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CpG-DNA-antigen conjugates: a new class of therapeutics?

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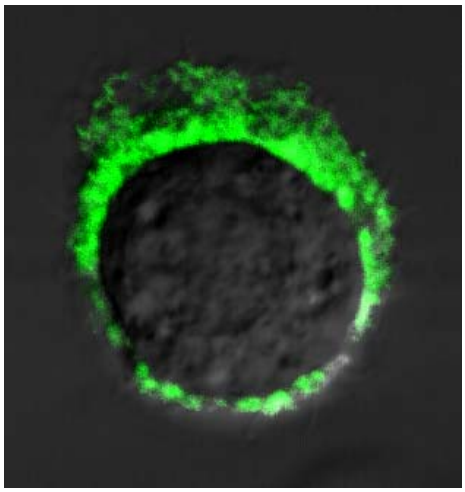


Meiner Frau Jean und unseren Söhnen Joshua und Kilian gewidmet.

Abbreviations:

#	product number
-/-	knock-out
2-ME	2-mercaptoethanol
A	adenosine
APC	antigen presenting cell(s)
APS	ammonium peroxodisulfate
β-gal	β-galactosidase
BCG	bacillus Calmette Guerin
BMDC	bone marrow derived dendritic cell(s)
°C	degree Celsius
C	cytosine
CD	cluster of differentiation
Ci	Curie
CL	crosslinker S-MBS
CpG	deoxycytidylate-phosophate-deoxyguanylate; immunostimulatory cytosine-guanosine motif
cpm	counts per minute
CTL	cytotoxic T cell(s)
Cu	copper
D	Dalton
DC	dendritic cell(s)
DNA	desoxyribonucleid acid
DPBS	Dulbecco´s phosphate buffered saline
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Flt3-ligand	FMS-like tyrosine kinase 3 - ligand
FPLC	fast protein liquid chromatography
G	guanosine
g	gram

GM-CSF	granulocyte/monocyte-colony stimulating factor
GpC	deoxyguanylate-phosophate-deoxycytidylate; non-immunostimulatory guanosine-cytosine motif
h	hour
HIV	human immundeficiency virus
IFN	interferon
IL	interleukin
k	kilo (10^3)
l	liter
LN	lymphnode(s)
LPS	lipopolysaccharides
µ	micro (10^{-6})
m	milli (10^{-3})
M	molar
mA	milliAmpere
mAb	monoclonal antibody
MHC	major histocompatibility complex
min	minute
n	nano (10^{-9})
ODN	oligonucleotide(s)
OVA	ovalbumin, chicken egg albumin
PAGE	polyacrylamide gel
PFA	paraformaldehyde polyoxymethylene
RT	room temperature
SDS	sodium dodecyl sulfate
sec	second
S-MBS	sulfo-maleimidobenzoyl-N-hydroxysuccinimide ester
T	thymidine
Temed	N,N,N´,N´-tetramethylethylendiamine
Th	T helper
TLR	toll-like receptor
Tris base	tris-hydroxymethylaminomethan
Tris Cl	tris-hydrochloride
UV	Ultraviolet

Contents

Picture on page 2: confocal microscopy picture of a J-774 cell that has engulfed fluorescent OVA-FITC conjugates

1 Introduction

1.1 Innate and adaptive immunity

Traditionally the immune system of vertebrates is divided into an innate and an adaptive component. While the adaptive immunity, characterized by specificity and memory, is mediated by clonally distributed T and B lymphocytes, the innate immunity was formerly thought to be a nonspecific first-line immune response consisting of antimicrobial peptides, the alternative complement pathway as well as cellular components such as macrophages and NK cells (Akira et al., 2001).
The effector mechanisms of innate immunity, however, do not only control the replication of an infecting pathogen and contain the infection during the initial steps of infection until specifically activated and clonally expanded T and B lymphocytes can generate an efficient immune response (Medzhitov and Janeway, Jr., 2000b). The innate immune system plays a much more important and fundamental role in host defense: cells of the innate immune system are capable of discriminating between pathogens and self (Akira et al., 2001) and, even more importantly, are responsible for initially activating the adaptive immune system (Medzhitov and Janeway, Jr., 2000b).

1.2 Interaction of innate and adaptive immune system

Antigen-presenting cells (APC), particularly dendritic cells (DC), play such a key role among the cells of innate immunity and effectively link innate and acquired immune-recognition systems. Immature DC are located in peripheral tissues including potential pathogen-entry sites where they constitutively sample and process their enviroment. Subsequently fragments of engulfed proteins, bound to molecules of the major histocompatibility complexes (MHC), are presented on the surface of an APC. Classical antigen-presentation studies revealed that endocytosed antigens are processed and presented on MHC class II while intracellular proteins, cut into peptides in the cytosol of an APC, are loaded onto MHC class I molecules in the endoplasmic reticulum before those complexes are transferred to the cell surface (Germain, 1994), (Banchereau and Steinman, 1998).

This strict dichotomy, however, has been challenged in recent years by several studies (Yewdell et al., 1999), (Heath and Carbone, 2001) that show that exogenous proteins can gain access to the cytosol by yet undefined pathways (cytosolic diversion) and can therefore be presented on MHC class I (Rock et al., 1990), (Kovacsovics-Bankowski and Rock, 1995), (Norbury et al., 1995), (York and Rock, 1996), (Rock, 1996). These processes, referred to as „cross-presentation“, explain why injection of various types of exogenous antigens efficiently prime MHC class I restricted CTL responses (Schirmbeck et al., 1994), (Jondal et al., 1996), (Brossart and Bevan, 1997). Especially immature DC (Shen et al., 1997), (Norbury et al., 1997), (Albert et al., 1998), furthermore macrophages (Castellino et al., 2000), (Singh-Jasuja et al., 2000) and – just recently discovered – B cells (Shirota et al., 2002) are particularily efficient in cross-presentation.

If, during an infection, an immature APC encounters bacterial products signalling infectious danger, these APC are activated and start to maturate by decreasing phagocytosis activity, upregulating inflammatory cytokine production and displaying a full set of costimulatory molecules such as CD40, CD80 and CD86 (Wagner, 1999).

T cells can recognize antigen fragments presented on MHC molecules of APC using their T cell antigen receptors (Banchereau and Steinman, 1998). To become activated T cells not only require the interaction of their antigen-specific T-cell receptor with the peptide-complexed major histocompatibility complexes, but in addition need activation signals mediated by costimulatory molecules and cytokines. In contrast, recognition of an antigen in the absence of these signals leads to permanent inactivation or apoptosis of the T cell (Medzhitov and Janeway, Jr., 2000b). The magnitude and quality of antigen presentation (signal1), of costimulatory molecule expression (signal 2) as well as the levels of induced cytokines (signal 3) in such an “immunological synapse”, a specialised area of contact between APC and T cells, greatly influence the outcome of adaptive T cell responses (Wagner, 2002), (Grakoui et al., 1999).

Naïve $CD4^+$ T cells, when stimulated with cognate antigens by APC, differentiate into two cell subsets: T helper 1 (Th1) and T helper 2 (Th2) cells. While interleukin 12 (IL-12) drives Th1 differentiation characterized mainly by cellular immunity and Th1 cells secreting interferon γ (INFγ), IL-4 induces Th2 differentiation – primarily humoral immunity and Th2 cells producing IL-4, IL-5, IL-10 and IL-13 (Akira et al., 2001), (Abbas et al., 1996).

Thus APC sensing infectious danger present not only the critical link between innate and acquired immunity but in fact control via cytokines and costimulatory molecules the extent as well as the quality (Th1 versus Th2) of emerging T cell responses (Wagner, 1999), (Abbas et al., 1996).

