vivo toxicity
III	 Cyclophosphamide  IL-2 linked to diphtheria toxin (DAB389IL-2)	Depletion of Tregs to remove inhibitory mechanisms and to enhance vaccine efficacy
IV	 Chemotherapy, γ -irradiation TLR ligand-containing cream	Induction of local inflammation to enhance T-cell infiltration and T-cell function

Figure 2. Additional immune interventions to enhance vaccine efficacy. In order to enhance the immunogenicity and efficacy of a peptide vaccine additional immune interventions can be applied. Ab: Antibody; APC; Antigen-presenting cell; TLR Toll-like receptor; Treg; T-regulatory cell.

Length of the peptide.

Unexpectedly, the length of the peptide used for vaccination strongly influences the magnitude of the induced CD8⁺ T cell response (Fig. 1B, III-IV). In a head-to-head comparison, a 35 amino acid long peptide containing the HPV16 E7₄₉₋₅₇ CD8⁺ T cell epitope induced a robust CD8⁺ T cell response capable of eradicating an established tumor, while the minimal CTL peptide (9 a.a. long) could not (75). One of the underlying mechanisms is that extension of these minimal CTL peptides to a longer peptide, increased the duration of Ag presentation *in vivo* and thereby enhanced the magnitude of the CD8⁺ T cell response and reviewed in (reviewed in (76) and C).

Additionally, the presentation of the CTL epitopes from the extended peptides requires Ag processing by professional APC, whereas the minimal CTL peptide can bind exogenously to MHC class I molecules. This might also explain why many of the first reported, so called “short synthetic peptides” performed so well. These “short synthetic peptides” were indeed relatively short when compared to whole antigens, but still were not the exact MHC class I binding peptides that - based on these animal models - are nowadays used in vaccine trials in humans. Actually, the vaccines used in these animal models could reach up to 27 amino acids in length (17-20,77-80) (Fig. 1B, III-IV) and, as such, should be qualified as Ag-processing dependent peptides that are more immunogenic than their exact MHC class I binding counterparts (75).

Using adjuvants to enhance immune reactivity.

The most devastating signal a T cell can receive is that through interaction of its T-cell receptor and a MHC-peptide complex presented on a non-activated and non-matured APC. T cell activation in the absence of co-stimulatory molecules or pro-inflammatory cytokines will generally result in the induction of tolerance (reviewed in (81)). The main hallmark of good vaccines is that they closely mimic the most successful natural triggers of DC activation. Therefore peptide vaccines have to be formulated with different kinds of adjuvants that enhance the magnitude, survival and polarization of the vaccine-induced T cells.

A very common vehicle and adjuvant for vaccination is oil-in-water emulsion (reviewed in (82)). The best known are: Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), and Montanide ISA-51. Besides CFA – that contains heat killed *Mycobacterium tuberculosis* – the other two adjuvants only contain mineral oil and their adjuvant effect is based on the formation of a depot from which Ag is slowly released into the system. Another promising adjuvant is Immuno Stimulatory Complexes (ISCOMs) ((83) and reviewed in (84)). ISCOMs are cage-like structures that are comprised of antigen, cholesterol, phospholipid, and saponin that targets the antigen and adjuvant components of the vaccine to both the endosomal and cytosolic pathways for antigen presentation.

Other ways to strongly activate DC are the use of Toll Like Receptor (TLR) ligands. These ligands mimic certain structures commonly found on the outside and inside of pathogens that can deliver danger signals upon binding to the TLR at the surface or inside the APC. Examples of the most common naturally occurring danger signals are: pathogen-derived RNA or DNA, lipoproteins/peptides, lipopolysaccharide (LPS), and peptidoglycan (reviewed in (85)). Inclusions of synthetic analogues of these TLR ligands into vaccines provide an ultimate way to enhance vaccine induced T cell responses via the activation of TLR on professional APC. So far many different TLR ligands have been exploited in vaccine studies in mice ((75,86) and Chapter 4+5). In human beings, monophosphoryl lipid A (a detoxified form of LPS) - that triggers TLR4 - was proven to be safe as a clinical vaccine adjuvant in combination with a recombinant protein vaccine against malaria (87). The use of CpG 7909 – a TLR9 ligand – in combination with a Melan-A minimal CTL peptide in IFA not only enhanced the magnitude of Melan-A specific CD8⁺ T cells compared to the minimal CTL peptide vaccine without CpG, it also changed the phenotype of these CD8⁺ T cells from IFN γ producing cells to IFN γ producing cells that were also granzyme and perforin positive (88). Direct coupling of the TLR ligands to Ag forms a next generation of vaccines (Fig. 1C, III). A pioneer study by Rammensee showed that the use of peptide linked to lipopeptides induced the same high affinity T cells as live influenza virus (89). Others have shown that coupling enhances Ag uptake by professional APC ((90) and Chapter 5), enhances Ab and T cell

