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Generation of $CD8^+$ T cell mediated protective immunity upon vaccination with soluble antigen: involvement of immunmodulatory factors like adjuvant or regulatory lymphocytes

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I. TABLE OF CONTENTS

II. INDEX OF FIGURES AND TABLES

III. ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AHR	Airway hyper- reactivity
AP-1	Activating protein-1
APC	Allophyco-cyanin
APC	Antigen presenting cells
BHI	Brain-heart infusion medium
BM	Bone marrow
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxy-fluoresceindiacetat succinimidyl ester
CFU	Colony forming unit
CMV	Cytomegalovirus
CTL	Cytotoxic T cell
DCs	Dendritic cells
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ELAM-1	Endothelcell-leucocyte-adhesion molecule-1
EDTA	Ethylendiamintetraacetate
EMA	Ethidiummonazid-bromide
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanat
FmTLR2	Flag-tagged murine TLR2
FoxP3	Forkhead box P3
GITR	Glucocorticoid-induced TNF receptor family-related protein
h	hour
HCV	Hepatitis C virus
HEK 293	Human embryonic kidney 293 cells
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus

hTLR2	human TLR2
IFN	Interferon
IL	Interleukin
IRAK	IL-1-receptor-associated kinase
i.p.	Intraperitoneal
IRF3	interferon regulatory factor 3
i.v.	Intravenously
JNK	c-Jun N-terminal kinase
kDa	kilodalton
KO	Gene knockout
LCMV	Lymphochoriomeningitis virus
L.m.	Listeria monocytogenes
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation marker 88
PE	Phycoerythrin
PFA	Paraformaldehyde
PLGA	Polylactide-polyglycolide
rpm	rounds per minute
RSV	Respiratory synciytial virus
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TAP	Transporter associated protein
TCR	T cell receptor
TEMED	N, N, N‘, N‘-Tetramethylethylendiamine
Th	T helper
TIR	Toll/IL-1-receptor
TIRAP (Mal)	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAM	TRIF related adaptor molecule
Treg	$CD4^+CD25^+$ regulatory T cell
TRIF (TICAM)	TIR domain containing adaptor inducing IFN

Tris	Tris-(Hydroxylmethyl-)Aminomethan
VSV	Vesicular stomatitis virus
wt	wildtype

1. INTRODUCTION

1.1. Vaccination

With the work of the english physician Edward Jenners the first mile stone was set for vaccination. He found that when he immunized individuals with for humans overly low virulent cowpox virus, they were resistant to the often lethal infection with small poxvirus. Many people see this historic event as the beginning of understanding immunological memory. From thereon, improved strategies have been evolved to generate safer and more efficient vaccines. Live vaccines including replicon-based vaccines, like alphaviruses, adenoviruses or pox viruses, which contain DNA, RNA or infectious particles are known to be highly immunogenic, characterized by strong $CD8^+$ T cell mediated immune responses. But critical decisions for the choice of strategy for vaccinations are not only dictated by the immunological properties of the vector system alone, also the immune status of an individual has to be considered, as replicating vectors can be potentially harmful for immunocompromised people. In addition, as several booster vaccinations are often needed to reach levels of effective protective immunity, strong immune responses against the vector might negatively interfere with the response against the target antigen. Efforts to make vaccines safer, especially by using non-replicating antigen structures (DNA, protein- or peptide-based vaccinations) unfortunately often result in substantial loss of immunogenicity. In order to improve immunogenicity of vaccines that so far only induce poor immune responses the enhancing effects of adjuvants have been explored. Several different adjuvants with measurable activity on immunogenicity are known, including mineral compounds (e.g. aluminium hydroxide), water-in-oil or oil-in-water emulsion (e.g. Freund's adjuvant), chemically or genetically inactivated bacterial toxins (e.g. *cholera* toxin), saponins or CpG oligonucleotides, just to list a few. However, until today only a few adjuvant applications have been approved for clinical use. This is mostly due to the fact that specific effects of adjuvant activity on protection and induction of long living memory T cells are still rare. Substantial progress has been made for analysis of T cell responses both in man and mouse. One important finding of these experiments was that antigen-specific T cells can differentiate into a variety of different subsets with distinct functions. Markers have now been identified to directly visualize these different T cell subsets and give new hope that with these more precise analysis the specific adjuvant effects will be better understandable and that this knowledge will provide rationales for improvement of vaccines.

1.2. Innate and adaptive immunity

It is well appreciated that tight and complex interactions between parts of the innate and the adaptive immune system are required to initiate and maintain protective immune responses. Both arms of the immune system contribute essential factors important for antigen-processing, -presentation, -recognition, T cell activation, differentiation and memory T cell development. But many of the underlying mechanisms are still not well understood.

The innate immune system represents the immediate immune response and consists of leukocyte populations that includes macrophages, monocytes, dendritic cells, neutrophils, mast cells or natural killer (NK) cells (Hsieh et al., 1993). Some innate cells are involved in discrimination between self and non-self, like NK-cells, others are crucial for presentation of foreign antigen to cells of the adaptive immune system. (Medzhitov and Janeway, 1998). Once cells of the innate immune system detect a pathogen, they can secrete cytokines that in turn deliver specific signals to immune cells in an autocrine or paracrine fashion, and other inflammatory mediators, which recruit more effector cells of both the innate and the adaptive immune system to the site of infection (Janeway and Travers, 1999). This can be followed by a late phase response, characterized by the presence of leukotrienes, cytokines and chemokines, which attract other cells that are part of the adaptive immune system (Downey, 1994). Due to their highly diversified receptors, cells of the adaptive immune system respond in a manner specific to the antigen that gets presented by the phagocytic or antigen-presenting cell (APC). The two main effector cells of the adaptive immune system are the B and T cells.

Upon recognition of antigen by the B-cell receptor (BCR) on B cells, these differentiate and proliferate in the B cell regions of secondary lymphatic organs to become antibody producing plasma cells. Soluble antibodies are able to specifically bind to antigen. Antibodies are needed e.g. for neutralization of bacterial toxins, opsonisation of pathogens, or they can activate the complement system, which can lead to antibody-mediated elimination of pathogens.

T cells, on the other hand, can be further divided in $CD4^+$ or $CD8^+$ T cells based on their co-receptor expression. Naïve $CD8^+$ T cells differentiate into cytotoxic T cells (CTLs) after recognition of antigen and the activation through co-stimulatory molecules. These CTLs, characterized by their lytic activity of infected target cells, make up a large fraction of $CD8^+$ T cells. $CD4^+$ T cells are known as T helper (Th) cells and based on their effector function they can be divided into Th1 and Th2 cells (Rogers et al., 1998). The first encounter with antigen and the type of antigen play the critical role for naïve $CD4^+$ T cells to differentiate into either Th1 or Th2 phenotypes (Morel and Oriss, 1998; Rogers et al., 1998; Romagnani, 1992). In

the presence of IL-4, Th precursors preferentially differentiate into Th2 T cells that secrete cytokines such as IL-4, IL-5, IL-10 and IL-13 (Medzhitov and Janeway, 1998; Morel and Oriss, 1998). In contrast, when IL-12 and/or IL-18 are secreted by APCs, Th precursors preferentially differentiate into Th1 cells, which secrete IL-2 and IFN among others (O'Garra and Murphy, 1996; Yoshimoto et al., 1999). Once this initial polarization of the response towards Th1 or Th2 phenotype is established, it can be self perpetuating and the Th1 cytokines can further enhance Th1 responses or down regulate Th2 differentiation, and vice versa (Morel and Oriss, 1998).

Most of the $CD4^+$ and $CD8^+$ T cells carry a T cell receptors (TCR) consisting of an and chain, with which they are able to recognize peptides presented by professional APCs. In recent years another T cell population has been described, the /TCR expressing T cells. This subpopulation makes up about 5% of overall T cells and they have been described to be involved in both innate and adaptive immunity. Naïve /T cells can respond very rapidly after encountering their specific ligand, a characteristic more typical for cells belonging to the innate immune system. However, because they express rearranged surface receptors and are able to develop into memory T cells, they also bear specific characteristics of cells of the adaptive immune system. Interestingly, in humans /T cells themselves have been implicated to function as APC, being able to present ligands to other /T cells (Modlin and Sieling, 2005).

1.3. Memory T cells

Activation of the adaptive immune system can lead to vigorous antigen-specific T cell proliferation. The expansion phase is followed by a contraction phase where more than 90% of the antigen-specific T cells die by apoptosis, leaving only a small pool of so called memory T cells. Programming of naïve T cells to become memory T cells takes place very early during the primary response. Already 24 hours of antigenic stimulation are sufficient to induce the program of clonal expansion, expression of effector function and differentiation into memory cells (Bevan and Fink, 2001; Masopust et al., 2004; Wong et al., 2004). Memory T cells can be immediately activated through re-encounter with antigen, resulting in rapid proliferation of antigen-specific T cells, therefore providing protection against re-encounter with the pathogen. Many studies have been performed to distinguish long living memory T cells via distinct markers from the pool of short living effector T cells. In humans this distinction was made using the markers CD45RO and CD45RA, because of the rapid proliferative activity of $CD45RO^+$ (memory) T cells after reencounter of antigen *in vitro*,

which is not found for $CD45RA^+$ (naïve) T cells. Also the migration pattern of T cells expressing CD45 isoforms is different; $CD45RA^+$ T cells migrate through lymphoid tissue, where they can encounter antigen, whereas $CD45RO^+$ T cells migrate through the whole body to be able to readily respond upon reencountering antigens. Further memory T cell differentiation was enabled through the markers CD62L, CD27 and CCR7. CCR7 is a homing receptor and in combination with CD45RA expression has been shown to distinguish two sets of memory T cells, central memory T cells (T_{CM} - $CCR7^{high}/CD45RA^{high}$) and effector memory T cells (T_{EM} - $CCR7^{low}/CD45RA^{high}$). T_{CM} cells are believed to be the main source for long-term maintenance of antigen-specific immune responses, whereas T_{EM} cells may be crucial for providing immediate protection (Sallusto et al., 1999). Detailed knowledge about the exact function of these memory T cell subpopulations is for example important to improve T cell-targeting vaccination strategies. With the recently identified marker CD127 (IL-7 receptor α-chain) in combination with CD62L the discrimination between effector and memory $CD8^+$ T cells could be made in the murine model, which so far was not possible because the markers applicable for human T cell phenotyping are not directly transferable to the mouse system. T_{CM} cells, which have the phenotype being $CD127^{high}/CD62L^{high}$ can be distinguished from T_{EM} cells ($CD127^{high}/CD62^{low}$) and effector T cells (T_E – $CD127^{low}/CD62L^{low}$)(Huster et al., 2004). The different memory T cell subpopulations also differ in their cytokine profiles; T_E (Curtsinger et al., 2003) and T_{EM} cells show immediate effector function characterized for example by production of IFNγ and TNFα, whereas T_{CM} cells do not produce these cytokines. Regarding the proliferative capacity, T_E cells show poor proliferative activity upon antigenic stimulation, the population is short lived and shows poor IL-2 production. Like it has been decribed for the human system, T_{CM} cells show high proliferative activity upon re-stimulation with antigen (Huster et al., 2004). With the help of the CD127 marker important information might be generated with respect to vaccine development. Especially, because already at early time points after vaccination the profile of CD127/CD62L distribution might indicate the quality of long term maintained immunity.

1.4. Antigen presentation

In order to activate T cells, antigen usually has to be taken up by APCs, processed and presented to the naïve T cell via MHC molecules. We can distinguish two major pathways, the MHC class I and class II pathway.

1.4.1. MHC class I pathway

MHC class I molecules are expressed on all nucleated cells and bind peptides that are generated from degradation of cytosolic antigens, which are subsequently presented to $CD8^+$ T cells. The MHC class I molecule consists of 2 chains, the α chain (α1-3) and the β2-microglobulin. The newly generated α chain of MHC class I molecules is first translocated into the endoplasmatic reticulum (ER), where it is stabilized by chaperones, like calnexin, until the light chain (β2m) can non-covalently attach to the complex. This leads to the release of calnexin and binding to the chaperone proteins calreticulum and tapasin. This entire complex is called TAP (transporter associated with antigen processing) loading complex. Cytosolic proteins get degraded by the proteasome, which cuts peptide fragments to a length of about 8-10 amino acids. These peptides get then transported into the ER, where they might bind to MHC class I molecule. The binding of peptide stabilizes the MHC class I complex; this results in the release from calreticulum, tapasin and TAP, and thereby allows transport through the Golgi complex to the cell surface (Abbas A., 2003).

1.4.2. MHC class II pathway

Expression of MHC class II molecules is only found on distinct subtypes of nucleated cells like B cells, DCs, macrophages, Langerhans cells and epithelial cells of the thymus. Antigen that gets recognized via MHC class II molecules can be derived for example from pathogens residing in intravesicular compartments or from proteins or toxins that are taken up from extracellular sources. Antigen arrives in endolysosomal vesicles, where after acidification a variety of proteases can get activated to degrade proteins into small peptide fragments.

MHC class II molecules consist of two chains, α (α 1 and α 2) and β (β1 and β2), which both participate in formation of the peptide binding groove. Like for MHC I, assembly of newly generated MHC II complexes occurs within the ER, where the complex is stabilized by the so-called invariant chain. A cytosolic sequence within the invariant chain targets MHC II molecules through the Golgi apparatus to the endosom-lysosomal compartment. Here, the invariant chain gets rapidly degraded, leaving a small peptide within the binding groove (CLIP). CLIP can be exchanged to antigen-derived peptide fragments with the help of certain

chaperons (DM, DO). These complexes can be released to the cell surface (Chapman et al., 1997).

1.4.3. Cross-presentation

Besides the direct route of antigen processing via the MHC class I presentation pathway an additional pathway has been identified, called cross-presentation. It describes the possibility to present MHC class I restricted epitopes derived from exogenous antigens to $CD8^+$ T cells. This was shown for the first time in the late 1970s by M. Bevan, who demonstrated $CD8^+$ T cell activation by immunization of mice with antigen-expressing cells, which lacked the restricting MHC molecules (Bevan, 1976). Not all APCs seem to be capable to perform cross-presentation; it might be restricted to just a few specialized immune cells, like DCs and macrophages. Proteins can enter the cross-presentation machinery by different means of endocytosis, including macropinocytosis, receptor-mediated endocytosis and phagocytosis (Ackerman and Cresswell, 2004). From there, antigen can be processed and loaded to MHC class I molecules by at least two different pathways, a TAP-dependent phagosome-to-cytosol pathway or a TAP-independent vacuolar pathway.

1.4.3.1. TAP-dependent pathway of cross-presentation

The TAP-dependent pathway describes the internalization of exogenous antigen into vacuolar compartments, which subsequently directly reaches the cytoplasm where it gets processed, translocated and loaded to MHC class I molecules, in order to get effectively cross-presented (Rock, 1996). This transfer of antigen into the cytosol occurs through endoplasmatic reticulum (ER)-mediated phagocytosis. There are currently two main models describing this process:

A) The ER membrane fuses with the plasma membrane during phagocytosis, which leads to the formation of a phagocytic cup, which after the cut off from the plasma membrane represents the initial phagosome (Gagnon et al., 2002; Houde et al., 2003). ER proteins then progressively disappear as the phagosomes mature into phagolysosomes. Sec61 is described as a pore complex that mediates retrotranslocation of peptides to the cytoplasmic face of the phagosome (Gagnon et al., 2002; Houde et al., 2003). The phagosome owns proteasomes, the peptide loading complex, consisting of TAP, tapasin, calreticulin, ERp57 and MHC class I-β2m dimers (Ackerman and Cresswell, 2004; Dick et al., 2002). Through the presence of this antigen-processing machinery the proteins can be further processed via the conventional pathway, meaning degradation of the protein through the proteasome and translocation of the

peptides to the ER via the TAP complex. TAP can also transport the peptides back to the phagosome where MHC class I molecules are present that can than bind the TAP-translocated peptides.

B) A different model suggests that at some point of the formation of the phagolysosome the ER becomes contiguous with the lumen of the phagosome. This leads to translocation of soluble antigen into the lumen of the ER, where the antigen undergoes ER-mediated degradation, involving Sec61-mediated retrotranslocation and proteasomal processing.

Both models are dependent on TAP, as experiments using TAP inhibitors, like US6, a protein inhibitor of peptide translocation interacting with the luminal domain of TAP, have shown that cross-presentation was abolished when TAP was not functional (Ackerman et al., 2003; Huang et al., 1996; Kovacsovics-Bankowski and Rock, 1995).

1.4.3.2. The vacuolar pathway

In contrast to the TAP-dependent pathway, a TAP-independent pathway has been described. The model for the TAP-independent pathway, or also called the vacuolar pathway, proposes that after internalization of exogenous antigens, they get degraded within the endocytic pathway by mechanisms consisting of reduction, unfolding and lysosomal proteoloysis (Ackerman and Cresswell, 2004; Chefalo and Harding, 2001). Cross-presentation in this model functions through the exchange of peptides bound to MHC class I complexes, which presumably recycle to the ER, with these newly generated peptides. The importance of this vacuolar pathway becomes obvious when analyzing viruses that infect APCs and have mechanisms to inhibit TAP dependent antigen presentation. Under these circumstances, it is crucial for the APC to have an “emergency exit” in form of the vacuolar pathway.

Important roles in this pathway have been described for Cathepsin (Cat S) and some heat shock proteins (HSP). Cat S is a cysteine protease that is preferentially expressed on APCs including DCs, macrophages, and B cells. Experiments using OVA incorporated into microspheres of poly-lactide poly-glycolide (PLGA) (which is cross-presented in an TAP-independent manner) showed that cross-presentation was decreased in Cat S – deficient BM DCs. For OVA-PLGA it was also shown that Cat S is crucial for the generation of H2-K^b restricted OVA peptide (SIINFEKL) in phagosomes. Therefore, Cat S might serve as the key protease in the TAP-independent pathway. Cat S has been also ascribed an important role during viral infections. During viral infections, antiviral cytokines like IFN are produced. IFN can induce Cat S expression, which in turn could lead to an increase of cross-presentation of viral antigens by the vacuolar pathway (Chapman et al., 1997).

In addition to Cat S, HSPs have been found to be involved in cross-presentation. In the past HSPs were known as molecular chaperons that are mainly involved in protein folding (Houry, 2001). But extensive studies have been made to understand the role of HSPs and it was discovered that HSPs also have immunological function and that certain HSPs can bind peptides in order to form immunogenic HSP-peptide complexes. It has been proposed that HSPs are involved in cross-presentation, but controversial results have been generated whether this involved the TAP-dependent or –independent pathway. It has been demonstrated that the exogenous HSPs can use the phagosome-to-cytosol pathway, perhaps by enhancing delivery of HSP-chaperoned peptides to the APC cytosol, thereby achieving access to proteasome-dependent processing. These results are strengthened by the discovery that HSP enhancement of peptide presentation is inhibited by brefeldin A, proteasome inhibitors and absence of TAP (Basu et al., 2001; Suto and Srivastava, 1995). On the other hand, contradictory studies suggest an involvement of HSP on the TAP-independent pathway, where the absence of TAP still leads to cross-presentation (Schirmbeck et al., 1997; Schirmbeck and Reimann, 1994). Recently is has been found that it is somehow dependent on the type of APC, which pathway of HSP mediated presentation pathway is accessible. For bacterial HSPs, like *E.coli* DnaK and *Mycobacterium tuberculosis* HSP70, it has been shown that indeed the cell type seems to be an important contributing factor. The group of Tobian et al. showed that these two bacterial HSPs take the vacuolar pathway in macrophages, demonstrated by the observation that cross- presentation was unaffected in the presence of brefeldin A or lactasine. In DCs, the same HSPs-mediated cross- presentation via the cytosolic pathway was shown by strict TAP dependency and inhibition of cross-presentation in the presence of brefeldin A (Tobian et al., 2004).

1.5. DC subpopulations

DCs are believed to be the major cell population able to efficiently cross-present antigen to $CD8^+$ T cells. DCs develop from $CD34^+$ progenitor cells in the bone marrow and migrate from there to the periphery (Banchereau, 1997). DCs have been subdivided into two major groups with different origin, namely myeloid and lymphoid DCs (Wu et al., 1996). However, in recent years more subclasses have been identified, but the exact lineage relationships are still a matter of debate. Plasmacytoid DCs are found in blood and in tissue and can produce large amounts of type I interferons, for example after encountering distinct TLR ligands. Two other DC subpopulations found in the blood are the $CD8^-CD4^-$ and $CD4^+$ DCs (Hochrein et al., 2001). Langerhans cells are known as DCs preferentially found in the skin; they are located

within the epithelium of the skin where they take up antigen or even whole pathogens, and migrate to the draining lymph nodes to present antigen to naïve T cells. One characteristic of this subpopulation is the presence of specialized intracellular vesicles, called Birbeck granules (Romani et al., 2003). A Langerhans cell-related DC population is the dermal DC, also known as interstitial DC. These cells differ from Langerhans cells by the absence of Birbeck granules and have significantly lower expression levels of the DEC205 (Henri et al., 2001). Another DC subpopulation is characterized by the expression of CD8□□. $CD8^{+}DEC205^{+}$ DCs are located in the T cell zone of secondary lymphoid organs (De Smedt et al., 1996; Henri et al., 2001), but can also be found in the thymus. Cross-presentation of antigen, which is well described for this population can lead to cross-priming or cross-tolerance depending on several additional factors, like expression of co-receptors or release of distinct patterns of cytokines (Belz et al., 2002). Co-stimulatory molecules, which are important for T cell-priming are listed in table 1.

DC	T cell
B7 family and coreceptors	
B7-1 (CD80)	CD28, CTLA-4 (CD152)
B7-2 (CD86)	CD28, CTLA-4 (CD152)
B7RP-1	ICOS (inducible immune costimulator)
PD-LI (B7-H1)	PD-1 (programmed cell death 1)
PD-L2	PD-2
B7-H3	Not known
TNF family ligand and receptors	
CD40	CD40L (CD154)
OX40L	OX40 (CD134)
4-1BBL	4-1BB (CD137)
TRANCE (RANK)	TRANCE (RANK-L)
CD27	CD27L (CD70)
CD30L (CD153)	CD30
Miscellaneous	
ICAM-1 (CD54)	LFA-1 (CD11a/CD18)
DC-SIGN (CD209)	ICAM-3 (CD50), ICAM-2 (CD102)
SLAM (CD150)	SLAM (CD150)
CD58	CD2

Table 1: Co-stimulatory molecules for DC-T cell signals (Lipscomb and Masten, 2002)

In addition to the expression of co-stimulatory molecules, DCs carry a set of chemokine receptors, which regulate their migration into tissue sites in response to inflammatory chemokines. Chemokine expression is changed as immature DCs, which can migrate along an inflammatory chemokine gradient, get in contact with proinflammatory cytokines and

antigen. This signals lead to maturation of DCs, which now downregulate inflammatory chemokine receptors (e.g. CCR1, CCR2, CCD5 and CXCR1) and upregulate lymphoid homing receptors (e.g. CCR4, CCR7 and CXCR4) and co-stimulatory molecules. For a certain time during inflammation DCs themselves can produce chemokines (e.g. RANTES, MIP-1a and MCP-1) to for example attract other DCs to the site of infection/inflammation. In order to attract T cells, DCs can generate other sets chemokines, like TARC, MDC and IP-10 (Lipscomb and Masten, 2002).

1.6. Toll like receptors – linking the innate with the adaptive immune system

It is long known that adaptive immune cells are the mediators of protective immunity, but also that generation of effective memory responses is dependent on certain cofactors that are provided by both innate and adaptive cells. Adjuvants, for example were found to improve immunogenicity during vaccination. But detailed information about the molecular mechanisms of adjuvants first could be gained by the discovery of so-called pattern-recognition receptors (PRRs), especially Toll like receptors (TLRs).

Toll was first identified in Drosophila. In the fly, control of infections relies exclusively on innate immune factors, and it was found that lack of Toll results in a specific lack of host defense against fungal infection (Takeda and Akira, 2005; Tzou et al., 2002). Shortly after the discovery of Drosophila Toll, the first human Toll like receptor, namely TLR4 was found. TLR4 was shown to recognize LPS, which is present in cell walls of Gram-negative bacteria (Poltorak et al., 1998; Takeuchi et al., 1999). Thereafter, extensive studies have been made to identify more human and murine TLRs. Until now, 11 members have been found, 9 (TLR1-9) of which are highly conserved between human and mouse (Takeda and Akira, 2005).

TLRs belong to the group of PRRs. They are type I transmembrane receptors consisting of an extracellular domain containing leucine-rich repeats (LRRs) and a cytoplasmic domain, which contains a sequence similar to that of the mammalian IL-1 receptor. TRLs are specifically stimulated by so called "pathogen-associated molecular patterns" (PAMPs) (Takeuchi et al., 1999). PAMPs are common integral structural components of microorganisms, which are conserved among certain subgroups of viruses, bacteria or fungi (Xu et al., 2005), but are not exclusively linked to pathogenic microorganisms. PAMP recognition by TLRs initiates signaling events resulting in the activation of innate host defense mechanisms.

TLRs are differently expressed on immune cells. Whereas TLR1, 2, 4, 5 and 6 traffic to the cell surface for engagement of extracellular PAMPs, TLR3, 7, 8 and 9 are found at

intracellular locations. As an overview, table 2 shows the expression characteristics and some known ligands of the different TLRs.

TLR	Location	ligand	microorganism
TLR1 (functionally associated with TLR2)	ubiquitous	Triacyl lipopeptides	
		soluble bacterial factors (*Mycobacteria*, *Neisseria*, *Borrelia*)	
TLR2*	surface membrane and phagolysosomes; myeloid, mast and NK cells; mDCs; T cells (□□ and □)	peptidoglycan and lipoteichoic acid	gram-postitive bacteria
		lipoarabinomannan	Mycobacteria
		glycosylphosphatidylinositol anchors	*Trypanosoma cruzi*
		phenol-soluble modulin	*Staphylococcus epidermis*
		Zymosan	fungi
		LcrV	*Yersinia ssp.*
		Glycolipids	*Treponema maltophilum*
		LPS	*Leptospira interrogans*
			Porphyromonas gingivalis
			Helicobacter pyroli
TLR3	intracellular in mDCs; NK cells	dsRNA	virus helminth
		poly (I:C)	
		siRNA	
		shRNA	
		endogenous: mRNA	
TLR4*	surface membrane of monocytes, mast cells and neutrophils; Golgi in gut epithelial cells; regulatory and □□ T cells; endothelial cells	LPS	gram-negative bacteria
		taxol	*Taxus brevifolia*
		HSP60 and HSP70	
		Extra domain A of fibronectin	
		Oligosaccharides of hyaluronic acid	
		Heparan sulfate	
		fibrinogen	
		F protein	RSV
TLR5	surface epithelial; NK cells; mDCs	flagellin	bacteria
		discontinuous 13 aa peptide	
TLR6 (functional associated with TLR2)	surface myeloid, mast and B cells	diacyl lipopeptides	
TLR7	endosomal: pDCs; B cells; eosinophils	imidazoquinoline	
		loxoribine	
		ssRNA	virus (e.g. influenza, VSV, HIV)
TLR8	endosomal: NK, T, myeloid cells	ssRNA	virus
TLR9	endosomal: pDCs; B and NK cells; surface of tonsillar cells	bacterial DNA (CpG DNA)	bacteria
		viral DNA	virus
		Malaria schizonts	*Plasmodium falciparum*
		host chromatin	
TLR11	murine uroeptithelium	Not known	uropathogenic bacteria

Table 2: TLRs and their ligands

*For TLR2 and TLR4 some ligands are doubtable because recombinant proteins produced from bacteria are used in these studies and these could contain potential contamination of TLR ligands (e.g. LPS) (Hopkins and Sriskandan, 2005; Pasare and Medzhitov, 2004; Takeda and Akira, 2005).

Priming of antigen-specific T cells is supported by TLRs. This can for example be achieved by recognition of PAMPs by TLRs expressed on professional APCs, especially on DCs. Several DC subsets reside in peripheral tissues where they can efficiently take up antigen (or even complete microbes). After uptake they migrate via the lymphatics to the draining lymph node. In the immature state DCs express low levels of MHC molecules, lack expression of co-stimulatory B7 molecules, and are not capable of priming naïve T cells. This phenotype changes dramatically after PAMP mediated TLR stimulation, which leads to maturation of the DCs, which are now characterized by high-level expression of MHC and co-stimulatory molecules. Mature DCs are fully capable of priming naïve T cells. In addition, TLR mediated signaling is responsible for induction of a variety of different cytokines and chemokines, which are further important cofactors modulating adaptive immune responses. These include for example IL-12, an important cytokine for the initiation of Th1-like immune response. Another important cytokine might be IL-6, which has been suggested to be necessary to overcome suppressive effects on T cell priming induced by regulatory $CD4^+CD25^+$ T cells (Treg cells). Upon secretion of IL-6 by DCs after activation through TLR stimulation, responder T cells seem to become refractory to suppression by Treg cells. This indirect mechanism allows that Treg cells can still have suppressive effects on other immune cells, resulting for example in prevention of auto-reactive T cell activation during an infection (Pasare and Medzhitov, 2004; Reis e Sousa, 2004). TLRs are believed to be one of the most crucial factors linking the innate and the adaptive immune system. Recent studies even imply a potential direct role of TLRs in memory T cell responses, because TLR2 has be shown to be expressed on the surface of activated and memory ($CD45RO^+$) $CD4^+$ T cells (Xu et al., 2005).

1.6.1. TLR signaling pathways

PAMP recognition via TLRs leads to a signaling cascade downstream of the TLR. After stimulation of TLRs the formation of homo- or heterodimers is induced.

Most TLRs form homodimers upon stimulation. An exception is TLR2, where heterophilic dimerization with TLR1 or TLR6 is required for responses to certain ligands (Ozinsky et al., 2000; Re and Strominger, 2001; Wyllie et al., 2000). Because the cytoplasmic portion of the TLRs bears similarities to the IL-1 receptor family, it is named Toll/IL-1 receptor (TIR) domain. This TIR domain contains three conserved amino acid sequences which are essential for the signaling pathway that takes place after dimerization of the TLRs (Takeda and Akira, 2005). A crucial adaptor molecule in the signaling pathway is MyD88 (myeloid-differentiation-primary-response-gene 88), which is a TIR domain-containing molecule and

has been shown to be essential for the induction of inflammatory cytokines like TNF and IL-12 through all TLRs. However, not all TLRs show the same pattern of gene expression profile upon activation. For example type 1 IFNs are induced after activation of TLR3 and TLR4, but not TLR2 and TLR5. Although activation of TLR7, 8 and 9 can also induce type 1 IFNs, the mechanism is distinct from the TLR3 and 4. Therefore, different pathways exists, which are generally subdivided into an MyD88 dependent and MyD88 independent pathways (Takeda and Akira, 2005).

1.6.1.1. MyD88 dependent pathway

MyD88 consists of a TIR domain and a death domain. The TIR domain of MyD88 can interact with that of the TLR and the death domain with that of the IL-1-receptors associated kinase (IRAK), (Medzhitov et al., 1998; Muzio, 1998). Stimulation of TLRs leads to recruitment of IRAK-4 to TLRs by MyD88. IRAK-4 further on phosphorylates IRAK-1, which in turn associates with TRAF6 (tumor necrosis factor TNF receptor associated factor 6) (Arch et al., 1998; Cao et al., 1996), which leads to the activation of two distinct pathways, resulting in the activation of AP-1 or NF-B transcription factors. In the absence of MyD88, as shown for MyD88-deficient mice, there is no production of inflammatory cytokines like TNF and IL-12p40 upon stimulation of the different TLR ligands. This demonstrates the important role of MyD88 for inflammatory cytokine production through all TRLs (Takeda and Akira, 2005).

1.6.1.2. MyD88 independent pathway

It has been shown that in MyD88-deficient macrophages there was no production of inflammatory cytokine upon TLR4 activation, but nevertheless NF-B was activated. This shows that even though the cytokine production depends on MyD88 there is also a MyD88 independent component in TLR4 signaling (Takeda and Akira, 2005).

The signal molecule involved in the MyD88 independent pathway is the TIRAP (TIR-domain containing adaptor protein) or MAL (MyD88-adaptor like protein). Like MyD88, TIRAP can interact with the TIR-domain of TLR4, which results in the induction of NF-B through IRAK-2 (Fitzgerald et al., 2001; Horng et al., 2001). Until now, only TLR2 and TLR4 are known to be able to signal via TIRAP. For TLR3 and 4 another adaptor molecule has been described, namely TRIF (TIR domain containing adaptor inducing IFN) or TICAM-1 (TIR containing adaptor molecule- 1) (Oshiumi et al., 2003; Yamamoto et al., 2002). In MyD88 deficient mice TRIF can induce activation of NF-B upon stimulation with Poly (I:C) or LPS.

In addition to the activation of NF-□B also the transcription factor IRF-3 (Interferon regulatory factor- 3) gets activated after TLR4 stimulation. Activation of IRF-3, which also gets activated by TLR3 results in IFN□ production. Therefore, it is proposed that both TLR3 and 4 can be activated in a MyD88 independent manner (Takeda and Akira, 2005).

1.6.2. TLRs - negative regulation

Although TLRs are known to be crucial mediators to support the first line of defense upon encounter with a pathogen, a careful balance has to be taken not to overstimulate the system. Several factors have been described regulating this careful balance, ranging from extracellular decoy receptors to intracellular inhibitors, membrane-bound suppressors, degradation of TLRs and TLR-induced apoptosis. For example soluble forms of TLR2 (sTLR2) and TLR4 (sTLR4) have been found in humans, which, even though not fully understood, likely block the interaction between TLR2 (or TLR4, respectively) and other co-receptor complexes, especially DM2 and CD14, resulting in the termination of TLR2 (or TLR4) signaling. Other negative regulators are the Toll-interacting protein (TOLLIP), which autophosphorylates IRAK1 and therefore blocks TLR2 and 4 signaling. Suppressor of cytokine signaling (SOCS1) suppresses IRAK, thereby affecting TLR4 and 9 signaling. Furthermore, the nucleotide-binding oligomerization protein 2 (NOD2) suppresses NF-kB and acts as negative regulator of TLR2 signaling (Liew et al., 2005). This shows the careful balance of positive and negative regulation by TLRs. Disruption of these counter balancing mechanisms might be involved in the pathophysiology of various diseases. For example, the malaria pigment hemozoin induces inflammatory responses through TLR9 and many auto-immune diseases like EAE (TLR4 – pertussis toxin recruits autoreactive T cells into the central nervous system), SLE (TLR9 – chromatin-IgG complexes activate B cells and DCs) or diabetes (TLR 2,3,4 and 9 – TLR ligands increase innate immunity) account for deregulation of this balance (Liew et al., 2005).

1.7. $CD4^+$ T cell help

As described above, stimulation of TLRs plays a crucial role during the generation of T cell responses. This seems also to be true for $CD8^+$ T cells. However, besides co-stimulatory factors provided by the APC, additional support by other cell populations seems to be important, especially on the generation of long living memory CD8+ T cells. One population are $CD4^+$ helper T cells. Until the late 1990s the common view was that for highly inflammatory stimuli, such as viral or bacteria infections, $CD4^+$ T cells were not needed for APC activation, because direct activation of the APC can be induced by inflammatory components of the pathogen (Ahmed et al., 1988; Buller et al., 1987; Liu and Mullbacher, 1989; Shedlock et al., 2003). On the other hand, non-inflammatory components, like for example osmotically loaded cells, were thought to be dependent on helper T cells because of the lack of stimulatory components (Bennett et al., 1997b). But more exact studies revealed that one has to strictly discriminate between primary and secondary immune responses when speaking about helper dependency. The observations of the past were biased, since the helper dependency of weak antigens was often measured after secondary restimulation, whereas for strong virus or bacterial induced immune responses the primary response was analyzed. Recent studies have shown that for almost all non-inflammatory or inflammatory immunogens absence of helper T cells during the priming phase does not alter the immune responses significantly. For example, the group of Shen compared primary $CD8^+$ T cell responses after immunization with a recombinant vaccinia virus (rVV33). They did not see differences in the immune responses when they compared mice that were either depleted of their $CD4^+$ T cells or not (Shedlock and Shen, 2003; Sun and Bevan, 2003). The same was true when comparing primary immune responses of MHC class II deficient mice that were immunized with a low dose of *Listeria monocytogenes*; both wildtype and $MHCII^{-/-}$ mice showed equal numbers of antigen specific $CD8^+$ T cell responses (Shedlock and Shen, 2003; Sun and Bevan, 2003). Still, there is data showing that in some experimental settings $CD4^+$ T cell help is required during the priming phase; for example infection with herpes simplex virus type-1 (HSV) lead to significantly decreased antigen specific $CD8^+$ T cell frequencies in $MHCII^{-/-}$ mice compared to wildtype animals (Behrens et al., 2004).