1.3 Pattern recognition receptors

To fulfill their role as first-line surveillance of the immune system APC posses germline-encoded receptors (Medzhitov and Janeway, Jr., 2000a). These so-called pattern recognition receptors (their number is estimated to be in the hundreds) recognize pathogen-associated molecular patterns including bacterial lipopolysaccharide, peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, double-stranded RNA and glucans (Medzhitov, 2001). Pattern recognition receptors belong to several structurally and functionally heterogeneous families of proteins, among them the toll-like receptors (Medzhitov and Janeway, Jr., 2000b), (Medzhitov and Janeway, Jr., 2000a).

In 1988 the first receptor of the toll family was identified in drosophila as a component of as signalling pathway that controls dorsoventral polarity in fly embryos (Anderson et al., 1985). Later on it was found that toll receptors in drosophila play an essential role in the fly´s immune response as well (Lemaitre et al., 1996), (Williams et al., 1997). Analysis of the toll gene revealed that it encodes a transmembrane protein with a large extracellular domain containing leucine-rich repeats and a cytoplasmic domain similar to the cytoplasmic domain of the mammalian IL-1 receptor (Gay and Keith, 1991). Signalling through this receptor – similar to the toll receptors in drosophila – leads to activation of the nuclear factor-kb and plays a key role in the induction of immune and inflammatroy responses in mammals as well (Belvin and Anderson, 1996), (Ghosh et al., 1998).

Since then several homologs of the drosophila toll proteins – named toll-like receptors (TLR) – have been identified in mammals (Medzhitov et al., 1997b), (Rock et al., 1998). Up to now nine different toll-like proteins in drosophila and at least ten mammalian TLR have been identified. Toll and some if not all of the mammalian TLR are involved in the initiation of innate immune activation in response to infection by detecting pathogen-specific molecular structures (Medzhitov and Janeway, Jr., 1997a), (Akira et al., 2001), (Bauer and Wagner, 2002).

1.4 The pattern recognition receptor TLR9

One of these TLR, in fact TLR9, recognizes unmethylated bacterial DNA and is able to distinguish bacterial DNA from self-DNA. TLR9-deficient mice are completely defective in their response to CpG-DNA – oligonucleotides (ODN) that mimic bacterial DNA – including proliferation of splenocytes, inflammatory cytokine production from macrophages and maturation of dendritic cells. The CpG-DNA mediated Th1 response *in vivo* is also abolished in TLR9 -/- mice (Hemmi et al., 2000). Furthermore TLR9 expression in human immune cells such as B cells and plasmacytoid DC correlates with responsiveness to bacterial CpG-DNA (Bauer et al., 2001). Interactions between CpG-DNA and TLR9 rapidly activate APC through the ancient Toll/IL-1-receptor signalling pathway. Thus, interactions between CpG-DNA and TLR9 effectively bridge innate and adaptive immunity (Wagner, 2002).

1.5 The discovery of immunostimulatory CpG-DNA

As far back as the 1700s, it was recorded that certain infectious diseases could exert a beneficial therapeutic effect upon malignancy. More than a century ago William B. Coley used a bacterial vaccine to treat primarily inoperable sarcoma accomplishing a cure rate of better than 10% (Wiemann and Starnes, 1994).

Since then, several kinds of cancer models were successfully treated with the attenuated mycobacteria bacillus Calmette Guerin (BCG) (Zbar and Rapp, 1974), (Bast, Jr. et al., 1974), (Bast and Bast, 1976) and extracts of BCG have become standard adjuvant immunotherapy in human superficial bladder cancer (Morales, 1976). In the attempt to identify the antitumor components of BCG extracts, Tokunaga and colleagues first established that it might be bacterial genomic DNA that accounts for the observed immune stimulation and antitumor effects (Tokunaga et al., 1984), (Yamamoto et al., 1988).

But not only DNA from BCG is stimulatory to the immune system. Naked bacterial DNA in general stimulates – in contrast to vertebrate DNA – *in vitro* proliferation of murine B cell lymphocytes (Messina et al., 1991). Furthermore synthetic single-stranded ODN from BCG-DNA facilitate immune responses – provided they contain a 6-base palindromic sequences centered around CpG dinucleotides, like GACGTC, GGCGCC or TGCGCA (Tokunaga et al., 1992), (Yamamoto et al., 1992a),

(Yamamoto et al., 1992b), (Yamamoto et al., 1994). In mammalian genomes these CpG-motifs as well as the necessary flanking sequences occur much less frequent (Bird, 1980), (Han et al., 1994) and are in addition commonly methylated at the 5 position of about 70% of the cytosines (Bird, 1987).

Subsequently it was shown – first in experiments with B cells – that ODN require an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines for optimal immunostimulatory properties (Krieg et al., 1995), (Sato et al., 1996). These singlestranded synthetic CpG-ODN share and probably mimic the stimulatory activity of bacterial DNA (Pisetsky, 1996), (Shimizu et al., 1997). It is reasoned that, by virtue of a 20-fold lower frequency of unmethylated CpG dinucleotides (termed „CpG suppression“) in mammalian versus bacterial DNA (Krieg, 1996a), cells of the innate immune system such as APC may have learned to use this structural difference to differentiate between self DNA and pathogen-derived DNA (Pisetsky, 1996), (Sparwasser et al., 1997a), (Lipford et al., 1997b). Bacterial DNA acts as a pathogen-associated molecular pattern and by interaction with the vertebrate pattern recognition receptor TLR9 signals the cells of the immune system the presence of “infectious danger” (Krieg, 1996b), (Krieg, 1996a), (Lipford et al., 1998).

1.6 CpG-DNA – a Th1-inducing adjuvant

CpG-DNA triggers – via the above mentioned interaction with TLR9 – a so-called Th1 immune response in vertebrates.

CpG-DNA activates APC, particularly dendritic cells and macrophages, and causes them to maturate into professional APC. They upreagulate CD40, CD80, CD86 and MHC class I and II on the cell surface, molecules necessary for costimulation (Hartmann et al., 1999), (Bauer et al., 1999), (Sparwasser et al., 2000), start to produce typical proinflammatory cytokines like tumor necrosis factor α (TNFα), IL-1, IL-6, IL-8, IL-12 and bioactive IL-12p70 as well as IL-15, IL-18 and type I interferons (IFNα, IFNβ, IFNγ) (Stacey et al., 1996), (Roman et al., 1997), (Sparwasser et al., 1998), (Wagner, 2001), (Hochrein et al., 2001), (Krug et al., 2001b), (Bauer et al., 2001), while typical Th2-cytokines (e.g. IL-4, IL-5) are suppressed (Chu et al., 1997), (Lipford et al., 2000b).

In B cells, also, CpG-DNA ODN trigger expression of costimulatory molecules and production of proinflammatory cytokines such as IL-6. Upon stimulation B cells

proliferate and secrete Th1-specific immunoglobulins (IgG_2), whereas Th2-typical immunolobulins like IgE, IgG_1 are restrained (Krieg et al., 1995), (Liang et al., 1996), (Yi et al., 1996), (Lipford et al., 1997a), (Bauer et al., 1999).

Not all kind of CpG-DNA, however, show all these described effects to the same extent. This has led to the distinction into CpG-A ODN and CpG-B ODN. While CpG-A ODN, defined by poly G motifs with phosphothioate linkages at the 5´ and 3´ ends around a phosphodiester CpG-containing core sequence, characteristically activate NK cells and induce production of type I interferons (among, of course, other common features of CpG-DNA) (Krug et al., 2001a), (Ballas et al., 1996), CpG-B ODN, fully phosphothioated ODN containing a CpG motif, display enhanced B cell stimulation (Krieg, 2002).