responses (91,92) and induces a more vigorous and protective T cell response to pathogens, with similar efficacy as a live vaccines (93). While the use of TLR ligands, and especially multiple TLR ligands (94,95), generally provides a potent APC stimulating environment needed for the induction of effector T cell responses, they may also unleash unwanted T-cell responses. For instance, in mice the use of TLR2 ligand (Pam3Cys) induces proliferation of Treg cells (96) and systemic application of CpG elicits potent regulatory responses by acting on a discrete, minor population of splenic DCs (97).

PHARMACOKINETICS OF PEPTIDE VACCINES DETERMINE OUTCOME

Of all the vaccinations with minimal CTL peptides (Fig. 1B), many resulted in the induction of a detectable but weak CD8⁺ T cell response and some peptide vaccines were even reported to induce T cell tolerance. Multiple (systemic high dose) vaccinations with the minimal CTL peptide GP₃₃₋₄₁ of LCMV in IFA resulted in CD8⁺ T cell tolerance (27) or deletion of GP₃₃₋₄₁ specific transgenic CD8⁺ T cells (26). This induction of CD8⁺ T cell tolerance has been attributed to the multiple high doses of peptide, which likely over-activated/stimulated the responding CD8⁺ T cells and subsequently induced apoptosis (27).

However, Toes et al. reported in an adenovirus tumor model that already a single injection with a low dose of the strong MHC class I binding CD8⁺ T cell peptide-epitopes of adenovirus (Ad5E1A or Ad5E1B) in IFA tolerized adenovirus-specific CD8⁺ T cells and enhanced tumor cell outgrowth (29,30). This result is not restricted to vaccination with these Ad5E1A or Ad5E1B minimal CTL peptides, as this also occurred after vaccination with the highly immunogenic Ovalbumin₂₅₇₋₂₆₄ minimal CTL peptide (Chapter 2).

Mechanism of peptide vaccine induced tolerance.

The tolerogenic feature of Ad5E1A has been attributed to the fact that the Ad5E1A minimal CTL peptide very rapidly distributes throughout the whole body in contrast to, for instance an immunogenic minimal CTL peptide, like HPV16 E7 (98). Of note, the Ad5E1A minimal CTL peptide displays a very strong binding affinity to its cognate MHC class I molecule, whereas the HPV16 E7 minimal CTL peptide displays only intermediate affinity to the MHC class I molecule (33). We realized that the injection of these high affinity minimal MHC class I binding peptides resulted in the binding to cell surface expressed MHC class I molecules of passenger lymphocytes in the dLN and showed that these lymphocytes export the peptide to more distant lymph nodes (Chapter 3). The slow release and thereby long duration of Ag presentation induced by the IFA adjuvant – more than 100 days (74) – in combination with

minimal CTL peptides presentation in non-inflammatory LN by non-professional APC, will provide an optimal environment to induce CD8⁺ T cell tolerance (99,100), or the deletion of specific CD8⁺ CTL when peptide vaccine-activated CD8⁺ T-cells present the minimal CTL peptide themselves- fratricide (101). These data suggest that the injection of modified minimal CTL peptides displaying a strongly increased peptide binding affinity for MHC class I molecules in an oil-in-water adjuvant such as IFA or Montanide ISA51, might work counterproductive.

Prevention of peptide vaccine induced tolerance.