But the special necessity of helper T cells was demonstrated when secondary immune responses to various immunogens were analyzed. To follow up the example described above, mice that were infected with *L.m.* or rVV33 and challenged with the same organism or boosted with recombinant *Listeria monocytogenes* (rLm33), respectively, showed reduced $CD8^+$ memory T cell responses when $CD4^+$ T cells were absent during the priming phase

(Shedlock and Shen, 2003; Sun and Bevan, 2003). That are only two examples among many that show that absence of $CD4^+$ T cell help during the priming phase does not seem to influence primary immune responses in terms of quantity. The quality of the immune response, however, seems to be heavily influenced by $CD4^+$ T cell help demonstrated by the crucial impact on the generation of long lasting memory $CD8^+$ T cells. The absolute requirement of $CD4^+$ T cells during the priming phase of $CD8^+$ T cell responses for effective memory T cell generation was further strengthened by experiments showing that the absence of $CD4^+$ T cells during secondary infection did not negatively effect memory $CD8^+$ T cell responses (Shedlock and Shen, 2003; Sun and Bevan, 2003). In the case of challenging mice with *L.m.,* $CD4^+$ T cell depletion during the challenge did even lead to increased $CD8^+$ T cell responses (Kursar et al., 2002). Bringing in the notion that $CD4^+$ T cells do not only support the $CD8^+$ T cell response but also have a function in regulating the latter, the complexity of $CD4^+$ T cell interactions with other cell populations becomes apparent. But in which way do $CD4^+$ T cells interact?

In defining factors that are involved in providing helper signals, cytokines were first thought to play a major role. One crucial cytokine, by which $CD4^+$ T-cells were thought to provide help, was IL-2. But this had to be reconsidered with the finding that CTL immunity to viruses could be generated in IL-2 knockout mice. Consequently, three different groups sought to find a cellular interaction molecule by which $CD4^+$ T cells execute their function. They found the interaction of CD40 with CD40L on APC to explain T cell help (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). With these findings the first model of CTL, APC and helper T cell interaction was described. It was proposed that activated $CD4^+$ T cells "license" APCs via CD40 (expressed on APCs) – CD40L (expressed on helper T cells) interaction to prime $CD8^+$ T cells. First, antigen specific interaction with APC activates $CD4^+$ T cells. Then activated $CD4^+$ T cell could in turn license several APC, which finally prime CTL. As a consequence, in this model it was not required that CTL and helper T cells had to recognize antigen on the same APC. The group of Tanchot argued that this was not a valid model. It is assumed that it takes about the same time for $CD4^+$ and $CD8^+$ T cells to get activated by APC and that it takes T cells about 24h to start to proliferate and another 2 to 3 days to migrate from the priming node to other sites. Therefore, a sequential interaction of APC, $CD4^+$ and $CD8^+$ T cell would not be in agreement with the priming kinetics of $CD8^+$ T cells (Behrens et al., 2004). To explain these conflicting results, another model was described, in which $CD4^+$ and $CD8^+$ T cells recognize antigen on the same APC. Thus, one $CD4^+$ T cell licenses one DC, which in turn leads to priming of one $CD8^+$ T cell.

It has been demonstrated that interaction between CD40 expressed on DCs and CD40L expressed on $CD4^+$ T cells led to activation of APCs. However, APCs from $CD40^{-/-}$ mice raised similar $CD8^+$ T cell responses than APC from wildtype mice showing that the helper effect was unaffected. Thus, the participating cellular partners of CD40 interaction had to be different. By demonstrating that mRNA levels of CD40 were upregulated in $CD4^+$ and $CD8^+$ T cells after activation, CD40-CD40L was proposed to happen via CD40 expressed on $CD8^+$ T cells and CD40L expressed on $CD4^+$ T cells (Bourgeois et al., 2002). The basic importance of CD40-CD40L interaction in providing helper signals remained unquestioned and was further strengthened by anti-CD40 monoclonal antibody treatment. In several studies treatment with anti-CD40 monoclonal antibody could restore $CD8^+$ T cell priming when helper T cells were absent. Yet, at the beginning of this year a third model for helper function has been described. In that model DCs activate $CD4^+$ T cells, which in turn get APCs themselves (Th-APCs). This is enabled by acquisition of synapse composed MHC class II, CD54 and CD80 molecules as well as bystander MHC class I – peptide complexes from DCs. The resulting Th-APC can activate other $CD4^+$ T cells to become Th-APCs and importantly directly prime $CD8^+$ T cells (Xiang et al., 2005).

Many advances in understanding T cell help have been achieved during the last years. Still many questions remain open. With the development of new bright cell dyes and advances in intra vital microscopy we will be able to investigate interactions of $CD4^+$ T cells in more detail. Recent and new models will have to prove themselves under these experimental conditions.

1.8. Regulatory T cells

Although negative selection of T lymphocytes in the thymus prevents emigration of most autoreactive T cells into the periphery, there are still other regulatory mechanisms to ensure inactivation of autoreactive T cells that might have escaped negative selection or that might get activated during immunmodulatory therapies. One specialized cell population that seems to play a dominant role to inhibit the activation of T cells by self-peptides are regulatory T cells (Treg). Treg cells still cannot be fully discriminated from normal naive or effector T cells, because there don't exist surface markers which are 100 percent specific for Treg cells. The majority of $CD4^+$ Treg cells expresses CD25 (IL-2Rα chain), CD62L, CD103, CD152, CTLA-4 and GITR (glucocorticoid-induced TNF receptor family-related protein) (Fehervari and Sakaguchi, 2004). Recently, the discovery of Foxp3, a member of the forkhead transcription factor family, identified a more distinct marker of Treg cells. Experiments using

mice with targeted deletion of Foxp3 showed that in the absence of Foxp3 no development of Treg cells could be observed (Cantor, 2004; Fontenot et al., 2003; Khattri et al., 2003). Extensive studies have been made to further characterize Treg populations, and so far two major groups of Treg cells have been distinguished, naturally occurring Treg cells and antigen-induced regulatory T cells.

1.8.1. Naturally occurring Treg cells

After their development in the thymus, natural Treg cells are found in peripheral tissues, where their main function might be to prevent activation of self-reactive T cells. Treg function has been described to be dependent on co-stimulatory signals like CD28-CD80/CD86 and CD40-CD40L, and their polyclonal TCR repertoire indicated that they can interact with a variety of different ligands, most likely self-peptide presenting MHC molecules (Belkaid and Rouse, 2005; Kumanogoh et al., 2001; Salomon et al., 2000). Co-stimulation seems to be required at two stages; first Treg cells need TCR ligation in combination with co-simulation to develop from their immature precursors into mature Treg cells. Second, CD28 seems to be crucial to maintain a stable pool of Treg cells in the periphery by promoting their self-renewal through homeostatic proliferation. In addition, survival of naturally occuring Treg cells seems to be dependent on IL-2, since IL-2R deficient mice lack Treg cells. Naturally occurring Treg cells can be discriminated from adaptive Treg cells (see below) by constitutive expression of CD25, whereas this expression is variable on adaptive Treg cells. Also in contrast to adaptive Treg cells, their function is relatively independent of IL-10 and only partially dependent on TGF (Oldenhove et al., 2003).

1.8.2. Antigen-induced regulatory $CD4^+$ T cells

Antigen-induced regulatory $CD4^+$ T cells also originate from the thymus, and it has been proposed that they mainly function to maintain homeostatic control over various adaptive immune responses. They might play a role in maintenance of self-tolerance, even though this has not yet been conclusively demonstrated. In contrast to naturally occuring Treg cells, antigen-induced regulatory $CD4^+$ T cells are generated in the periphery upon stimulation. Based on the activation signals it has been proposed that they can develop either from classical T cell subsets or eventually also from naturally occurring Treg cells. Antigen-induced regulatory $CD4^+$ T cells, unlike natural Treg cells, seem not to need co-stimulation to develop or survive. Instead, once activated they seem to need further activation through antigen for survival, a term called cell-contact dependency, because of the requirement of

physical contact between antigen-induced regulatory $CD4^+$ T cells, APC and the responding T cell. Survival of antigen-induced regulatory $CD4^+$ T cells also seems to be dependent on IL-2. Furthermore, antigen-induced regulatory $CD4^+$ T cells can be further discriminated via their profile of cytokine secretion; "Tr1 cells" produce high levels of IL-10 and TGF□, and require IL-10 for their differentiation and function. On the other hand there are Th3 cells that produce IL-10, TGF□ and IL-4 and need TGF□ for suppressive function (Oldenhove et al., 2003).

So far three models for the mechanism of suppression by antigen-induced regulatory $CD4^+$ T cells have been described, even though it is not clear if this suppression mechanisms can act singularly or if it is the sum of simultaneous activation of all three mechanisms that lead to suppression. The first model describes that after antigen-specific recognition of the target DCs, regulatory T cells function via inhibition of up-regulation of the co-stimulatory molecules CD40, CD80/86 on DCs (Liu et al., 1999; Vigouroux et al., 2004). A second model for activation of regulatory T cells stresses the importance of TGF□ and IL-10 secretion. IL-10, which can affect several signaling pathways in DCs, leads to inhibited secretion of proinflammatory cytokines by monocytes, macrophages and immature DCs, just to name a few effects (Groux et al., 1997).

A third mechanism of suppression has been found for $CD4^-CD8^-$ regulatory T cells, which kill effector $CD8^+$ T cells (the model is described for $CD8^+$ T cells responsible for rejection after allotransplantaion) by FAS-FASL interaction (Zhang et al., 2000).

1.9. Listeria

Listeria monoctogenes (*L.m.*) is a gram-positive bacterium, and is probably one of the best characterized human pathogens. *L.m.* has been extensively used to study and understand both the innate and the adaptive immune system. One of the advantages is that the infection can be easily monitored. Adaptive immunity after infection with *L.m.* is characterized by a dominant MHC class I mediated $CD8^+$ response, which peaks at day 7/8 after primary infection. The MHC class I epitopes that are known to induce $CD8^+$ T cell responses in mice on an $H\text{-}2K^d$ background are the dominant $LLO_{91\text{-}99}$ and the subdominant $p60_{217\text{-}225}$ epitope, whereas the dominant MHC class II restricted $LLO_{188\text{-}201}$ response has been identified in $H\text{-}2^b$ mouse strains. LLO plays a crucial role in the escape of bacteria from the phagosomal compartment, and is important for spreading of the bacteria in neighboring cells. P60 is a hydrolase involved in bacterial septation. The bacterial burden after primary infection with a sublethal dose of *L.m.* (approximately 0.1 x LD_{50}) peaks at day 3, and after steady decrease no bacteria are detectable at day 7 in an immunocompetent host. Both □/□ and □/□ $CD8^+$ T cells have an

essential role in clearance, although the effect is more pronounced for the γ/δ population. Another cell type that seems to be involved in killing of *Listeria* are neutrophils.

Due to the induction of a pool of effective memory $CD8^+$ T cells the immune responses after re-encounter with *Listeria* are much more rapid; $CD8^+$ T cell recall responses after infection with a high dose of *L.m.* (approximately 10 x LD_{50}) peak at day 5 and viable bacteria are usually cleared by day 2 in an immunocompetent host.

L.m. is a well-established infectious model, therefore infection with *L.m.* is widely used to better understand the mechanisms involved in generation of innate immunity and the linkage between innate and adaptive immunity. For example, infection with *L.m.* results in characteristic cytokine release; the cytokines IL-12 and IL-18 are produced by macrophages, which synergistically induce NK cells and dendritic cells to produce IFNγ. This leads to subsequent activation of macrophages and neutrophils to kill *L.m.* via production of listericidal molecules, such as NO. To study the involvement of these cytokines in host defense against *L.m.*, IL-12, IL-18 and IFNγ knockout mice were used and infected with low dose of *L.m.* Studies demonstrated that the knockout mice were highly susceptible to infection with *L.m.*, thus proposing a role of IL-12, IL-18 and IFNγ in the early host defense against this pathogen (Edelson and Unanue, 2002; Seki et al., 2002). *Listeria* infection was also used to understand the role of different TLRs in innate immunity. For this purpose different TLR knockout mice were infected with *L.m.* and their ability to clear the infection and establish primary as well as long-lasting immune responses was compared. TLR2 and TLR4 knockout mice for example could clear bacteria as efficiently as wildtype animals after primary infection with low dose of *Listeria*, but is was shown that TLR2 and TLR2/4 knockout mice showed reduced serum levels of IFNγ and IL-12. These results suggest that TLR2 might be involved in IL-12 dependent IFNγ-production after *L.m.* infection, but also show that in the absence of TLR2 other molecules seem to be sufficient for control of *L.m.* infection (Edelson and Unanue, 2002; Seki et al., 2002).

A molecule that plays a major role in nearly all TLR signaling pathways, is MyD88. To further access the role of TLRs in innate immunity, MyD88 knockout mice were infected with *L.m.* In the absence of MyD88, mice were highly susceptible to *L.m.* infection. MyD88 is also known to play a critical role in IL-18R-mediated IL-18 signaling that induces IFNγ-production from IL-12-stimulated innate or acquired immune cells, and it was shown that MyD88 knockout mice lack IL-12 and IFNγ-production. Thus, it can be concluded that one of the crucial roles of TLRs in innate immunity after *L.m.* infection is to provide soluble factors like IFNγ for efficient bacterial clearance (Edelson and Unanue, 2002; Seki et al., 2002).

1.10. Aim of this PhD work

Soluble antigens, like synthetic peptide-epitopes or purified proteins are usually characterized by poor $CD8^+$ T cell immunogenicity.

With this thesis work, I addressed the question of whether $CD8^+$ T cell immunogenicity towards soluble proteins can be improved by the adjuvant activity of Toll-like receptor (TLR) ligands, and to what extend $CD4^+$ T cell help or regulatory T cells further modulate immune responses under those conditions.

In order to achieve this goal, the following experimental tools and systems had to be generated and explored:

1) Whereas synthetic peptide-epitopes could be purchased from industrial providers, purified proteins from the target pathogen - *Listeria monocytogenes* (*L.m.*) - needed to be newly generated. Listeriolysin O (LLO) was purified from recombinant overexpression in *E.coli*. In addition, I generated a recombinant fusion protein in which LLO is covalently linked to the TLR2 ligand V7. Because of the lytic activity LLO has in higher concentrations on cells, I mutated the recombinant LLO proteins towards non-lytic forms, the $LLO_{492T\ A}$ and the $LLO_{492T\ A}$-V7.

2) To determine the influence of antigen size (peptide versus full protein), adjuvant (CpG or V7), and the route of application, extensive immunization and dose titration experiments following subcutaneous and systemic (intravenous) administration had to be performed.

3) Measurement of the antigen-specific $CD8^+$ T cell priming and memory T cell generation/ differentiation was done by MHC multimer analysis and intracellular cytokine stainings. Basically all necessary reagents (like MHC multimers as well as the development of multicolour flow cytometry) were self generated and established for this purpose.

4) Analysis of the dependency of $CD4^+$ T cell help on $CD8^+$ T cell priming was performed by $CD4^+$ T cell depletion or experiments in MHC class II knockout mice. Therefore, in house antibody generation (GK1.5) and mouse breeding was required.

5) The influence of $CD25^+$ regulatory T cells on $CD8^+$ T cell priming was analyzed by anti-CD25 antibody mediated depletion.

6) Effector functions, especially cytotoxicity and protection capacity, were measured by in vivo cytotoxicity assays and infection with living *L.m.*

2. MATERIAL AND METHODS

2.1. Material

2.1.1. Equipment

Affinity chromatograph	Äkta Prime, Amersham Biosciences Europe GmbH	Freiburg, Germany
Flow cytometer (Cyan)	Daco Cytomation	Freiburg, Germany
Centrifuge	Biofuge fresco, Heraeus	Hanau, Germany
Centrifuge	Sorvall® RC 26 Plus	Hanau, Germany
Centrifuge	Varifuge 3.0RS, Heraeus	Hanau, Germany
Flow cytometer (FACSCalibur)	BECTON DICKINSON	Heidelberg, Germany
FPLC	Amersham Biosciences Europe GmbH	Freiburg, Germany
Heating block	Thermomixer compact	Eppendorf
Laminar flow hood	HERA safe, Heraeus	Hanau, Germany
Incubator	Cytoperm 2, Heraeus	Hanau, Germany
Microscope	Axiovert S 100, Zeiss	Jena, Germany
Neubauer counting device	Schubert	Munich, Germany
Photometer	BioPhotometer	Eppendorf
Shaker	Multitron® Version 2, INFORS AG	Bottmingen, Switzerland
Thermocycler	T3 Thermocycler, Biometra®	Göttingen, Germany
Waterbath	Lauda ecoline 019, LAUDA Dr. R. Wobster GmbH & co. KG	Lauda-Königshofen, Germany

2.1.2. Lab ware

Cell strainer (100mm Nylon)	BD Falcon™	BD Biosciences, Belgium
Nylon filter	Hopfinger	Munich, Germany
Syringe (2 ml)	BD Discardit™ II	BD Labware, France
Tissue culture dish (60x15mm)	Easy Grip™	BD Labware, France
24 well plate	NUNC	NUNC A/S, Denmark
96 well plate	NUNC	NUNC A/S, Denmark

2.1.3. Reagents

Acrylamide/Bis 30%	Biorad	Munich, Germany
β-Mercaptoethanol	Invitrogen	Paisley, U.K.
Bovine Serum (BSA)	SIGMA- ALDRICH	Steinheim, Germany
Kanamycin	SIGMA- ALDRICH	Steinheim, Germany
CpG-DNA (1668)	TIB Molbiol	Berlin, Germany
Cytofix/Cytoperm	BD Biosciences, Pharmingen	San Diego, USA
DNAse I	SIGMA- ALDRICH	Steinheim, Germany

DMSO	SIGMA- ALDRICH	Steinheim, Germany
Ethidiummonazide bromide (EMA)	GIBCO™, Invitrogen Corporation	Paisley, U.K.
FETAL CALF SERUM	seromed®, Biochrom AG	Berlin, Germany
Gentamycin	GIBCO™, Invitrogen Corporation	Paisley, U.K.
HEPES	GIBCO™, Invitrogen Corporation	Paisley, U.K.
1kb DNA ladder	Life Technologies	Paisley, U.K.
Ethidiumbromide	Life Technologies	Paisley, U.K.
Golgi plug	BD Biosciences, Pharmingen	San Diego, USA
IPTG	SIGMA- ALDRICH	Steinheim, Germany
Leupeptin	SIGMA- ALDRICH	Steinheim, Germany
L-Glutamin	GIBCO™, Invitrogen Corporation	Paisley, U.K.
Lysozyme	SIGMA- ALDRICH	Steinheim, Germany
Mutagenesis Kit	Stratagene	Amsterdam, Netherlands
Ni-NTA agarose beads	QIAGEN GmbH	Hilden, Germany
PBS	Biochrom AG	Berlin, Germany
Penicillin	GIBCO™, Invitrogen Corporation	Paisley, U.K.
Pepstatin	SIGMA- ALDRICH	Steinheim, Germany
PermWash	BD Biosciences, Pharmingen	San Diego, USA
pET-27b vector	MERCK BIOSCIENCES GmbH	Bad Soden, Germany
PFA	SIGMA- ALDRICH	Steinheim, Germany
Pfu DNA polymerase	Stratagene	Amsterdam, Netherlands
QIAprep Spin Miniprep Kit	QIAGEN GmbH	Hilden, Germany
QIAquick gel Extraction Kit	QIAGEN GmbH	Hilden, Germany
RPMI 1640	Gibco Life Technologies	Gaithersburg MD, USA
See Blue 2x protein ladder	Invitrogen	Paisley, U.K.
Streptavidin-PE	Invitrogen	Paisley, U.K.
Streptomycin	GIBCO™, Invitrogen Corporation	Paisley, U.K.
T4 DNA Ligation Kit	Life Technologies	Paisley, U.K.
TA Cloning Kit	Invitrogen	Paisley, U.K.
Temed	Biorad	Munich, Germany
Trypan Blue solution	SIGMA- ALDRICH	Steinheim, Germany
X-Gal	Life Technologies	Paisley, U.K.

2.1.4. Competent cells

BL21 Codon (DE3) Plus	Stratagene	Amsterdam, Netherlands
One Shot Top 10 Kit	Invitrogen	Paisley, U.K.

2.1.5. Antibodies

Antibody	*Clone*	*company*
Fc block (rat anti-mouse CD16/CD32)	2.4 G2	BD Biosciences, Pharmingen, San Diego, USA
Rat anti-mouse CD4 PerCp	L3T4	BD Biosciences, Pharmingen, San Diego, USA
Rat anti-mouse CD8 PE		BD Biosciences, Pharmingen, San Diego, USA
Rat anti-mouse CD8 APC	CT-CD8a	Caltag Laboratories GmbH, Hamburg, Germany
Rat anti-mouse CD62L APC	MEL-14	BD Biosciences, Pharmingen, San Diego, USA
Rat anti-mouse CD62L FITC	MEL-14	BD Biosciences, Pharmingen, San Diego, USA
Rat anti-mouse CD127 APC	A7R34	eBioscience
Rat anti-mouse IFN	XMG1.2	BD Biosciences, Pharmingen, San Diego, USA
Rat anti-mouse TNF	MP6-XT22	BD Biosciences, Pharmingen, San Diego, USA
Rat IgG1 isotype control immunglobulin	R3-34	BD Biosciences, Pharmingen, San Diego, USA

2.1.6. Tetramers

MHC-I Tetramers for the detection of Ag-specific $CD8^+$ T cells were routinely produced at the laboratory of Prof. Busch according to well-established protocols (Busch et al., 1998b). Depending on the mouse inbred strain and the respective MHC-allele, the following peptide loaded MHC-I Tetramers were used:

H2-K^d#45/ m$_2$m/ LLO_{91-99} PE (BALB/c)

H2-K^b#45/ m$_2$m/ $OVA_{257-264}$ PE (BL6)

2.1.7. Peptides (Affina Immuntechnik GmbH, Berlin, Germany)

LLO_{91-99} (1mg/ml): GYKDGNEYI

$LLO_{188-201}$ (1mg/ml): RWNEKYAQAYPNVS

$OVA_{257-264}$ (1mg/ml): SIINFEKL

2.1.8. Solutions

Ammoniumchloride - Tris

NH_4Cl	0.17M
Tris-HCl (pH 7.5)	0.17M

The ratio NH_4Cl : Tris-HCl is 9 : 1.

10mM and 200mM Imidazol (in PBS, pH 8.0)

The 10mM and 200mM solutions are diluted from a 1M solution:

Imidazol	8.51g
dH_2O	125ml

2.1.9. Medium

Mini Expression medium

LB Medium	20ml
Glucose (40%)	200µl
Kanamycin (stock 30mg/ml)	20µl

CTG medium (in 1L dH_2O)

$CaCl_2$ x $2H_2O$	7.35g
Glycerol	100ml
Thymidine	50mg

The medium was autoclaved and stored at 4°C.

Complete DMEM medium

DMEM	500ml
10% FSC	50ml
5% SC+	25ml

Complete RPMI medium (RP10+)

RPMI	500ml

10% FSC	50ml
5% SC+	25ml

SC+ cell culture supplement (in 1L RPMI medium)

-Mercaptoethanol	1ml
Gentamycin (50mM)	20ml
HEPES	23.83g
L-Glutamin	4g
Penicillin/Streptomycin	200ml

SOC medium

SOB	100ml
20% glucose	2ml

NZY medium (in 500ml ddH_2O, pH 7.5)

NaCl	2.5g
$MgSO_4$ x $7H_2O$	1g
Yeast extract	2.5g
NZ amine	5g

2.1.10. Buffers

FACS buffer (in 1L PBS, pH 8.0)

0.5% BSA	5g
0.02% NaN_3	660 l

Lysis buffer (in 200ml PBS, pH 8.0)

50mM HEPES	10ml
150mM NaCl	1.75g
1% Triton-X-100	2ml
Lysozym	2mg/ml

4x running buffer (in 2L dH_2O)

Tris	24g

Glycine	115.2g
SDS	8g

5x TBE buffer (in 2L dH_2O, pH 8.0)

Tris Ultra	108g
Boric acid	55g
EDTA (0.5M)	40ml

2x Sample buffer (SB)

dH_2O	10ml
0.5M Tris pH 6.8	10ml
10% SDS	20ml
Glycerol	10ml
DTT	1.543g
Bromphenole blue	0.1g

2.1.11. Gels

Running gel (SDS PAGE)

dH_2O	7ml
1.5M Tris pH 8.8	4.38ml
30% Acryl 1% bis	5.8ml
10% SDS	170µl
Temed	8.8µl
10% fresh APS	170µl

Stocking gel (SDS page)

dH_2O	6.2ml

0.5M Tris pH 6.8	2.5ml
30% Acryl 1% bis	1.2ml
10% SDS	100µl
Temed	5µl
10% fresh APS	100µl

Agarose gel

Agarose	0.45g
TBE buffer	40ml
ethidium bromid	1µl

2.2. Methods

2.2.1. Protein production

To generate LLO and p60 protein from *Listeria monocytogenes*, cDNA of the wanted proteins was extracted from *Listeria monocytogenes*. This cDNA was amplified and cloned into an expression vector (BL21 (CD3) CP) to be able to recombinantly express the proteins in *E.coli*.

2.2.1.1. DNA purification from **L.m.** *colony*

To generate *Listeria*-derived proteins, DNA from *Listeria* cultures was purified. For that purpose 20µl of the isolates *Listeria monocytogenes* (*L.m.*) 10403s from ATCC (Rockville, USA) were inoculated in 6ml BHI medium grown until the optical densitiy reached an OD_{600nm} of 0.05- 0.1. Bacteria were then diluted with PBS to a cell suspension of $5x10^3$ cells/ml and 10µl of this suspension was plated out on BHI plates and incubated (37°C, overnight). The next day, a single colony was picked with a Q-tip and transferred to an Eppendorf tube containing 1ml of NaCl. The suspension was then diluted to MacFarland of 1 and then centrifuged (5', 7500rpm). Thereafter the supernatant was discarded and the pellet was stored at –20°C. The DNA was extracted using the QIAamp DNA-Minikit following the manufacturer's protocol for tissue preparation.

2.2.1.2. Primer design for LLO and p60

Following primers were designed for the *Listeria*-derived proteins LLO, $LLO_{492A\to T}$ and p60.

LLO primer 1:	5' GGCTCGAGTTCGATTGGATTATCTACACT 3'
LLO primer 2:	5' GGGGATCCAGATGCATCTGCATTCAATAAA 3'
$LLO_{492A\to T}$ primer 1:	5' GGTTTAGCTTGGGAATGGGCGAGAACGGTAATTGATG 3'
$LLO_{492A\to T}$ primer 2:	5' CATCAATTACCGTTCTCGCCCATTCCCAAGCTAAACC 3'
p60 primer 1:	5' GGGATCCGAGCACTGTAGTAGTCGAAGCTGG 3'
p60 primer 2:	5' GGCTCGAGTACGCGACCGAAGCCAAC 3'

The nucleotide sequence of LLO and p60 can be looked up at www.nbci.nlm.nih.gov, entries U25452 and AF500184 respectively. The primers were designed using the 2+4 rule, meaning that the annealing temperature of the primers can be calculated using their amount of GC/AT. Per C/G within the sequence 4°C and per A/T 2°C are calculated. Optimally, the annealing temperature should be between 55°C and 68°C and the amount of GC should be between 40 and 60%.

2.2.1.3. PCR preparation

The PCR samples were prepared as follows:

Primer 1 (1:10 diluted)	2.5µl
Primer 2 (1:10 diluted)	2.5µl
dNTPs	5µl
10x puffer	5µl
DNA	1µl
pfu polymerase	1µl
dH_2O	33µl

annealing:	94°C	5’
	80°C	2’ (hot start)
elongation:	94°C	2’
	56°C	2’
	72°C	2.5'
end:	72°C	5’
	4°C	∞

The elongation step was repeated for 30 times.

After the PCR a poly-A tail was added. The vector pCR2.1 used in the subsequent cloning step contains single 3’ deoxythymidine (T) residues and can therefore ligate efficiently with the 3’ A-overhangs of the PCR inserts. For that purpose the PCR product was incubated with 5µl dATPs and 1µl Taq Polymerase for 10’ at 72°C.

2.2.1.4. Agarose gel

DNA fragments can be separated through agarose gel electrophoresis, because negatively charged DNA segments migrate towards the anode under the influence of the applied electric field. This migration leads to separation of DNA fragments according to their size. To visualize the DNA fragments an intercalating substance (ethidiumbromid) is added to the gel. The fluorescent DNA bands can then be detected by UV-light at 280nm.

50µl of the PCR product (after Taq step) was mixed with 10µl 6x Orange G Dye and 30µl of this mix was loaded on an agarose gel. 5µl of a 1kb DNA ladder was loaded to mark the size of the bands.

2.2.1.5. Gel extraction

To use the DNA for further cloning steps, the DNA band was cut out of the agarose gel and extracted from the gel using the Qiagen gel extraction kit following the manufacturers protocol.

2.2.1.6. Ligation of PCR product with plasmid vector

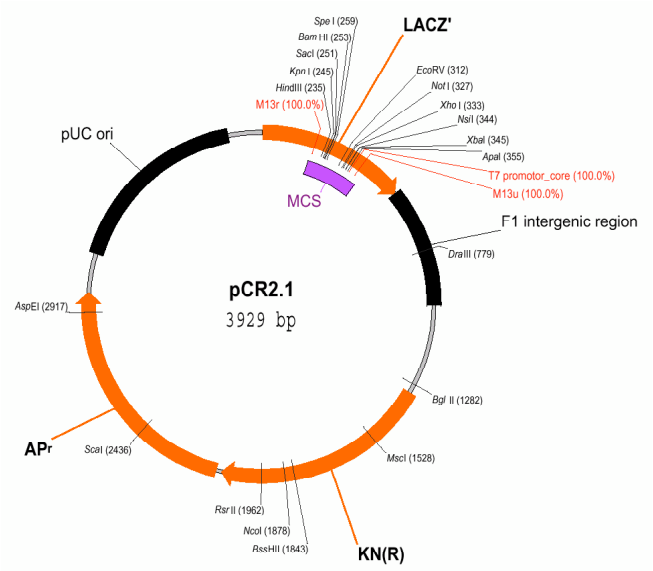


Figure 1: pCR2.1 vector map

Through the TA-ligation the PCR product can be inserted directly into a plasmid vector.

The ligation was performed as followed:

ddH_2O	4µl
10x Ligation buffer	1µl
pCR 2.1 vector	2µl
PCR product	2µl
T4 DNA Ligase	1µl

The mix was incubated overnight at 14°C.

2.2.1.7. Transformation into One Shot competent cells

To test if the TA-ligation was successful a transformation of the ligation product in One Shot competent cells was performed:

One Shot competent cells	50µl
Ligase product	5µl

DNA was transferred into cells by heat shock (30'', 42°C), followed by incubation on ice for 30 minutes. 250µl SOC medium was then added to the cells and the mix was incubated for 1 hour at 37°C.

LB agar plates containing ampicillin were coated with 40µl X-Gal. The transformation mix was then plated onto the plates and incubated overnight at 37°C. The vector pCR2.1 contains a gene (*lacZ*- gene) coding for the enzyme β-galactosidase. This enzym can transform X-Gal into a blue dye. If the ligation worked the gene for the β-galactosidase is destroyed and the colonies are white. Without the insert the β-galactosidase can be expressed and the colonies are blue. White colonies were picked and grown in 6ml LB medium containing 6µl ampicillin. The DNA was extracted via a mini preparation following the manufacturers QIAprep Spin Miniprep Kit protocol.

2.2.1.8. Enzymatic digest

Since the PCR product can be inserted into the vector in either orientation, the plasmid was analyzed by restriction mapping for orientation. The correct recombinant plasmid was then purified and used for further subcloning. Another reason for the digestion is to linearize the PCR2.1 vector containing the DNA product to make it suitable for cloning in the expression vector pET27b. To do so, also the pET27b vector was digested to create a cloning site. For our experiments the enzymes XhoI and BamHI were used because they comprise unique cloning sites in both the pET27b vector and the pCR2.1 vector.

XhoI digest

dH_2O	5.5µl
TA-Ligation product	
or pET27b vector	3µl
Reaction buffer 2	1µl
XhoI	0.5µl

The digest was incubated overnight at 37°C. The next day a buffer exchange was performed following the QIAquick PCR Purification Kit Protocol. At the end the DNA was eluted in 25µl dH_2O.

BamHI digest

DNA	25µl
Reaction buffer 3	3µl
BamHI	2µl

The digest was incubated for 3 hours before the product was run over an agarose gel to confirm the expected size.

2.2.1.9. Subcloning of DNA in expression vector

dH_2O	5µl
vector	3µl
5x DNA dilution buffer	2µl
2x T4 DNA ligation buffer	10µl
T4 DNA ligase	1µl

The mix was incubated for 15 minutes at room temperature. After that it was kept on ice until the transformation followed.

2.2.1.10. **E. coli** *DH5α competent cells*

The transformation was performed using DH5α competent cells. To generate DH5α competent cells, 2µl of a stock of these cells mixed with 100µl LB medium, were plated out and incubated overnight at 37°C.

The next day single colonies were incubated overnight in 5ml LB medium in a shaker at 37°C. This cell suspension was used to inoculate 500ml of LB medium and was grown until it reached an OD_{590} of 0.3. The whole culture was immediately cooled on ice and centrifuged (5', 5000rpm, 4°C). The supernatant was discarded and the pellet resuspended in 125ml ice-cold CTG medium and was incubated on ice for 20 minutes. Subsequently, the cells were centrifuged again and the supernatant was discarded. The pellet was resuspended gently in 40ml CTG medium, and aliquots were immediately shock frozen in liquid nitrogen before they were stored at –80°C.