CpG-DNA used as adjuvant in combination with antigen enhances the immune response against these antigens and supports – independent of T cell help – priming of antigen-specific cytotoxic T cells (CTL) (Lipford et al., 1997a), (Bendigs et al., 1999), (Sun et al., 1998), (Sparwasser et al., 2000), (Krieg, 2000), (Wagner, 2001).

The therapeutic effect of CpG-ODN could been documented on a variety of established murine tumor models including B cell lymphoma, IE7 fibrosarcoma, B16 melanoma, and 3LL lung carcinoma. In these tumors repeated peritumoral injection resulted in complete rejection or strong inhibition of tumor growth (Weiner et al., 1997), (Kawarada et al., 2001).

Also in vaccination protocols (e.g. successful vaccination of mice and orangutans against hepatitis B; human trial under way) CpG-DNA proved to be an effective adjuvant (Davis et al., 1998), (Davis et al., 2000), (Weiner, 2000). As vaccine adjuvant in DNA-vaccines CpG-DNA is a stronger Th1-inducing adjuvant for the induction of B cell and T cell responses than the gold standard, CFA, as measured by its ability to drive the differentiation of CTL and IFNγ secreting T cells (Krieg, 2002), (Krieg and Davis, 2001).

In a murine model of asthma, where a pathological Th2-biased immune reaction is prevalent, ODN displaying a CpG-motif prevented airway eosinophihilia, Th2 cytokine induction, IgE production and bronchial hyperreactivity when coadministrated with antigen – even in previously sensitized mice (Kline et al., 1998).

Among many additional findings CpG-DNA is capable to revert preexisting Th2 immune responses: it conveys protective immunity in BALB/c mice against Leishmania major, a model for a lethal Th2-driven disease – even if administered as

late as 20 days after lethal infection (Zimmermann et al., 1998). More recently, it could be shown that CpG-DNA significantly prolongs survival of mice inoculated with scrapie prion bearing brain homogenates from infected mice (Sethi et al., 2002).

1.7 Goal of this doctoral thesis

CpG-DNA yields therapeutic potential as adjuvant in vaccination strategies for cancer, allergy and infectious diseases (Klinman et al., 1999), (Wagner, 1999), (Krieg, 2000). Therefore, refinement of such CpG-DNA vaccination formulas, that is, reducing allergenicity and toxicity while at same time improving immunogenicity is of particular interest.

In this search of better vaccination strageties the idea arose to covalently link antigen to immunostimulatory DNA (Tighe et al., 2000a). It was hypothesized that conjugation of CpG-DNA to protein antigens would strongly enhance their immunogenicity because both antigen and adjuvant would be co-delivered to the same APC – according to a mechanism that has been described for antigen-antibody immune complexes (Schuurhuis et al., 2002). Furthermore cells of the innate immune system would only be activated by CpG-DNA in the presence of antigen, thereby diminishing undesired unspecific activation and minimizing unwanted side effects mediated by bystander cells that have been activated by free ODN. Also, it seemed

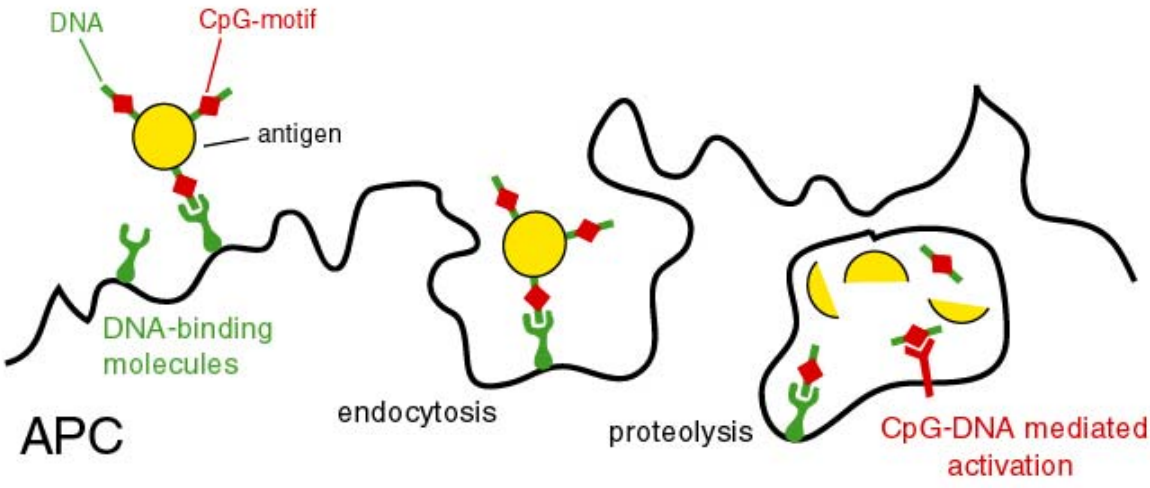


Figure 1: Proposed mechanism of engulfment: DNA aided uptake of conjugate into antigen-presenting cells (APC) followed by CpG-DNA mediated activation of APC

reasonable to believe that lower amounts of CpG-DNA and antigen would be required to achieve the same magnitude of specific immune response – once, due to physical delivery of both protein and adjuvant to the same cell, secondly, because the antigen-linked ODN could serves as a shuttle mechanism for antigen-uptake since free DNA is endocytosed in an active manner (Hacker et al., 1998) (figure 1).
Based on these considerations we wanted to address the issue whether CpG-DNA-antigen conjugates are superior to the simple mixture of both, and – if so – to what extent. Can covalently linked ODN mediate the uptake of conjugates? What are the implications of increased antigen-uptake? Could such a vaccination formula be of clinical relevance for future therapy regimens?

2 Materials and Methods

2.1 Companies

Materials, reagents and chemicals were obtained from following companies:

American Type Culture Collection, Rockville, MD, USA
Amersham Biosciences, Freiburg
Becton Dickinson, Heidelberg
Biochrom, Berlin
Calbiochem, San Diego, CA, USA
Corning, Corning, NY, USA
Eurocetus, Amsterdam, The Netherlands
Fluka, Buchs, Switzerland
Greiner bio-one, Frichenhausen
Labsystems Oy, Helsinki, Finland
Merck, Darmstadt
Molecular Probes, Leiden, The Netherlands
Nunc, Wiesbaden
PAA Laboratories GmbH, Cölbe
PAN Biotech, Aidenbach
Pharmingen, Heidelberg
Pierce, Bonn
RDI Apotheke (TU München), München
Research Genetics, Huntsville, AL, USA
Roth, Karlsruhe
Sigma, Deisenhofen
TIB Molbiol, Berlin
TriLink Biotechnologies, La Jolla, CA, USA
Whatman, Maidstone, England

2.2 Reagents and chemicals

2.2.1 Media and cell culture components

2-ME (143 mM)	PAN, # MB-11080101
FCS	PAN, # P21-1312
Flt3-ligand (human)	courtesy of Dr. Hochrein, Institute of Medical Microbiology, Immunology and Hygiene, TU München
GM-CSF	generated as described (Zal et al., 1994)
Hepes	Roth, # 9105.2
recombinant IL-2 (human)	kindly provided by Eurocetus
Instamed	Biochrom, # T 125-10
NaCl	Roth, # 3957.2
$NaHCO_3$	Merck, # 106329
Penicillin, streptomycine, L-glutamine	PAA, # P11-013
RPMI 1640	PAN, # P04-17500

2.2.2 Reagents for conjugation

OVA	Sigma, # A-2512
FITC-labelled OVA	Molecular Probes, # O-23020
Timothy grass pollen allergen 5 (phleum, Phl p 5)	courtesy of Prof. Dr. Ollert, Department of Dermatology, Klinikum Rechts der Isar, TU München
β-galactosidase	Sigma, # G-3153

For conjugation following phosphothioated sulfhydryl-modified ODN were custom-synthesized from TriLink Biotechnologies (the ODN 2216-S is only phosphothioated at 5´- and 3´- end; “p” indicates phosphodiester-linkages):

1668-S	5´-S-TCCAT**GA<u>CG</u>TT**CCTGATGCT-3´
1720-S	5´-S-TCCAT**GA<u>GC</u>TT**CCTGATGCT-3´
1668pG-S	5´-S-TCCAT**GA<u>CG</u>TT**CCTGGGGGG-3´
2216-S	5´-S-GGGpGp**GpAp<u>CpG</u>pApTp<u>CpG</u>pTpCpG**GGGGG-3´

ODN were synthesized at the 15 µmol scale aliquoted in 10 tubes (usually 250 nmol ODN as lyophilized solid per tube) in a non-reduced state to prevent inactivation of reduced ODN.