The studies described above indicate that the pharmacokinetics of the peptides used for vaccination are important to evaluate the capacity of a peptide vaccine to induce an effective immune response. In addition, help in the form of agonistic CD40 Ab (Figure 2,I) initially enhanced the magnitude of the Ad5E1A-specific (102) CD8⁺ T cell responses induced by peptide in IFA vaccines, however, this treatment did not prevent the tolerization of the responding CD8⁺ T cells at later stages (74). While the αCD40 antibody will be cleared rapidly from the system and as such is not able to activate the APC, the CD8⁺ T cell continuously receives signals by the minimal CTL peptide leaking out of the IFA depot for more than 100 days (74) presented by non-activated APC. Importantly, the provision of continuous help by co-injection of a minimal Th peptide (Fig. 1B, II) in IFA, rescues CD8⁺ T cells from tolerance, indicating that CD4⁺ T cell help is not only important in enhancing the CD8⁺ T cell response as has been reported previously (23), but also in preventing the induction of CD8⁺ T cell tolerance (Chapter 2). These results teach us that both the APC activating signals and the T-cell activating signals (peptide) should be matched both in time and location.

MULTI-EPITOPE VACCINES TO PREVENT IMMUNE ESCAPE

Many vaccination trials are performed with only a single minimal CTL peptide designed to fit only one HLA class I molecule and are therefore not widely applicable to many patients of different genetic backgrounds (Fig. 1B, I). Such a strategy not only narrows down the overall force that can be undertaken against an infected/transformed cell but also constrains the efficacy of the vaccines in the case of Ag loss variants or specific down regulation of HLA class I molecules by the target cell.

This was exemplified by the sequential HLA and Ag loss variants of subsequent metastases of melanoma in a patient reported by Yamshchikov et al. (103). The first escape-variant showed selective loss of three HLA class I molecules. The second metastasis displayed the specific down-regulation of a HLA molecule involved in the presentation of a Mart-1

specific peptide, while still presenting a tyrosinase-derived epitope. Accordingly, Lehmann et al. reported that CTL lines that recognized the first metastasis did not cross react with the second metastasis, due to the selective loss of the HLA molecules to which the CTL lines were restricted (104). New CTL clones which specifically recognized the second metastasis were directed to a different HLA class I molecule and to a different antigen compared to the first metastasis.

Multiple peptide-epitopes vaccines.

The most effective strategy for vaccination is the use of multiple CTL and Th epitopes of a given protein Ag so that they can be presented by many different HLA (Class I and II) molecules, reducing the chance of outgrowth of Ag and/or HLA class I loss variants. This can be achieved by vaccination with the whole Ag as a vaccine; either by overlapping long peptides that span the whole protein (Fig. 1B, V); or by using recombinant or synthetic proteins (Fig. 1A). Furthermore, attacking the cell via multiple Ag, would further enhance the strength and as such the success of the vaccine.

For instance, the therapeutic use of overlapping peptides (27-34 a.a. long, Fig. 1B, V) spanning the entire Cottontail Rabbit Papilloma Virus (CRPV) E6 and E7 proteins was reported to induce regression of established warts induced by CRPV and a reduction of the CRPV viral load (105). A similar vaccine consisting of overlapping peptides (27-34 a.a.) spanning the sequence of HPV16 E6 and E7 induces CD4⁺ and CD8⁺ T cell responses in cervical cancer patients (Melief and Van der Burg, unpublished data). Also longer peptides, ranging from 44-182 a.a. in length were effective in inducing strong Ab and T cell responses in mouse and human malaria model systems (106-110). In addition, linking of two long HPV16 E7 peptides to create the synthetic protein (98 a.a. long) of HPV16 E7 was shown to be highly effective in clearing an established tumor (111).

Besides the use of multiple antigens to target virally infected cells or tumor cells, the choice of proteins may contribute to the clinical success of vaccination. It is likely that proteins less susceptible to mutations (structural proteins of HIV) or proteins that are needed to maintain the oncogenic form of the cell would provide better target antigens. Examples would be anti-apoptotic proteins such as survivin (112), bcl-2 (113), bcl-xL (114), and Mcl-1 (115). Accordingly, telomerase reverse transcriptase (hTERT) (116) or proteins that are involved in resistance to chemotherapeutic agents such as TRAG-3 (taxol resistance gene) (117) might constitute appropriate target antigens for T-cells to attack malignant cells.