2.2.1.11. Transformation into DH5α competent cells

DH5α competent cells	100µl
Ligation product	10µl

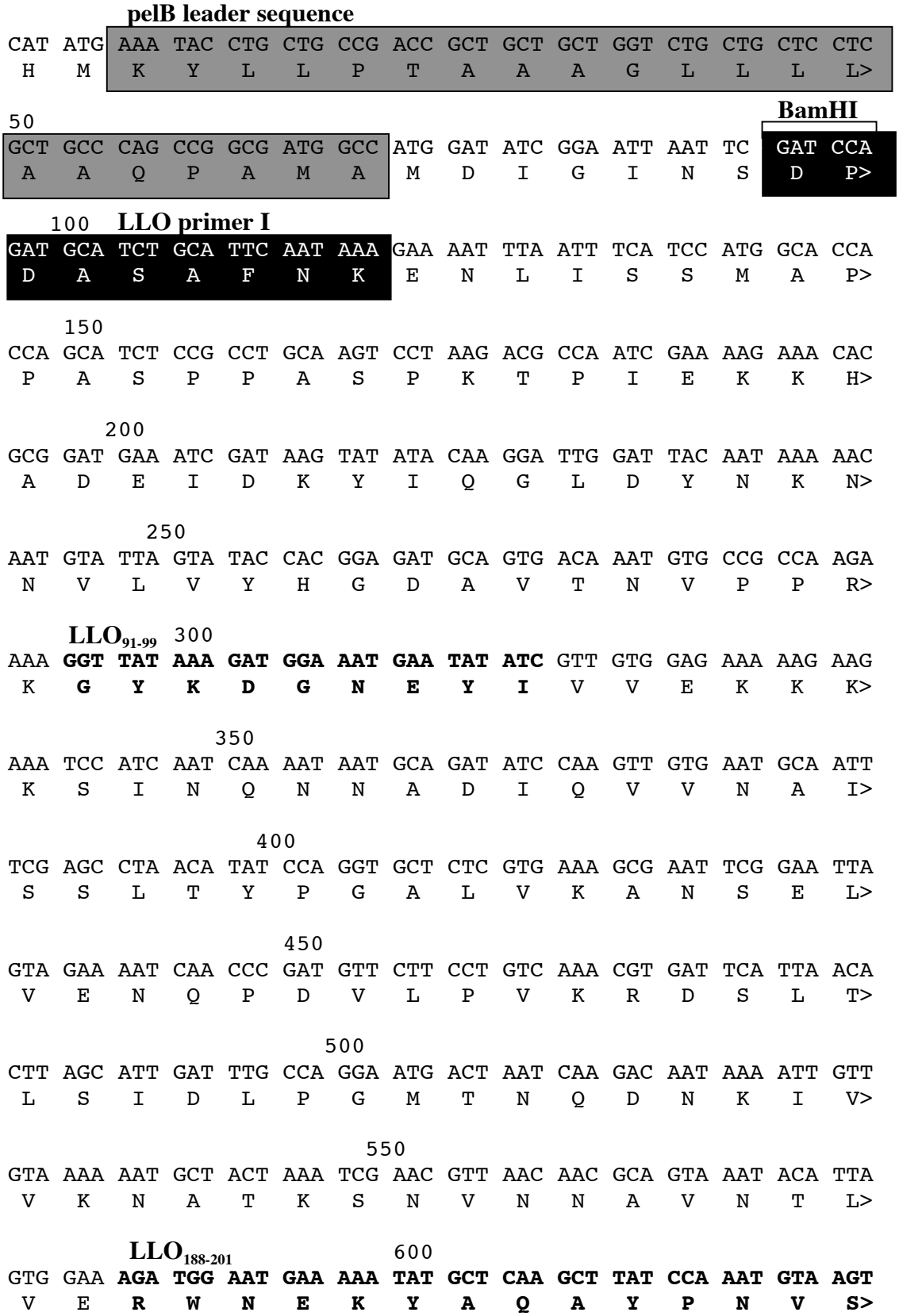
The mix was incubated for 30 minutes on ice, followed by a heat shock treatment (1', 42°C). After that the mix was incubated on ice (2') before adding 150µl NZY medium and incubating it for 1 hour at 37°C. The mix was then plated on LB agar plates that contained kanamycin.

After an overnight incubation at 37°C single clones were picked at the next day and incubated in 6ml LB medium containing 6µl kanamycin (37°C, overnight). The next day, a mini preparation was made as described before and the DNA was digested (2-3 hours, 37°C) to search for positive clones:

dH_2O	5µl
DNA	3µl
Reaction buffer 2	1µl
XhoI	0.5µl
BamHI	0.5µl

Sequencing of the positive clones led to following sequences:

LLO sequence:



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                                650
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 A   K   I   D   Y   D   D   E   M   A   Y   S   E   S   Q   L>

                                    700
ATT GCA AAA TTT GGT ACG GCA TTT AAA GCT GTA AAT AAT AGC TTG AAT
 I   A   K   F   G   T   A   F   K   A   V   N   N   S   L   N>

                                        750
GTA AAC TTC GGC GCA ATC AGT GAA GGG AAA ATG CAA GAA GAA GTC ATT
 V   N   F   G   A   I   S   E   G   K   M   Q   E   E   V   I>

                                            800
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 S   F   K   Q   I   Y   Y   N   V   N   V   N   E   P   T   R>

                                                850
CCT TCC AGA TTT TTC GGC AAA GCT GTT ACT AAA GAG CAG TTG CAA GCG
 P   S   R   F   F   G   K   A   V   T   K   E   Q   L   Q   A>

                                                    900
CTT GGA GTG AAT GCA GAA AAT CCT CCT GCA TAT ATC TCA AGT GTG GCA
 L   G   V   N   A   E   N   P   P   A   Y   I   S   S   V   A>

                                                        950
TAT GGC CGT CAA GTT TAT TTG AAA TTA TCA ACT AAT TCC CAT AGT ACC
 Y   G   R   Q   V   Y   L   K   L   S   T   N   S   H   S   T>

                                                            1000
AAA GTA AAA GCT GCT TTT GAC GCT GCC GTA AGT GGG AAA TCT GTC TCA
 K   V   K   A   A   F   D   A   A   V   S   G   K   S   V   S>

                                                                1050
GGT GAT GTA GAA CTG ACA AAT ATC ATC AAA AAT TCT TCC TTC AAA GCC
 G   D   V   E   L   T   N   I   I   K   N   S   S   F   K   A>

                                                                    1100
GTA ATT TAC GGT GGC TCC GCA AAA GAT GAA GTT CAA ATC ATC GAC GGT
 V   I   Y   G   G   S   A   K   D   E   V   Q   I   I   D   G>

                                                                        1150
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 N   L   G   D   L   R   D   I   L   K   K   G   A   T   F   N>

                                                                            1200
CGG GAA ACA CCA GGA GTT CCC ATT GCC TAT ACA ACA AAC TTC TTA AAA
 R   E   T   P   G   V   P   I   A   Y   T   T   N   F   L   K>

GAC AAT GAA TTA GCT GTT ATT AAA AAC AAC TCA GAA TAT ATT GAA ACA
 D   N   E   L   A   V   I   K   N   N   S   E   Y   I   E   T>

1250
ACT TCA AAA GCT TAT ACA GAT GGA AAA ATC AAC ATC GAT CAC TCT GGA
 T   S   K   A   Y   T   D   G   K   I   N   I   D   H   S   G>

 1300
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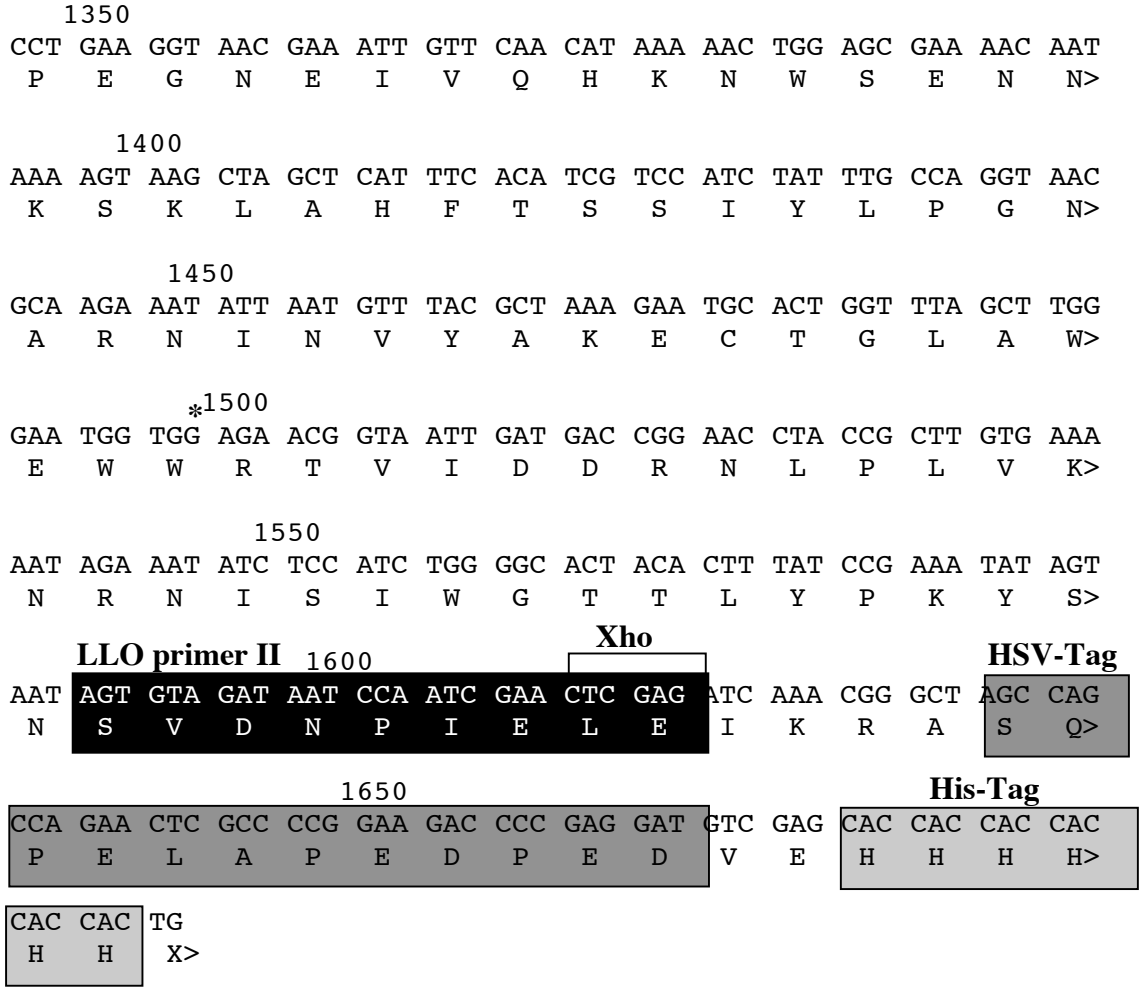
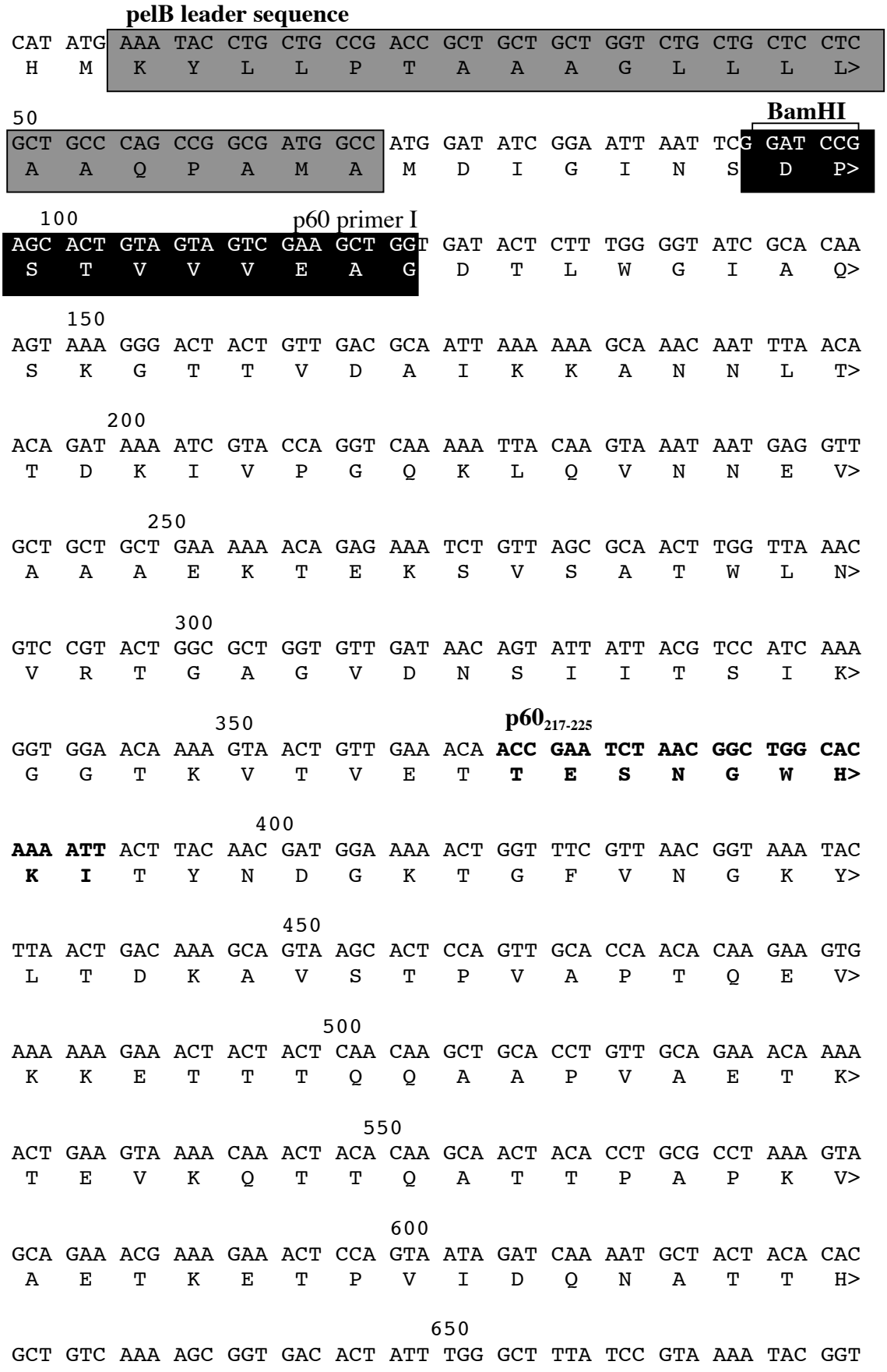


* Mutation at bp 492: trp ala

p60 sequence:



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                                  700
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 V   S   V   Q   D   I   M   S   W   N   N   L   S   S   S   S>

                                      750
ATT TAT GTA GGT CAA AAG CTT GCT ATT AAA CAA ACT GCT AAC ACA GCT
 I   Y   V   G   Q   K   L   A   I   K   Q   T   A   N   T   A>

                                          800
ACT CCA AAA GCA GAA GTG AAA ACG GAA GCT CCA GCA GCT GAA AAA CAA
 T   P   K   A   E   V   K   T   E   A   P   A   A   E   K   Q>

                                              850
GCA GCT CCA GTA GTT AAA GAA AAT ACT AAC ACA AAT ACT GCT ACT ACA
 A   A   P   V   V   K   E   N   T   N   T   N   T   A   T   T>

                                                  900
GAG AAA AAA GAA ACA GCA ACG CAA CAA CAA ACA GCA CCT AAA GCA CCA
 E   K   K   E   T   A   T   Q   Q   Q   T   A   P   K   A   P>

                                                      950
ACA GAA GCT GCA AAA CCA GCT CCT GCA CCA TCT ACA AAC ACA AAT GCT
 T   E   A   A   K   P   A   P   A   P   S   T   N   T   N   A>

                                                         1000
AAT AAA ACG AAT ACA AAT ACA AAC AAT ACT AAT ACA CCA TCT AAA AAT
 N   K   T   N   T   N   T   N   N   T   N   T   P   S   K   N>

                                                             1050
ACT AAT ACA AAC TCA AAT ACT AAT ACG AAT ACA AAC TCA AAT ACG AAT
 T   N   T   N   S   N   T   N   T   N   T   N   S   N   T   N>

                                                                 1100
GCT AAT CAA GGT TCT TCC AAC AAT AAC AGC AAT TCA AGT GCA AGT GCT
 A   N   Q   G   S   S   N   N   N   S   N   S   S   A   S   A>

                                                                     1150
ATT ATT GCT GAA GCT CAA AAA CAC CTT GGA AAA GCT TAT TCA TGG GGT
 I   I   A   E   A   Q   K   H   L   G   K   A   Y   S   W   G>

                                                                         1200
GGT AAC GGA CCA ACT ACA TTT GAT TGC TCT GGT TAC ACT AAA TAT GTA
 G   N   G   P   T   T   F   D   C   S   G   Y   T   K   Y   V>

TTT GCT AAA GCG GGT ATC TCC CTT CCA CGT ACA TCT GGC GCA CAA TAT
 F   A   K   A   G   I   S   L   P   R   T   S   G   A   Q   Y>

1250
GCT AGC ACT ACA AGA ATT TCT GAA TCT CAA GCA AAA CCT GGT GAT TTA
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1300
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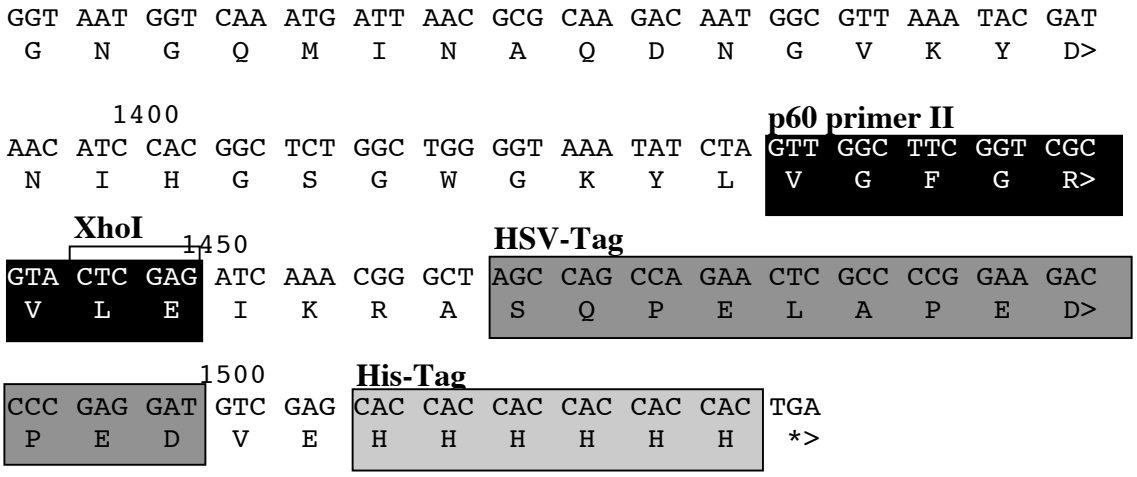
 V   F   F   D   Y   G   S   G   I   S   H   V   G   I   Y   V>