S-MBS	Pierce, # 22312
L-cysteine	Roth, # 3467.1

2.2.3 Antibodies

Allophycocyanin-labelled anti-CD11c mAb	Pharmingen, # 550261
Biotinylated anti-mouse IL-$12_{p40/p70}$ detection Ab	Pharmingen, # 554476
Biotinylated anti-mouse IL-6 detection Ab	Pharmingen, # 554402
Biotinylated anti-mouse TNFα detection Ab	Pharmingen, # 554415
FITC-labelled anti-CD40 mAb	Pharmingen, # 553790
FITC-labelled anti-CD86 mAb	Pharmingen, # 553691
Purified anti-mouse IL-$12_{p40/p70}$ capture Ab	Pharmingen, # 551219
Purified anti-mouse IL-6 capture Ab	Pharmingen, # 554400
Purified anti-mouse TNFα capture Ab	Pharmingen, # 557516

For FACS analysis antibodies were diluted 1:100 in FACS staining buffer and cells were stained in 25 µl in eppendorf tubes. Corresponding isotype controls were also purchased from Pharmingen.
To detect cytokines using ELISA antibodies were diluted as described in 2.11.2.

2.2.4 Oligonucleotides

Nuclease-resistant phosphothioate-modified ODN were custom-synthesized at a 2 µmole scale by TIB Molbiol (the ODN 2216 is only phosphothioated at 5´- and 3´-end; “p” indicates phosphodiester-linkages). The sequences used were:

1668	5´-TCCAT**GACGTT**CCTGATGCT-3´
1720	5´-TCCAT**GAGCTT**CCTGATGCT-3´
1668pG	5´-TCCAT**GAGCTT**CCTGGGGGG-3´
2216	5´-GGGpGp**GpApCpGpApTpCpGpTpCpG**GGGGG-3´

ODN were solubilized in sterile ultra pure water to a final concentration of 250 µM, filter-sterilized, aliquoted and stored frozen at –20°C.

2.2.5 Further reagents and chemicals

Acetic acid	Roth, # 7332.2
30% Acrylamide / 0.8% bisacrylamide	Roth, # 3029.1
APS	Sigma, # A-6761
Bromophenol blue	Sigma, # B-5525
Chlorophenolred-β-D-galactopyranoside	Calbiochem, # 220588
DTT	Roth, # 6908.2
EDTA	Sigma, # E-9884
Eosine	Merck, # 115935
Ethanol 96% (v/v)	RDI Apotheke, # 1-135
Ethidium bromide	Sigma, # E-8751
FITC-labelled OVA	Molecular Probes, # O-23020
Formaldehyde solution 37% (v/v)	Merck, # 104003
Glycerol	Roth, # 3783.1
Glycine	Roth, # 3790.2
$KHCO_3$	Sigma, # P-4913
2-ME (14.3 M)	Sigma, # M-6250
Methanol	Roth, # 7342.1
$MgCl_2$ hexahydrate	Fluka, # 63065
$Na_2{}^{51}CrO_4$	Amersham Biosciences, CSJ1
Na_2CO_3 (waterfree)	Merck, # 1.06398.1000
NH_4Cl	Merck, # 1145
Nonidet P-40	Sigma, # N-3516
OPD	Sigma, # P-9187
OVA	Sigma, # A-2512
PFA	Sigma, # P-6148
SDS	Sigma, # A-6761
SIINFEKL (OVA peptide 257-264)	custom-synthesized by Research Genetics
Silvernitrate	Roth, # 6207.1
Sodium-azide	Merck, # 106688
Sodium-thiosulfate	Sigma, # S-8503

Streptavidin-horseradish peroxidase	Amersham Biosciences, # RPN 4401 V
Sulfuric acid	Roth, # 9316.1
Temed	Roth, # 2367.1
Tris base	Roth, # 5429.3
Tris Cl	Roth, # 9090.3
Trypanblue	Sigma, # T-6146
Tween 20	Roth, # 9127.2
Xylene blue	Sigma, # X-4126

2.3 Media

Complete RPMI 1640

RPMI 1640 was supplemented with 10% (v/v) heat-inactivated (30 min at 57°C) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycine, 2 mM L-glutamine and 50 µM 2-ME.

Complete Clicks RPMI 1640

Instamed powder for 10 l, 23.83 g Hepes, 11.75 g $NaHCO_3$ was solved in 10 l aqua bidest. The pH was adjusted to 7.2, Clicks RPMI 1640 was filter-sterilized and aliquoted in 500 ml bottles and kept at 4°C. Prior to use Clicks RPMI 1640 was supplemented with 10% (v/v) heat-inactivated (30 min at 57°C) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycine, 2 mM L-glutamine and 50 µM 2-ME.

2.4 Buffers and solutions

Buffers and solutions were kept at 4°C unless otherwise stated.

Agarose-loading buffer (6x)

Aqua bidest was added to 30% (v/v) glycerine, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene blue.

Ammonium chloride lysis buffer

Aqua bidest was added to 0.83% (w/v) NH_4Cl (0.15 M), 0.1% (w/v) $KHCO_3$ (10.0 M) and 0.0037% (w/v) 0.1 M EDTA and pH was titrated to 7.3. Buffer was filter-sterilized and stored at RT.

10% APS solution

Aqua bidest was added to 10% (w/v) APS. Aliquotes of 200 µl were stored frozen at –20°C.

Conjugation buffer

For each conjugation a freshly made 50 mM EDTA-DPBS buffer was used. 10 ml of a 0.5 M EDTA solution were added to 90 ml DPBS and the pH was adjusted to 7.0. Solution was used at RT.

Developing solution

For each staining procedure a freshly made developing solution was used. Na_2CO_3 solution was added to 0.2% (v/v) sodium-thiosulfate 10x stock solution and 0.05% (v/v) formaldehyde solution. Solution was used at RT.

Dulbecco´s phosphate buffered saline

DPBS was purchased from PAN Biotech (# P04-36500).

0.5 M EDTA solution

14.6% (w/v) EDTA was solubilized in aqua bidest at pH 8.0 and subsequently filter-sterilized.

ELISA washing solution

PBS powder was solubilized in aqua bidest. Subsequently 0.1% (v/v) Tween 20 was added.

Eosine staining solution

Aqua bidest was added to 0.4% (w/v) eosine, 0.05% (w/v) sodium azide, 8.1% (w/v) NaCl and 10% FCS and subsequently filter-sterilized.

Ethanol solution

Aqua bidest was added to 50% (v/v) ethanol. Solution was stored at RT.

Ethidium bromide stock solution

Aqua bidest was added to 0.05% (w/v) ethidium bromide.

Ethidium bromide working solution

Aqua bidest was added to 0.1% ethidium bromide stock solution. Solution was stored at RT protected from light and changed after one month of use.