EXPERT COMMENTARY

Minimal CTL peptide vaccinations are widely used against multiple forms of cancer and other diseases. However, these peptide-based vaccines have so far induced little to no clinical or immunological responses (reviewed in (31,32)). We are convinced that the translation of peptide vaccination from early pre-clinical mouse studies (17-20,27,77-80,118) to human clinical studies was flawed by an imperfect understanding of the mechanisms that play a role in successful peptide vaccination. First, while studies in human subjects almost always explore the efficacy of vaccines consisting of the exact HLA class I binding peptides (Fig. 1B, I), most of the groundbreaking studies which showed that “short synthetic peptide” vaccines could be effective in pre-clinical mouse studies, used peptides that were not exact MHC class I binding peptides. In general, the peptides were much longer and could reach up to 27 a.a. in length (17-20,77-80) (Fig. 1B, III-IV) and are therefore likely to be retained in the local draining lymph node where they are processed and presented by professional APC to CD8⁺ T cells ((75) and reviewed in (76)). Additionally, some of the “short synthetic peptides” contained also a CD4⁺ T cell epitope in the peptide sequence (17,53) (Fig. 1B, IV). These two factors – the predominant local presentation of peptides and the provision of CD4⁺ T-cell help (53,75)- which are lacking in most of the peptide vaccines used in human subjects, have greatly contributed to the enhanced efficacy of the early pre-clinical “short synthetic peptide” vaccines tested in rodents.

Second, the efficacy of many of the synthetic minimal CTL peptide vaccines that did seem to induce a proper CTL response, was assessed in prophylactic settings only (20,27,77,118). Importantly, the mice were lethally challenged when the CD8⁺ T cell response was at its maximum and, therefore, able to rapidly deal with the infection/tumor challenge (20,27,77,118). In patients, peptide vaccinations are given in a situation of established diseases, requiring first and foremost a need to build up a highly effective Ag-specific CD4⁺ and CD8⁺ T cell response, subsequently this immune response needs to be maintained for a long period of time to combat the disease. Notably, the early studies demonstrating that the use of minimal CTL peptide vaccines could induce tolerance when provided in oil-in-water formulations (IFA) (26,27,29,30) should have made us cautious in using minimal peptide-epitopes for vaccination. As we now know, the longer variants of these minimal CTL peptides provide a much safer form of vaccination (Chapter 2) (Fig. 1B, III). Moreover, since human beings display up to 8 different HLA class II molecules it is highly likely that these synthetic long CTL peptides (or proteins, Fig. 1A) contain CD4⁺ T cell epitopes for one or more HLA class II molecules (62,65) (Fig. 1B, IV-V) and this will ensure full activation of professional APC allowing optimal priming of CD8⁺ T cells (50,51). Covalent linking of synthetic TLR ligands to these synthetic peptide vaccines may be used to increase the magnitude of the CD8⁺ T cell

response ((90,91,93) and Chapter 5) (Fig. 1C, III). Hence, for design of a successful peptide vaccine, it is important to adhere to a number of principles shown in Table 1.

Table 1. Basic principles for peptide vaccination

Multiple epitopes	Vaccine containing multiple epitopes induces a broad (CD4+ and CD8+) T cell repertoire that reduces the chance of immune escape variants	(62,65,103-105, 142-144)
Broad applicability	A (peptide) vaccine should be applicable for patients irrespective of their HLA type	(105)
Ag targeting to professional APC	Vaccines should contain peptides that predominantly target professional APC in order to prevent the possibility that T cells become tolerized by peptide presentation on non-professional APC	(75,81) and Chapter 3,5
Extended duration of Ag presentation	Peptide vaccines should install sustained duration of Ag presentation <i>in vivo</i> for optimal (CD8+) T cell clonal expansion to occur	(145-147) and Chapter 2,3
Include CD4+ T cell help	Concomitant induction of CD4+ and CD8+ T cell responses enhances and maintains strong CD8+ T cell effector responses	(21,23,28,52-57, 61-69,148-150) and Chapter 2 &4
Include strong APC activating signals	Provision of strong APC activating signals induces optimal activation and expansion of effector T cells	(49-51,74,75, 85-95,102) and Chapter 3,4,5
Co-localization of Ag and APC activation	Ag presentation and APC activating signals should be closely matched in time and location	(89-93) and Chapter 2,3,5