1350
```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G N G Q M I N A Q D N G V K Y D>
1400
AAC ATC CAC GGC TCT GGC TGG GGT AAA TAT CTA GTT GGC TTC GGT CGC
N I H G S G W G K Y L V G F G R>
p60 primer II
XhoI
1450
GTA CTC GAG ATC AAA CGG GCT AGC CAG CCA GAA CTC GCC CCG GAA GAC
V L E I K R A S Q P E L A P E D>
HSV-Tag
1500
CCC GAG GAT GTC GAG CAC CAC CAC CAC CAC CAC CAC TGA
P E D V E H H H H H H *>
His-Tag

2.2.1.12. Transformation into BL21 (DE3) CP competent cells

For subsequent recombinant expression of the protein in *E.coli*, the DNA was transformed into BL21 (DE3) CP competent cells:

BL21 (DE3) CP competent cells	25µl
DNA	2µl

The mix was first incubated on ice (30'), followed by heat shock treatment (30'', 42°C), then incubated on ice for 2 minutes before adding 250µl SOC medium. The transformation mix was incubated for 1h at 37°C with gentle shaking, and was thereafter plated on LB-agar plates containing kanamycin.

2.2.1.13. Mini expression

To confirm if the protein can be expressed in *E.coli,* a mini expression was performed. For this purpose 3-4 colonies from the overnight transformation were picked and added to mini expression medium.

The colonies were incubated in a shaker at 37°C until they reached an OD_{600nm} of 0.3-0.4.

At the OD_{600nm} of 0.7-0.8 IPTG (100µg /ml) was added to induce expression of the protein through the activation of the T7 polymerase.

Both control and 3 hour samples were centrifuged (3', 9000rpm) washed once with 1ml PBS and the pellet was stored at –20°C until used for analysis via a SDS gel.

2.2.1.14. SDS gel

Analysis of protein bands was performed by denaturating SDS- polyacrylamidelectrophoresis. The proteins were separated in 10% SDS gels under reducing conditions. The gels were made through the polymerization of acrylamid and methyl-bis-acrylamid, which form a three dimensional net. Depending on the degree of cross-linking, the proteins are moving in the gel after an electric field is applied. The polymerization is initiated through ammoniumperoxidsulfat (APS) and tetraethylendiamin (TEMED). To separate the proteins via the size and to eliminate other factors like the steric conformation sodium-dodecyl-sulfat (SDS) is used. SDS attaches to the protein and destroys its steric structure. In this process the proteins are more or less linearized and are getting negatively charged depending on their size. Most of the time proteins, like for example antibodies contain several subunits that are

held together through disulfide bridges. For analysis of these subunits the disulfide bridges are cut by heating up the samples with DTT.

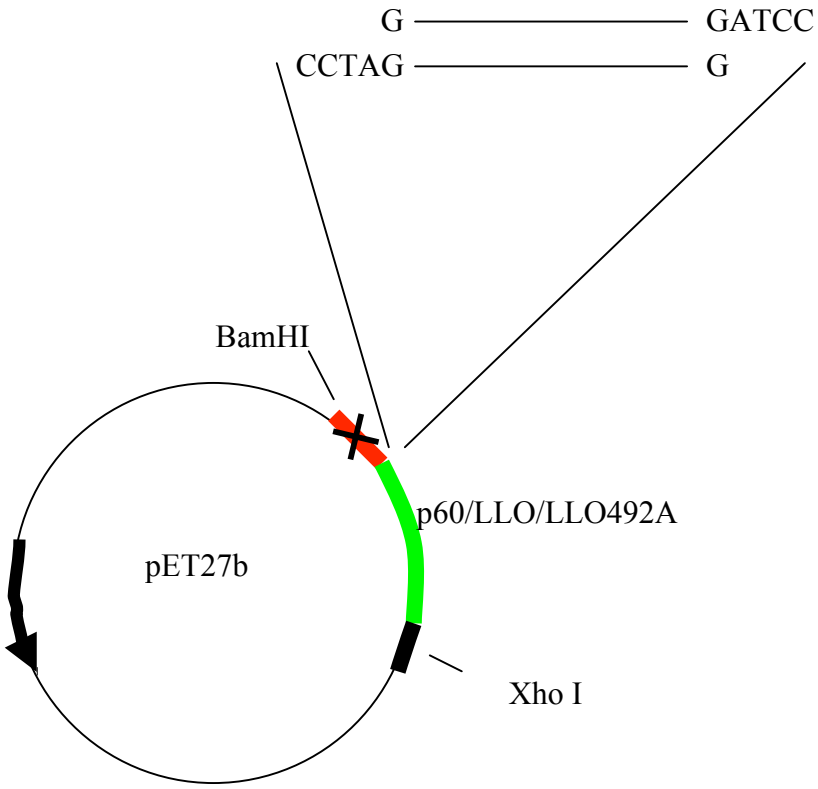
To make the gel the running gel is poured into the gel device up to 2/3 of the height of the plate. The gel is immediately coated with dH_2O. When the gel is polymerized the stocking gel is added on top and the comb is placed into the gel. After polymerization of the gel the comp is removed and the gel is placed into a frame (Bio Rad). The whole device is filled with 1x running buffer and all remaining air bubbles are removed.

The sample pellets were vortexed with 100µl dH_2O. After that the samples were mixed with 2xSB and together with the marker incubated for 5 minutes at 96°C before they were transferred onto ice for two minutes. The samples were than loaded onto the gel.

The gel was run for the first half hour at 100V, for the samples to accumulate at the lower line of the stocking gel, and thereafter at 170V.

After that the gel was stained with coomassie blue for 2 hours before putting it in destaining solution overnight. To dry the gel it was incubated for 1 hour in a glycerol solution and than clamped in a drying device.

2.2.2. Generation of the V7 conjugates



To generate V7 conjugates, the vector encoding the protein was linearized to anneal the DNA encoding the V7 peptide to the N-terminal end of the linearized vector. For that purpose a BamHI digest was performed using the DNA from the mini preparation.
The DNA was digested for two hours and the last half hour 1µl of Shrimp alkaline Phosphatase was added to avoid recircularization.
2µl of each oligonucleotide were added to 6.5µl of dH_2O and incubated for 2 minutes at 95°C before the mix was put on ice for 10 minutes.
Following reagents were added thereafter:

5x buffer (forward)	3µl
T4 kinase	2µl
ATP (10mM)	1.5µl

The mix was incubated for 30 minutes in a 37°C hot water bath and both oligonucleotide mixes were annealed by mixing and incubating them for 10 minutes at 65°C in a heating block. The sample was than slowly cooled down in the heating block for another 20 minutes and another 30 minutes at room temperature.
Thereafter the oligonucleotides were ligated with the linearized vector as followed:

Insert (oligonucleotide mix)	5µl
vector	2µl
dH_2O	1µl
5x DNA dilution buffer	2µl
2x T4 DNA ligation buffer	10µl
T4 DNA ligase	1µl

The ligation was performed at room temperature for 15 minutes and then put on ice.
The product was further purified over a column following the protocol of the Qiagen gel extraction kit. The product was again digested with BamHI to distinguish between vectors were the oligonucleotides were correctly inserted (missing the BamHI site) and the ones missing the insert. The positive ligation products were then used for transformation of DH5α competent cells.

2.2.3. Recombinant protein production

After successful mini-expression of recombinant protein, we produced the protein in larger scale for further experiments.

For that following protocol was established:

LB medium	1L
glucose solution (40%)	10ml
antibiotic	1ml (650µg kanamycin, 6.5ml LB medium)
bacteria	1ml (7ml LB medium, 20µl bacteria)

The bacteria were grown overnight until they reached an OD_{600nm} of 0.9-1.1. IPTG (100µg/l) was added to the culture to induce protein expression.

The bacteria were centrifuged (3750rpm, 20', 4°C), washed once with PBS before resuspension in 30ml lysis puffer. The lysate was incubated for one hour at room temperature before storing it at –80°C overnight.

The next day the lysates were thawed at 30°C. 80µl of DNAse was added to each tube and incubated for one hour at room temperature.

Then the lysates were centrifuged (12000 rpm, 20',4°C) and pooled. 1% of 10mM Imidazol was added together with the Ni-NTA-agarose beads and the solution was rotated in 50ml Falcon tubes in an overhead rotator for 2 hours (4°C). The expressed proteins contain a His Tag that is encoded in the pET27b vectors. The Ni-NTA-agarose beads bind to this His Tag of the recombinant protein. The sample was washed three times with PBS (pH8.0, 1500 rpm, 15',4°C) and transferred onto a H10-10 column. The fractions were eluted with 200mM Imidazol, which disrupts the binding between the Ni-NTA-agarose beads to the His-tag region. The fractions were pooled, concentrated and the protein was further purified via FPLC. Leupeptide, pepstatin, NaAzide and EDTA were added to inhibit protease activity or bacterial growth. To confirm the purification of the protein, SDS gel analysis was performed.

The concentration was determined by using a BCA assay. 12.25ml of BCA assay reagent A was mixed with 250µl of BCA assay reagent B. 1ml of the mix was vortexed with 20µl of the standard samples or the protein sample. After 30 minutes of incubation at 37°C the absorption was measured at OD_{562}.

2.2.4. Mutagenesis

In order to reduce the lytic activity of the LLO protein one amino acid was exchanged.

The primers $LLO_{492A\rightarrow T}$ primer 1 and $LLO_{492A\rightarrow T}$ primer 2 were designed and a PCR was done as followes (preparations 1-3):

10x buffer	5µl
DNA	2µl
primer 1	1.25µl
primer 2	1.25µl
dNTP's	1µl

To preparation 1, 1µl pfu was added and all preparations were filled up to a volume of 50µl with dH_2O.

The PCR program was as described:

annealing:	95°C	30'
elongation:	95°C	30'
	55°C	1'
	68°C	12'
end:	4°C	∞

The elongation step was repeated for 20 times.

After the PCR a digest was performed, preparations 1 and 2 were incubated with 1µl Dpn1 (1h, 37°C) and run over an agarose gel. Dpn1 is a endonuclease that is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. Because the DNA, isolated from an *E.coli* strain, is dam methylated it is susceptible for Dpn1 digestion.

2.2.5. Antibody purification

Hybridoma cells producing CD25 (PC61) antibody or CD4 (GK1.5) antibody were cultured in complete DMEM medium. Supernatant was collected and the antibody was purified using a ProteinG sepharose column. The antibody was eluted from the column with an elution buffer, which has a very low pH. To avoid denaturation of the antibody, the fractions are

collected into tubes containing 1M Tris buffer, which has a high pH to neutralize the pH again.

Afterwards the antibody was dialyzed overnight against PBS and the protein concentration was measured using a BCA assay following the protocol as described above. To further test the size and specificity of the antibody, the antibody was run over a SDS gel and used to stain cells for further FACS analysis.

2.2.6. Immunization protocol

2.2.6.1. Listeria *infection*

6ml of BHI medium were inoculated with 20µl of the glycerol stock of *Listeria monocytogenes* and the bacteria were grown until they reached an OD_{600nm} of 0.05 - 0.1.

The bacterial count per ml was calculated with the following formula:

L.m. 10403s: OD_{600nm} 0.1 = 1.1 x 10^8 bacteria/ml

L.m. OVA: OD_{600nm} 0.1 = 8.0 x 10^7 bacteria/ml

For primary infection mice were infected intravenously (*i.v.*) with 1 x 10^3 bacteria (*Lm 10403s*) or 2 x 10^3 bacteria (*Lm OVA*) in a volume of 200µl, whereas for recall infection they were infected with a 100 fold higher dose.

2.2.6.2. Protein immunization

Mice were immunized subcutaneously (*s.c.*) with 10µg of the given protein in a volume of 50µl. If CpG was also applied, it was mixed to protein in a concentration of 5nmol.

2.2.6.3. $CD4^+$ T cell and $CD25^+$ cell depletion

$CD4^+$ cell depletion

For CD4 depletion mice were injected intraperitoneally (*i.p*) with 200µl PBS containing 150µg GK1.5 antibody on days –3,-2,-1,+2,+5 before/after immunization.

$CD25^+$ cell depletion

For CD25 depletion mice were injected *i.p.* with 200µl PBS containing 250µg PC61 antibody on days –5,-1,+2,+5 before/after immunization.

2.2.7.1. Spleen preparation

Spleens were removed and homogenated with a syringe plunger over a cell strainer into $RP10^+$ medium. After centrifuging the homogenate (7', 1500rpm) the erythrocytes were lysed with ACT buffer (7', RT) and centrifuged again. The cells were again filtered over a Nylon filter and counted. Cells were then ready for staining.

2.2.7.2. Blood preparation

Mice were bled via tail veins and the blood was collected into tubes containing 100µl PBS and 10µl heparin to inhibit blood clotting. Erythrocytes were lysed in 10ml ACT buffer for 10 minutes at room temperature. Thereafter, cells were washed once with PBS (1500rpm, 7', 4°C), filtered and used for staining.

2.2.7.3. Lysis of erythrocytes with LLO/LLO-V7 protein

Mice were bled via tail veins and the blood was collected into tubes containing 100µl PBS and 10µl heparin to inhibit blood clotting. 10µl of erythrocytes were incubated with 50µl of serial 1:2 dilutions of LLO or LLO-V7 (starting with 5mg) in a 96 well plate. As control, 10µl of erythrocytes were incubated with 50µl of ACT or PBS. After 15' incubation at 37°C, cells were briefly centrifuged (2', 1500rpm) to evaluate cell lysis.

2.2.8. Cell counting

Cells were counted at a 1:20 dilution. 10µl of cell suspension was mixed with 90µl PBS and 100µl Trypan blue solution (0.4%, Sigma) and counted in a Neubauer counting device. Two quadrates were counted and the cell number was calculated using the following formula:

N (cells/ml) = mean of the two quadrates x dilution factor x 10^4

2.2.9. Staining protocols

2.2.9.1. Tetramer staining

For tetramer staining $6x10^6$ cells were used per sample and the staining was performed in a 96 well plate. First the cells were incubated with 50µl Fc block (1:100) and EMA (1:1000, 20', under light) to block Fc-receptors and stain dead cells, respectively. Cells were then washed once with FACS in a total volume of 200µl (2', 1500rpm) and incubated with 50µl of antibody mix (1:50, 60', in the dark and on ice). The samples were washed three times with FACS buffer, fixed in 1% PFA, and stored in the dark at 4°C before further analysis.

2.2.9.2. Intracellular cytokine staining

Splenocytes were prepared as described above. For *in vitro* restimulation, $1x10^7$cells/ml cells were incubated in $RP10^+$ medium in a 24 well plate with peptide at a concentration of 1µg/ml for 2 hours at 37°C. Golgi plug was added at a concentration of 2µg/ml to inhibit the transport of intracellular cytokines through the Golgi apparatus. After 3 hours of incubation, cells were transferred from the 24 well plate in 15ml Falcon tubes and centrifuged (3', 1500rpm), and transferred to a 96 well plate.

Cells were then incubated with 50µl Fc block- EMA mix (20', under light and on ice), washed three times with FACS puffer and stained with 50µl of the surface markers CD8a and CD62L (20',in the dark on ice). After that the cells were treated with 100µl Cytofix/Cytoperm to permealize the cell walls (20',in the dark on ice). Cells were then washed three times with PermWash puffer before they were stained with 50µl of intracellular antibodies (30', in the dark on ice). Finally, cells were washed again three times, and fixed with 1% PFA until used for analysis.

2.2.9. Viable bacteria

At given time points mice were sacrificed, spleen and liver were taken out. The organs were prepared in 5ml PBS as described above and 100µl of the cell suspension was mixed with 900µl 0.1% Triton-X-100 solution to break the cells open and free the *Listeria*.

1:10 and 1:100 dilutions were made before plating out triplets of 10µl of each dilution on BHI plates. The plates were incubated overnight at 37°C and *Listeria* colonies were counted the next day.

2.2.10. *In vivo* cytotoxicity assay

Splenocytes were prepared as described above. After cell counting, cells are divided equally and incubated for one hour with or without peptide (10^{-7} molar). Cells were washed three times with PBS and incubated with CFSE (10', 37°C). The cell pool that was incubated with peptide was stained with a high concentration of CFSE (5µM), the other cell pool with a low concentration of CFSE (0.5µM). To bind excessive CFSE the reaction was stopped by adding $RP10^+$ medium and incubated on ice for 5 minutes. After three washing steps with PBS, both cell pools are mixed, resuspended in PBS, and injected intravenously. After the indicated time points, the mice are bled and the blood was prepared as described before. Percentage of antigen specific cell lysis is calculated as followed:

%lysis = 100- ((%$CFSE^{high}$/ %$CFSE^{low}$ x 100) x (%$CFSE^{low}$/ %$CFSE^{high}$ of naïve control))

3. RESULTS

3.1. Generation of necessary tools

Goal of this study was to modulate the immune system during immunization with purified antigens to achieve long-lasting $CD8^+$ memory T cell responses. We used peptide or protein for immunization together with different TLR stimulating adjuvants and monitored changes in memory $CD8^+$ T cell responses with these different settings. The peptides were readily available by industrial providers, whereas the proteins and protein conjugates had to be generated.

3.1.1. Protein production

We generated recombinant proteins LLO and p60, both derived from *Listeria monocytogenes*. Protection after infection with *L.m.* is mediated by $CD8^+$ T cells targeting *Listeria*-derived antigens that are directly secreted into the cytosol of infected cells (like p60 and LLO). LLO_{91-99}-specific $CD8^+$ T cells confer protection to *L.m.* in BALB/c mice and therefore, we chose the LLO protein as target antigen. LLO has lytic activity on cells in higher concentrations, which might limit the applicability of purified LLO as a safe vaccine. To circumvent this potential problem, a mutated non-lytic form of LLO was generated in addition, the $LLO_{492T\ A}$. The subdominant epitope $p60_{217-225}$ of p60 also plays an important role for *Listeria*-specific $CD8^+$ T cell responses in BALB/c mice. This gave us the chance to study immunization protocols inducing *Listeria*-derived immunity with a protein different from LLO, potentially allowing to make more general conclusions. Recombinant p60 protein was generated similar to LLO. We wanted to study the stimulatory effect of different TLR ligands (TLR9 and TLR2 ligands) as adjuvants on protective $CD8^+$ T cell responses. CpG (TLR9 ligand) was purchased from an industrial provider, LLO-V7, $LLO_{492T\ A}$-V7 and p60-V7 fusion proteins (TLR2 ligands) were generated by ourselves, and OVA-1668 conjugates were kindly provided by Antje Heit.

3.1.1.1. Generation of Listeria*-derived LLO and p60 DNA*

Starting with the known sequences of LLO and p60, we designed DNA-primers that lay shortly after the start codon and before the stop codon in order to amplify the coding sequence. PCRs were performed using DNA purified from a single *Listeria monocytogenes* colony. After running the PCR product over an agarose gel, distinct bands with the approximate sizes of 1500bp and 1400bp for LLO and p60, respectively (Figure 2) could be

identified, which matches perfectly with the expected lengths of 1515bp (LLO) and 1374bp (p60).

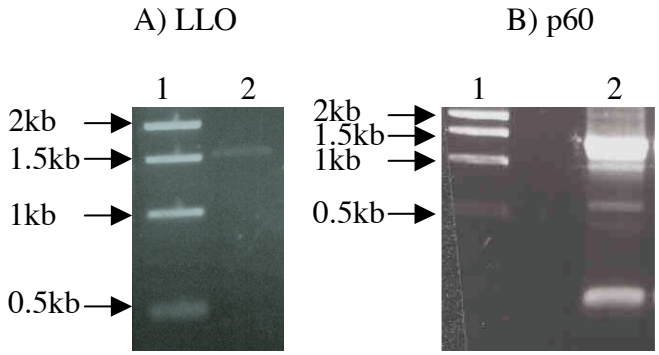


Figure 2: LLO and p60 PCR products

Agarose gel with 1kb linear DNA ladder (1), amplified products LLO (A,2) and p60 (B,2) after staining with ethidium bromide and visualization with UV light.

The PCR products were further purified after extracting bands of 1500bp (LLO) and 1400bp (p60) from the gel, following standard procedures. LLO and p60 DNA was further used to modify vectors. $LLO_{492T\square A}$ was generated by mutagenesis, and LLO-V7, $LLO_{492T\square A}$-V7 and p60-V7 by insertion of V7 oligonucleotides. The correctness of the all DNA products was confirmed by sequence analysis.

3.1.1.2. Protein purification

LLO, $LLO_{492T\square A}$, LLO-V7, $LLO_{492T\square A}$-V7, p60 and p60-V7 DNA were inserted into pET27b expression vectors, which support recombinant expression into the periplasmatic space of *E.coli*. Recombinant proteins were further purified via a His-Tag affinity chromatography and FPLC. Expression of recombinant proteins was analyzed by SDS gel electrophoresis, followed by Coomassie blue staining to visualize the bands. Comparison with the marker bands showed that proteins with the sizes of about 60kd were generated. Referring to the literature calculations of the molecular weight for p60 and LLO protein revealed 58kd for LLO (Erdenlig et al., 1999), and 60kd for p60 (Yu et al., 2004). The slight deviation between the calculated weight and the size of the bands shown on the gel can be explained by the fact that proteins do not necessarily run at the length that is calculated.

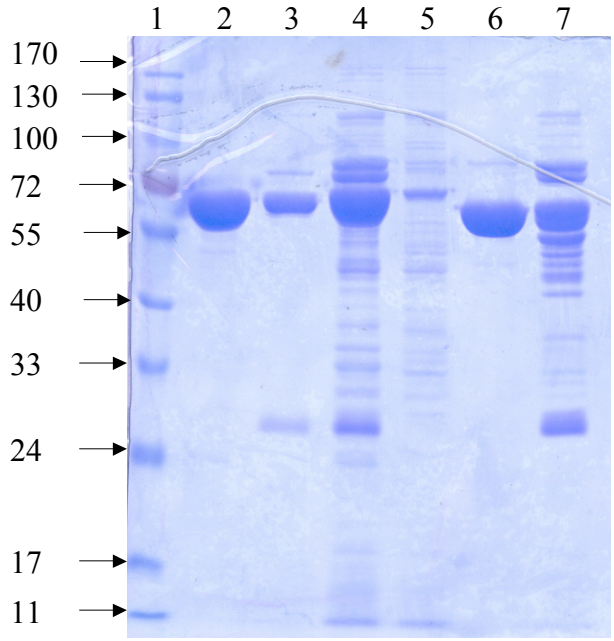


Figure 3: Protein size analysis via SDS PAGE

Following proteins were loaded on the gel:
Marker (1), LLO (2), LLO-V7 (3), $LLO_{492T\Delta A}$ (4), $LLO_{492T\Delta A}$-V7 (5), p60 (6) and p60-V7 (7). Arrows indicate known sizes of marker bands.

3.2. Role of antigen source (peptide/ full protein), route of application and CpG adjuvant impact $CD8^+$ T cell immunogenicity

We compared different antigens and their ability to efficiently prime $CD8^+$ T cell responses; we used the LLO_{91-99} peptide for immunization, as well as LLO, p60 and OVA protein. To test the role of adjuvant, we used CpG (TLR9), which is known to induce maturation of DCs.

Because antigen uptake and presentation of processed antigen is a crucial factor for efficient priming, we needed to analyze which application route, *i.v.* versus *s.c.*, is best to induce efficient $CD8^+$ T cell priming. After local application, antigen has to be taken up by APCs, which transport it to the lymph nodes where it can be further processed and presented. Systemic application most likely causes rapid dilution of the antigen, and probably will also target different APCs as compared to local application. Most studies analyzing systemic immunization indicate that antigen gets preferentially taken up and processed in the spleen. In the case of peptide immunization, the antigen does not need to be processed, and therefore, also other cell types might present the epitope to T cells. Therefore, it is of great importance to find out, which of these two application routes serve to be better for $CD8^+$ T cell priming.

3.2.1. *S.c.* immunization with LLO_{91-99} peptide

Mice were *s.c.* immunized with 100µg of LLO_{91-99} peptide (50µl/mouse) in the presence or absence of 5nmol CpG (*s.c.*). Seven days after immunization, LLO_{91-99}-specific T cell frequencies were compared to the frequencies achieved after *i.v. L.m.* infection with a low dose (0.1 x LD_{50}). Comparing the frequencies of activated antigen-specific $CD62L^{low}$ LLO_{91-99} $tetramer^+$ T cells (LLO_{91-99} and LLO_{91-99} plus CpG) with a group that underwent infection with *L.m.* revealed that both groups of peptide immunized mice showed very weak LLO_{91-99}-specific $CD8^+$ T cell responses (<0.1%) compared to about 3% LLO_{91-99}-specific, $CD62L^{low}$ $CD8^+$ T cells after *i.v.* infection with *L.m.* (Figure 4).

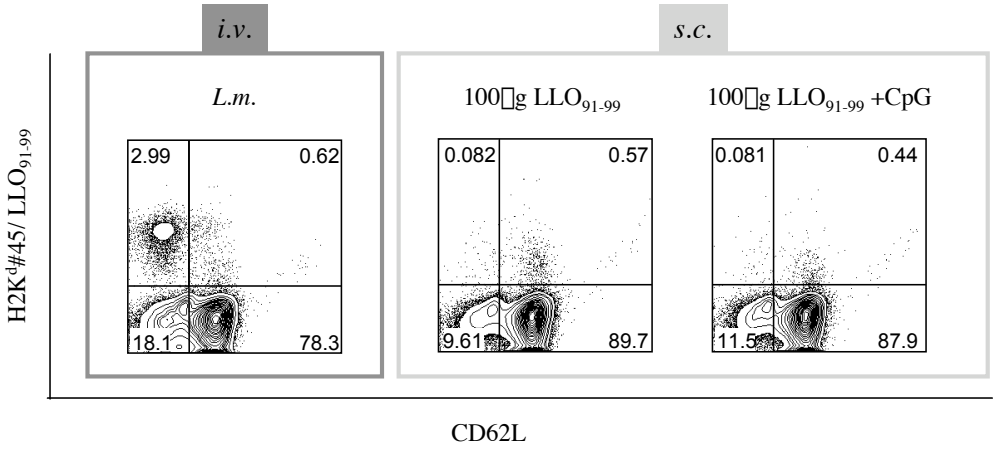


Figure 4: Adjuvant effect on peptide immunization

BALB/c mice were immunized s.c. with 100µg LLO_{91-99} peptide with or without 5nmol of CpG (s.c.). Control mice were i.v. infected with $1x10^3$ L.m. Seven days after immunization splenocytes were taken, isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer and APC-conjugated CD8α mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cell and represent data from at least 3 mice per group.

Between the group immunized with peptide alone and the one that additionally was treated with CpG, no difference in antigen-specific $CD8^+$ T cell frequencies was detectable. That result shows that LLO_{91-99} peptide leads to weak antigen-specific $CD8^+$ T cell responses, which cannot be increased by the addition of CpG and therefore, this immunization protocol is not useful for vaccination.

3.2.2. Immunization with protein

3.2.2.1. Limitation of LLO for i.v. *application*

Application of protein- in contrast to peptide-immunization requires access of the antigen to the processing/presentation machinery of professional APCs in order to result in $CD8^+$ T cell priming. As mice can die of heart failure after *i.v.* application of LLO, we first performed dose response experiments to test the susceptibility of mice to LLO. For this purpose, we applied 0.1, 1, 10, 100 g of purified full-length LLO protein *i.v.*, as well as the mutated $LLO_{492T\ A}$, which is known to have lost the lytic activity through the mutation. To exclude that the lethal effect of LLO was due to toxic components of the protein purification procedure, we also immunized mice with 0.1, 1, 10, 100 g of p60 protein. LLO and $LLO_{492T\ A}$ protein treated mice survived 0.1 and 1 g LLO protein, but died within 30 seconds after application of 10 g or 100 g protein. p60 protein treated groups survived all doses applied (Figure 5).

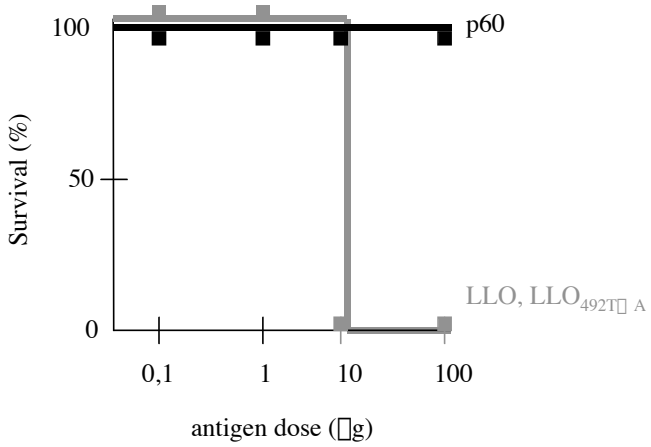


Figure 5: Dose dependent survival

Mice were immunized i.v. with the indicated doses of LLO, $LLO_{492T\ A}$ (grey line) or p60 (black line) protein. For each antigen dose 3 mice were used.

This result indicated that the lethal effect of LLO is not due to the lytic activity of LLO nor the protein preparation procedure, but must be due to other, yet unknown factors specific to the sequence of LLO. Furthermore, it becomes obvious that systemic application of LLO full-length protein or protein modifications is limited to very low antigen doses.

3.2.2.2. LLO protein with/without adjuvant

One of the crucial factors for efficient $CD8^+$ T cell priming is antigen uptake, processing and presentation by professional APCs. Using protein immunization, especially in addition with CpG application, it is of importance to find out whether antigen delivery through systemic or local application leads to better $CD8^+$ T cell priming. Mice were immunized *i.v.* or *s.c.* with 1µg LLO protein. Higher doses could not be applied, because mice die after *i.v.* immunization with doses of 10µg and more (see above) . Therefore, we chose to use 1µg LLO protein for both groups (*i.v.* and *s.c.*) to be able to compare the different application routes. CpG was mixed to the protein at a concentration of 5nmol. Seven days after immunization, mice were sacrificed and LLO_{91-99}-specific tetramer staining was performed on splenocytes. *I.v.* immunization with LLO resulted in relatively low frequencies of LLO_{91-99}-specific $CD8^+$ T cells. Although the addition of CpG resulted in the generation of a substantial amount of activated ($CD62L^{low}$) T cells and LLO_{91-99}-specific $CD8^+$ T cell responses increased, the frequencies were still rather low. In contrast, after *s.c.* immunization with LLO distinct LLO_{91-99}-specific T cell populations were readily detectable. Even in the absence of addition of adjuvant, about 0.3% LLO_{91-99}-specific $CD62L^{low}$ T cells were identified. Addition of CpG further increases the frequency of LLO_{91-99}-specific $CD8^+$ T cells to more than 1%.

Comparison of *i.v.* versus *s.c.* immunization demonstrates that local immunization leads to higher $CD8^+$ immune responses than systemic application. The positive effect of CpG is weak for systemic application of LLO protein, but upon local immunization CpG leads to a 3-fold increase of LLO_{91-99}-specific $CD8^+$ T cells (Figure 6).

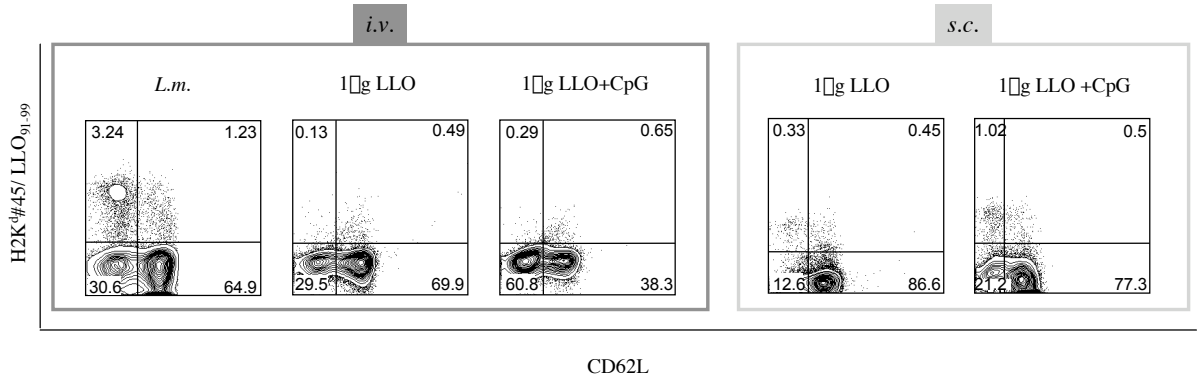


***Figure 6: I.v.* versus *s.c.* application of LLO protein**

BALB/c mice were immunized either i.v. or s.c. with 1µg LLO with/without 5nmol CpG. Control mice were infected with 1x10³ L.m. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer and APC-conjugated CD8a mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (n= 2-3 mice/ group).

Using local application of LLO protein, there is no limitation for the amount of protein used for immunization, as has been described above for systemic application. Therefore, we performed a dose titration of LLO protein to find out if we can further increase the LLO_{91-99}-specific $CD8^+$ T cells responses after application of higher amounts of LLO protein. Figure 7 shows that there is a steady increase in LLO_{91-99}-specific $CD8^+$ T cell frequencies from 1, 5 to 10 g LLO protein. However, after application of higher amounts (50 g) of LLO protein LLO_{91-99}-specific $CD8^+$ T cell frequencies decrease. Control mice immunized with CpG or PBS did show barely detectable LLO_{91-99}-specific $CD8^+$ T cell frequencies (≤ 0.1% LLO_{91-99}^{high}, $CD62L^{low}$ $CD8^+$ T cells). Therefore, for all further experiments 10 g LLO protein was the favored amount to use for *s.c.* immunization.

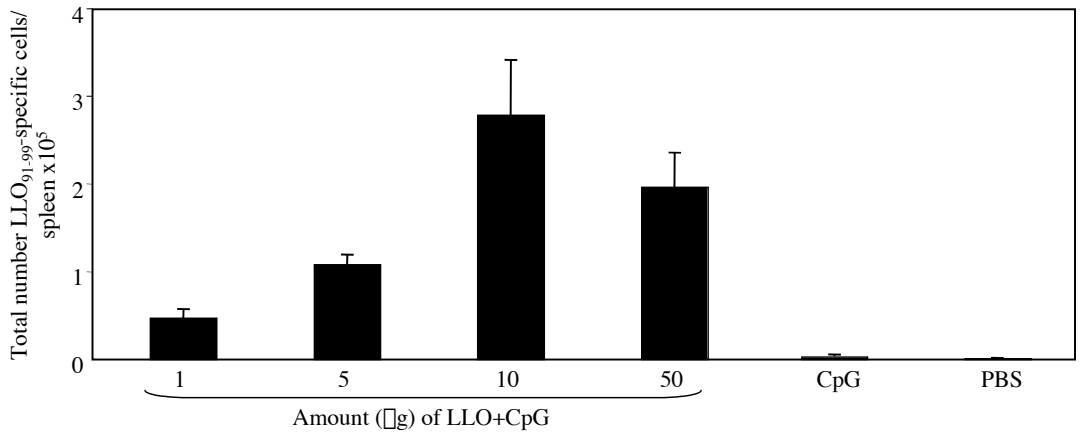


Figure 7: S.c. titration of LLO protein

BALB/c mice were immunized either s.c. with indicated amounts of LLO with 5nmol CpG. Control mice immunized s.c. either with 5nmol CpG or PBS. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer and APC-conjugated CD8a mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (n= 2-3 mice/ group).