FACS staining buffer

0.4% (v/v) of a 0.5 M EDTA solution and 2% (v/v) heat-inactivated (30 min at 57°C) FCS were added to DPBS.

2% FCS-DPBS buffer:

10% (v/v) heat-inactivated (30 min at 57°C) FCS was added to DPBS.

Fixation solution

Aqua bidest was added to 50% (v/v) methanol and 12% (v/v) acetic acid. Solution was stored at RT.

FPLC elution buffer

800 ml aqua bidest was added to 200 ml DPBS and subsequently filter-sterilized giving a 0.2x DPBS buffer.

L-cysteine solution

20 mg L-cysteine was suspended in 200 µl DPBS giving a 825 mM L-cysteine solution. Solution was used at RT.

Na_2CO_3 solution

Aqua bidest was added to 6% (w/v) waterfree Na_2CO_3. Solution was stored at RT.

1% PFA solution

1% (w/v) PFA was solubilized in DPBS at 56°C over night and filter-sterilized.

1% PFA-trypanblue solution

0.05 mg/ml trypanblue was added to 1% PFA solution.

Reducing solution

7.7 mg DTT were suspended in 1 ml DPBS giving a 50 mM DTT-DPBS solution. Solution was used at RT.

10% SDS solution

Aqua bidest was added to 10% (w/v) SDS. Solution was stored at RT.

SDS-loading buffer pH 6.8 (4x)

Aqua bidest was added to 1.58% (w/v) Tris Cl (100mM), 8% (w/v) SDS (280mM), 0.2% bromophenol blue, 20% (v/v) glycerol and 3.08% (w/v) DTT (200mM). The pH was adjusted to 6.8.

SDS-running buffer

Aqua bidest was added to 0.3% (w/v) Tris base (25mM), 1.5 (w/v) glycine (200mM), 1% (v/v) 10% SDS solution. Solution was stored at RT.

SIINFEKL peptide stock solution

1 mM SIINFEKL was solved in complete Clicks RPMI 1640, filter-sterilized and stored in aliquots at -20°C.

SIINFEKL peptide working solution

For each chromium release assay a freshly prepared SIINFEKL peptide working solution was used. 1 µl SIINFEKL peptide stock solution was solved in 1 ml complete Clicks RPMI 1640 giving a 0.1 µM SIINFEKL peptide working solution.

Silvernitrate solution

For each staining procedure a freshly made silvernitrate solution was used. Aqua bidest was added to 0.4% (w/v) silvernitrate and 0.076% (v/v) formaldehyde solution. Solution was used at RT.

Sodium-azide solution

0.03% (w/v) sodium-azide was suspended in DPBS.

Sodium-thiosulfate 10x stock solution

Aqua bidest was added to 0.2% (w/v) sodium-thiosulfate. Solution was stored at RT.

Sodium-thiosulfate solution

For each staining procedure a freshly made sodium-thiosulfate solution was used. Aqua bidest was added to 10% (v/v) sodium-thiosulfate 10x stock solution. Solution was used at RT.

Sterile ultra pure water

Sterile ultra pure water was purchased from Biochrom (# L0015)

TBE-running buffer (5x)

Aqua bidest was added to 5.4% (w/v) Tris base (445mM), 2.75% (w/v) boric acid (445 mM) and 2% 500mM EDTA solution. Prior to use buffer was diluted with aqua bidest giving a 1x TBE-running buffer.

1.5 M Tris base buffer pH 6.8

Aqua bidest was added to 18.2% (w/v) Tris base and the pH was adjusted to 6.8. Solution was stored at RT.

1.5 M Tris base buffer pH 8.6

Aqua bidest was added to 18.2% (w/v) Tris base and the pH was adjusted to 8.6. Solution was stored at RT.

Z-buffer

DPBS was added to 0.7% (v/v) 2-ME, 9mM $MgCl_2$, 0.125% Nonidet P-40 and 0.15mM chlorophenol red β-galactoside.

2.5 Further materials

0.2 µm Anotop 10 Plus filter	Whatman, # 6809-3122
0.22 µm 150 ml Bottle Top filter	Corning, # 430626
Bio-gel P-2 gel fine	Biorad, # 150-4114
Econo 10DG columns	Biorad, # 732-2010
Maxisorb microtiter plates	Nunc, # 439454
Modified Lowry protein assay	Pierce, # 23240
Protein weight marker	Biorad, # 161-0372
Superdex 75HR column	Amersham Biosciences, # 17-1047-01

2.6 Devices and instruments

Following instruments and technical devices were used:

Centrifuges	Heraeus instruments, Hanau
Cobra II auto gamma counter	Packard, Frankfurt/Main
Confocal microscope (LSM 510)	Carl Zeiss, Jena
Electronic precision balances	Sartorius, Göttingen
Electrophoresis instruments	Biometra, Göttingen
ELISA washer	Skatron Instruments, Norwegen
Emax precision microplate reader	Molecular Devices, Menlo Park, CA, USA
FACS Calibur flow cytometer	Becton Dickinson, Heidelberg
Fluorescence Measurement System	Millipore, Eching
FPLC unit	Amersham Biosciences, Freiburg
Gas incubators	Heraeus instruments, Hanau
Gel drying equibment	Biorad, München; Bachofer, Reutlingen
Gradient Former	Biorad, München
Heatblock	Gebr. Liebisch, Bielefeld
Laminar flow benches	Heraeus instruments, Hanau

Microcentrifuges	Heraeus instruments, Hanau
Microscopes	Carl Zeiss, Jena
pH-Meter	Wissenschaftlich-Technische Werkstätten, Weilheim
Photometer	Amersham Biosciences, Freiburg
Pipetboy	Becton Dickinson, Heidelberg
Pipettes	Biohit, Köln; Thermo Lifesciences, Egelsbach
UV-documentation system	Hitachi, Düsseldorf; Mitsubishi, Ratingen; CS1 Cybertech, Berlin
Vaccum concentrator	Bachofer, Reutlingen
Vortexer	Scientific Industries, Bohemia, NY, USA
Water bath	Gesellschaft für Labortechnik, Burgwedel

2.7 Computer programs

Following computer programs were used in addition to specialized software provided with equipment mentioned above:

Adobe® Illustrator 8.0

Adobe® Photoshop 6.0

Microsoft® Excel 2000

Microsoft® Word 2000

SPSS Inc. SigmaPlot 2000

2.8 Mice

Female C57BL-6 mice were purchased from Harlan Winkelmann, Borchen. All animals were kept under specific pathogen-free conditions in macrolon cages at 23 – 25°C, 60% humidity and a 12 h /12 h day-night cycle and were fed with natural diets for mice (Harlan-Winkelmann GmbH, Borchen) and tap water containing 2% HCl. Mice were used at 6 – 8 weeks of age (18 – 20 g bodyweight) in accordance with an animal experiment permission of the Bavarian government (# 211-2531-66/97).

TLR9 -/- mice were kindly provided by S. Akira (Hemmi et al., 2000).

2.9 *In vitro* culture

Cells were cultured and incubated at 37°C and 5% CO_2 in gas incubators unless otherwise stated. Cell lines and BMDC were maintained in complete RPMI-1640, whereas primary LN cells were cultured in complete Clicks RPMI 1640. In some assays Flt3-ligand cultured BMDC were incubated in the media obtained by culturing – referred to as "conditioned media". To exclude dead cells during counting eosine staining solution was used. Cell-culture approved plastic came from Greiner bio-one (Frichenhausen), Nunc (Wiesbaden), Corning (Corning, NY, USA) and Becton Dickinson (Heidelberg).

2.9.1 Cell lines

ANA-1

ANA-1 (H-2^b), a macrophage cell-line from C57BL-6 mice, was purchased from the American Type Culture Collection.