FIVE-YEAR VIEW

In our opinion the use of synthetic vaccines comprising multiple CD4⁺ and CD8⁺ T-cell epitopes is only the start of the next generation of therapeutic vaccines (Fig. 1B, V). There is a need for additional regimens to further optimize the efficacy of the vaccine (Figure 2). A generic method to improve both the magnitude and the efficacy of the vaccine-induced T-cell response is to use longer peptides (75) (Fig. 1B, III-V). While most peptide vaccinations are already applied in oil-in-water adjuvants such as Montanide/ISA-51, occasionally supplemented with TLR-ligands such as MPL or CpG ((87,88) and reviewed in (31,85)), the inclusion of the immune potentiating TLR ligands will become general practice.

Furthermore, vaccines exploiting antigens covalently linked to TLR ligands ((91,93,119) and Chapter 5) (Fig. 1C, III) increase the magnitude of the T-cell response as well as polarize the vaccine-induced immune response into a type 1 helper T cell/CTL response and are therefore likely to find their way into the clinic.

Vaccines are likely to be used in combination with the injection of T cell growth hormones such as IL-2 (43,120), IL-15 (121,122) or cytokines complexed to specific Abs or soluble receptors (123-125) resulting in enhanced *in vivo* efficacy, while greatly reducing the toxicity of these hormones (Figure 2, II). Pre-existing Ag-specific T regulatory T cells that can be boosted by the vaccine itself (126-128), should be removed first by for instance cyclophosphamide pre-treatment (129) or by the use of recombinant IL-2 linked to diphtheria toxin (DAB389IL-2) (130,131) (Figure 2, III). This will provide the right environment to further improve vaccine induced T cell response.

Notably, other emerging strategies to enhance this vaccine-induced T-cell response are injection of antibodies that either target specific members of the TNF receptor family, such as OX40 (132,133) and 4-1-BB (134) (Figure 2, I). Recently it was shown that successful peptide vaccines able to induce vast numbers of gp100-specific CD8⁺ T-cells in melanoma patients, failed to induce concomitant clinical responses (135). This lack of clinical efficacy could be alleviated by blocking the inhibitory signal given to T cells using blocking antibodies against CTLA-4 (136,137) (Figure 2, I). Note, however, that the use of aCTLA-4 might, in the case that self-proteins are the target antigen, also result in the induction of auto-immunity (137). Alternative strategies can be the use of PD-L1 blocking Ab (reviewed in (138)). Additionally, inhibition of immune regulatory proteins in T cells - such as Cbl-b - could further improve the expansion and effectiveness of the T cell responses (Chapter 6).

In contrast to the treatment of hematological cancers, the homing of immune cells to solid tumors is essential but often fails to occur due to immunosuppressive mechanisms (139). Immunotherapy trials that combine vaccines together with modalities that are able to induce pro-inflammatory signals, either induced by chemotherapy (140), radiation, or by application of creams that contain TLR ligands on accessible lesions (141) are likely to be more successful in recruiting effector T cells (Figure 2, IV).

We therefore expect that the first successful product will be a completely synthetic inflammatory product comprising multiple Ag determinants - provided as long peptides or synthetic/recombinant proteins (Fig. 1A) - that will be ingested by professional APC and following Ag processing stimulates both CD4⁺ and CD8⁺ T cells. This product that is likely to be given in combination with one or more therapeutic modalities that reduce immunosuppression and enhance T cell expansion as well as T cell homing.

KEY ISSUES

- The lack of a profound insight in the mechanisms of peptide-induced CD8+ T-cell response by minimal CTL peptides, explains in part their failure to induce clinical responses in human vaccination trials.
- The induction of a strong T-cell response by (peptide) vaccines requires that both Ag presentation and the APC activating signals have to be matched both in time and in location.
- An inherent risk of (peptide) vaccines is that not only beneficial helper CD4+ T cells but also detrimental (pre-existing) regulatory T-cells are stimulated by vaccines.
- The immunotherapy of cancer will be a multi-modality approach in which synthetic (peptide) vaccines will be combined with strategies to decrease immune suppression, enhance T-cell expansion, effector potential, and T-cell homing.