3.2.2.3. p60 protein with/without adjuvant

To exclude that the enhanced immune responses after *s.c.* immunization in comparison to *i.v.* immunization is protein specific, we repeated similar experiments using p60 protein. Infection experiments with *L.m.* suggest that p60-specifc immune responses are not as immunodominant as for LLO. Titration experiments had shown that immunization with 10µg of p60 resulted in very weak $p60_{217-225}$-specific $CD8^+$ T cell responses (≤ 0.08% $p60_{217-225}^{high}$ $CD62L^{low}$ $CD8^+$ T cells, data not shown) and therefore, we decided to immunize mice with initially relatively high dose of 100µg of p60 protein (*i.v.* and *s.c.*). As control, a group of mice was infected with *L.m.* *L.m.* infection led to about 1% $p60_{217-225}$-specific $CD8^+$ T cells. *I.v.* immunization, either with or without CpG resulted in very weak antigen-specific $CD8^+$ T cell responses (0.16%). When p60 protein alone or in addition with CpG was applied locally, this procedure resulted in about 0.2% (0.16+/-0.05%) and about 0.5% (0.31+/-0.14%) antigen-specific $CD8^+$ T cell frequencies, respectively (Figure 8). These data demonstrate that although immunization with p60 full-length protein appears to be less immunogenic for priming and expansion of $p60_{217-225}$-specific $CD8^+$ T cells (as compared to LLO), the dependencies on application route and adjuvant effects are comparable to the results obtained for LLO immunization. Local application seems to be much more efficient for $CD8^+$ T cell priming and also CpG-mediated enhancement of epitope-specific $CD8^+$ T cells is only seen in the setting of local immunization.

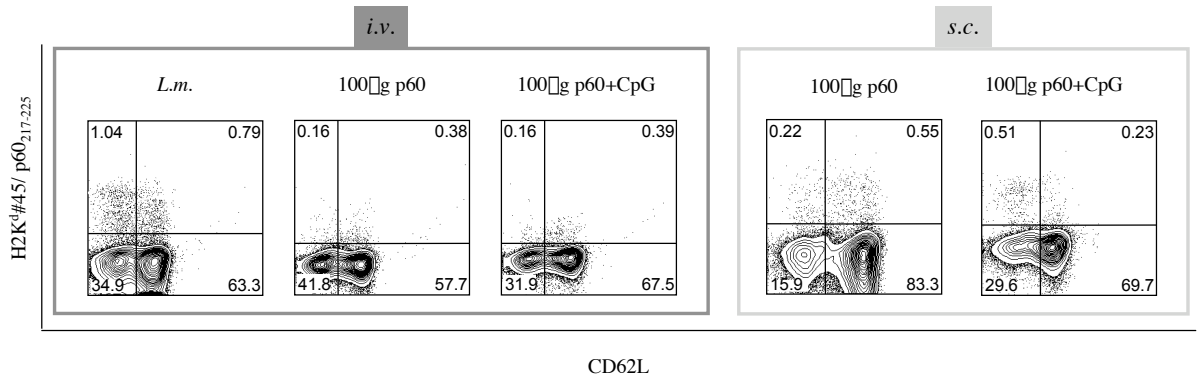


***Figure 8: I.v.* versus *s.c.* application (p60)**

BALB/c mice were immunized with 100µg p60 i.v. or s.c. As control, mice were immunized with 1x10^3 L.m. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled $p60_{217-225}$-tetramer and APC-conjugated CD8α mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells and are representative of 3 mice per group.

3.2.2.4. Covalently linked CpG conjugates improve the immune responses

S.c. application of CpG in combination with full-length protein enhances immune responses (see above). It has been shown by other groups that direct conjugation of CpG-DNA to OVA (OVA-1668) leads to even higher OVA-specific $CD8^+$ T cell frequencies than just adding CpG to the OVA protein. To confirm this result, we immunized BL6 mice with 10µg of OVA-1668 conjugate or 10µg OVA plus 5nmol CpG (*i.v.* or *s.c.*). As control, we infected mice with a sublethal dose (0.1 x LD_{50}) of *L.m.OVA*, which recombinantly express ovalbumine. Seven days after immunization or infection, $OVA_{257-264}$ (= SIINFEKL) specific $CD8^+$ T cell responses were analyzed by tetramer staining. *L.m.OVA* infected mice showed SIINEFKL-specific frequencies of about 4%, whereas systemically treated mice either with OVA plus CpG or OVA-1668 conjugate showed frequencies of 0.26% (+/-0.12%) and 0.67% (+/-0.43%), respectively. For the locally immunized mice, frequencies of about 0.2% (0.19+/-0,04%; OVA plus CpG) and 4.16% (OVA-1668 conjugate) were measured. Thus, direct conjugation of OVA with CpG improves the antigen-specific immune responses. This effect can be already seen with *i.v.* application (4-fold increase), but is much more pronounced after *s.c.* application of the protein (18-fold increase) (Figure 9).

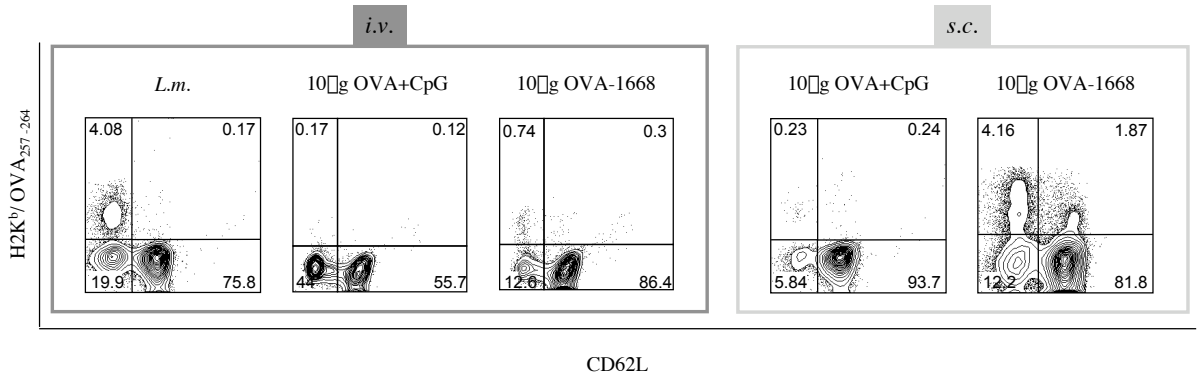


Figure 9: Comparison of OVA plus CpG versus OVA-1668 application

BL6 mice were immunized i.v. or s.c. with either 10µg OVA plus CpG or OVA-1668 conjugate. As control, one group was infected with $2x10^3$ L.m.OVA. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled $OVA_{257-264}$ tetramer and APC-conjugated CD8α mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (n=3 mice/group).

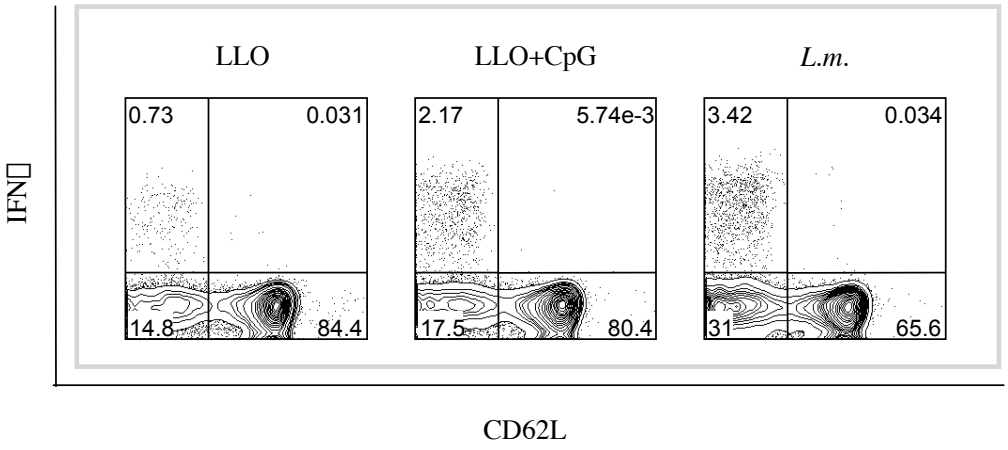
These data indicate that also upon immunization with OVA-1668 conjugate local application is the favored route of immunization, because it results in strong antigen-specific $CD8^+$ T cell frequencies. Direct conjugation of full protein with CpG especially reduces the amount of

antigen needed to induce maximal T cell responses. Therefore, we tried to directly conjugate also recombinant LLO and p60 protein with CpG, but due to technical problems conjugations were unsuccessful.

3.2.3. Correlation of frequencies of antigen-specific T cells and functionality

Our data show that *s.c.* immunization with LLO, p60 and OVA protein in addition with CpG leads to increased antigen-specific $CD8^+$ T cell responses compared to protein vaccination alone. In order to correlate the increased numbers of antigen-specific $CD8^+$ T cells with increased effector function, IFN-production was determined by ICCS. To be able to make a fair comparison, splenocytes used for ICCS were also used to stain for LLO_{91-99}–specific tetramer frequencies. Mice were immunized *s.c.* with 10g LLO protein with or without 5nmol of CpG or infected with *L.m.* (0.1 x LD_{50}). Seven days later, IFN-production of splenocytes was analyzed. As negative controls, splenocytes were incubated with DMSO alone (data not shown, background levels were always below 0.04% IFN-producing $CD8^+$ T cells).

A)



B)

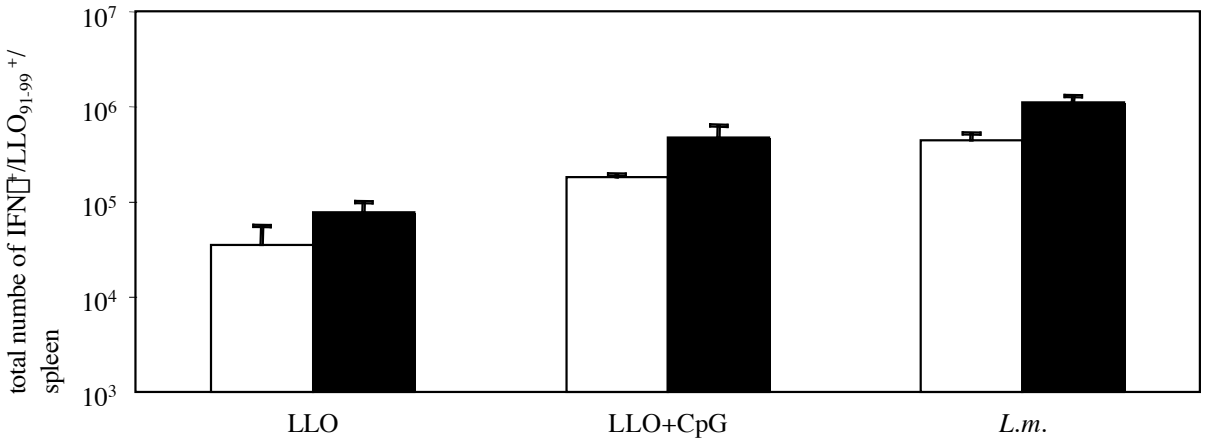


Figure 10: Frequencies of IFNγ-producing- and LLO_{91-99}-specific $CD8^+$ T cells after immunization with LLO with or without CpG

BALB/c were immunized s.c. with 10µg LLO with/without 5nmol CpG. Control mice were infected with $1x10^3$ L.m. After one week mice were sacrificed and splenocytes were incubated with DMSO or LLO_{91-99} peptide for 5 hours. For the final 3 hours Golgi-Plug was added to the cultures. Cells were surface stained with PE-conjugated anti-CD8α and APC-conjugated anti-CD62L mAb and intracellularly with FITC-conjugated IFNγ. For tetramer staining splenocytes were stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer and APC-conjugated CD8α mAb. Life/death discrimination was done by EMA staining. Background frequencies and numbers of IFNγ-producing cells determined in cultures containing DMSO were subtracted from frequencies and numbers derived from cultures with peptide. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs depict mean +/- SD for LLO_{91-99}-specific IFNγ- (open bars) and LLO_{91-99}-specific (filled bars) $CD8^+$ T cells of three individually analyzed mice per experimental group (B). The experiment shown is representative for at least 5 similar experiments.

Frequency analyses and calculations of total numbers of IFN-secreting $CD8^+$ T cells showed that T cells derived from LLO immunized mice produced only little amounts of IFN, whereas LLO plus CpG immunized mice and *L.m.* infected mice had relatively high frequencies of IFN-producing $CD8^+$ T cells (Figure 10A and B).

The direct comparison of total numbers of IFN-secreting $CD8^+$ T cell and LLO_{91-99}-specific $CD8^+$ T cells showed that the tetramer levels were slightly higher for all groups analyzed. This result indicates that nearly all LLO_{91-99}-specific $CD8^+$ T cells differentiated into effector cells.

To further analyze the effector function capacity of in vivo generated antigen-specific T cells, we performed *in vivo* cytotoxicity assays (CTX). Mice were again immunized with 10mg LLO protein with or without 5nmol of CpG or infected with *L.m.* (0.1 x LD_{50}). Seven days later, splenocytes of naïve mice were given *i.v.* (50% loaded with LLO_{91-99} peptide and stained brightly with CFSE, 50% unloaded and stained dim with CFSE). At indicated time points (5 and 20 hours) mice were bled and the number of CFSE expressing cells was analyzed. Naïve mice were used as negative control. These control mice do not lyse LLO peptide loaded $CFSE^{bright}$ cells or unloaded control cells ($CFSE^{low}$), as they do not bear LLO-specific cytotoxic cells.

Mice previously immunized with LLO showed CTL specific lysis of about 4% after 5 hours (reduction of $CFSE^{bright}$ peak), whereas mice immunized with LLO plus CpG or *L.m.* showed lysis of around 30%. After 20 hours, lysis in LLO immunized mice increased to about 15%, in LLO plus CpG or *L.m.* treated mice to about 80% (Figure 11A and B).

Like it was found for IFNg production as measurement of effector function, LLO immunization leads to poor *in vivo* lysis compared to LLO plus CpG. LLO plus CpG immunization reached a CTL capacity comparable to *L.m.* infected mice.

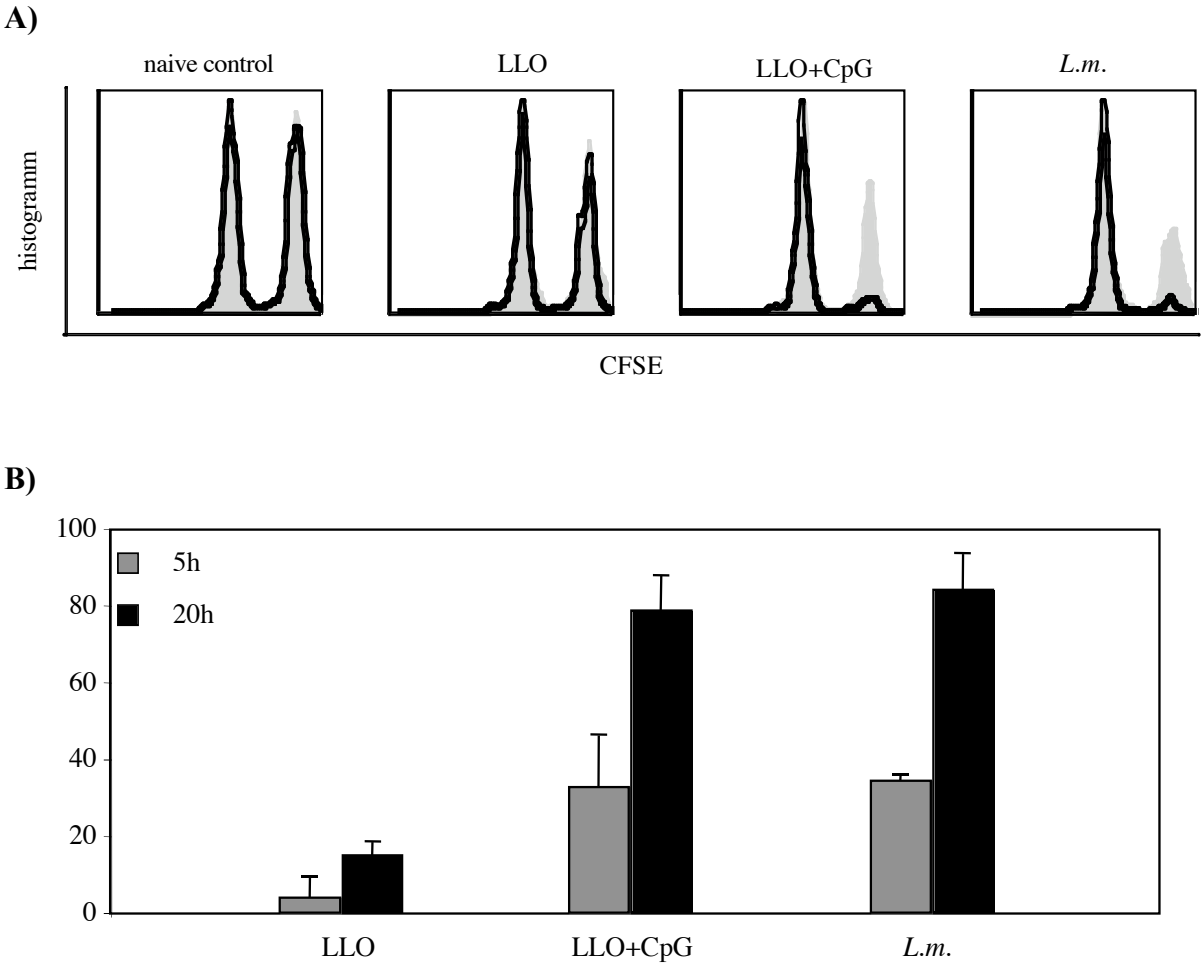


Figure 11: Enhanced CTL activity after addition of CpG

Mice were immunized s.c. with 10µg LLO with/without 5nmol CpG. Control mice were infected with 1x10³ L.m. Seven days after immunization mice were infected with a mixture of splenocytes from naïve mice; half of the splenocytes was labeled with low dose of CFSE (0.5µM) and the other half was loaded with LLO_{91-99} peptide and labeled with high dose of CFSE (5µM). At 5 (grey bars) and 20 (black bars) hours after cell transfer mice were bled via tail vein incision and the number of CFSE labeled cells was analyzed. To show the CTL activity over time, the histograms for 5 (filled with grey) and 20 (black line) hours are shown as overlay (A). Bar graphs depict mean +/- SD for LLO_{91-99}-specific lysis of three individually analyzed mice (B).

3.2.4. DCs are the cell population mediating priming of antigen-specific $CD8^+$ T cells

The data so far show that priming of $CD8^+$ T cells is not efficient after s.c. immunization with peptide, but can be improved when protein is used. Furthermore, additional application of CpG has a strong influence, leading to enhanced $CD8^+$ T cell responses. It has been demonstrated recently that CpG signaling through TLR9 mediates activation and maturation of DCs, as well as increased efficacy in cross-presentation of antigen to $CD8^+$ T cells. Using full-length proteins for vaccination and priming of $CD8^+$ T cells requires cross-presentation

and -priming. This suggests that the positive adjuvant effects by CpG are mainly mediated by DCs, which are believed to be the only cell population capable of efficient cross-priming. To test whether DCs are really crucial for $CD8^+$ T cell priming in the vaccination protocols established for our studies, we compared T cell responses in mice in the presence or absence of $CD11^+$ DCs. In order to deplete DCs, CD11c DTR (diphteria toxin receptor) transgenic mice were used. CD11c is a surface molecule mainly expressed by DCs. CD11c DTR transgenic mice carry the CD11c promotor linked to human DTR. Injected DT (diphteria toxin) binds to the DTR, leading to rapid cell death.

Available CD11c DTR transgenic mice were on a BL6 ($H2^b$) background, and are therefore not useful for the monitoring of LLO immunization ($H2K^d$ restriction of LLO_{91-99}). To overcome this problem, CD11c DTR transgene mice were crossed with BALB/c mice and the off-spring (F1 generation- $H2^b$/ $H2^d$) was used. Mice were immunized with 10 g LLO plus CpG, one group was treated with DT one day before immunization to deplete DCs. Seven days after immunization frequencies of LLO_{91-99}-specific T cells in mice lacking DCs were near the detection limit. Mice with functional DCs developed robust immune responses. The same can be concluded when comparing total numbers of $CD8^+$ LLO_{91-99}-specific T cells. There was a decrease in cell number of about 2 log scales when DCs were absent. This result indicates that indeed DCs seem to be the main population mediating efficient $CD8^+$ T cell priming upon vaccination with full-length LLO.

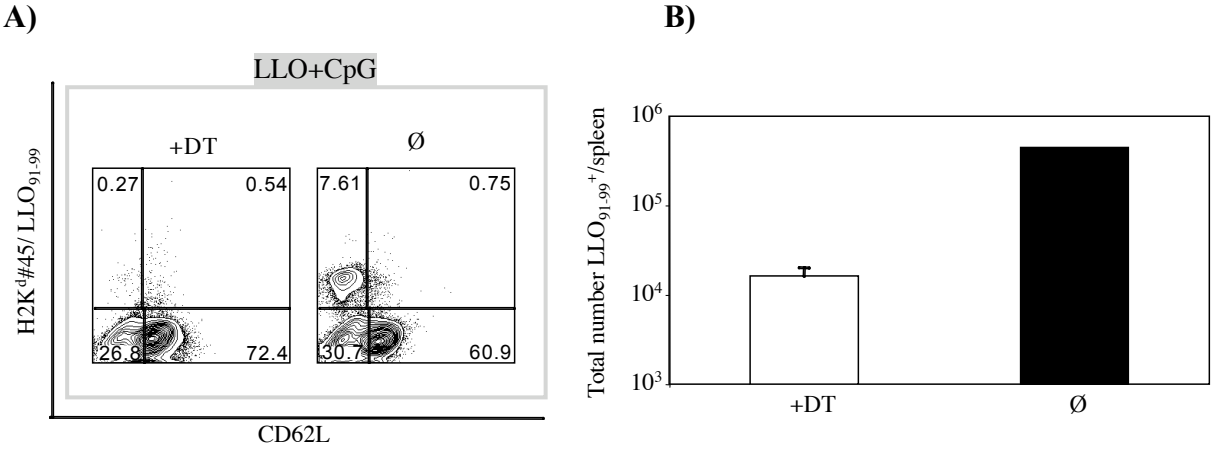


Figure 12: DCs are crucial for $CD8^+$ T cell cross-priming

CD11c DTR transgene mice (F1 generation) were immunized s.c. with LLO plus CpG. The indicated group was treated i.p. with DT one day before immunization. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer and APC-conjugated CD8 mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs represent mean values +/- SD for spleen cells of three individually analyzed mice (B).

3.3. Role of CD4+ T cells for establishment of immune response after protein immunization

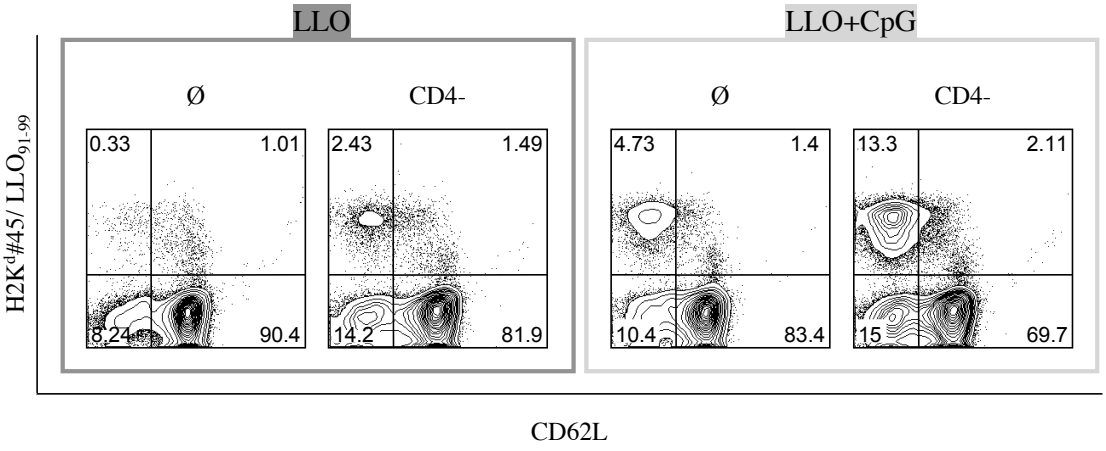
In our immunization setting with LLO protein we demonstrated that the addition of CpG enhances immunogenicity in many respects; it leads to increased tetramer-specific CD8+ T cell responses and these antigen-specific T cells produce a substantial amount of IFN and lyse target cells as effective as *L.m.* infected mice. The positive CpG effect most likely acts on the level of DCs, resulting in efficient cross-presentation of the antigen to CD8+ T cells.

Different groups have shown for various infection models that CD4+ T cell help is an important factor for the efficient generation of antigen-specific CD8+ T cells. Lack of CD4+ T cells during the time of priming of CD8+ T cells results in impaired CD8+ dependent protection against secondary infection (Kursar et al., 2002; Sun and Bevan, 2003). Having these data in mind, the question arose what impact CD4+ T cells have on the generation of CD8+ T cell mediated primary and memory responses in our immunization setting. Especially the superior immunogenicity of full-length protein in comparison to peptide immunization might mediate a participation of antigen-specific CD4+ T cells. On the other hand, the strong adjuvant effects by CpG might be able to overcome any dependency of CD8+ T cell priming and memory T cell generation on CD4+ T cell help.

3.3.1. Effect of CD4+ T cell depletion on antigen-specific CD8+ T cell frequencies after LLO plus CpG immunization

To evaluate the involvement of CD4+ T cell help in our vaccination setting, we depleted mice of CD4+ T cells. Depletion was performed by intraperitoneal (*i.p.*) application of CD4 depleting antibody on days -3, -2, -1 before immunization (day 0) and on days +2 and +5 after immunization, following a recently published protocol showing T helper dependency of CD8+ memory T cell generation (Janssen et al., 2003). Again, mice were immunized with 10 g LLO protein with or without 5nmol of CpG. One week later, splenocytes were analyzed for antigen-specific CD8+ T cells. The collected data for this important experiment are summarized in Figure 13. When looking at the antigen-specific CD8+ T cells both in frequencies and total numbers, we could not find a CD4+ dependent decreases in antigen-specific CD8+ T cell populations like it might have been expected from published data. Most surprisingly, upon CD4+ T cell depletion LLO_{91-99}-specific T cell frequencies were even substantially enhanced. This could be observed for immunization with LLO protein alone or in combination with CpG.

A)



B)

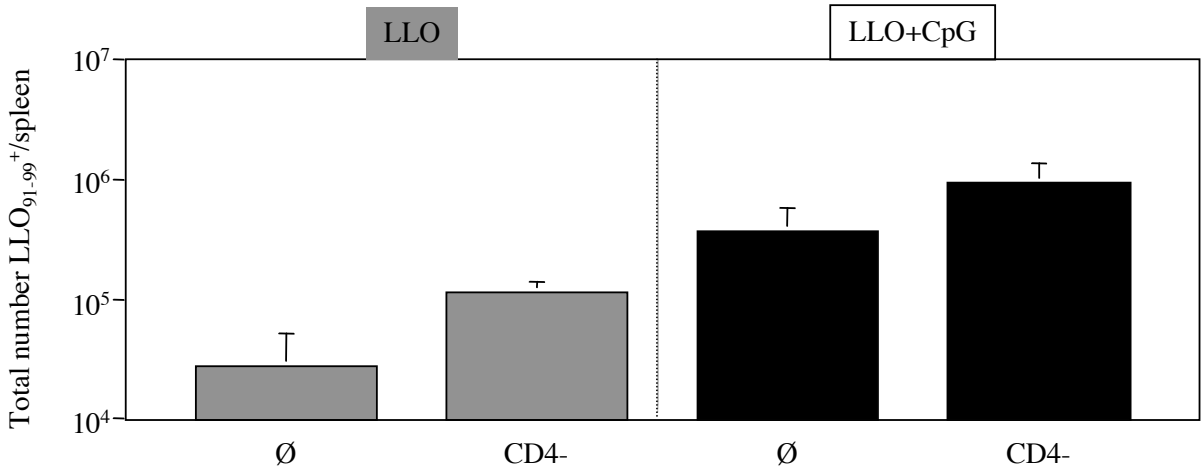


Figure 13: Frequencies and numbers of LLO_{91-99}-specific $CD8^+$ T cells after $CD4^+$ T cell depletion

BALB/c mice were immunized with 10µg LLO or LLO plus 5nmol CpG. Indicated groups got treated with 150µg GK1.5 i.p. at days –3, -2, -1, +2, +5 before/after immunization. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer and APC-conjugated CD8α mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs represent mean values +/- SD for spleen cells of three individually analyzed mice. Results are representative of at least 4 independent experiments (B).

These experiments indicate that $CD4^+$ T cell depletion can enhance LLO-targeted immune response upon full-length protein vaccination in the absence of additional adjuvant. Providing a further strong stimulus to the APC through CpG together with $CD4^+$ T cell depletion additionally enhances the response. Therefore, in our setting it seems that $CD4^+$ T cells might exert inhibitory function on the generation of $CD8^+$ T cell responses, because the absence of $CD4^+$ T cells leads to an unexpected enhancement of $CD8^+$ T cell responses.

3.3.2. Effect of $CD4^+$ T cell depletion on functionality after LLO plus CpG immunization

Driven by the surprising finding that $CD4^+$ T cell depletion enhances LLO-specific $CD8^+$ T cell responses upon protein vaccination, we decided to determine whether the enlarged antigen-specific cell populations consisted of functional effector T cells. As an important effector function antigen-induced IFNγ-production of mice that were immunized seven days earlier with LLO or LLO plus CpG protein with or without $CD4^+$ T cell depletion was measured. The results of these experiments are summarized in Figure 14. In direct correlation with tetramer staining data, IFNγ-producing $LLO_{91\text{-}99}$-specific T cells increased when $CD4^+$ T cells were depleted. The enhancing effect of $CD4^+$ T cell depletion was greater in mice that got immunized with LLO plus CpG, as compared to LLO immunization alone, which also reflects the previous observations made by MHC class I tetramer staining.

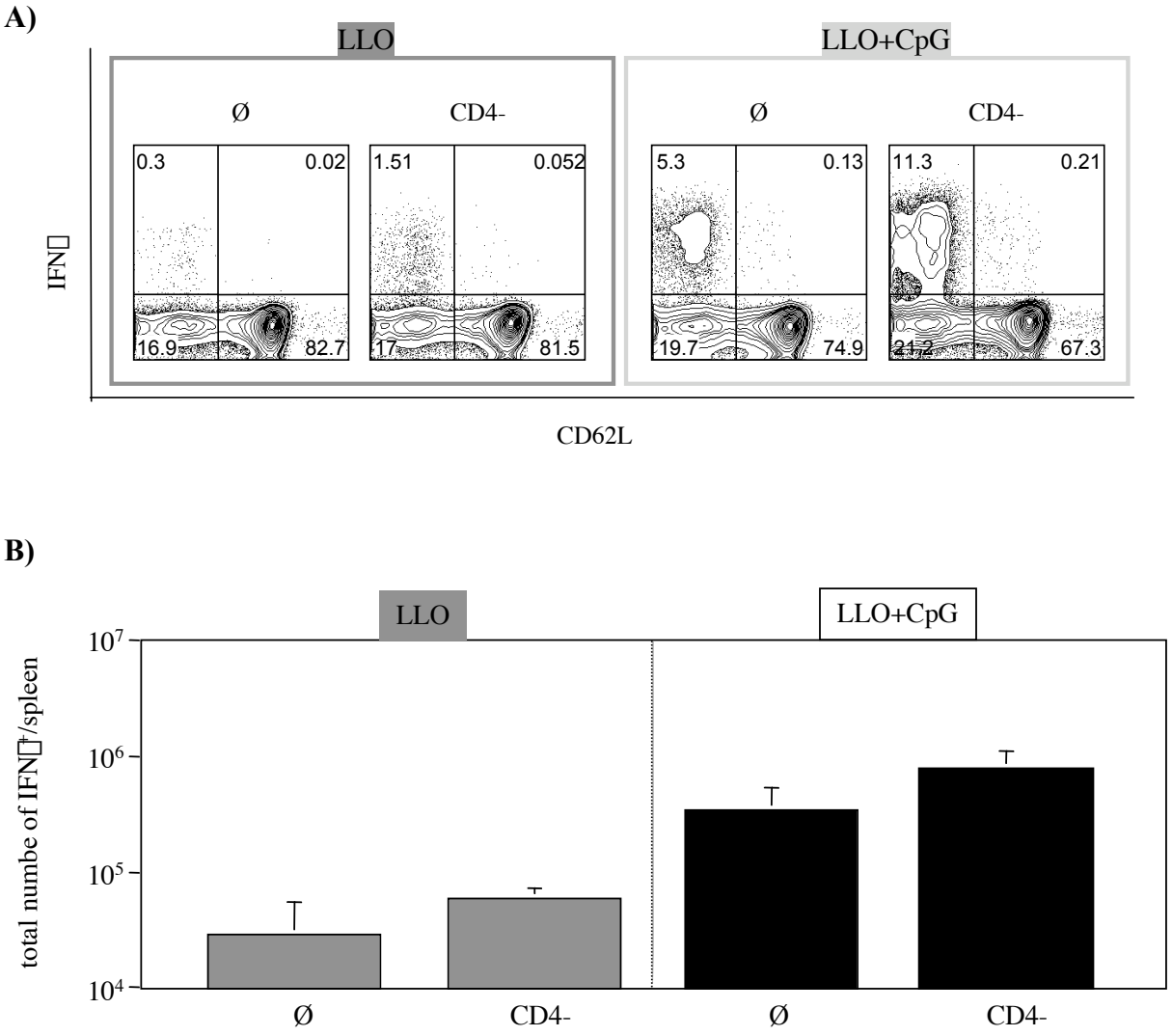


Figure 14: Frequencies and numbers of IFN-producing LLO_{91-99}-specific $CD8^+$ T cells after $CD4^+$ T cell depletion

BALB/c were immunized s.c. with 10g LLO with/without 5nmol CpG. Indicated groups got treated with 150g GK1.5 i.p. at days –3, -2, -1, +2, +5 before/after immunization. After one week, mice were sacrificed and splenocytes were incubated with DMSO or LLO_{91-99} peptide for 5 hours. For the final 3 hours Golgi-Plus was added to the cultures. Cells were surface stained with PE-conjugated anti-CD8 and APC-conjugated anti-CD62L mAb and intracellularly with FITC-conjugated IFN. Life/death discrimination was done by EMA staining. Background frequencies and numbers of IFN-producing cells determined in cultures containing DMSO were subtracted from frequencies and numbers derived from cultures with peptide. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs depict mean +/- SD for LLO_{91-99}-specific IFN $CD8^+$ T cells of three individually analyzed mice per experimental group (B). The experiment shown is representative for at least 3 similar experiments.

To test additional effector functions of antigen-specific $CD8^+$ T cells directly *in vivo*, we performed *in vivo* CTX assays. In LLO immunized mice, depletion of $CD4^+$ T cells resulted in enhanced capability to kill target cells, both after 5 and 20 hours of incubation with target cells. However, compared to CTL activity of *L.m.* infected animals, lysis was still relatively low. In mice immunized with LLO plus CpG, $CD4^+$ T cells depletion did not substantially enhance the in vivo killing capacity. However, LLO plus CpG immunization resulted already in a killing of target cells as good as did *L.m.* infection (both at 5 and 20 hours). We interpret these data as a consequence that the assay system seems not to be able to discriminate differences in antigen-specific killing above a certain frequency of effector T cells (Figure 15).

Taken together, with the data generated so far we can conclude the following:

1) LLO alone is a weak antigen, with little immunogenicity, inducing low frequencies of antigen-specific T cells with effector function, and immunization with LLO protein cannot, or only insufficiently, provide protection.

2) Addition of CpG together with LLO immunization leads to enhanced immunogenicity and increased frequencies of $CD8^+$ T cells with vigorous effector function.

3) $CD4^+$ T cell depletion leads to enhanced immune responses in both groups (immunization with LLO or LLO plus CpG).

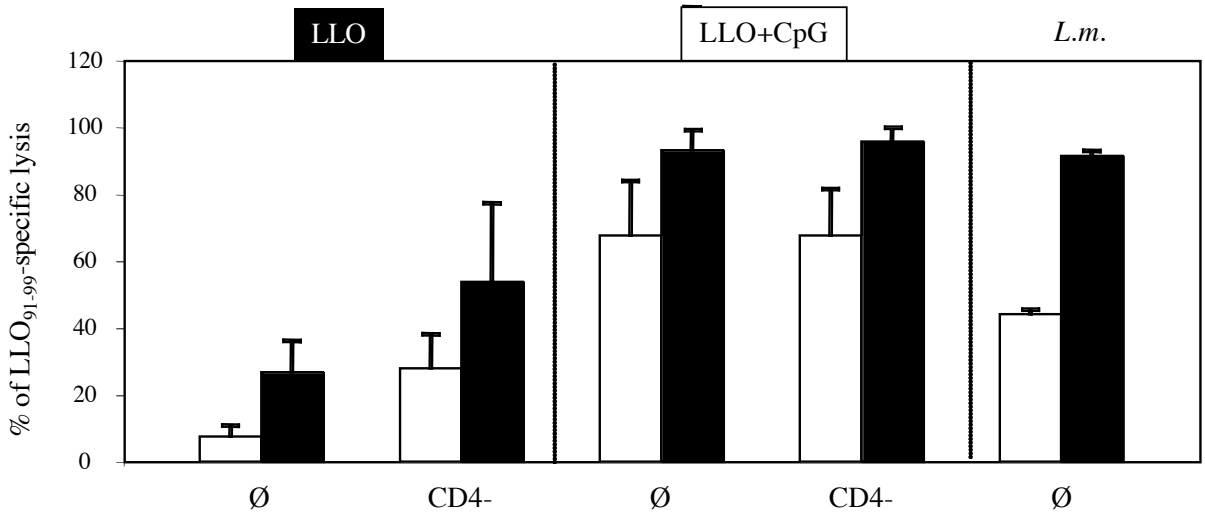


Figure 15: Specific lysis of LLO_{91-99}-specific $CD8^+$ target cells

Mice were immunized s.c. with 10µg LLO with/without 5nmol CpG. As control mice were infected with $1x10^3$ L.m. Seven days after immunization mice were infected with a mix of splenocytes from naïve mice; half of the splenocytes was labeled with low dose of CFSE (0.5µM) and the other half was loaded with LLO_{91-99} peptide and labeled with high dose of CFSE (5µM). At 5 (white bars) and 20 (black bars) hours after cell transfer mice were bled via tail vein incision and the number of CFSE labeled cells was analyzed. Bar graphs depict mean +/- SD for LLO_{91-99}-specific lysis of three individually analyzed mice.

3.4. Influence of homeostatic effects through T cell depletion

The finding that $CD4^+$ T cell depletion leads to an overall increase of antigen-specific $CD8^+$ T cells upon immunization was unexpected. One possible explanation for this observation might be that activated $CD8^+$ T cells undergo additional homeostatic proliferation induced by $CD4^+$ T cell depletion. $CD4^+$ T cells make up 20-30 percent of the lymphocytes and depletion of these cells suddenly opens a substantial amount of "space", which could promote proliferation of remaining $CD8^+$ T cells to fill up the space again.

3.4.1. $CD4^+$ T cell depletion does not lead to homeostatic proliferation

To test whether homeostasis is the cause for the increased numbers of antigen-specific cells $CD8^+$ after $CD4^+$ T cell depletion, we depleted naïve BALB/c mice carrying the surface marker Thy1.2 of their $CD4^+$ T cells using the same depletion protocol as used for the immunization (days –3, -2, -1, +2, +5 before/ after cell transfer). Instead of immunizing the mice at day 0, recipient mice were injected with CFSE labeled Thy1.1 positive splenocytes of naïve donor mice. As a control, recipient mice that did not undergo $CD4^+$ T cell depletion, but were otherwise treated the same way were used (Figure 16A). Through staining for Thy1.1 positive cells we were able to identify the transferred cells in the splenocytes of recipient mice seven days later, and their CFSE staining profile indicated whether these cells underwent proliferation or not. We found that $CD4^+$ T cell depletion did not lead to any detectable proliferation of transferred cells within this short period of time.

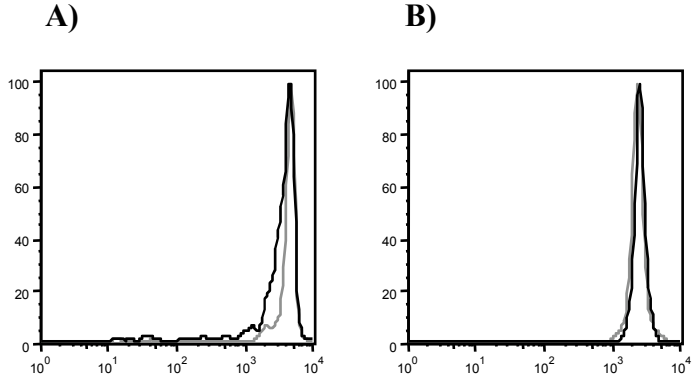


Figure 16: Adoptive transfer of naïve or antigen- specific T cells

Thy1.1 (A) or LLO_{91-99}-specific (B) cells were labeled with CFSE and transferred i.v. in naïve or anti-CD4 mAb treated mice. One week later this cells were tracked by staining splenocytes of the donor mice with PE-conjugated Thy1.1 or PE-labeled LLO_{91-99} tetramer and CFSE staining to analyze the proliferative activity of the transferred cells. To determine proliferative differences, histograms are shown as overlay presenting proliferation of transferred cells in undepleted mice (gray line) and depleted mice (black line).

This finding makes it unlikely that the space created through $CD4^+$ T cell depletion induces "unspecific" homeostatic proliferation of $CD8^+$ T cells. However, there still could be a difference in homeostatic behavior between naïve and recently activated $CD8^+$ T cells.
In order to exclude this possibility, we repeated similar experiments and transferred recently activated LLO_{91-99}-specific T cells from a LLO_{91-99}-specific T cell line labeled with CFSE. Again, as it can be seen in Figure 16B, no homeostatic proliferation of these cells after transfer in mice that got $CD4^+$ T cell depleted could be observed.
Based on these data we believe that the increase of antigen-specific $CD8^+$ T cell frequencies seen after $CD4^+$ T cell depletion and immunization with LLO protein is unlikely to be mediated by homeostatic effects.

3.5. Contribution of $CD25^+$ regulatory T cells for establishment of protective immune response

According to our data, $CD4^+$ T cells must be able to mediate inhibitory effects on $CD8^+$ T cell expansion, because depletion of this population leads to enhanced immune responses. A regulatory role of $CD4^+$ T cells is described for a subset of this population, namely $CD4^+CD25^+$ T cells, that constitute 5-15% of peripheral $CD4^+$ T cells. Therefore, it could be possible that the enhanced immunogenicity after $CD4^+$ T cell depletion is mostly due to the absence of the $CD4^+CD25^+$ subset and not the overall loss of $CD4^+$ T cells.

3.5.1. Effect of $CD25^+$ cell depletion on antigen-specific $CD8^+$ T cell frequencies after LLO plus CpG immunization

To evaluate whether loss of the whole $CD4^+$ T cell compartment or just the subset of $CD4^+CD25^+$ T cells causes the enhancement of $CD8^+$ T cell responses, we used another depletion strategy targeting $CD25^+$ T cells. Application of anti-CD25 depleting antibodies was done on days -5, -1, +2, +5 before/after immunization (day 0), following a recently published protocol showing $CD4^+CD25^+$ T cell restriction on $CD8^+$ memory T cell responses (Kursar et al., 2002; Sun and Bevan, 2003). As controls, mice were undepleted or depleted for $CD4^+$ T cells as described above. The frequencies and total numbers of antigen-specific $CD8^+$ T cells of the LLO immunized groups seven days after immunization were determined. As shown in Figure 17, LLO immunization alone leads to weak antigen-specific immune responses as detected by tetramer staining for LLO_{91-99}-specific T cells (0.21+/-0.04%). The frequencies were even lower for the $CD25^+$ cell depleted groups (0.15-/-0.03%), but increased up to about 2% (1.52+/-0.28%) for the $CD4^+$ T cell depleted group. The application of LLO plus CpG

further enhances these effects to frequencies of about 3% (2.46+/-0.95%; non-depleted), about 7% (7.37+/-0.55%; $CD25^+$ cell depleted) and about 3.5% (3.53+/-1.95%; $CD4^+$ T cell depleted group).

These data indicate that in the case of vaccination with LLO protein alone $CD25^+$ regulatory T cells are not capable to significantly suppress LLO-specific $CD8^+$ T cell responses. However, there seem to be some other $CD4^+$ T cells that can exert such a suppressive effect, since overall depletion of $CD4^+$ T cells clearly leads to enhanced $CD8^+$ T cell responses.

Immunization in combination with a strong adjuvant like CpG shows a different picture. Under these conditions, $CD25^+$ cell depletion alone strongly enhances LLO_{91-99}-specific T cell populations, even more than after total $CD4^+$ T cell depletion. This might be due to the fact that $CD25^+$ cell depletion diminishes suppressive regulatory T cells but still the positive contribution of help by conventional $CD4^+$ T cells is present.

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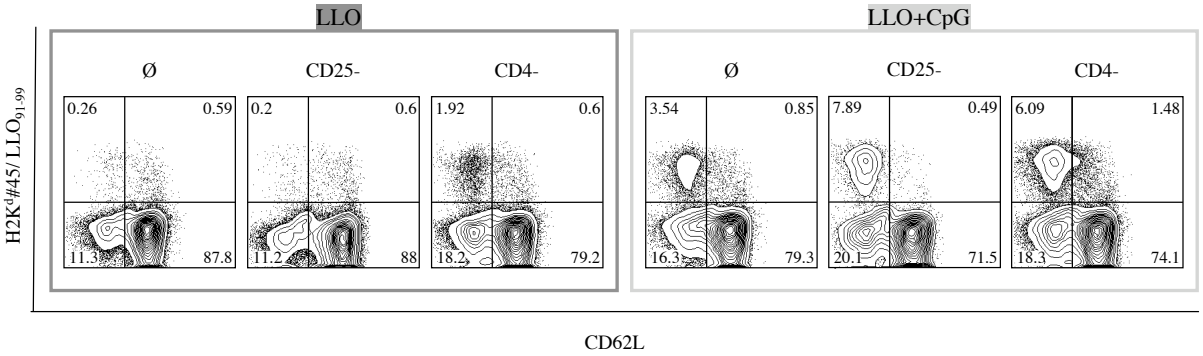
A)



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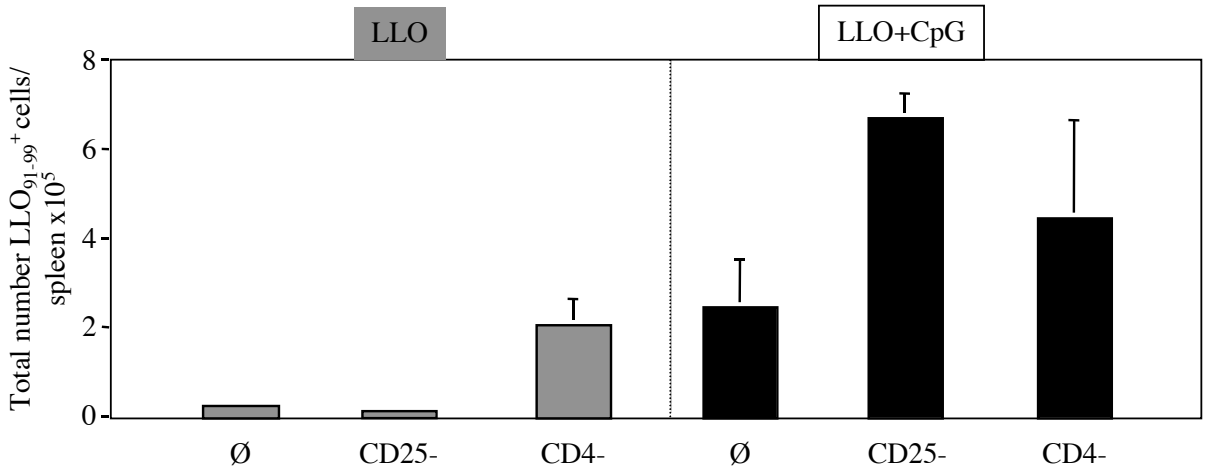


Figure 17: Frequencies and numbers of LLO_{91-99}-specific $CD8^+$ T cells after $CD4^+$ T cell or $CD25^+$ cell depletion

BALB/c mice were immunized with 10µg LLO or LLO plus 5nmol CpG. Indicated groups got treated with 150µg GK1.5 i.p. at days –3, -2, -1, +2, +5 or 250µg anti-CD25 mAb at days –5, -2, +2, +5 before/after immunization. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer and APC-conjugated CD8α mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs represent mean values +/- SD for spleen cells of three individually analyzed mice. Results are representative of at lease 4 independent experiments (B).

3.5.2. Effect of $CD25^+$ cell depletion on functionality after LLO plus CpG immunization

The increase in frequencies of antigen-specific $CD8^+$ T cells after immunization with LLO plus CpG and $CD25^+$ cell depletion raises the question whether this stimulatory effect of $CD25^+$ cell depletion also correlates with enhanced effector function of induced T cells.