B3Z

B3Z (H-2^b), a somatic T cell hybridoma generated by fusing the OVA/K^b-specific cytotoxic clone B3 from C57BL-6 mice with a lacZ-inducible derivative of BW5147 fusion partner, was kindly provided by Dr. B. L. Kelsall (National Institutes of Health, Bethesda, MD, USA) (Karttunen et al., 1992).

EL-4

EL-4 (H-2^b), T lymphoma cells from C57BL-6 mice, was purchased from the American Type Culture Collection.

J774A.1

J774A.1 (H-2^d), macrophage cells derived from a tumor in a female Balb/c mouse, was purchased from the American Type Culture Collection.

RAW 264.7

RAW 264.7 (H-2^d), a macrophage cell-line derived from an Abelson murine leukemia virus-induced tumor in Balb/c mice, was purchased from the American Type Culture Collection.

2.9.2 Generation of bone marrow derived dendritic cells

Generation of Flt3-ligand DC

Flt3-ligand supplemented BMDC were generated as described with slight modification (Brasel et al., 2000). Briefly, bone marrow cells of C57BL-6 mice were flushed out of femurs and tibiae, centrifuged and red blood cells were lysed for 2 min with ammonium chloride lysis buffer at 37°C. After washing, cells were cultured at 1,5 x 10^6 cells per ml in media complemented with 200 ng/ml human Flt3-ligand. Cells were used after 10 days of culture.

Generation of GM-CSF cultured BMDC

GM-CSF supplemented BMDC were generated as described with slight modification (Inaba et al., 1992). Briefly, bone marrow cells of C57BL-6 mice were flushed out of femurs and tibiae, centrifuged and red blood cells were lysed for 2 min with ammonium chloride lysis buffer at 37°C. After washing, cells were cultured at 5 x 10^5 cells per ml in 10 ml media complemented with 4 ng/ml GM-CSF (Zal et al., 1994) in 100 mm tissue culture petri dishes. After three days of culture 10 ml media complemented with 4 ng/ml GM-CSF was added. On day 6 10 ml was taken off, centrifuged, supernatant was removed and cells were resuspended in 10 ml of fresh media complemented with 4 ng/ml GM-CSF. The in the petri dish remaining 10 ml were added, leaving adherent cells behind, and cells were cultured for additional two days in a new petri dish. Cells were used on days 7 to 10 of culture.

2.10 Conjugation of oligonucleotides to antigen

Conjugation of OVA to ODN was established on the basis of a protocol previously described (Cho et al., 2000). Covalent linking of ODN to protein requires a small

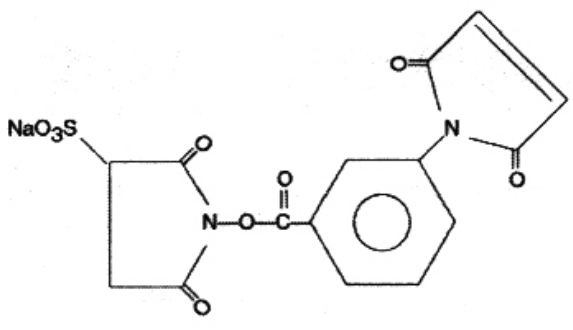


Figure 2: m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Pierce, bulletin 0438w)

molecule – a crosslinker – that targets reactive groups both, on protein and ODN. In this study the bi-functional crosslinker S-MBS was used (figure 2). It possesses a NHS-ester group that targets primary amino groups (figure 3A). However, an α-amine group is only available at the N-terminal of a protein.

Therefore the side-reaction with the ε-amine group of lysine comes to the fore leading to a stable amide linkage. The proteins used possess several lysines (OVA: 20 lysines; phleum: 24 lysines; β-galactosidase: 32 lysines) that might be available for conjugation. On the other side S-MBS has a maleimide group that is most selective for sulfhydryl groups (figure 3B). Therefore sulfhydryl-modified ODN were used. A stable thioether bond is formed between the maleimide group of the crosslinker and the sulfhydryl residue. Furthermore, the crosslinker S-MBS possesses a sulfonate group for water solubility allowing the use of aqueous solutions. A major competing side reaction, however, is hydrolysis of the crosslinker in aqueous solutions (figure 3C).

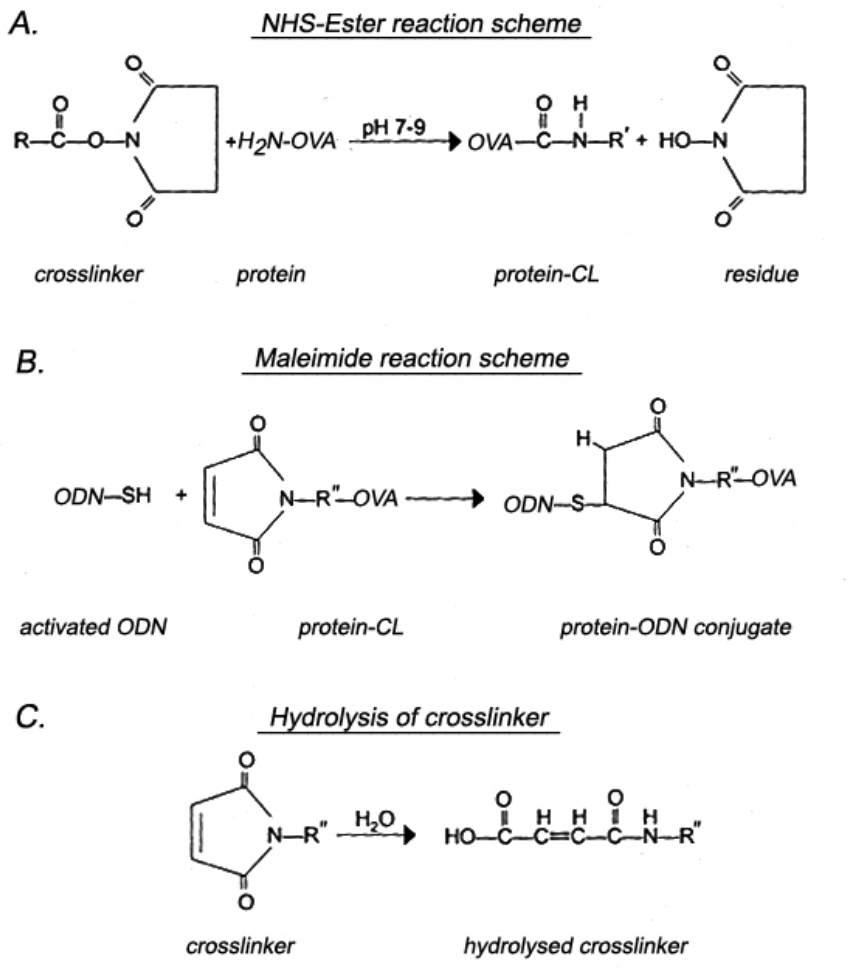


Figure 3: Crosslinker reaction schemes: A, reaction of the NHS-Ester with amino-residues of OVA. B, reaction of maleimide with sulfhydryl-modified ODN. C, hydrolysis of crosslinker (modified after Pierce, bulletin 0438w)

When conjugating antigen to ODN (figure 4), protein is first incubated with crosslinker. To prevent excessive linkage of the crosslinker to sulfhydryl groups of cysteine and formation of protein multimers, EDTA is added to the conjugation buffer. Before reduced (activated) ODN is added to the linker-modified protein solution protein-unbound S-MBS is removed by chromatography to prevent loss of activated ODN by binding to protein-unbound S-MBS. At the end of the incubation period cysteine is added to quench remaining reactive maleimide groups. Elimination of uncoupled ODN from the resulting conjugates is achieved by a second chromatography step.