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8

Nederlandse
samenvatting

NEDERLANDSE SAMENVATTING

Met vaccinaties kunnen veel ziektes voorkomen worden, zoals de mazelen, de rode hond en de griep. Echter een vaccin voor het voorkomen van kanker is veel moeilijker om te maken. Dit komt omdat de meeste kankers verschillend van elkaar zijn. Het is niet mogelijk één vaccin te maken tegen verschillende soorten kankers. Vaccineren tegen kanker, kan dus pas wanneer je al kanker hebt, dit wordt therapeutische vaccinatie genoemd. Door middel van het injecteren van peptiden kun je het immuunsysteem activeren. Peptiden zijn stukjes eiwit. Wanneer peptiden gebruikt worden die specifiek zijn voor de kankercel, kun je het immuunsysteem activeren om zo de kanker op te ruimen. Gedurende mijn promotie onderzoek, heb ik het effect van de lengte van deze peptiden op de activatie van het immuunsysteem en de gepaard gaande antikanker respons onderzocht. Daarnaast heb ik onderzocht hoe we peptide vaccinaties verder kunnen optimaliseren door het toevoegen van verschillende soorten adjuvantia (immuun stimulerende stoffen) aan het vaccin.

Het immuunsysteem

Het immuunsysteem bestaat uit een scala aan cellen met verschillende functies. Een van de belangrijkste cellen van het immuunsysteem zijn de professionele antigeen presenterende cellen (APC), waarvan de dendritische cellen (DC) de meest gespecialiseerde zijn. DC hebben lange uitlopers, dendrieten genaamd, waarmee ze pathogenen (virussen, bacteriën, of parasieten) of dode (kanker) cellen kunnen oppakken en internaliseren. De DC knipt vervolgens de eiwitten - van de pathogenen of de kankercellen - in kleine peptiden van 8-10 aminozuren groot. Het peptide wordt samen met een ander eiwit – MHC – als een complex gepresenteerd. Het MHC heeft een groeve waar dit peptide precies in past. Het peptide, samen met het MHC wordt op het cel oppervlak als peptide:MHC complex gepresenteerd aan andere cellen van het immuunsysteem, de CD8⁺ T cellen. Wanneer de CD8⁺ T cel een peptide sequentie herkent op het oppervlak van de DC, wordt de CD8⁺ T cel geactiveerd voor dit specifieke peptide. De CD8⁺ T cel vermenigvuldigt waardoor er een groot leger aan CD8⁺ T cellen ontstaat wat specifiek is voor dat ene peptide. Vervolgens migreert de CD8⁺ T cel naar de door de pathogeen geïnfecteerde cellen of de kankercellen om deze vervolgens op te ruimen. CD8⁺ T cellen herkennen hun target cel aan de buitenkant. Iedere cel presenteert op het oppervlak, peptiden van alle eiwitten die zich in de cel bevinden als peptide:MHC complex. Indien een cel met een virus is geïnfecteerd of tot een kankercel is getransformeerd, worden dus ook peptiden van de eiwitten die daar betrokken bij zijn gepresenteerd. Zodra de CD8⁺ T cel het peptide:MHC complex herkent op de cel waarvoor het geactiveerd is, wordt deze cel opgeruimd.

Peptide vaccinatie

Kanker specifieke peptiden die op de kankercel gepresenteerd worden als peptide:MHC complex, kunnen ook gebruikt worden voor vaccinatie. Het is per slot van rekening hetzelfde peptide waarmee de CD8+ T cel geactiveerd wordt om vervolgens de kankercel op te ruimen. Deze peptiden van 8-10 aminozuren lang worden korte peptiden genoemd. Het induceren van een antikanker respons met deze korte peptide is niet optimaal. Wanneer een peptide van 30 aminozuren lang wordt gebruikt voor vaccinatie - waar dezelfde sequentie van 8-10 aminozuren in zit - blijkt de CD8+ T cel respons veel sterker te zijn dan wanneer het korte peptide voor vaccinatie wordt gebruikt. Het ontrafelen van dit fenomeen was een van het belangrijkste onderwerp van mijn promotie onderzoek

Dit proefschrift

Het effect van korte en lange peptiden vaccinaties hangt af van of het peptide in een waterige oplossing met een adjuvant (hoofdstuk 2) of in een olieachtige substantie - wat meestal in patiënten wordt gebruikt - wordt toegediend (hoofdstuk 3). De vorm van toediening bepaalt de duur van presentatie van de peptiden aan het immuunsysteem. Het peptide in het waterige vaccin wordt voor ongeveer 2 tot 10 dagen aan het immuunsysteem gepresenteerd (hoofdstuk 2) terwijl de peptiden die in een olieachtige substantie worden toegediend voor meer dan 60 dagen aan het immuunsysteem worden gepresenteerd (hoofdstuk 3).