As it has been seen for tetramer staining within the LLO immunized groups, there is an increase in IFNγ-producing $CD8^+$ T cell numbers upon $CD4^+$ T cell depletion, but not when mice were depleted for $CD25^+$ cells. Within the LLO plus CpG treated groups we found higher numbers of IFNγ-producing $CD8^+$ T cell in the LLO plus CpG treated mice compared to mice immunized with LLO alone. We further found enhanced numbers of IFNγ-producing $CD8^+$ T cell in $CD4^+$ T cell depleted mice and even more in $CD25^+$ cell depleted mice. These data correlate perfectly with the results generated with $LLO_{91\text{-}99}$-specific tetramer staining indicating that treatment of mice with anti-CD25 depleting antibodies can largely enhance the $CD8^+$ T cell immunogenicity toward LLO protein vaccination, provided that a strong adjuvant stimulus is present.

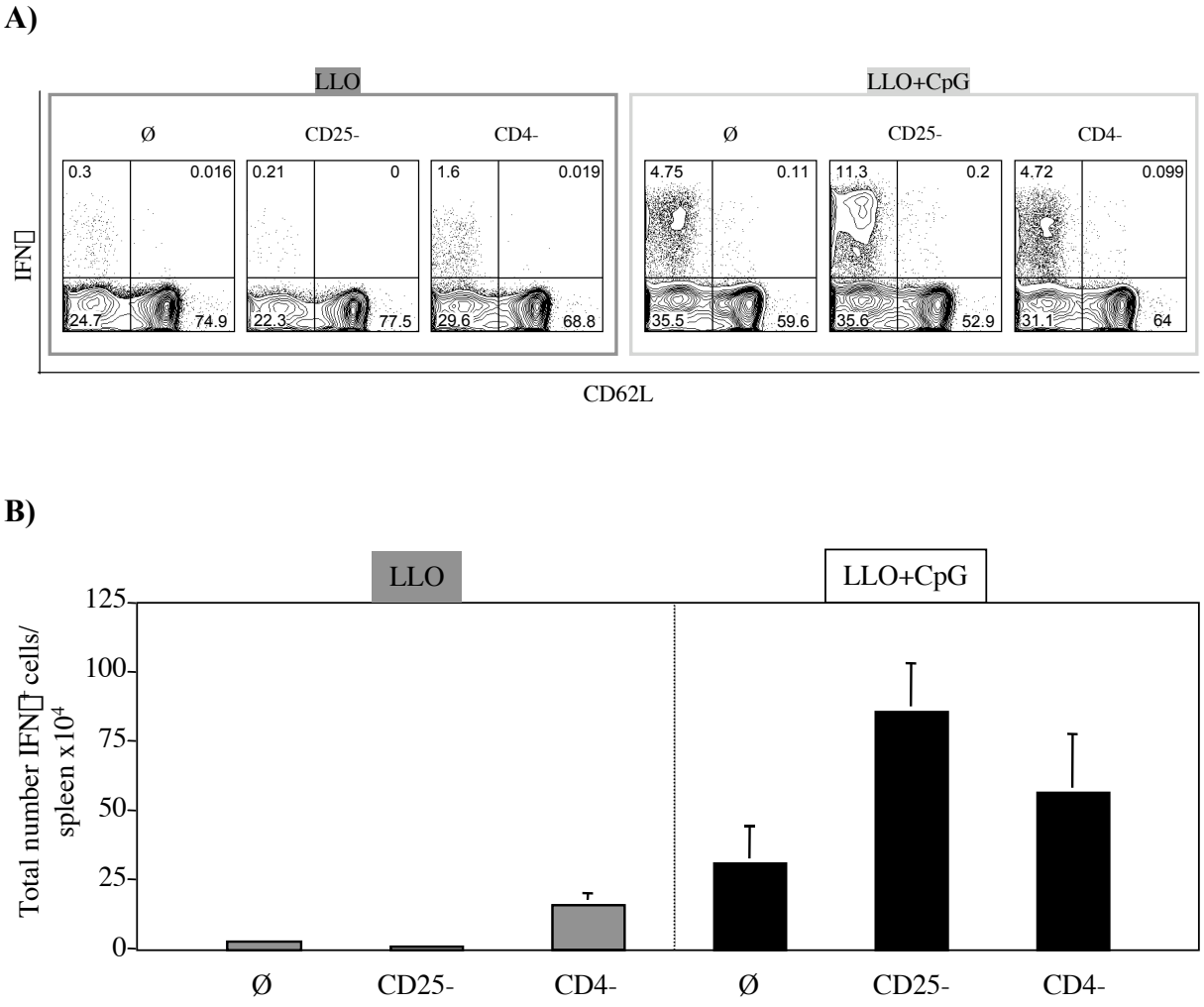


Figure 18: Frequencies and numbers of IFN-producing LLO_{91-99}-specific $CD8^+$ T cells after $CD4^+$ T cell or $CD25^+$ cell depletion

BALB/c were immunized s.c. with 10g LLO with/without 5nmol CpG. Indicated groups got treated with 150g GK1.5 i.p. at days –3, -2, -1, +2, +5 or anti-CD25 mAb at days –5, -2, +2, +5 before/after immunization. After one week mice were sacrificed and splenocytes were incubated with DMSO or LLO_{91-99} peptide for 5 hours. For the final 3 hours Golgi-Plus was added to the cultures. Cells were surface stained with PE-conjugated anti-CD8 and APC-conjugated anti-CD62L mAb and intracellularly with FITC-conjugated IFN. Life/death discrimination was done by EMA staining. Background frequencies and numbers of IFN-producing cells determined in cultures containing DMSO were subtracted from frequencies and numbers derived from cultures with peptide. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs depict mean +/- SD for LLO_{91-99}-specific IFN $CD8^+$ T cells of three individually analyzed mice per experimental group (B). The experiment shown is representative for at least 3 similar experiments.

3.5.3. Effect of CD25$^+$ cell depletion on antigen-specific CD8$^+$ memory responses after LLO plus CpG immunization

The obvious next question to address was whether CD25$^+$ cell depletion also leads to increased memory responses or if the positive effect seen after CpG treatment is restricted to the primary immune response. Therefore, *in vivo* CTX assays were performed to determine the maintenance of antigen-specific lysis 5 weeks after primary immunization.

Mice were immunized and mAb depleted as indicated and 5 weeks later they were injected with naïve splenocytes that were incubated with LLO_{91-99} peptide and labeled with CFSE.

After 20 hours of in vivo target cell exposure there was no significant difference in the antigen-specific lysis within the groups of LLO immunized mice either plus additional CD4$^+$ or CD25$^+$ cell depletion. Interestingly, in the groups of LLO plus CpG immunized mice CD4$^+$ depletion during the primary response increased the lysis indicating that through this treatment also the number or quality of LLO_{91-99}-specific CD8$^+$ effector memory T cells gets substantially enhanced. This positive effect on effector memory T cells was even more pronounced in the group of mice treated with anti-CD25 depleting antibodies.

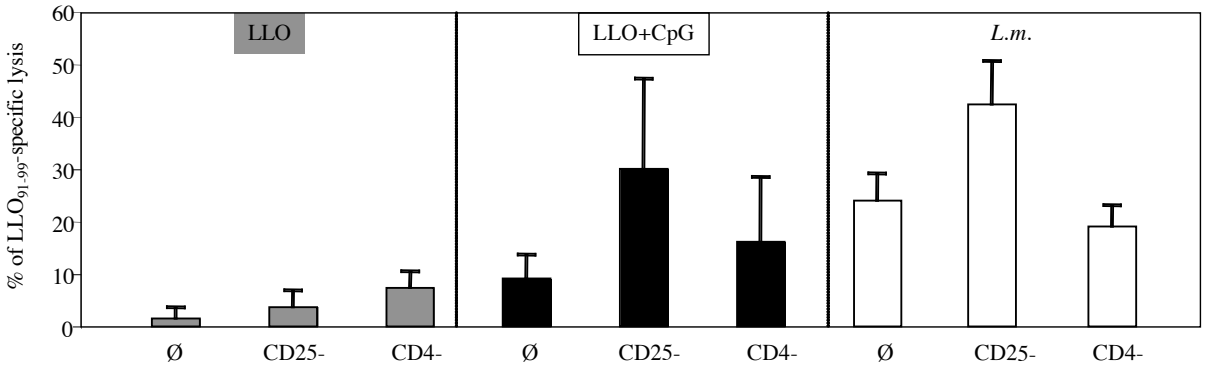


Figure 19: Specific lysis of LLO_{91-99}-specific CD8$^+$ target cells

Mice were immunized s.c. with 10µg LLO with/without 5nmol CpG. Control mice were infected with 1x10^3 L.m. Indicated groups got treated with 150µg GK1.5 i.p. at days –3, -2, -1, +2, +5 or anti-CD25 mAb at days –5, -2, +2, +5 before/after immunization. 35 days after immunization mice were infected with a mix of splenocytes from naïve mice; half of the splenocytes was labeled with low dose of CFSE (0.5µM) and the other half was loaded with LLO_{91-99} peptide and labeled with high dose of CFSE (5µM). At 5 and 20 hours after cell transfer mice were bled via tail vain incision and the number of CFSE labeled cells was analyzed. Bar graphs depict mean +/- SD for LLO_{91-99}-specific lysis of three individually analyzed mice.

In contrast, *L.m.* infected animals depleted of $CD4^+$ T cells during the primary immune response showed reduced levels of lysis, confirming recently published data. On the other hand, depletion of $CD25^+$ T cells increased LLO_{91-99}-specific effector memory T cells, again indicating that conventional $CD4^+$ T cells and $CD25^+$ regulatory T cells both participate in the modulation and regulation of immune responses.

Taken together, our data demonstrate that $CD25^+$ cell depletion during vaccination with LLO plus CpG not only leads to elevated antigen-specific frequencies and effector function in the primary immune response but also induces a potent pool of memory T cells.

3.5.4. Effect of $CD25^+$ cell depletion on protection after LLO plus CpG immunization

Depletion of $CD25^+$ cells during immunization with LLO protein plus CpG enhances numbers of effector memory T cells, which could be a promising strategy to improve the protective capacity of protein vaccinations. To address this question, mice immunized with LLO plus CpG in the absence of $CD25^+$ cells were challenged with high dose of *L.m.* As indicated in Figure 20, mice were immunized with LLO or LLO plus CpG in the presence or absence of depleting mAb against CD4 or CD25. As a control, one group of mice was infected with a low dose of *L.m.* (0.1 x LD_{50}). Five weeks after immunization mice were challenged with a high dose of *L.m.* (2 x LD_{50}). Three days later, tissue homogenate of spleens was plated out, and viable bacteria were counted the following day.

Control groups showed expected results: primary *L.m.*-infected mice were effectively protected against *Listeria* challenge, as demonstrated by the absence of any viable bacteria in the spleens of these mice. On the other hand, unimmunized naïve control mice are unprotected and showed high numbers of viable bacteria in the spleen. Compared to this group, mice that got immunized with LLO demonstrated slightly decreased numbers of viable bacteria, but overall there was no significant difference in the level of protection. Similarly, depletion of $CD4^+$ T cells or $CD25^+$ cells in mice immunized with LLO protein alone did not lead to any changes in the bacterial burden. Strikingly, when comparing mice immunized with LLO plus CpG with the control groups, a quite substantial protective effect of this vaccination becomes apparent. Both $CD4^+$ and $CD25^+$ cell depletion during vaccination decreased the number of viable bacteria recovered from infected organs more than two log scaled (as compared to naïve infected controls).

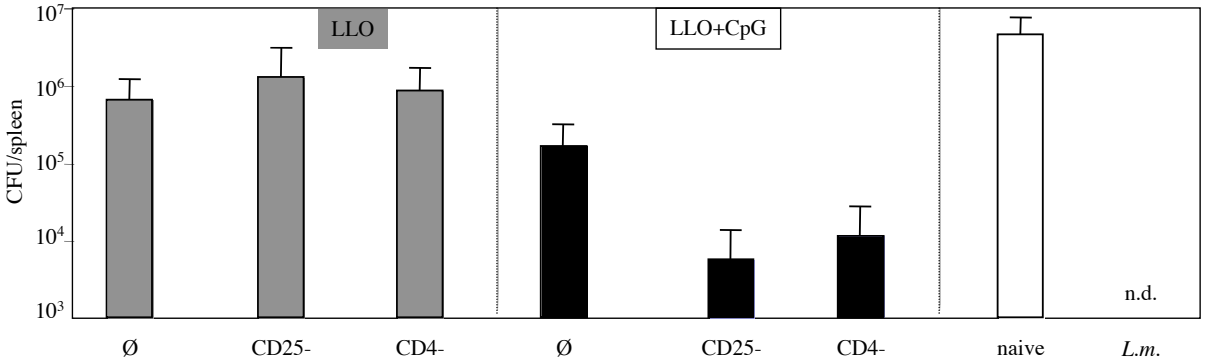


Figure 20: Level of protection after challenge with L.m.

Mice were primary immunized with 10µg LLO or LLO plus CpG. Indicated groups were additional treated with mAb during primary immune response. Mice that got infected with low dose of L.m. (0.1 x LD_{50}) served as control group. 5 weeks after immunization, all groups got challenged with a high dose of L.m. (2 x LD_{50}). Naïve mice, treated with the high dose again served as control. Splenocytes were treated with 0.1% Triton-X solution and plated out in triplets in serial dilutions on BHI plates. The following day CFU was analyzes by counting bacterial colonies. Numbers shown are the mean from 3 mice/ group (n.d.= not detectable).

Taken together, these data determining the effect of LLO protein vaccination on protection against infection with life bacteria perfectly correlate with the analysis of effector memory T cell functionality (*in vivo* CTL assays) shown before. Protein vaccination in the absence of CpG adjuvant does not induce significant levels of protective immunity; depletion of $CD4^+$ or $CD25^+$ T cells does not affect this observation. Addition of CpG improved immunogenicity, resulting in weak but detectable protection. However, depletion of $CD4^+$ T cells and to a larger extent depletion of $CD25^+$ cells substantially increases the induction of protection against *Listeria* infection.

3.5.5. Effect of $CD25^+$ cell depletion on generation of T_{EM} cells after LLO plus CpG immunization

The described results show that depletion of $CD25^+$ cell during the priming phase leads to enhanced immune responses both during the primary and memory phase. We know from recent data that the outcome of effective $CD8^+$ T cell mediated memory responses depends not only on the number of memory $CD8^+$ T cells generated, but also on the type of memory T cells. In mice and human, so far two distinct memory T cell populations can be discriminated, T_{CM} (central memory) cells and T_{EM} (effector memory) cells. T_{CM} ($CD127^{high}/CD62L^{high}$) cells are characterized by high proliferative capacity but do not have immediate effector function,

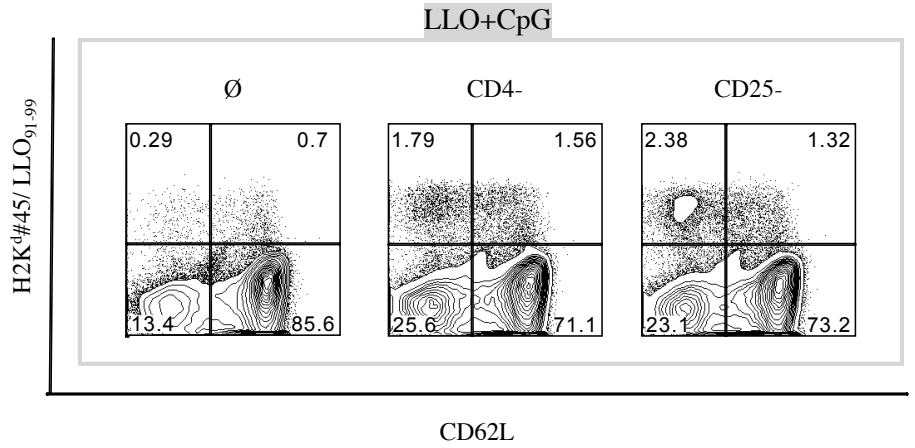
T_{EM} ($CD127^{high}/CD62L^{low}$) cells poorly proliferate but show immediate effector function. It is well known that the absence of $CD4^+$ T helper cells in bacterial and viral models (Shedlock and Shen, 2003; Sun and Bevan, 2003) results in reduced memory responses, explained by a lack of T_{EM} (Huster et al., 2004). Lack of $CD25^+$ regulatory $CD4^+$ T cells on the other hand enhanced protective memory responses, but dependency on distinct memory T cell subpopulations has so far not been described (Dikopoulos et al., 2004; Suvas et al., 2003; Toka et al., 2004).

In our system, $CD25^+$ cell depletion as well as $CD4^+$ T cell depletion enhanced protection, indicating that the lack of regulatory $CD25^+$ T cells induced by $CD4^+$ T cell depletion exceeds potential negative effects of lack of $CD4^+$ T cell help. We hypothesized that the distribution of T_{CM} and T_{EM} cells might be specifically influenced by the depletion of $CD25^+$ regulatory T cells.

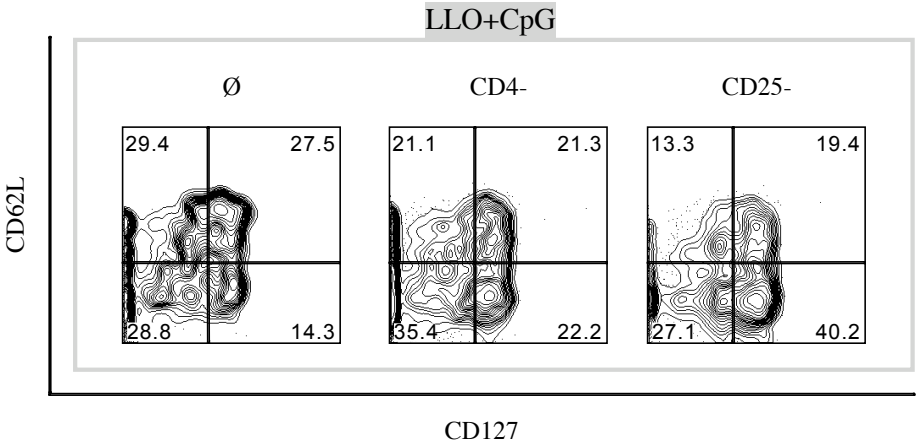
To test this, we immunized mice in the absence or presence of either $CD4^+$ or $CD25^+$ cells with LLO plus CpG. 12 days later, already in the contraction phase, different subpopulations of memory T cells can be distinguished by their CD127 and CD62L expression.

The frequencies of LLO_{91-99}–specific $CD8^+$ T cells 12 days after immunization are higher in $CD4^+$ or $CD25^+$ depleted mice compared to undepleted mice. Comparing the distribution of T_{CM} and T_{EM} cells, we could demonstrate that the percentage of T_{EM} cells increased in the absence of $CD4^+$ T cells and even more in the absence of $CD25^+$ cells (Figure 21C). The number of T_{CM} cells decreased when $CD4^+$ T cells or $CD25^+$ cells were depleted (Figure 21D). These were highly significant findings showing that in the absence of $CD4^+$ cells and even more in the absence of $CD25^+$ cells the distribution of T_{CM} and T_{EM} cells is shifted towards more T_{EM} cells and less T_{CM} cells compared to the number in mice that are not depleted. Therefore, these experiments provide important data linking the magnitude of T_{EM} cells to the level of protective immune responses. T_{CM} cells on the other hand do not seem to be the memory population that mediates protection against *L.m.*

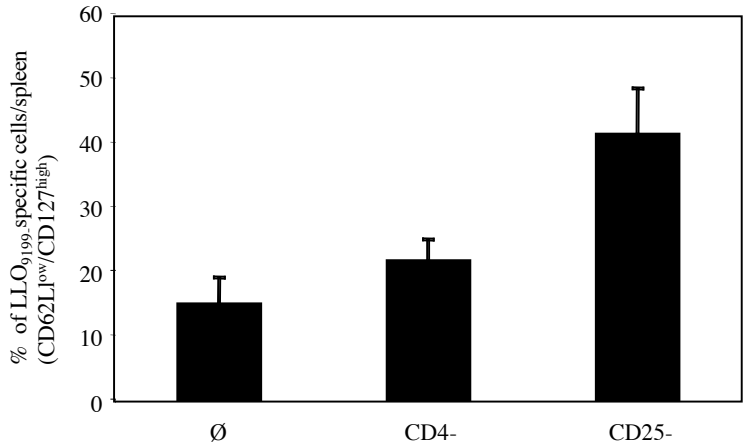
A)


LLO+CpG
Ø
CD4-
CD25-
0.29
0.7
13.4
85.6
1.79
1.56
25.6
71.1
2.38
1.32
23.1
73.2
H2K^{d}#45/ LLO$_{91-99}$
CD62L

B)


LLO+CpG
Ø
CD4-
CD25-
29.4
27.5
28.8
14.3
21.1
21.3
35.4
22.2
13.3
19.4
27.1
40.2
CD62L
CD127

C)


60
50
40
30
20
10
0
% of LLO$_{9199}$-specific cells/spleen (CD62L^{low}/CD127^{high})
Ø
CD4-
CD25-

D)

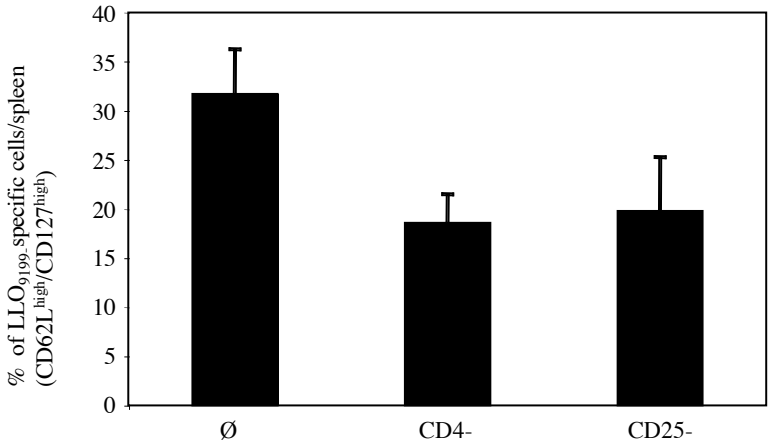


Figure 21: Frequencies of LLO_{91-99}-specific $CD8^+$ T cells after $CD4^+$ T cell or $CD25^+$ cell depletion (d12)

BALB/c mice were immunized with 10µg LLO plus 5nmol CpG. Indicated groups got treated with 150µg GK1.5 i.p. at days –3, -2, -1, +2, +5 or 250µg anti-CD25 mAb at days –5, -2, +2, +5 before/after immunization. 12 days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer, Alexa 405-conjugated anti-CD8α mAb and APC-conjugated CD127 mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (A) or $CD8^+$ LLO_{91-99}-specific T cells (B). Each dot plot is representative for 9-15 mice per group. Bar graphs represent mean values +/- SD for spleen cells of five individually analyzed mice. Results are representative of at least 3 independent experiments (C, D).

3.5.6. $CD4^+$ and $CD25^+$ effects are similar to LLO for OVA-1668 immunization in BL6 mice

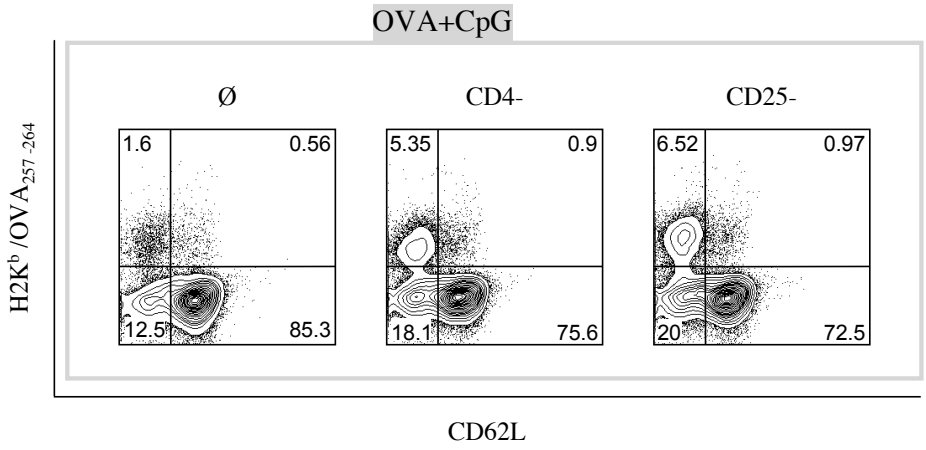
Antibody depletion as a tool to study immune responses has the advantage to selectively eliminate specific populations at desired time-points. On the other hand, this method has limitations concerning the efficiency, as not all cells may be depleted, and the transience, since the depleted populations successively recover. To overcome these limitations, we decided to use MHC class $II^{-/-}$ mice to confirm our data.

Since available MHC class $II^{-/-}$ mice are on the BL6 background, we first needed to transfer our basic experimental setting to BL6 mice. As there are no known $H2^b$ restricted *L.m.* epitopes for $CD8^+$ T cells, we decided to use OVA-1668 as a model antigen to monitor antigen-specific immune responses, because this also enabled us to use infection with *L.m.* recombinantly expressing OVA as control. The immunodominant K^b restricted OVA epitope SIINFEKL was used for analysis. As described before, we depleted mice with $CD4^+$ T cell or $CD25^+$ cell depleting antibodies and immunized with 10µg OVA-1668 conjugate. Seven days

later, splenocytes were stained with $K^b/OVA_{257-264}$-specific tetramer. The number of antigen-specific $CD8^+$ T cells constantly increased between the groups of immunized BL6 mice alone, $CD4^+$ T cells depleted, and $CD25^+$ cells depleted mice. OVA-1668 immunization alone already induced 1.6% of SIINEFKL-specific $CD8^+$ T cells. Comparing this group with the ones that got depleted for $CD4^+$ T cells or $CD25^+$ cells, there was a 3-fold increase in frequencies of $CD4^+$ T cell depleted mice and a 4-fold increase in the $CD25^+$ cell depleted group. The effect is even more pronounced when comparing total numbers of antigen-specific $CD8^+$ T cells per spleen; the $CD4^+$ T cell depleted group shows a 6-fold increase and the $CD25^+$ cell depleted group a 9-fold increase of antigen-specific $CD8^+$ T cells compared to the undepleted group.

In summary, the effects on epitope specific T cells upon protein immunization in combination with $CD4^+$ T cell or $CD25^+$ cell depletion and CpG treatment are reproducible in different mouse strains (BL6 and BALB/c) and when using different antigens (OVA and LLO). This finding strongly supports the notion of a general mechanism underlying the observed effects.

A)



B)

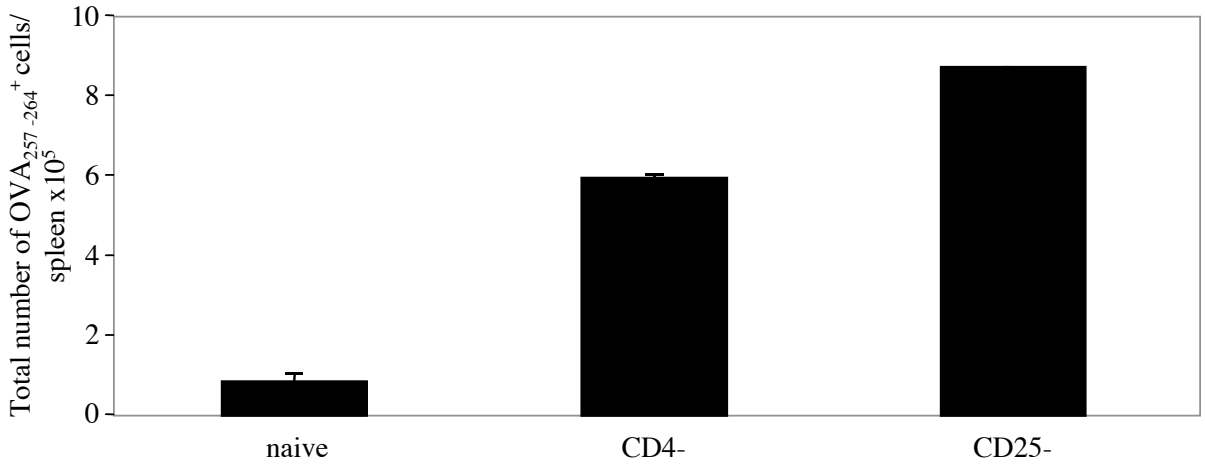


Figure 22: Frequencies and numbers of $OVA_{257-264}$-specific $CD8^+$ T cells after $CD4^+$ T cell or $CD25^+$ cell depletion

BL6 mice were immunized with 10µg OVA-1668. Indicated groups got treated with 150µg GK1.5 i.p. at days –3, -2, -1, +2, +5 or anti-CD25 mAb at days –5, -2, +2, +5 before/after immunization. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled $OVA_{257-264}$ tetramer and APC-conjugated CD8α mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs represent mean values +/- SD for spleen cells of three individually analyzed mice (B).

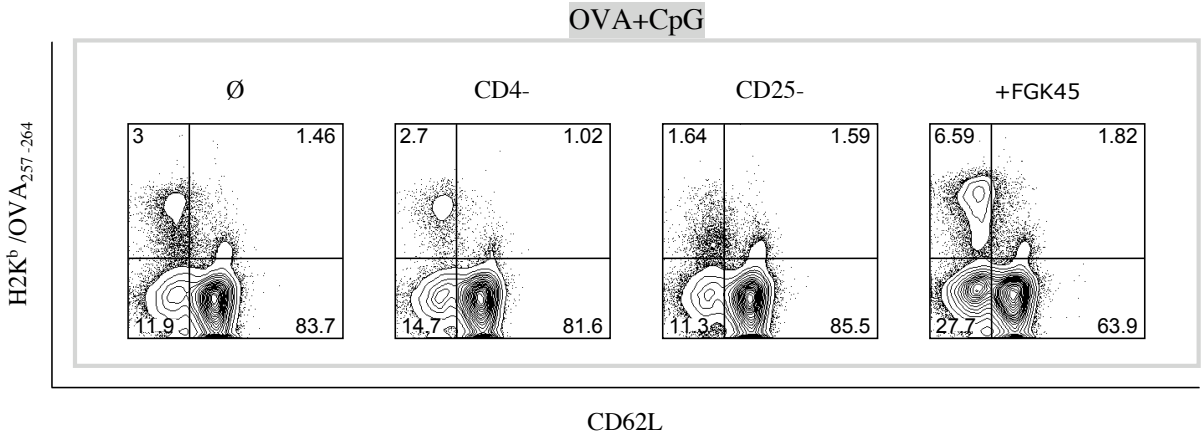
Next we wanted to determine whether the same effect of increased immunogenicity can be observed in MHC class II$^{-/-}$ mice. Analysis of splenocytes of naïve MHC class II$^{-/-}$ mice showed that approximately 2-4% of cells still stained positive for the CD4 T cell marker. Therefore, we additionally treated one group of immunized mice with $CD4^+$ T cell depleting mAbs in order to analyze which effect these remaining $CD4^+$ cells exert on $CD8^+$ immune responses. So far we hypothesized that mainly naturally arising $CD25^+CD4^+$ T cells were

depleted. In MHC class $II^{-/-}$ mice, $CD4^+CD25^+$ Treg cells should be absent by definition and additional treatment of the MHC class $II^{-/-}$ mice with the $CD25^+$ cell depleting mAb could only deplete other $CD25^+$ cell populations.

As a control whether changes in $CD8^+$ T cell immune response in MHC class $II^{-/-}$ mice are due to the lack of $CD4^+$ T cell help, a group of mice got treated with CD40 stimulating antibody (FGK45) to mimic one of the most important costimulatory activator mediating $CD4^+$ T cell help. CD40L is expressed on $CD4^+$ T cells and binds directly to CD40 on the APC. This leads to enhanced expression of costimulatory molecules on the APC and finally to improved T cell activation. Application of FGK45 can restore in part the CD4 helper function. To provide CD40 stimulation to the APCs, mice were treated for 5 consecutive days with the mAb starting at the day of immunization. $CD4^+$ T cell- and $CD25^+$ cell depletion was performed as described before. Seven days after *s.c.* immunization with 10 g of OVA-1668 conjugate splenocytes were taken for analysis.

As summarized in Figure 23, SIINEFKL-specific $CD8^+$ frequencies were increased in the FGK45 treated group, and no difference could be observed in the other groups, no matter whether they were treated with anti-$CD4^+$ or -$CD25^+$ mAb. These results provide several important informations: $CD4^+$ T cell depletion has no effect on $CD8^+$ T cell immunogenicity in MHC class $II^{-/-}$ mice when compared to the untreated group. This is expected because MHC class $II^{-/-}$ mice should not have any functional $CD4^+$ T cells in their system. $CD25^+$ cell depletion also shows no effect, which might indicate that in a normal wildtype mouse the observed depletion effects are mainly mediated by naturally arising $CD4^+CD25^+$ T cells, which are not present in the MHC class $II^{-/-}$mice. The increase of SIINEFKL-specific $CD8^+$ frequencies after application of FGK45 demonstrates that $CD8^+$ T cells are characterized by a general activation defect in this knockout system because they show normal antigen-specific proliferation when the right co-stimulation is given.

A)



B)

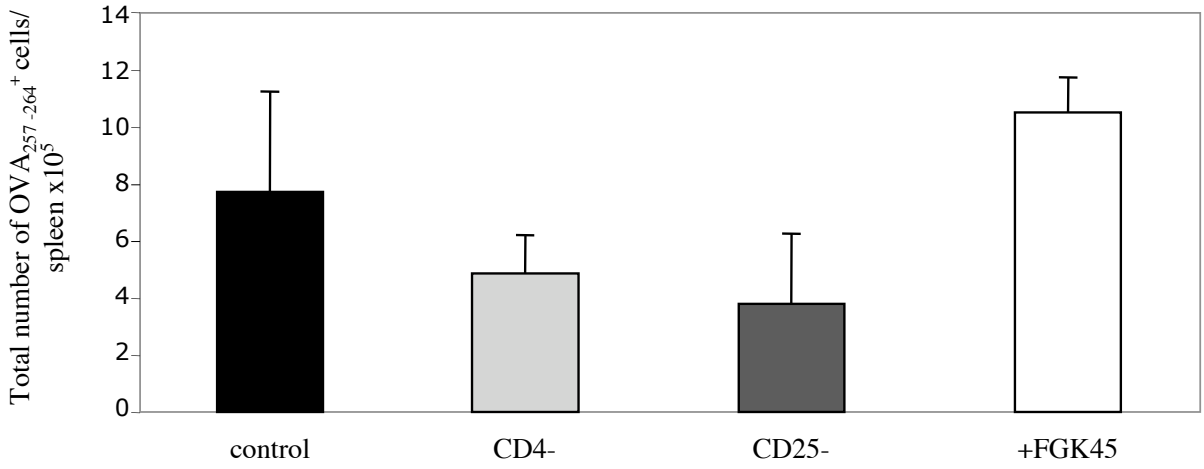


Figure 23: Frequencies and numbers of OVA$_{257-264}$-specific CD8$^+$ T cells after CD4$^+$ T cell or CD25$^+$ cell depletion

MHC class II$^{-/-}$ mice were immunized with 10µg OVA-1668. Indicated groups got treated with 150µg GK1.5 i.p. at days –3, -2, -1, +2, +5 or 250µg anti-CD25 mAb at days –5, -2, +2, +5 before/after immunization. FGK45 mAb was applied at 5 subsequent days beginning with 100µg at day 0 and 50µg the following 5 days. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled OVA$_{257-264}$ tetramer and APC-conjugated CD8α mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on CD8$^+$ T cells (A). Bar graphs represent mean values +/- SD for spleen cells of three individually analyzed mice (B). The experiment shown is representative for two individual experiments.

3.6. Modulation of immune response via different TLR signaling

TLRs play a very important role in innate immunity and as we demonstrated above, strong effects on the outcome of adaptive immune response can be observed after specific stimulation of TLR9 with CpG. We next wanted to analyze whether similar effects can be observed after stimulation of different TLRs. “V7” is a peptide from the LcrV protein. LcrV is a *Yersinia*-derived protein and it has been shown that the “V7” peptide sequence of this protein can efficiently signal through TLR2 leading to increased IL-10 production by macrophages. We therefore generated recombinant fusion proteins based on V7 peptide sequence and LLO, $LLO_{492T\ A}$ or p60 proteins, and analyzed the immune responses elicited after immunization with these conjugates.

3.6.1. Reduced functionality of $LLO_{492T\ A}$ -V7 and p60-V7

First, we tested conjugates for their function *in vitro* and measured NF B-dependent ELAM-1 promotor luciferase activity of HEK293 cells after stimulation with $LLO_{492T\ A}$-V7 or p60-V7. LLO-V7 protein is not feasible for this assay because LLO shows strong toxicity by its lytic activity on cells. The experiments were performed in collaboration with Dr. C. Kirschning. Description of the experimental procedures are published by Kischning et al. (Kirschning et al., 1998).

Although LPS is known to be a weak stimulator of TLR2 and its function is more ascribed for induction of TLR4 signaling, we first wanted to exclude the possibility that contamination with LPS in our protein purifications does stimulate TLR2. For this purpose HEK293 cells were incubated together with the recombinant protein and the LPS inhibitor Polymyxin B (PmB). As can be seen in Figure 24, “group 3” representing HEK293 cells that were transfected with different human and murine TLRs and stimulated with LPS and PmB showed indeed decreased luciferase activity for all TLRs tested. Since direct measurement of LPS amounts indicate that they do not exceed 1 g LPS content as used in this assay for stimulation (data not shown), we excluded the possibility that LPS contamination leads to stimulation of the TLRs tested. HEK293 cells that got stimulated with $LLO_{492T\ A}$-V7 showed increased luciferase activity when cells were transfected with hTLR2 (human TLR2) or FmTLR2 (flag-tagged murine TLR2), but not with the human or murine TLR4 or TLR3, which was used as control. Also when HEK293 cells got stimulated with the $LLO_{492T\ A}$ protein alone we could only measure background luciferase activity for all TLRs tested.

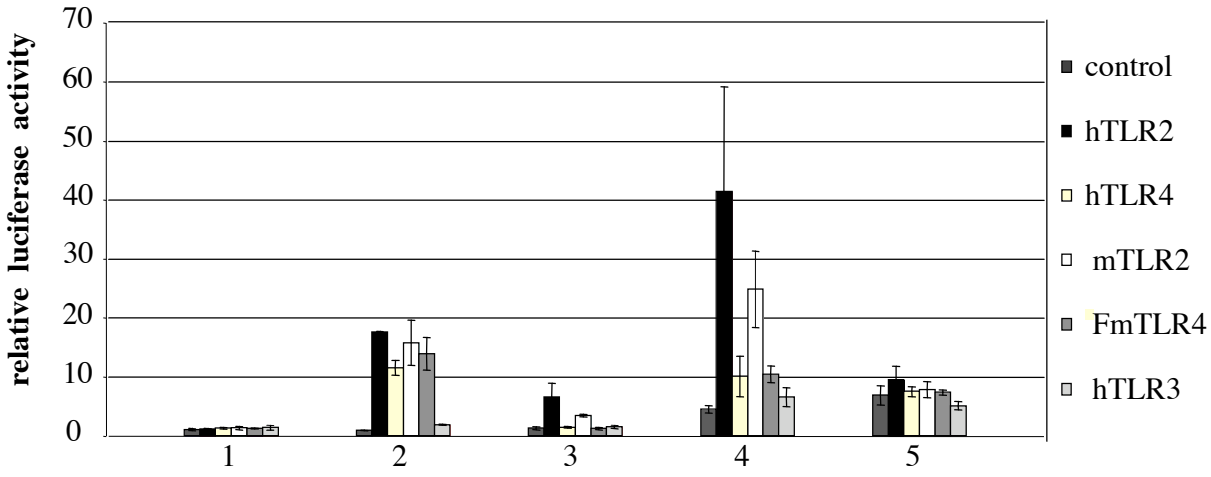


Figure 24: NF□B-dependent ELAM-1 promotor luciferase activity of HEK293

HEK293 cells were stimulated as followed and were transfected with different human (hTLRs, hTLR4 and hTLR4) and murine (mTLR2 and FmTLR4) TLRs: 1) unstimulated, 2) LPS, 3) LPS+ PmB, 4) $LLO_{492T\square A}$-V7+PmB, 5) $LLO_{492T\square A}$+PmB.

The same picture could be seen when HEK293 cells were incubated with p60-V7 protein. Again, an increase in luciferase activity was observed when cells were transfected with hTLR2 or FmTLR2, but not after transfection with other TLRs. Incubation with p60 protein alone did not lead to stimulation via the tested TLRs. Therefore, it can be concluded that the $LLO_{492T\square A}$–V7 and p60-V7 conjugates were both functional TLR ligands and were both specific only for signaling via TLR2. Based on these data it seems feasible that V7 protein conjugates can be used as TLR2 stimulators to evaluate the effect of stimulation of another TLR on the generation of memory $CD8^+$ T cell responses.

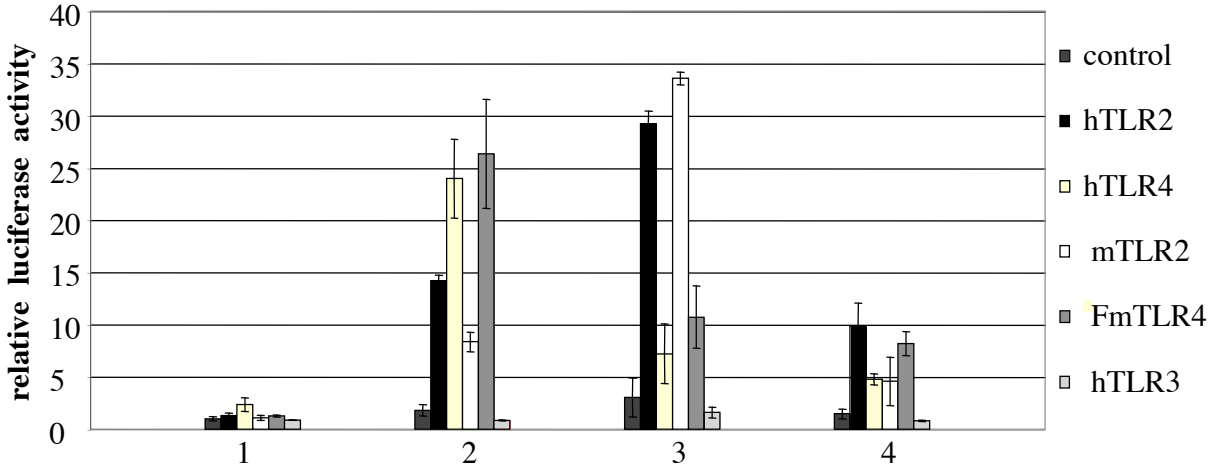


Figure 25: NF□B-dependent ELAM-1 promotor luciferase activity of HEK293

HEK293 cells were stimulated as followed and were transfected with different human (hTLRs, hTLR4 and hTLR4) and murine (mTLR2 and FmTLR4) TLRs: 1) unstimulated, 2) LPS, 3) p60-V7+ PmB, 4) p60+PmB.

3.6.2. Signaling via TLR2 decreases primary immune response

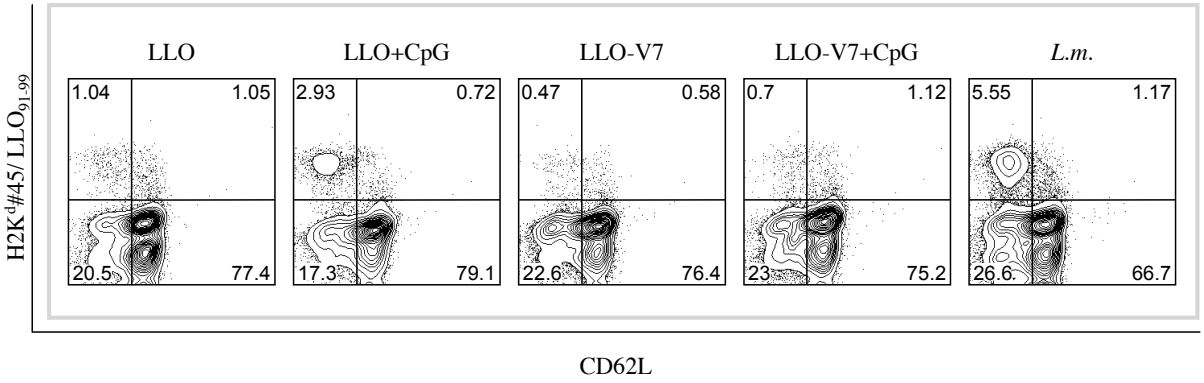
Above described *in vitro* signaling experiments demonstrated that LLO- and p60-V7 fusion proteins trigger TLR2 mediated events in a V7 dependent manner. This allowed us to compare the *in vivo* effects on $CD8^+$ T cell priming in the presence of V7/ TLR2 activity with the effect of CpG. As it has been described recently, V7 can trigger TLR2-dependent IL-10 production of macrophages, thereby mediating preferentially immunosuppressive activities *in vivo*, as demonstrated by infection experiments using *Yersinia* (Sing et al., 2002). CpG on the other hand can activate macrophages and DCs to produce proinflammatory cytokines such as TNF□, IL-1 and IL-6 and effector cytokines such as IL-12 and IL-15 (Wagner, 2001). Therefore, we hypothesized that the immune responses after application of LLO-V7 differ from immunization with LLO plus CpG.

To test this hypothesis, mice were immunized *s.c.* with LLO or LLO-V7 protein in the absence or presence of 5nmol CpG. As shown in Figure 26, immunization with LLO protein alone resulted in $LLO_{91\text{-}99}$-specific $CD62L^{low}$ $CD8^+$ T cell frequencies around 1% (1+/-0.15%). Upon immunization with LLO-V7, frequencies were significantly reduced by about 50% (0.44+/-0.08%). Co-application of CpG together with LLO wildtype protein largely increased the $LLO_{91\text{-}99}$-specific $CD62L^{low}$ $CD8^+$ T cell population as described before (2.48+/-0.65%). In contrast, additional adjuvant presence of CpG only marginally increased $LLO_{91\text{-}99}$-specific T cell frequencies during LLO-V7 vaccination. When analyzing at the level of total numbers of antigen-specific $CD8^+$ T cells in spleens, the effects were even more pronounced. Addition of CpG increased the numbers of antigen-specific $CD8^+$ T cells during vaccination with wildtype

LLO, whereas the CpG-mediated enhancement was only very weak for the LLO-V7 treated group.

Taken together, these data indicate that the *in vivo* effects on CD8+ T cell responses mediated by V7 differ substantially from CpG. V7 alone as adjuvant seems to suppress LLO specific $CD8^+$ T cell responses and this suppressive effect cannot be overcome by co-administration of CpG.

A)



B)

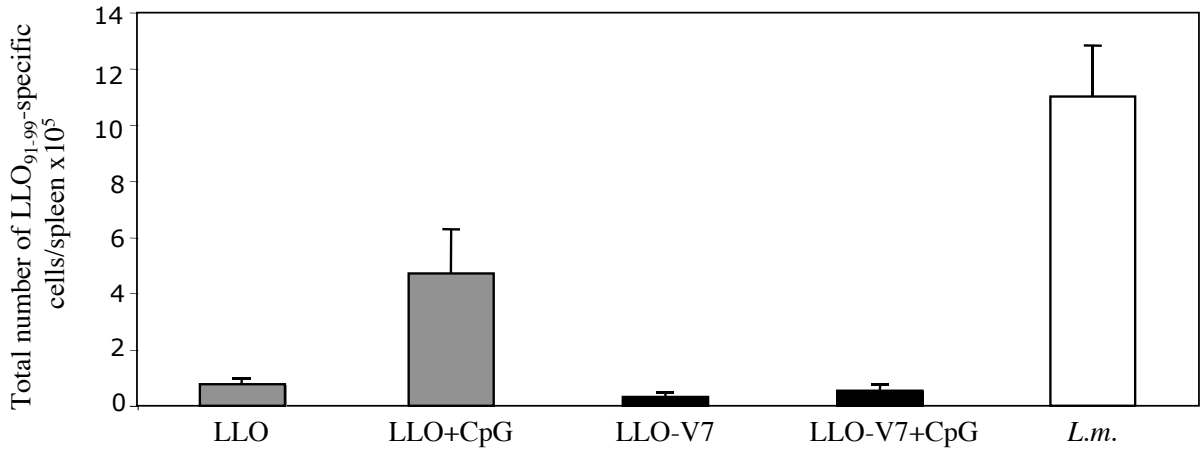


Figure 26: Frequencies and numbers of LLO_{91-99}-specific $CD8^+$ T cells after immunization with LLO or LLO-V7

BALB/c mice were immunized with 10□g LLO, LLO-V7 with/without 5nmol CpG. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer and APC-conjugated CD8□ mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs represent mean values +/- SD for spleen cells of three individually analyzed mice (B). The experiment shown is representative for three individual experiments.

To exclude the possibility that the weak immunogenicity of LLO-V7 fusion protein is mediated by structural changes of the LLO protein itself, we performed several control experiments:

If the fusion of LLO to V7 would severely alter the overall structure of the LLO protein, then the fusion protein should loose its hemolytic activity. As shown in Figure 27, incubation of erythrocytes with LLO or LLO-V7 demonstrates dose dependent and very similar lytic activity, making it unlikely that the V7-fusion changed the LLO molecule severely.

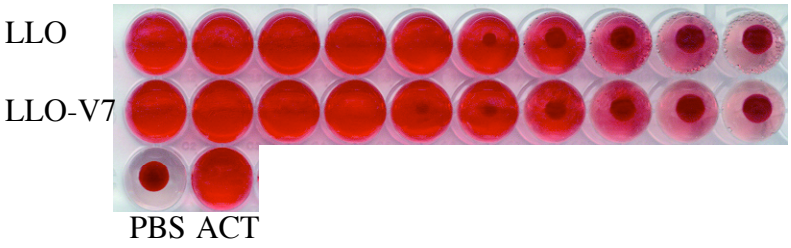


Figure 27: Specific lysis of erythrocytes with LLO and LLO-V7

BL6 mice were bled via tail vein incision. Cells were incubated for 15' with LLO or LLO-V7 with concentrations starting with 5□g/well followed by serial 1:2 dilutions. ACT and PBS treated cells served as control.

In order to demonstrate that the suppressive effects are really related to the V7 sequence, we generated an additional fusion protein LLO-V5. The V5 sequence is almost identical to V7, but has been described to be inactive for TLR2-signaling. As shown in Figure 28, immunization with LLO-V5 protein results in basically identical LLO_{91-99}-specific $CD8^+$ T cell responses in the presence or absence of CpG. These data clearly focus the suppressive effect of LLO-V7 to the V7 sequence, and in addition also show that LLO fusion proteins can give rise to identical T cell responses as compared to the wildtype potein.

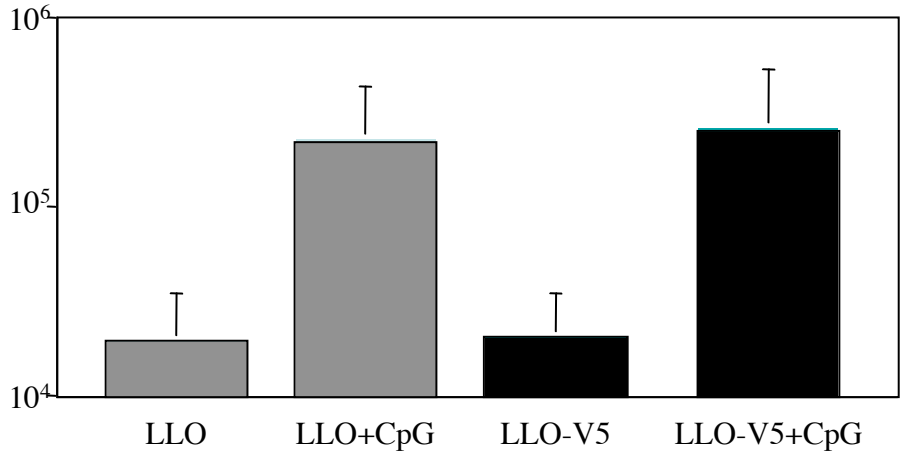


Figure 28: Numbers of LLO_{91-99}-specific $CD8^+$ T cells after immunization with LLO or LLO-V5

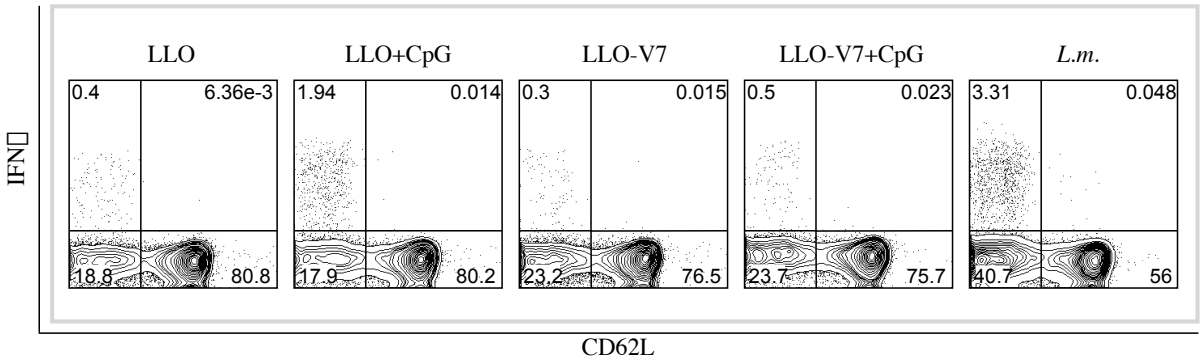