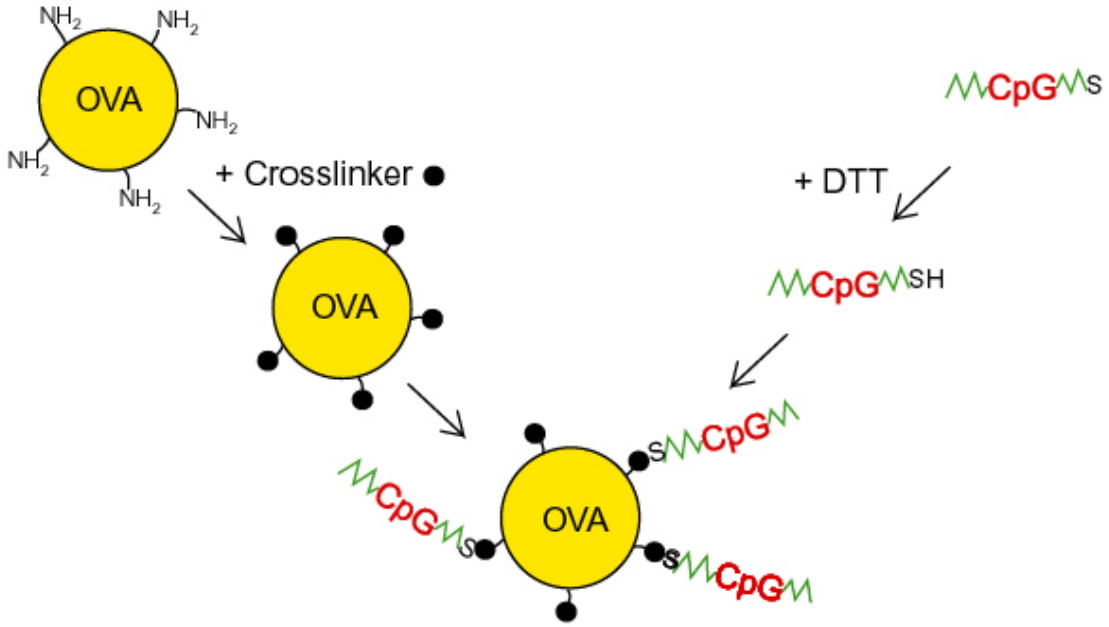


Figure 4: Conjugation of CpG-ODN to OVA

2.10.1 Preparation of gel chromatography columns

According manufacturer´s instructions two gel chromatography columns were prepared: one for the removal of protein-unbound S-MBS from S-MBS linked to protein, another one for elution of activated ODN without the used reducing agent DTT. 6 g of Biorad P-2 dry gel matrix was added to 100 ml DPBS in a beaker and degased at 95°C for 1 h in a water bath. Even slurry was poured into empty 10DG columns and columns were rinsed with DPBS until gel matrix had settled. The gel bed volume was usually about 6 ml. To prevent drying of gel matrix a filter cover was added on top. Columns were equilibrated with at least three bed volumes of DPBS

and – immediately prior to use – with at least three bed volumes of conjugation buffer. For each purification run a maximum of 1 ml solution was added on these columns. After use columns were rinsed with DPBS and sodium-azide solution to prevent bacterial growth. Columns were kept at 4°C and discarded after two months.

2.10.2 Conjugation protocol

The protein to be conjugated was incubated with a 10 fold molar excess of the crosslinker S-MBS in a conjugation buffer for 1 h protected from light at RT. To compensate dilutional effects during conjugation and to prevent excessive hydrolysis of the crosslinker – a side-reaction particularly in dilute protein solutions that abolishes its function – the protein concentration of this solution was kept between 5 to 30 mg/ml. Thereafter the protein / crosslinker solution was centrifuged at 1.8 x 10^4 g for 1 min and, to remove unbound S-MBS, was applied on a Biorade P-2 gel column following elution with conjugation buffer. 500 µl fractions were collected and the absorbance of dilutions in DPBS was measured with a photometer at 280 nm with DPBS as reference. The sulfhydryl-modified ODN were reduced for 10 min solving each required tube in 200 µl reducing solution. Subsequently DTT was removed by chromatography over a Biorade P-2 gel chromatography column. Again, 500 µl fractions were collected and the absorbance of dilutions in DPBS at 260 nm was measured with a photometer with DPBS as reference. The amount of protein and activated ODN in each fraction was calculated and fractions with highest concentrations were used for conjugation. The activated ODN were incubated at 5 fold molar excess with the linker-modified protein at RT protected from light for 2.5 h. Thereafter L-cysteine solution was added at a 50 fold molar excess to the amount of protein used to quench remaining reactive crosslinker groups. The protein-ODN solution was filter-sterilized and stored at -20°C until purification.

2.10.3 Purification and storage of conjugates

Free ODN in the protein-ODN solution were removed by gel chromatography using FPLC. A Superdex 75HR chromatography column was equilibrated in FPLC elution buffer over night at 4°C. The protein-ODN solution was thawed, again filter-sterilized, and 1 ml of solution was applied on the column for each run. 500 µl fractions were collected and the absorbance of the eluent was constantly monitored at 280nm. Fractions with highest concentrations of protein-ODN conjugate were pooled and

aliquoted. Aliquots were either kept at -20°C or – if concentration was required – frozen with liquid nitrogen, lyophilized for 3 to 4 h at 35°C and then stored at -20°C.

2.10.4 Analysis of conjugates using gel electrophoresis

In polyacrylamide gel electrophoresis, proteins migrate from the cathode to the anode through a gel matrix. The migration rate depends on protein size, shape, charge and gel pore size. However, under denaturing conditions – by heating the protein samples in the presence of a low molecular weight thiol (like DTT) and SDS – the protein´s shape is disrupted and SDS is bound to protein in a constant-weight ratio, leading to identical charge densities which makes it possible to separate those proteins according to their molecular weight. By comparison with known molecular weight protein standards the molecular weight of proteins can therefore be determined (Gallagher, 1999).

For analysis, purified conjugates were applied on a 6-20% gradient SDS-PAGE, silverstained and ratio of bound ODN on protein was determined. To prove that molecular weight changes of proteins observed on the SDS-PAGE were due to protein-bound ODN and to show efficient removal of unbound ODN from conjugates 4-15% gradient non-denaturing, non-reducing PAGE were run, consecutively silverstained or visualized using ethidium bromide staining.

2.10.4.1 Casting polyacrylamide gels

When casting polyacrylamide gels monomeric acrylamide forms polymeric acrylamide chains that are cross-linked by N,N´-methylenebisacrylamide. Increased acrylamide concentration hereby results into decreased pore size. The polymerisation reaction is initiated by the addition of ammonium persulfate and catalyzed by Temed that generates free radicals from ammonium persulfate.

According to the Laemmli method of discontinuous gel electrophoresis, buffers of different pH and composition were used for the stacking and the gradient gel resulting in the generation of a discontinuous pH and voltage gradient in the gels. This technique concentrates the protein samples at the border between the stacking and the gradient gel into narrow bands thus achieving higher resolution (Gallagher, 1999).

When casting a 6-20% gradient SDS-PAGE or a 4-15% gradient non-denaturing, non-reducing PAGE the following substances (table 1) were transferred in three

Polyacrylamide gels: substances for each gel	**Gradient gel**				**Stacking gel**
	4%	6%	15%	20%	4%
1.5 M Tris base buffer pH 8.6	1.75 ml	1.75 ml	1.75 ml	1.75 ml	
1.5 M Tris base buffer pH 6.8					0.625 ml
Aqua bidest	4.4 ml	4 ml	1.2 ml		3.68 ml
10% SDS solution		70 µl		70 µl	(50 µl)
Glycerol (warm)			0.6 ml	0.6 ml	
30% Acrylamide / 0.8% bisacrylamide	0.9 ml	1.3 ml	3.5 ml	4.7 ml	0.65 ml
Temed	5 µl	5 µl	5 µl	5 µl	5 µl
10% APS solution	35 µl	35 µl	35 µl	35 µl	25 µl

Table 1

different tubes (two tubes for each percentage of the gradient gel and one tube for the collecting gel). Note, that for the 4-15% gradient non-denaturing, non-reducing PAGE also no SDS was used in the stacking gel.