Bij het waterige vaccin zijn een aantal parameters belangrijk voor een antikanker respons (hoofdstuk 2). Dit zijn het aantal CD8+ T cellen geïnduceerd door het vaccin, het APC type dat het peptide presenteert en de sterkte van binding van het peptide aan het MHC. Een kort peptide dat niet zo goed bindt aan het MHC wordt maar 2 dagen door het immuunsysteem gepresenteerd aan de CD8+ T cellen. Hierdoor worden maar weinig CD8+ T cellen geïnduceerd en is er geen goede antikanker respons. Een langere duur van presentatie van 10 dagen, wordt bereikt na vaccinatie met: een niet zo sterk MHC bindend lang peptide of met een sterk MHC bindend kort of lang peptide. Vaccinaties met deze peptiden induceren allemaal veel CD8+ T cellen. Echter de antikanker respons is het meest optimaal voor de beide langere peptiden. Deze peptiden worden namelijk door DC gepresenteerd, welke de meest professionele APC zijn in het immuunsysteem. Het sterk MHC bindend kort peptide wordt gepresenteerd door professionele en niet-professionele APC. Ondanks de inductie van veel CD8+ T cellen, zijn deze CD8+ T cellen niet goed in staat om de kanker op te ruimen. Het gebruik van lange peptiden voor vaccinaties tegen kanker lijkt dus altijd een sterkere immuunrespons te induceren dan het korte peptide.

Bij het olieachtige vaccin hebben we een kort en lang peptide dat sterk aan het MHC kan binden met elkaar vergeleken (hoofdstuk 3). De duur van presentatie speelt hierbij geen rol

aangezien beide peptiden voor meer dan 60 dagen gepresenteerd worden. De CD8+ T cel respons na vaccinatie met het korte peptide is kortdurend. Op dag 10 zijn de CD8+ T cellen te detecteren, op dag 30 zijn deze echter volledig verdwenen. Wanneer het lange peptide wordt gebruikt voor vaccinatie, zijn de CD8+ T cellen nog wel aanwezig op dag 30. Het verschil in het effect van korte en lange peptide vaccinaties ligt aan het APC type dat het peptide presenteert en aan de locatie waar het peptide wordt gepresenteerd. Het lange peptide wordt in de lymfeklier vlak bij de plek van vaccinatie door DC gepresenteerd. Echter bij het korte peptide zijn ook andere cellen betrokken bij de presentatie van het peptide, zoals B- en T cellen. Daarnaast wordt het korte peptide ook in het gehele lichaam door B- en T cellen gepresenteerd. Presentatie van het korte peptide door deze cellen kan leiden tot tolerantie of dood van de CD8+ T cel. Dit is precies wat we zien na vaccinatie met het korte peptide. Concluderend, wanneer een olieachtige substantie wordt gebruikt voor peptide vaccinatie, is alleen het lange peptide vaccin in staat om CD8+ T cellen te induceren die langdurig aanwezig blijven. Daarom is dit lange peptide vaccin beter om mee te vaccineren dan het korte peptide vaccin.

In hoofdstuk 4 staat het onderzoek beschreven van hoe een waterig lang peptide vaccin bestaande uit een peptide dat zowel CD8+ als CD4+ T cellen kan induceren, verbeterd kan worden door het toevoegen van een adjuvant. We hebben hiervoor verschillende soorten adjuvantia getest. Het onderzoek toonde aan dat de mate waarin het adjuvant de DC kon activeren niet bepalend was of er ook een goede CD8+ T cel respons werd geïnduceerd. Het aantal door het vaccin geïnduceerde CD8+ T cellen was ook hier belangrijk voor de antikanker respons maar niet doorslaggevend. Er waren drie adjuvantia die evenveel CD8+ T cellen induceerden, echter was maar één adjuvant goed in het induceren van CD8+ T cellen die ook de kanker konden opruimen. Belangrijke parameters van een goed antikanker vaccin zijn, dat het vaccin CD8+ T cellen induceerde die IFN γ produceerden en dat het vaccin tegelijkertijd veel CD4+ T cellen activeerde.