BALB/c mice were immunized with 10µg LLO, LLO-V5 with/without 5nmol CpG. Seven days after immunization, spleen cells were counted and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO_{91-99} tetramer and APC-conjugated CD8α mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs represent mean values +/- SD for spleen cells of three individually analyzed mice (B).

3.6.3. Decreased number of functional $CD8^+$ T cells after TLR2 stimulation

We just described that adjuvant activity of the TLR2-ligand V7 led to decreased $CD8^+$ immune responses. We next wanted to determine whether the quantitative differences also correlated with decreased functionality.

When comparing the levels of IFNγ-secretion after restimulation of the splenocytes with LLO_{91-99} peptide, we found that the difference between the LLO and the LLO-V7 immunized group was not as marked as could be seen for the tetramer frequencies. Usually about 50% of LLO_{91-99}-tetramer positive cells also respond in the IFNγ assay in LLO immunized mice. Although the tetramer staining data indicated substantial lower numbers in LLO-V7 immunized mice, the frequencies of IFNγ-responding T cells were almost comparable to the LLO immunized mice. Differences become more pronounced when comparing the groups that additionally received CpG treatment. Again the LLO plus CpG treated mice showed an increase in frequencies of IFNγ-secreting $CD8^+$ T cells, whereas the frequencies and absolute numbers were 3-4 fold reduced for the group that got immunized with LLO-V7 plus CpG.

A)



B)

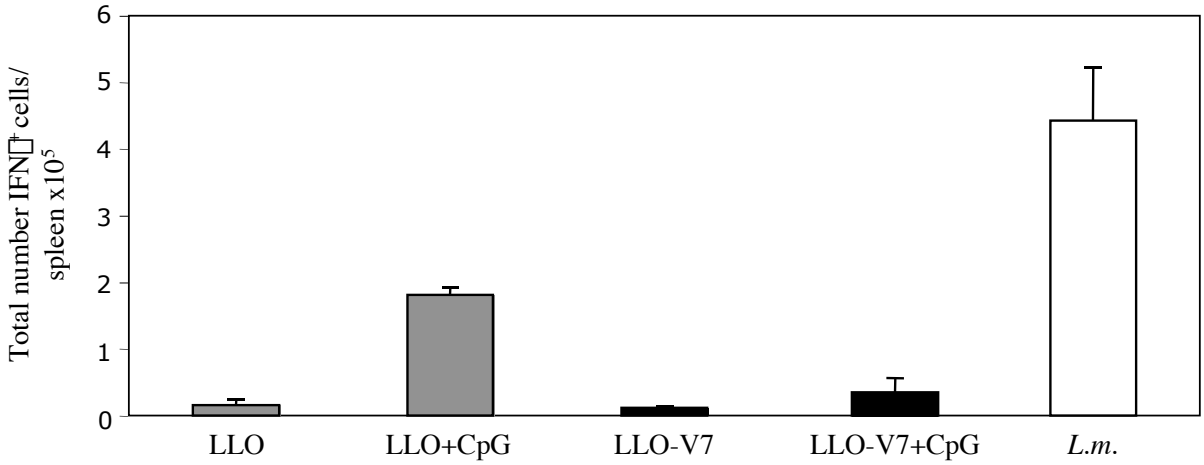


Figure 29: Frequencies and numbers of IFN-producing LLO_{91-99}-specific $CD8^+$ T cells after immunization with LLO or LLO-V7

BALB/c were immunized s.c. with 10g LLO, LLO-V7 with/without 5nmol CpG. After one week mice were sacrificed and splenocytes were incubated with DMSO or LLO_{91-99} peptide for 5 hours. For the final 3 hours Golgi-Plus was added to the cultures. Cells were surface stained with PE-conjugated anti-CD8 and APC-conjugated anti-CD62L mAb and intracellularly with FITC-conjugated IFN Life/death discrimination was done by EMA staining. Background frequencies and numbers of IFN-producing cells determined in cultures containing DMSO were subtracted from frequencies and numbers derived from cultures with peptide. Dot plots shown are gated on $CD8^+$ T cells (A). Bar graphs depict mean +/- SD for LLO_{91-99}-specific IFN $CD8^+$ T cells of three individually analyzed mice per experimental group (B). The experiment shown is representative for 2 similar experiments.

To test T cell functionality in another setting, we performed *in vivo* cytotoxicity assays. Naive splenocytes that were loaded with LLO_{91-99}-specific peptide and labeled with CFSE were injected into mice one week after immunization, and CFSE staining of blood lymphocytes was analyzed after 5 and 20 hours after injection. When looking for antigen-specific lysis (Figure 30), we cannot describe a marked difference between the groups of LLO or LLO-V7 immunized mice at 5 or 20 hours after stimulation. In both groups the lysis is rather low, and close to the level of significance for this assay. However, there is an increase of lysis for both groups for 5 and 20 hours, correlating with the presence of low numbers of LLO_{91-99}-specific $CD8^+$ T cells. Differences get more clear when comparing groups that additionally got treated with CpG. Already at 5 hours after stimulation there is a significant decrease of killing rates for the group of mice that got immunized with LLO-V7 plus CpG when compared to the LLO plus CpG treated group. CTLs of LLO-V7 plus CpG treated mice were not able to eliminate any of the antigen carrying cells at 5 hours after stimulation, whereas in the group of LLO plus CpG immunized mice there was already up to 40% epitope specific killing. When looking at 20 hours after *in vivo* exposure, now the group of LLO-V7 plus CpG treated mice showed a clear increase in killing rates when compared to the 5 hours time point, but still much lower compared to the LLO plus CpG treated group. Taken together, these data on functional analysis of LLO_{91-99}-specific $CD8^+$ T cells upon LLO or LLO-V7 immunization indicates that in the presence of V7 functional $CD8^+$ T cells (IFNγ-production, cytolytic activity) can be generated, but their overall number and frequency is much more lower as compared to mice immunized with wildtype LLO protein.

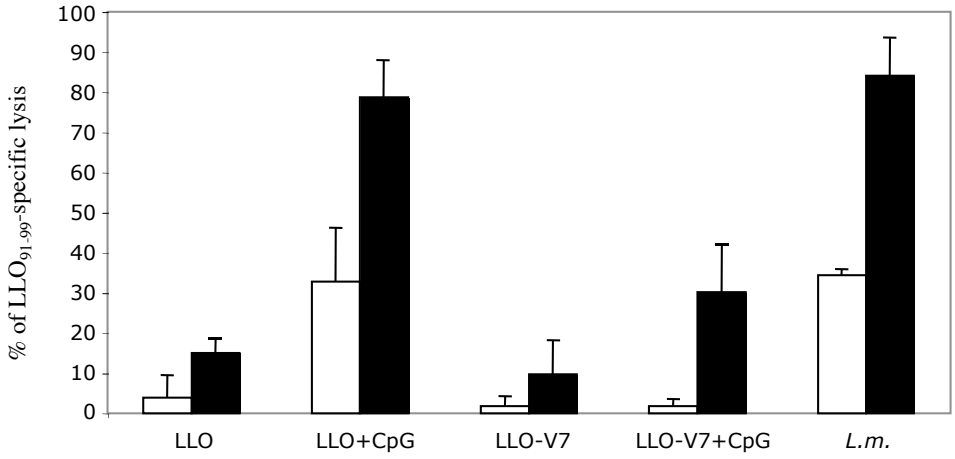


Figure 30: Specific lysis of LLO_{91-99}-specific $CD8^+$ target cells

Mice were immunized s.c. with 10µg LLO, LLO-V7 with/without 5nmol CpG. Seven days after immunization mice were infected with a mixture of splenocytes from naïve mice; half of the splenocytes was labeled with low dose of CFSE (0.5µM) and the other half was loaded with LLO_{91-99} peptide and labeled with high dose of CFSE (5µM). At 5 (white bars) and 20 (black bars) hours after cell transfer mice were bled via tail vein incision and the number of CFSE labeled cells was analyzed. Bar graphs depict mean +/- SD for LLO_{91-99}-specific lysis of three individually analyzed mice.

3.6.4. Effect of $CD4^+$ T cell or $CD25^+$ cell depletion on antigen-specific $CD8^+$ T cell frequencies after immunization

We showed before that $CD4^+$ T cell depletion had a strong effect on primary immune responses, no matter if a relatively weak or strong immunogen (LLO versus LLO plus CpG) was used. $CD25^+$ cell depletion showed an enhancing effect on the immune responses for the groups additionally treated with CpG. Based on these finding, we wanted to determine whether similar effects of $CD4^+$ or $CD25^+$ cell depletion could be found for vaccination with LLO-V7. Because the depletion effect was most prominent upon additional treatment with CpG, we chose to use LLO-V7 plus CpG immunization for these experiments. As described above, we immunized with 10µg LLO-V7 plus CpG and depleted additional groups of either their $CD4^+$ or $CD25^+$ T cells. As control, one group got immunized with LLO plus CpG.

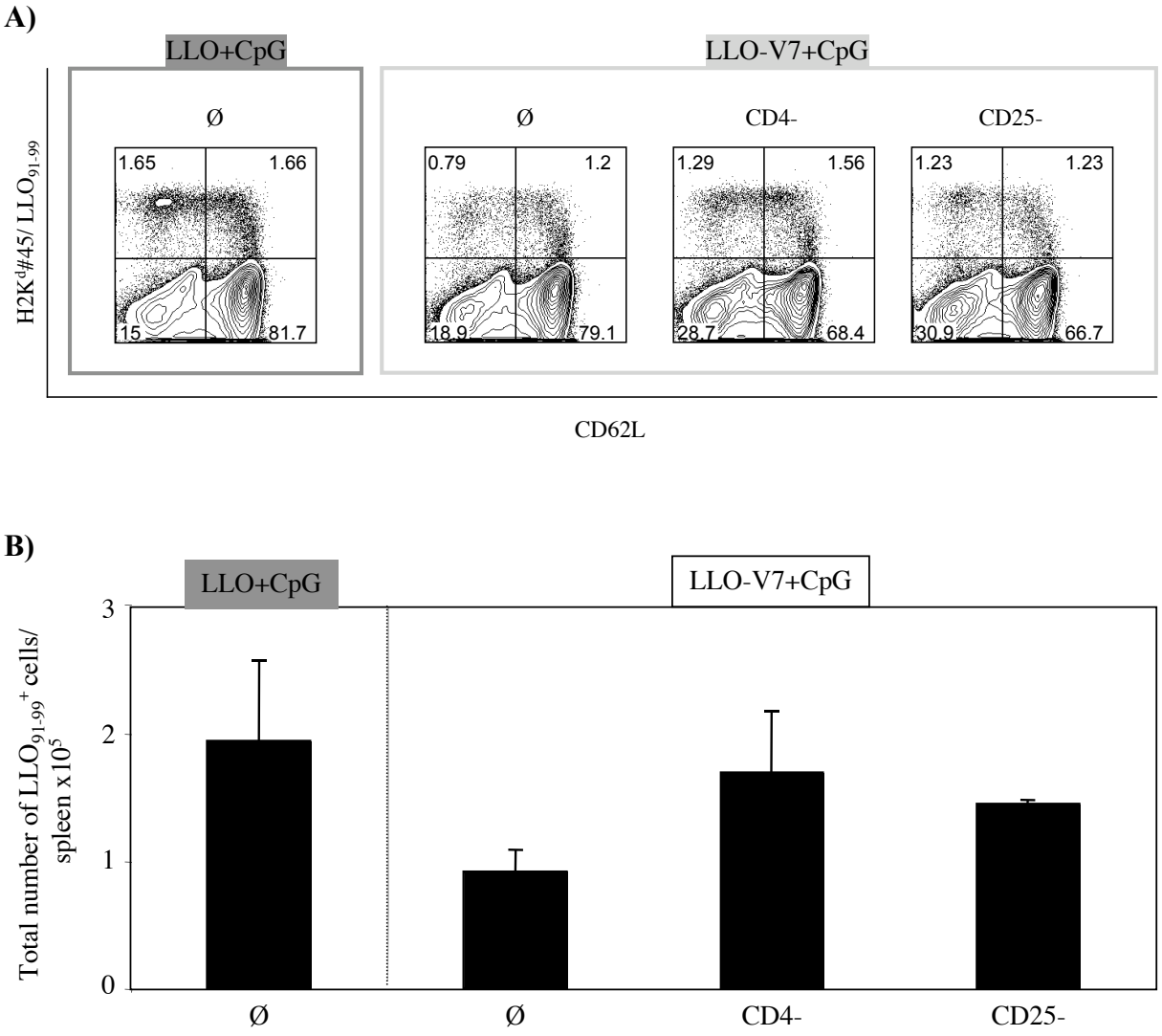


Figure 31: Frequencies and numbers of LLO$_{91-99}$-specific CD8$^+$ T cells after CD4$^+$ T cell or CD25$^+$ cell depletion

BALB/c mice were immunized with 10µg LLO plus 5nmol CpG or LLO-V7 plus 5nmol CpG. Indicated groups got treated with 150µg GK1.5 i.p. at days –3, -2, -1, +2, +5 or 250µg anti-CD25 mAb at days –5, -2, +2, +5 before/after immunization. Seven days after immunization, spleen cells were isolated and stained with FITC-conjugated anti-CD62L mAb, PE-labeled LLO$_{91-99}$ tetramer and APC-conjugated CD8α mAb. Cells were analyzed by flow cytometry. Life/death discrimination was done by EMA staining. Dot plots shown are gated on CD8$^+$ T cells (A). Bar graphs represent mean values +/- SD for spleen cells of three individually analyzed mice. Results are representative of at least 2 independent experiments (B).

As summarized in Figure 31, there was a slight enhancement of LLO$_{91-99}$-specific CD8$^+$ frequencies and absolute numbers in the absence of CD4$^+$ or CD25$^+$ cells, but still never reaching the numbers found for mice immunized with wildtype LLO plus CpG.

Taken together, these data indicate that depletion of CD4$^+$ or CD25$^+$ cells has similar enhancing effects on LLO-V7 as compared to immunization with LLO wildtype protein;

however, the overall frequencies of LLO_{91-99}–specific $CD8^+$ T cells are in general much lower in LLO-V7 immunized mice, even in the absence of $CD4^+$ T helper cells or $CD25^+$ T_{reg} cells. This finding further demonstrates that the main target population mediating the suppressive effects of V7 is not a $CD4^+$ or $CD25^+$ regulatory T cell.

4. DISCUSSION

Our data demonstrate that immunization with peptide in the presence or absence of adjuvant induced only weak $CD8^+$ T cell responses. Using local application of full-length protein in addition with the TLR9 stimulating adjuvant CpG, $CD8^+$ T cell-mediated immune responses were significantly improved resulting in protective immunity towards *Listeria monocytogenes*. In this vaccination setting, $CD4^+$ T cell help seems to play a weaker role in differentiation and development of memory $CD8^+$ T cell responses. Most interestingly, Treg cells appear to have a very strong inhibitory influence on T cell differentiation. In contrast to CpG, fusion of the same protein to a TLR2 ligand diminishes $CD8^+$ T cell-mediated immune responses. Thus, the choice of TLR stimulating adjuvant has major implications for the outcome of immnogenicity.

4.1. Weak immunogenicity after peptide immunization with/ without adjuvant

The major goal of this study was to vaccinate mice with purified *Listeria monocytogenes* (*L.m.*)-derived antigens, which are known to be only weakly immunogenic, and to modulate the immune system by TLR agonists as adjuvant to convert these weak antigens to induce protective memory $CD8^+$ T cell responses. We first evaluated the effects during immunization of mice with LLO_{91-99} peptide. LLO_{91-99} is the best known immunodominant *Listeria*-epitope (H2-K^d restricted), and LLO_{91-99}-specific $CD8^+$ T cell responses have been shown to confer efficient protection towards *Listeria* infection.

The advantage of using peptides for vaccination is that they can be easily synthesized in large quantities.

Both *i.v.* and *s.c.* application of LLO_{91-99} peptide alone did not induce measurable $CD8^+$ T cell responses, which is in line with observations made before by others. However, also addition of CpG as adjuvant could not significantly enhance the LLO_{91-99}-specific $CD8^+$ T cell frequencies. Since MHC class I molecules are expressed on all nucleated cells, LLO_{91-99} peptide can bind to MHC class I molecules expressed not only on professional APCs but also on other nucleated cells. Efficient stimulation of T cells requires an antigenic stimulus through the TCR (signal 1) as well as a second signal delivered by costimulatory molecules (signal 2). When peptide is expressed on MHC class I molecules on “resting” APCs, T cells receive signal 1 but not signal 2, which leads to T cell anergy (Karpus et al., 1994; Rocha et al., 1995). Another explanation for the lack of measurable $CD8^+$ T cell responses upon

immunization with peptide alone beside T cell anergy could be CTL exhaustion/tolerance. It has been described by several groups that at high antigen loads CTLs can get depleted (Moskophidis et al., 1993; Zajac et al., 1998). Tolerogenic effects of MHC-I restricted peptide immunization have further been linked to the absence of $CD4^+$ T cell help (signal 3). Some studies have shown that this type of tolerance can be partially converted in efficient $CD8^+$ T cell priming by activation of CD40 (Diehl et al., 1999). We have some experimental evidence that indeed induction of tolerance occurs during vaccination with LLO_{91-99} peptide. Challenge experiments (upon previous immunization with LLO_{91-99} peptide) with low dose of *L.m.* resulted in lower antigen-specific $CD8^+$ T cell frequencies in comparison to control groups that have not been pre-challenged with peptide (data no shown).

All our peptide-immunization studies were performed with an amount of 100 g peptide, as it has been suggested also by other groups (Kursar et al., 2002; Toka et al., 2005). However, it still can be possible that this particular antigen dose is not optimal for T cell activation. It could be that either lower amounts, because of the reasons described above (CTL exhaustion/ tolerance), or higher amounts of peptide (to increase the chances of binding to professional APCs) are needed to improve $CD8^+$-specific immune response. Similar reasons might also explain why the addition of CpG does not lead to improved $CD8^+$ T cell priming. CpG is known to induce DC maturation by binding to TLR9. Therefore, CpG can act as a strong inducer of signal 2. But because the LLO_{91-99} peptide is not directly linked to the adjuvant, binding of the peptide and CpG might not occur on the same cell, resulting in loss of targeted adjuvant effects. The possibility remains that LLO_{91-99} peptide given at higher concentrations may lead to stronger immune responses by increasing the chances of the synchronal binding of CpG and peptide to the same APC. In a recent study, the authors demonstrated that local administration of 50–500 g of GP33 (glycoprotein of LCMV) emulsified in IFA (incomplete Freund's adjuvant) can lead to generation of protective CTL responses without inducing tolerance. They interpret their result that by local application in combination with IFA an antigen depot is generated, from where peptide slowly reaches the draining lymph nodes. Here, peptide-loaded professional APCs are able to induce optimal T cell activation by providing both TCR engagement and costimulatory signals (Aichele et al., 1995). In line with this findings, the group of Kursar et al. showed that 100 g LLO_{91-99} peptide emulsified in IFA could induce antigen-specific $CD8^+$ T cell responses when used as boost immunization after *L.m.* infection (Kursar et al., 2002). Another group tested the adjuvant effect of CpG using peptide immunization with the peptide analog of MART-1/Melan-A_{26-35}. They found strong antigen-specific immune responses upon immunization (Miconnet et al., 2002). Referring to

these data, it remains unclear why immunization with the peptide LLO_{91-99} in the presence of CpG is so inefficient. It might be possible that LLO_{91-99} gets rapidly diluted throughout the body after local application. Perhaps IFA also prevents peptides from rapid dilution (and/or degradation) and therefore can enhance their immunogenicity.

4.2. *S.c.* application of LLO protein enhances CTL activity

We next analyzed whether immunization with purified full-length LLO protein leads to better $CD8^+$ T cell priming, because in this setting the protein has to pass the entire recognition-, uptake-, processing- and presentation-machinery through professional APCs until it might result in efficient presentation of LLO_{91-99} peptide to T cells. In this setting, also activation of T helper cells might contribute to generation of effective $CD8^+$ T cell responses providing the necessary third signal in addition to signal 1 and 2. There are currently three main models trying to explain how the interaction of $CD4^+$ T cells and APCs can enhance $CD8^+$ T cell priming.

1) Antigen specific interactions with the APC activate $CD4^+$ T cells. These activated $CD4^+$ T cell in turn "license" the same or other APCs to further prime $CD8^+$ T cells. As a consequence, in this model CTL and helper T cell recognition of antigen on the same APC is not required (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998).
2) $CD4^+$ and $CD8^+$ T cells recognize antigen on the same APC. During early activation, CD40 is expressed on $CD8^+$ T cells and CD40L on $CD4^+$ T cells, which might allow a direct interaction between these two T cell subsets (Bourgeois et al., 2002).
3) DCs activate $CD4^+$ T cells, which in turn become APCs themselves (Th-APCs). This is enabled by acquisition of synapse composed MHC class II, CD54 and CD80 molecules as well as bystander MHC class I – peptide complexes from DCs. The resulting Th-APC might activate other $CD4^+$ T cells to become Th-APCs or directly primes $CD8^+$ T cells (Xiang et al., 2005).

The first two models, which are most frequently discussed, both require CD40 (expressed on APCs or $CD8^+$ T cells) -and CD40L (expressed on $CD4^+$ T cells) interactions to provide the optimal stimulus for the APC to induce $CD8^+$ T cell priming.

Also the route of application has strong influences on $CD8^+$ T cell priming. We compared local (*s.c.*) with systemic (*i.v.*) application of LLO after immunization with full-length LLO protein. We were limited to use amounts that did not exceed 1□g of LLO protein for *i.v.* experiments, because at higher doses systemic application showed to be lethal for mice. The

exact reason for this toxicity is still unclear. A report from the late 1970 showed already that intravenous immunization of rats with LLO induced bradycardia and inhibition of atrio-ventricular conduction. In addition, cessation of the spontaneous beating and degeneration of cultured fetal mouse heart cells has been observed (Takeda et al., 1978).

Local application of full-length LLO protein led to higher LLO_{91-99}-specific $CD8^+$ frequencies than *i.v.* immunization. For both application routes the additional treatment with CpG as adjuvant enhanced the antigen-specific frequencies. In all cases, full-length LLO protein was not covalently bound to CpG. Therefore, *i.v.* immunization leads to dilution of LLO and CpG upon entering the blood stream, and the chances that LLO and CpG get taken up and recognized by the same APC are rather low. By applying LLO and CpG *s.c.*, CpG is in closer proximity to LLO, increasing the chances that LLO and CpG get recognized by the same APCs. Also the protein/CpG mixture is localized in a kind of depot under the skin after *s.c.* immunization. Therefore, DCs that recognize the antigen together with the CpG stimulus might attract other DCs to the site of immunization.

Consistent with these findings, we observed similar results when immunizing with full-length p60 or OVA protein. Upon infection with *L.m.*, $p60_{217-225}$ represents a T cell epitope with intermediate dominance. Dose titration experiments indicated that an amount of 100 g was needed to induce measurable antigen-specific $CD8^+$ T cell frequencies (data not shown). Therefore, the full-length p60 protein was applied for further studies in a higher dose than full-length LLO protein. *I.v.* application with or without CpG only induced barely detectable antigen-specific $CD8^+$ T cell frequencies. As could be seen for full-length LLO protein, *s.c.* immunization of full-length p60 protein together with CpG resulted in increased frequencies. We obtained similar results when immunizing with OVA protein. In this immunization setting, we were able to further test the aspect of "close proximity" of CpG and the target protein on the outcome of the immune responses. We directly compared the immune responses of OVA covalently conjugated with CpG (OVA-1668) with non-covalently linked OVA plus CpG. Already *i.v.* immunization led to increased antigen-specific $CD8^+$ frequencies after application with OVA-1668 compared to OVA plus CpG. This is in perfect agreement with out previous speculations that upon systemic immunization of protein that is not directly linked to the adjuvant, both protein and adjuvant get highly diluted, thereby decreasing the chances of synchronal binding to the same APC. Upon immunization with OVA-1668 both components can synchronously bind to the APC, which leads to improved immune responses.

After local immunization, the antigen-specific $CD8^+$ T cell response induced by application of OVA-1668 conjugate dominated the response that was achieved by immunization with OVA

plus CpG. Similar results have been already reported by others before, all indicating that covalently conjugated CpG to antigen creates a more potent immunogen than mixture of antigen with similar amount of CpG (Cho et al., 2000; Maurer et al., 2002; Shirota et al., 2000). In addition, others have shown that uptake of conjugated OVA by DCs was more efficient than that of OVA mixed with CpG (Datta et al., 2004). This effect appears to be mediated by DNA-binding receptors expressed by APCs (Shirota et al., 2001). Since these promising data based on conjugation of OVA with CpG might indicate a great advance in the development of $CD8^+$ T cell based vaccines, we wanted to generate similar conjugates with p60 and LLO to test whether conjugation of these proteins also leads to enhanced immunogenicity. Unfortunately, we were unsuccessful to generate sufficient amounts of CpG conjugates, most probably due to the fact that the binding chemistry is substantially different from ovalbumin.

In summary, our data demontrate that local application for all tested proteins seems to be the favored route for efficient $CD8^+$ T cell priming, which is most likely explained by the fact that all crucial components for CD8+ T cell priming (protein, adjuvant, and APC) get best targeted to T cell areas in the draining lymph nodes.

To further test whether immune responses can be enhanced through treatment with an optimal ratio of protein and CpG, dose titration experiments of full-length LLO protein using a constant amount of CpG were performed (1, 5, 10 and 50 g of protein mixed with 5nmol CpG). Enhanced numbers of LLO_{91-99}-specific $CD8^+$ T cells could be detected to up to 10 g of LLO plus CpG, higher doses (50 g) led to decreased immune response. A possible explanation for this finding might be that immunization with such a high antigen dose causes overstimulation and antigen-induced T-cell death. Considering the fact that LLO might have some toxicity for lymphocytes, it also could be a possible scenario that direct effects of full-length LLO protein induce apoptosis of the lymphocytes (Carrero et al., 2004).

4.2.1. DCs are the mediators for induction of CTL responses in this setting

We could demonstrate that addition of CpG leads to increased $CD8^+$ T cell responses after *s.c.* immunization with purified full-length proteins. We reasoned that this positive effect is induced by binding of CpG to TLR9 on DCs, resulting in the maturation of DCs and cross-presentation of the processed antigen by DCs. DCs are characterized by the expression of CD11c. In CD11c DTR transgenic mice, targeted deletion of $CD11c^+$ DCs can be induced. Using the CD11c DTR transgenic mouse model only very weak $CD8^+$ T cell responses could

be detected after immunization with LLO plus CpG after depletion of DCs in transgenic mice, whereas in non-depleted transgenic mice a robust response could be induced. These results would be in line with the interpretation that indeed DCs are the mediators to induce cross-priming of $CD8^+$ T cells. However, one has to keep in mind recent findings where CD11c expression has been reported for activated intraepithelial lymphocytes and $CD8^+$ T cells and that *in vivo* activated CD11c-DTR transgenic $CD8^+$ T cells displayed a substantial proportion of CD11c promoter activity (Huleatt and Lefrancois, 1995; Jung et al., 2002). Since the depletion effects of DT last only 2-3 days, and since activation of $CD8^+$ T cells and subsequent CD11c expression requires some time, it seems unlikely that DT-mediated depletion of activated $CD8^+$ T cells is the main cause of low frequencies of antigen-specific $CD8^+$ T cells in the CD11c-DTR transgenic model.

4.3. T helper cell dependency of primary $CD8^+$ T cell responses upon immunization with LLO in the absence or presence of CpG

The superior $CD8^+$ T cell immunogenicity of full-length protein versus peptide vaccination could be due to the induction of additional antigen specific $CD4^+$ T cell responses, which provide enhancing factors by conventional T cell help. To test this hypothesis we performed $CD4^+$ T cell depletion experiments and could show that antigen-specific $CD8^+$ T cell responses were even enhanced after $CD4^+$ T cell depletion, both after immunization with full-length LLO protein and LLO plus CpG. This could be also confirmed on the level of functional T cell assays, e.g. by increased IFN production. This is a rather surprising finding and somewhat contradictory to other studies. Other groups have shown that in the absence of $CD4^+$ T cells during the priming phase, the primary response was not altered when compared to wildtype animals. Some studies even show reduction of $CD8^+$-specific immune responses in the absence of $CD4^+$ T cells. For example, infection with herpes simplex virus type-1 (HSV) leads to significantly decreased antigen-specific $CD8^+$ T cell frequencies in $MHCII^{-/-}$ mice compared to wildtype animals. Experiments with $CD4^{-/-}$ mice showed that primary immune responses were decreased after *L.m.* or LCMV infection (Tyznik et al., 2004). Thus, this is the first report showing that there are increased primary immune responses in the absence of $CD4^+$ T cells. The increase in effector T cell responses also correlate with increased CTL activity in $CD4^+$ T cell depleted animals upon immunization with full-length LLO protein as demonstrated by *in vivo* CTX assays. Although we could not detect a further increase of lytic activity in the absence of $CD4^+$ T cells in LLO plus CpG treated mice, the percentage of lysis was comparable to that observed for animals infected with *L.m.*

Obviously, the assay system is not be able to discriminate differences in antigen-specific killing above a certain frequency of effector T cells.

4.3.1. Does treatment with CpG circumvent the need for T cell help?

By positively influencing both signal 1 (antigen in the context of MHC class I) and signal 2 (costimulatory molecules), the LLO plus CpG vaccine might effectively compensate for CD40/CD40L dependent "licencing" interactions between DCs and T helper cells. Therefore, $CD8^+$ T cell priming in the presence of CpG as an adjuvant might be less $CD4^+$ T cell dependent, which has been already suggested by others (Cho et al., 2000; Schirmbeck et al., 2003; Vabulas et al., 2000). Also for studies on human cells it has been demonstrated that PDCs, which express TLR9, showed quick up-regulation of costimulatory molecules CD40, CD80, and CD86 and produced IFN upon stimulation with CpG ODN (Lore et al., 2003), suggesting that TLR-mediated signals might be able to limit the strict requirement for $CD4^+$ T cell help (Albert et al., 2001; Bennett et al., 1997a). It has also been proposed that TLR9 signaling directly supports the efficiency of cross-priming, most likely due to complementary pathways to MyD88 signaling. These pathways might activate the MHC class I- related antigen processing machinery (Datta et al., 2003).

4.3.2. NKT cells might have suppressive function upon immunization with LLO

For full-length LLO protein immunized mice, the explanation for the increased immune responses after $CD4^+$ T cell depletion is more complex. LLO alone shows already some intrinsic immunogenicity and induces LLO_{91-99}-specific $CD8^+$ T cell responses when applied alone. This could for example be explained by the induction of helper T cells. Therefore, it is even more surprising that $CD4^+$ T cell depletion leads to increased immune responses. In the case of immunization with full-length LLO protein alone, no additional stimulus is added that could contribute to DC activation in the absence of CD4 T cell help, the third signal needed for effective $CD8^+$ T cell priming. Thus, LLO alone might contain factors that have positive effects on $CD8^+$ T cell priming. Possible LS contaminations in the protein purification might attribute to stimulation of TLR4 or other PRRs. Normally, concentrations of about 25 g/kg body weight LPS are used for *in vivo* administration in mice (Baumgarten et al., 2001; Frost et al., 2004). Contamination measurements exhibited LPS contaminations of less than 1ng. We further excluded any relevant stimulatory LPS contaminations by external addition of LPS in concentrations that exceeded the measured LPS contaminations of LLO more than 50-fold to the LLO protein. These relatively "high" concentration of LPS (50ng LPS/mouse) did not lead to enhanced immune responses, minimizing potential side-effects of LPS