In hoofdstuk 5 staat het onderzoek beschreven van het mechanisme waarom koppeling van een lang peptide aan een adjuvant, een betere CD8+ T cel respons induceert dan het niet gekoppelde vaccin. De reden hiervoor is dat door de koppeling meer peptiden in de DC terecht komen. Het adjuvant gedeelte van het complex wordt namelijk gebonden door receptoren op de DC. Hierdoor wordt dus tegelijkertijd het peptide door de DC opgenomen. De receptoren die betrokken waren bij de opname van het adjuvant bleken andere receptoren te zijn dan die verantwoordelijk zijn voor de activatie van de DC via dit adjuvant. Waarschijnlijk zijn algemene ('scavenger') receptoren betrokken bij de opname van deze adjuvantia.

In hoofdstuk 6 staat het onderzoek beschreven van de antikanker respons in muizen die het cbl-b gen missen. De onderzoeksvraag was of afwezigheid van het cbl-b gen, en daardoor het

eiwit, de antikanker T cel respons zou versterken. Afwezigheid van cbl-b leidt tot CD8+ T cellen die hypergevoelig zijn. Deze muizen waren in staat om spontaan grote hoeveelheden kanker cellen op te ruimen zonder dat ze gevaccineerd hoefden te worden. Het remmen van cbl-b in CD8+ T cellen tijdens een (peptide) vaccinatie in kanker patiënten zou daarom een additionele therapie kunnen zijn voor het induceren van een betere antikanker respons.

Samenvattend hebben we met dit onderzoek beter inzicht gekregen in hoe korte en lange peptiden vaccins werken. Daarnaast hebben we laten zien waarom lange peptiden vaccins een betere CD8+ T cel respons induceren dan korte peptiden vaccins. De keuze van het adjuvant speelt ook een cruciale rol in de effectiviteit van het vaccin en het koppelen van het adjuvant aan het lange peptide kan het effect van het vaccin verder versterken. Deze data zijn van groot belang voor het verdere ontwikkelen van toekomstige peptiden vaccins.

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Dankwoord

DANKWOORD

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Curriculum vitae

CURRICULUM VITAE

Martijn Sander Bijker werd geboren op 15 juli 1978 in Lisse. In 1996 behaalde hij zijn VWO diploma op het Northgo College in Noordwijk. Hierna begon hij aan een Scheikunde opleiding aan de Vrije Universiteit in Amsterdam. Tijdens deze studie voltooide hij een stage op de afdeling Biochemie en Moleculaire Biologie onder begeleiding van Marco Siderius en Pim Mager. Dit onderzoek was gericht op het vinden van eiwitten die betrokken waren bij signalering van osmotische stress in gist. Vervolgens heeft hij een stage gedaan in het veld van de dendritische cel biologie bij Tanja de Gruijl en Rik Scheper op de afdeling Pathologie in het VU Medisch Centrum. Na deze stage vertrok hij voor een periode van 8 maanden naar San Diego in de Verenigde Staten om bij Stephen Schoenberger op het La Jolla Institute for Allergy and Immunology te werken aan het programmeren van CD8+ T cellen. In 2002 haalde hij het doctoraal examen waarna hij in 2003 begon aan zijn promotie onderzoek op de afdeling Immunohematologie en Bloodtransfusie bij de sectie Tumorimmunologie in het Leids Universitair Medisch Centrum. Dit onderzoek werd begeleid door Prof. Dr. C.J.M. Melief, Dr. S.H. van der Burg en Dr. R. Offringa. In januari 2008 zal hij gaan werken aan de rol van Neuropeptide op T regulatoire cellen in het 'Garvan Institute' in Sidney, Australië onder begeleiding van Prof. Dr. F. Mackay.

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