contamination through unwanted TLR4 stimulation (data not shown). Thus, other yet unknown factors must contribute to the stimulatory effect of LLO protein alone and furthermore to the generation of effective $CD8^+$ mediated immune responses in the absence of $CD4^+$ T cells. Overall, our experiments point to a dominant inhibitory function mediated by a cell population among $CD4^+$ T cells. Possible candidates might be $CD4^+$ NKT cells or regulatory T cells.

$CD4^+$ NKT cells are a specialized subset of T cells that express a number of NK cell markers. They have a skewed □□ T cell receptor repertoire restricted to the nonclassical MHC class I-like CD1d molecule (Park et al., 2005; van Dommelen et al., 2003). Upon stimulation of NKT cells, they can secrete cytokines that are important for the differentiation of Th1 and Th2 subsets. Therefore, NKT cells are involved in a broad variety of diseases, including autoimmune diseases and tumor development, among others (Akbari et al., 2003; Moodycliffe et al., 2000; Tamada et al., 1997; Terabe et al., 2000). Because of their ability to promote either Th1 or Th2 immune responses, they can be involved in prevention or pathogenesis of inflammatory diseases. One example is the involvement of NKT cells in different viral infections. □-galactosylceramide (□-GalCer) has been identified as ligand for the semi-invariant TCR of NKT cells in association with CD1d. Activation of NKT cells by □-GalCer results in improved viral clearance or prevention of virus-induced disease described for hepatitis B virus, diabetogenic encephalomyocarditis virus, and respiratory syncytial virus (Exley et al., 2001; Johnson et al., 2002; Jonjic et al., 1994). But NKT cells do not regulate the responses to all viruses in a similar manner. In contrast to the above described functions, NKT cells have been described to possess negative regulatory function in the immune response to LCMV infection. Absence of NKT cells using CD1d-deficient mice resulted in higher levels of IL-2, IL-4 and IFN□after LCMV infection compared to wildtype mice. In a cancer model using nonregressor CT26 syngeneic colon carcinoma it could be demonstrated that the absence of $CD4^+$ T cells reduced the number of lung metastases of CT26 tumors in syngeneic mice. Using $CD1d^{-/-}$ mice, the same result could be obtained, thus linking the suppressive effect on the lung metastasis to $CD4^+$ NKT cells (Park et al., 2005).

In our vaccination protocol using $CD4^+$ T cell depletion, $CD4^+$ NKT cells are also depleted, which may exhibit suppressive functions that are abolished after depletion. In order to experimentally address this question, immunization experiments should be for example performed in CD1d deficient mice. Unfortunately, these mice were not yet available on a proper genetic background for our studies (BLAB/c). Therefore, these experiments could not be included into this study.

The possible involvement of $CD4^+$ regulatory T cells will be discussed later.

4.3.3. Treatment with CpG upon immunization with LLO can induce long-lasting memory $CD8^+$ T cell responses in $CD4^+$ T cell depleted mice

Our data indicate that the enhancing effects seen after $CD4^+$ T cell depletion are not restricted to primary immune responses, but they are also transferred into the memory phase. Based on the literature, there are currently mostly data showing that the absence of $CD4^+$ T cells during primary immune responses leads to decreased memory responses (Shedlock and Shen, 2003; Sun and Bevan, 2003). This might be also true for immunization with full-length LLO protein alone, but surprisingly challenge of LLO plus CpG immunized mice with a high dose of *L.m.* did not result in diminished protection - the opposite was the case; protective immunity was even enhanced. Thus, our data are somehow in contrast to the common view that conventional $CD4^+$ T cell help are essential during early T cell priming to program the differentiation of $CD8^+$ T cells to develop into memory $CD8^+$ T cells. It has also been described that the number of memory $CD8^+$ T cells that develop in the absence of $CD4^+$ T cell help gradually decline, suggesting that their self-renewing capacity is reduced (Sun et al., 2004). What could be the underlying mechanism for our observation that $CD4^+$ T cell depletion leads to enhanced antigen-specific $CD8^+$ T cell expansion as well as memory T cell differentiation?

We first ruled out that potential homeostatic effects are the reason for this phenotype just by filling up the "space" created though $CD4^+$ T cell depletion. We injected naïve or activated antigen-specific CFSE labeled cells in $CD4^+$ T cell depleted or undepleted animals. After seven days, the time point where mice were usually analyzed for primary immune response, we could not detect proliferation of the transferred CFSE labeled cells indicating that no homeostatic proliferation occurred in the absence of $CD4^+$ T cells.

A more recent publication followed up the question of whether CpG, which is a potent inducer of primary $CD8^+$ T cell responses in the absence of $CD4^+$ T cells, can also influence memory T cell differentiation. The group of Bullock used MHC class $II^{-/-}$ mice to show that memory $CD8^+$ T cells that were generated in the absence of $CD4^+$ T cell help were capable of proliferation and effector function after immunization with MHC class $II^{-/-}$ DCs. They immunized mice with $OVA_{587-264}$-pulsed DCs from MHC class $II^{-/-}$ mice that were treated with CpG and challenged with a recombinant vaccinia virus expressing OVA. They linked this effect to CD70 (CD27L), which is expressed on DCs. Under normal priming conditions, CD40L-mediated upregulation of CD70 on DCs is likely to be involved in licensing $CD8^+$ T cells to expand. This effect was also seen in MHC class $II^{-/-}$ mice when DCs were treated with

CpG (Bullock and Yagita, 2005). This mechanism could be a possible explanation why the need for helper T cells can be partially circumvented by the stimulation of the DCs with CpG. Although these experimental settings substantially differ from ours, because in their system $CD4^+$ T cells are absent both during primary and recall responses, these data might also provide possible explanations for our findings.

4.4. $CD4^+$ Treg cells do not mediate suppressive effect of $CD4^+$ T cells upon i mmunization with LLO

As described above, $CD4^+$ T cell depletion during immunization with LLO leads to enhanced antigen-specific $CD8^+$ T cell responses. To test whether Treg cells that constitute 5-15% of peripheral $CD4^+$ T cells might be involved in this phenomenon, we performed $CD25^+$ T cell depletion experiments.

In contrast to $CD4^+$ T cell depletion, $CD25^+$ cell depletion did not augment the $CD8^+$ mediated immune responses. Therefore, in the case of LLO protein immunization $CD4^+$ T cells seem to exhibit inhibitory functions that are not mediated though $CD4^+CD25^+$ regulatory T cells, but seem to be contributed by other $CD4^+$ cell subpopulations. In addition to the already discussed $CD4^+$ NKT cells, also antigen-induced regulatory $CD4^+$ T cells might be a candidate cell population involved in the suppressive effects. In contrast to naturally occurring Treg cells, antigen-induced regulatory T cells have variable expression of CD25, do not express Foxp3, and do not need co-stimulation for development or survival, but are also able to inhibit $CD4^+CD25^-$ T cell proliferation to the same extent as do naturally occurring Treg cells. The regulatory capacity of both naturally occurring Treg cells and antigen-induced regulatory $CD4^+$ T cells is at least in part cell contact dependent and can be overcome by exogenous IL-2 (Bluestone and Abbas, 2003; Oldenhove et al., 2003; Vieira et al., 2004). Because of their varible CD25 expression, antigen-induced regulatory $CD4^+$ T cells probably are not getting efficiently depleted upon treatment with anti-CD25 mAb, but likely get depleted upon treatment with anti-CD4 mAb. Therefore, this regulatory population might mediate the suppressive effect seen after LLO immunization alone, which is abolished after $CD4^+$ T cell depletion.

4.4.1. Treg cells exhibit negative regulatory function upon immunization with LLO plus CpG

$CD25^+$ cell depletion during primary responses after LLO plus CpG immunization augmented numbers of antigen-specific $CD8^+$ T cell, both on cellular and functional levels. Interestingly, the $CD25^+$ cell depletion induced enhancement exceeded the effects achieved by $CD4^+$ T cell depletion. When comparing long lasting memory responses, again $CD25^+$ cell depleted mice showed better protection levels than $CD4^+$ T cell depleted animals upon challenge with high dose of *L.m.*

This observation is consistent with the current literature. For example, it was demonstrated for the mouse model of malaria infection that depletion of Treg cells protected them against death induced by *Plasmodium yoelii* (Hisaeda et al., 2004). Treg cells were additionally found to be involved in disease progression after viral infection. For example, depletion of Treg cells in mice infected with either HSV, Friend leukemia virus or HCV led to enhanced antigen-specific $CD8^+$ T cell responses (Boettler et al., 2005; Cabrera et al., 2004; Dittmer et al., 2004; Suvas et al., 2003)**.** However, Treg cells do not only contribute to the outcome of primary immune responses but also affect memory T cell responses, as it has been shown for infection with *L.m.*, HSV and leishmania (Kursar et al., 2002; Mendez et al., 2004; Suvas et al., 2003). Also in our immunization setting, $CD25^+CD4^+$ Treg cells seem to be crucially involved in suppression of $CD8^+$ T cell proliferation during the effector phase. We used MHC class $II^{-/-}$ mice for immunization to further demonstrate that naturally occurring Treg cells indeed participate in the observed suppression. These mice don't have conventional T helper cells or naturally occurring Treg cells by definition, because they lack MHC class II-restricted $CD4^+$ T cells. Because MHC class $II^{-/-}$ mice were only available on the $H2^b$ background, we could not use the LLO protein for immunization (until now, no immunodominant $H2^b$-restricted LLO epitope has been identified) and instead used OVA-CpG DNA conjugate (OVA-1668). We could show previously in experiments using wildtype BL6 animals that the immune responses after OVA-1668 and additional treatment with anti-CD4 or anti-CD25 mAb showed the same effects as compared to experiments using BALB/c mice for immunization with LLO plus CpG; antigen-specific $CD8^+$ T cell responses were increased in the absence of $CD4^+$ T cells and even more in the absence of $CD25^+$ cells. In contrast, $CD25^+$ cell depletion in MHC class $II^{-/-}$ mice did not alter the immune responses compared to undepleted MHC class $II^{-/-}$ mice. However, treatment of MHC class $II^{-/-}$ mice with CD40 stimulating mAb substantially increased K^b/SIINFEKL-specific $CD8^+$ T cell frequencies, indicating that typical T cell help-mediated signals can positively affect immunogenicity in this model.

In conclusion, the data from experiments in MHC class $II^{-/-}$ mice indicate that indeed naturally occurring Treg cells seem to mediate negative regulatory function on immune responses towards full-length LLO protein in the presence of CpG as adjuvant.

4.5. Fusion of TLR2 ligand V7 to LLO results in decreased immunogenicity upon immunization

To test whether stimulation of a TLR different from TLR9 leads to similar effects, we repeated our experiments using LLO-V7 and p60-V7 fusion proteins. “V7” is a peptide from the LcrV protein. LcrV is a *Yersinia*-derived protein, and it has been shown that the “V7” peptide sequence of this protein can efficiently signal through TLR2 leading to increased IL-10 production by macrophages (Sing et al., 2002). Consistent with these data, luciferase assays measuring ELAM-1 promoter activity revealed that only TLR2 transfected cells responded to stimulation with $LLO_{492T\ A}$ -V7 or p60-V7, demonstrating that the V7 fusion proteins only signals via TLR2. LLO and LLO-V7 proteins have similar lytic activity on erythrocytes, suggesting that by fusing V7 to LLO the overall structure of LLO is not altered. Immunization with LLO-V7 leads to decreased antigen-specific $CD8^+$ responses, as well as reduced epitope-specific lysis by antigen-specific T cells as compared to LLO immunized mice. Addition of CpG could augment these responses slightly, but not to the same extent as achieved after immunization with LLO and CpG. Thus, stimulation of TLR2 through V7 seems to trigger a signaling cascade resulting in suppression of $CD8^+$ T cell responses.

Consistent with our data, a recent study showed that different TLR ligands induce distinct immune responses. The group used immunostimulatory DNA (ISS-ODN) as ligand for TLR9, Pam_3Cys as TLR2 ligand, and OVA as antigen to study the outcome of experimental asthma under the different TLR stimulations. They found that upon stimulation with Pam_3Cys a Th2-associated antibody and cytokine profile was induced with induction of IgG2a responses and the production of cytokines like IL-13, GM-CFS and IL-1□. In contrast, immunization with ISS-ODN resulted in a Th1-like antibody and cytokine profile with pronounced IgG1 responses and secretion of IL-12, IL-18- IL-27 and IFN□□. Priming of mice with Pam_3Cys/OVA or ISS-ODN/OVA followed by OVA challenges aggravated airway hyper-reactivity (AHR) or led to improved AHR, respectively (Redecke et al., 2004). Therefore, depending on the type of TLR triggering, Th1/Th2 differentiation can be strongly influenced, which might also be the reason for our results. Although both TLR2 and TLR9 are known to signal via the Myd88 dependent pathway, TLR2 uses at least one additional adaptor protein (Toll-IL-1R domain containing adaptor molecule). Whether this or further signaling

molecules might explain the diversity seen with this two TLR ligands remains to be elucidated.

It has been shown by another group that V7/ TLR2 interaction leads to IL-10 production by macrophages. IL-10 is known to be involved in Th2 cytokine responses and therefore it can be speculated that IL-10 production has a suppressive effect on Th1 like cytokine production resulting in decreased $CD8^+$ T cell responses (Sing et al., 2002).

4.5.1. Treg cells only partly contribute to the suppressive effect of LLO-V7

We performed $CD4^+$ T cell and $CD25^+$ cell depletion on LLO-V7 immunized animals to find out whether the suppressive effects, which are thought to be mediated through V7/TLR2 interaction, could be also due to suppression induced through regulatory T cells. Because significant effects of depletion were seen for the LLO plus CpG immunized animals, we also used LLO-V7 plus CpG immunization to address this question. As expected, $CD4^+$ and even more $CD25^+$ cell depletion led to increased antigen-specific $CD8^+$ T cell responses. However, these responses did not exceed the responses achieved by LLO plus CpG immunization.

The group of Netea et al. showed that TLR2 plays a major role during *Candida albicans* infections. Using $TLR2^{-/-}$ mice, they showed that mice were less susceptible to infection with *Candida albicans*. Analysis of cytokine levels of $TLR2^{-/-}$ mice revealed decreased IL-10 levels, but elevated IFN production (Netea et al., 2004). The same is true using infection with *Yersinia enterocolitica*. Again, $TLR2^{-/-}$ mice are more resistant to the infection (Sing et al., 2002). Therefore, also in these models TLR2-dependent release of IL-10 is likely to be the mechanism of immunosuppression. IL-10 derived from dendritic cells has been also shown to be necessary for the proper development of Treg cells (Montagnoli et al., 2002). On the other hand, Treg cells produce IL-10 to decrease cellular defense. Using the model of *Candida albicans* infections, it could be demonstrated that depletion of Treg cells decreased fungal outgrowth in the kidney day seven after infection by 10-fold (Montagnoli et al., 2002). Our data do not show such a dramatic effect after $CD25^+$ cell depletion, but still we see enhanced $CD8^+$ immune responses. Therefore, also in this setting Treg cells seem to negatively regulate $CD8^+$ T cell responses. However, we have no experimental hints for a direct interaction of V7 with Treg cells.

Another important finding of this experiment was that even by adding a strong stimulus like CpG together with LLO-V7 immunization, the antigen-specific $CD8^+$ T cell response is still diminished compared to LLO plus CpG treatment. Thus, providing a Th1-biased stimulus cannot revert the effect achieved through V7/TLR2 interactions. This effect is even more

pronounced when comparing $CD25^+$ cell depleted immunized animals. Even under this depleting conditions that show strong effects under CpG treatment, the responses of LLO-V7 immunized animals cannot be reversed by CpG.

Various studies have demonstrated that activation of two different TLRs lead to synergistic effects on the immune responses. For example, TLR9 stimulation (CpG DNA) and TLR3 stimulation (LPS) synergistically induce TNFα-production in RAW264.7 cells and J774 cells, mainly through activation of NF-κB (Yi et al., 2001). Using human DCs, it could be demonstrated that synergistic stimulation of TLR3 or TLR4 with TLR8 leads to the induction of significantly higher amounts of IL-12, IL-23 and Delta-4 when compared to levels induced through stimulation of single TLRs (Napolitani et al., 2005). Sato et al. have reported that costimulation of mouse peritoneal macrophages with TLR2 ligand (mycoplasma lipopeptide; MALP-2) and TLR4 ligand (LPS) resulted in a synergistic increase of TNFα-production (Sato et al., 2000). TLR9 and TLR4 synergy has been analyzed in more detail, and it was shown that they synergize to enhance TNFα-secretion from mouse macrophages at a post-transcriptional level (Gao et al., 2001). Most of these studies are based on *in vitro* models, concentrating on single effects, like TNFα-production. Therefore, these results cannot be directly translated to the *in vivo* situation, although it is likely that synergistic TLR stimulations can occur also *in vivo*, as pathogens contain several different TLR agonists that trigger TLRs in different cellular compartments. However, so far there are no reports on synergistic effects of TLR2 and TLR9, and it needs to be elucidated whether stimulation via two “counterparts” (TLR2/Th2 and TLR9/Th1) together will be capable to improve immune responses. Based on our data, it seems more likely that one of the TLR signals (in our case TLR2-mediated signal) dominates over the other ones or even has the capability to suppress additional signals (TLR9-meditated signal).

4.6. Conclusion

In summary, our data indicate that immunization with epitope-oligopeptides does not induce efficient $CD8^+$ T cell priming, no matter if an adjuvant is present or not.

Using full-length protein, the antigen has to enter the whole uptake-, processing- and presentation-machinery of professional APCs, in order to result in efficient $CD8^+$ T cell responses. The adjuvant CpG acts as a positive enhancer of $CD8^+$ T cell cross-priming, most likely by induction of DC maturation, upregulation of costimulatory molecules, and supporting cross-presentation pathways. Treg cells seem to be the main suppressors of the immune responses upon immunization with purified soluble proteins plus CpG, since their depletion greatly improves long-lasting protective immunity. Also $CD4^+$ T cell help affects the efficacy of cross-priming, thereby CD40-CD40L interactions still seem to play a major role. Interestingly, the positive impact of taking away the suppressive effect of Treg cells on memory T cell generation appear to largely exceed the effect of $CD4^+$ T cell help.

A crucial factor for positive modulation of T cell immunization based on cross-priming is the choice of the stimulating TLR ligand as adjuvant. We could show that by using CpG (TLR9 ligand), antigen-specific $CD8^+$ T cell-mediated immune responses could be enhanced, whereas by using V7 (TLR2 ligand) they were decreased.

These findings will be useful to improve certain vaccines and vaccination strategies. Our novel results, that long-lasting protective immunity can be generated T helper cell independent in the absence of $CD25^+$ Treg cell have great implications for vaccine development especially for $CD4^+$ cell deficient individuals (e.g. HIV patients). Having the new marker combination to distinguish certain memory subpopulations we can estimate the pool of protective memory cells and therefore the protective capacity of the vaccine already at an early time point upon immunization.

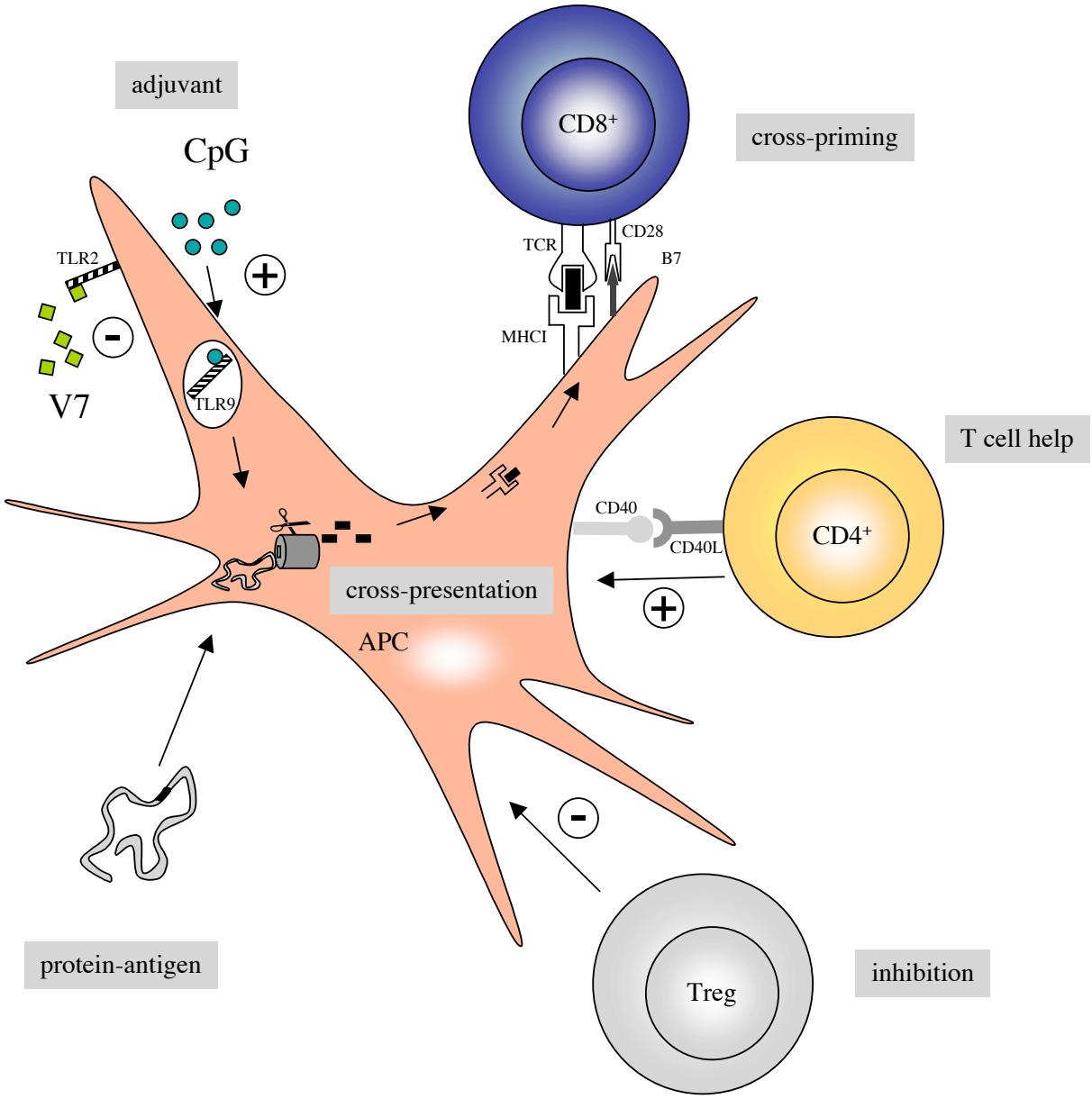


Figure 32: Model

This cartoon summarizes our main findings and interpretations extrapolated from our data. Several components are crucial for optimal cross-presentation and -priming of $CD8^+$ T cells. CpG adjuvant activity, optimal antigen delivery to DCs, and CD40/CD40L mediated $CD4^+$ T cell help positively (⊕) promotes development of protective immunity. TLR2 signaling triggered by V7 and Treg activity negatively (⊖) interfere with primary and/or memory $CD8^+$ T cell development.

5. SUMMARY

In times of globalization and the resulting contact to ever-new pathogens, it becomes even more important to understand how exactly protective immunity is induced to be able to generate more potent vaccinations. $CD8^+$ T cells are crucial for protection against intracellular pathogens. Until today, vaccines capable to induce long lasting $CD8^+$ T cell responses are usually based on living intracellular organisms. However, live vaccines can be potentially harmful, especially for immunocompromised individuals. In order to improve the safety of vaccines, the goal of many laboratories is to dissect the components derived from live vaccines, which are responsible to effectively induce long lasting protective immunity.

Our aim of this study was to use purified *Listeria monocytogenes* (*L.m.*)-derived antigens and to modulate the immune system to promote the development of preferentially $CD8^+$ effector memory T cells (T_{EM}), which have recently shown to be the major population mediating protective immunity against *L.m.* We chose the *L.m.*-derived protein LLO and the LLO_{91-99} peptide as targets, because LLO-specific T cell responses have been demonstrated to confer efficient protection in this model. Furthermore, strong LLO_{91-99}- specific immune responses are induced, increasing the chances to analyze also more subtle changes within the induced cell populations. While immunization with peptide alone or in combination with adjuvant did not lead to measurable $CD8^+$ immune response, the full protein LLO induced readily detectable antigen specific $CD8^+$ T cell numbers. Thereby, the route of application played a crucial role, since LLO_{91-99}-specific immune responses were much higher after local application of LLO and almost absent after systemic administration. Priming of LLO_{91-99}-specific $CD8^+$ T-cell responses upon immunization with the purified protein requires access of antigen to the MHC class I cross-presentation pathway. As TLR9 ligands like bacterial CpG-DNA are believed to be able to enhance cross-presentation by DCs, adjuvant effect of CpG-DNA were analyzed. Indeed, CpG functioned as a strong stimulatory component, since mice treated with LLO and CpG as adjuvant showed almost equal levels of LLO_{91-99}-specific T cell immune response as compared to *L.m.* infected animals. Immunization with LLO alone induced only weak immune responses. The superior $CD8^+$ T cell immunogenicity of full protein versus peptide vaccination could be due to the induction of additional antigen specific $CD4^+$ T cell responses, which provide enhancing factors by T cell help. To test the $CD4^+$ T helper dependency, we performed antibody mediated $CD4^+$ T cell depletion. To our surprise, LLO_{91-99}-specific $CD8^+$ T cell frequencies were even increased in $CD4^+$ T cells depleted mice. Since $CD4^+$ lymphocytes consist besides conventional $CD4^+$ T cells also of a substantial

fraction of regulatory T cells (Treg cells, $CD4^+CD25^+$), we analyzed which of these populations account for the unexpected increase of LLO_{91-99}-specific $CD8^+$ T cell numbers. Using a $CD25^+$ depleting antibody, we could show that the absence of $CD25^+$ T cells led to enhanced LLO_{91-99}-specific $CD8^+$ T cell responses in combination with CpG as adjuvant, as good as after $CD4^+$ T cell depletion, identifying $CD25^+$ Treg cells as inhibitory cell population during T cell priming. Challenge experiments with a high dose of *L.m.* indicated that $CD25^+$ T cell depleted mice were long term protected against *L.m.* infection, even more effectively than $CD4^+$ T cell depleted mice. This result correlates with the observation that $CD25^+$ depleted animals showed increased numbers of T_{EM} cells. Experiments using MHC class $II^{-/-}$ mice further confirmed these findings: $CD8^+$ T-cell responses in MHC class $II^{-/-}$ mice were not effected by anti-$CD25^+$ depletion; in contrast, treatment with a CD40 stimulating antibody to provide signals mimicking $CD4^+$ T cell help substantially enlarged antigen-specific $CD8^+$ T cell responses. Taken together, these data demonstrate that both $CD4^+$ lymphocyte subsets affect memory T cell generation, with Treg cells being capable to reduce not only the population size, also the memory T cell differentiation pattern is largely affected. To our knowledge, this is the first report linking regulatory T cell activity to memory T cell differentiation.

To address the question whether other TLR ligands are also able to support cross-priming and enhance $CD8^+$ T cell immunogenicity, we generated purified LLO protein covalently linked to V7 peptide. V7 is a *Yersinia*-derived oligopeptide and has been shown to confer signaling via TLR2. Interestingly, V7 also induces IL-10 secretion from macrophages, already indicating that the modulatory capabilities might differ from CpG. Indeed, $CD8^+$ immune responses were diminished when compared to LLO treated animals. This phenotype could be only partially reverted by additional application of CpG, indicating that the V7 effect might not only be mediated on the level of the APC. One candidate cell population were again the Treg cells. We found that depletion of $CD25^+$ T cells upon vaccination with LLO-V7 plus CpG resulted in increased frequencies, but not to the same extent as observed for LLO plus CpG treated mice. This finding indicates that in this setting Treg cells partly seem to negatively regulate $CD8^+$ T cell responses.

In conclusion, we show for the first time that the activity of TLR-ligands as $CD8^+$ T cell priming adjuvant can be strongly influenced by naturally occurring Treg cells. In addition, Treg downmodulation in combination with TLR stimulation might represent a novel approach to specifically support differentiation of $CD8^+$ T cells to T_{EM} cells. These results provide

important implications for the design of more effective T cell vaccines when immunizing with purified proteins.

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