Since APS is starting polymerisation it was added just immediately prior to casting the gels. To cast the gradient gels a Gradient Former following manufacturer's instructions was used.

After polymerisation gels were inserted in gel chambers and, in case of the 6-20% gradient SDS-PAGE, equilibrated with SDS-running buffer. Samples were thereafter diluted in DPBS and SDS-loading buffer to a final volume of 10 to 25 µl, heated for 5 min at 95°C and then centrifuged for 1 min at 1.65 x 10^4 g. Usually 2 µg of unconjugated protein or protein / S-MBS mixture of the fraction used for conjugation and 4 µg of conjugated protein-ODN before and after the FPLC purification and 3 µl of a prestained protein weight marker were applied on these gels.

When running the 4-15% gradient non-denaturing, non-reducing PAGE TBE-running buffer (1x) was used. Samples were diluted in DPBS and agarose-loading buffer to a final volume of 10 to 30 µl and centrifuged for 1 min at 1.65 x 10^4 g. Samples were not heated. Usually 12 µg of protein – either unconjugated, as protein / S-MBS mixture (of the fraction used for conjugation) or conjugated (before and after the FPLC purification) were applied on these gels.

Gels were run at 30 mA per gel. Subsequently a silverstain was performed. One of the 4-15% gradient non-denaturing, non-reducing PAGE was also visualized under UV-light after incubation with ethidium bromide.

2.10.4.2 Silverstain

Binding of silver to chemical groups (e.g. sulfhydryl and carboxyl residues) of protein can be used for protein staining in polyacrylamide gels. Therefore proteins in gels are fixed to prevent elution, exposed to silver nitrate and subsequently developed (Sasse and Gallagher, 2000).
Gels were put in a developing dish and treated with the following solutions for the indicated time periods:

Fixation solution	30min
Ethanol solution	3 x 30 min
Sodium-thiosulfate solution	1 min
Aqua bidest	3 x 20 sec
Silvernitrate solution	20 min
Aqua bidest	2 x 30 sec
Developing solution	until bands were clearly visible
Fixation solution	at least 15 min to stop staining

Gels were washed with aqua bidest, scanned and dried between seran wrap and Whatman filter paper (Roth, Karlsruhe) for documentation.

2.10.4.3 Ethidium bromide stain

Ethidium bromide is commonly used for visualization of double- and single-stranded DNA in agarose gels, but can also be used in polyacrylamide gels. The dye intercalates between stacked bases of nucleic acids and fluoresces red-orange (560nm) under UV light (Voytas, 2002).
Gels were stained with ethidium bromide working solution protected from light for at least 15 min, washed with aqua bidest, illuminated under UV light and photo-documented.

2.10.5 Analysis of protein content of conjugates

Protein concentration of conjugates was determined using the modified Lowry protein assay according to manufacturer's instructions. The microtiter plate protocol was adjusted to fit the filter set available on the microplate reader used. As shown in figure 5 measurement at 650 nm with reference wavelength 450 nm in a Emax precision microplate reader is linear and therefore equivalent to the reading obtained in quarz cuvettes in a photometer at 750 nm.

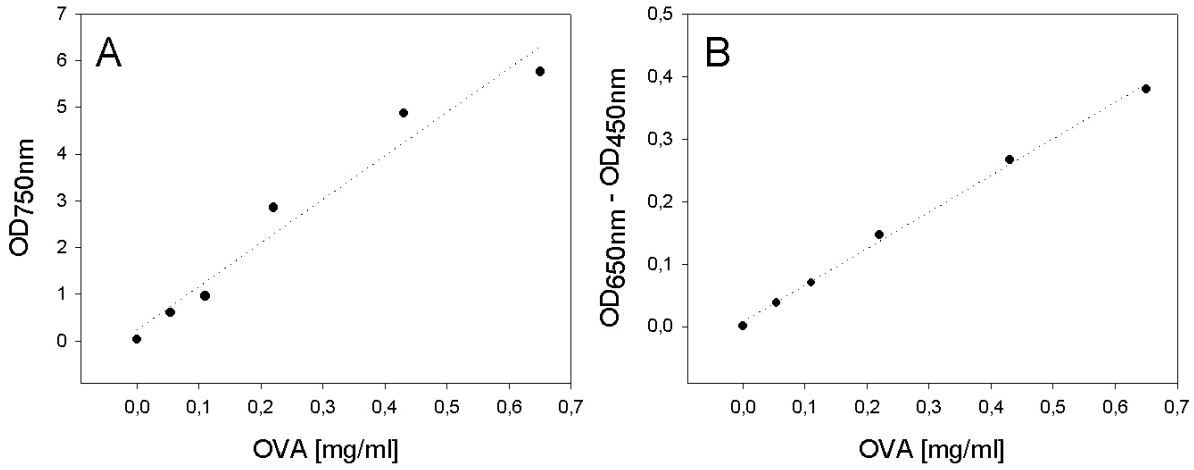


Figure 5: Microtiter plate modification of Lowry protein assay can be used for protein determination: optical densities (filled dots) obtained after OVA protein samples of known concentrations were treated in the Lowry protein assay. For measurement either a photometer at 750 nm (A) – as originally described in the manufacturer´s protocol – or a Emax precision microplate reader at 650 nm, with 450 nm as reference wavelength (B) was used. dotted line: linear regression graph.

2.10.6 Fluorescence intensity of FITC-labelled conjugates

To standardize fluorescence intensity of FITC-labelled conjugates and unconjugated FITC-labelled OVA, serial dilutions in triplicates of these substances in a 96 well microtiter plate were read in a fluorescence reader and relation of fluorescence intensity was determined.

2.11 Functional studies using conjugates

2.11.1 Analysis of co-stimulatory molecule expression

The ability of covalently linked CpG-DNA to upreagulate co-stimulatory molecules on APC *in vitro* was studied by flow cytometry.

In flow cytometry single cells in a liquid medium flowing past excitation sources are analysed regarding size (forward scatter), granulation (sideward scatter) and light emission after excitation (Sharrow, 2002). Measurement of these light emissions allows for example quantitative description of uptake of fluorescent protein or antibody-stained surface marker expression in single cells.

1 x 10^6 Flt3-ligand cultured BMDC per ml conditioned media were incubated with 17.6 µg/ml OVA alone, mixed or conjugated to 1µM CpG-DNA. Cells were cultured for 24 h, transferred into eppendorf tubes and centrifuged at 5 x 10^3 g for 5 min. Supernatant was removed, cells were washed with ice-cold FACS staining buffer and stained with CD11c mAb, CD40 mAb or CD86 mAb for 45 min. Subsequently cells were washed with ice-cold FACS staining buffer and fixed in 250 µl 1% PFA solution. Cells were analysed on a FACSCalibur flow cytometer acquiring at least 4 x 10^4 events per sample. FACS data was analysed using CellQuest software.

2.11.2 Detection of cytokines

For the serum determination of cytokines *in vivo* mice were challenged intraperitoneally with 5nmol/mouse CpG-DNA conjugated or mixed with OVA (107.5µg) in DPBS. 2 h later blood of mice was collected, centrifuged and cytokine concentrations (TNFα, IL-12) were determined in the serum as described below.

For detection of cytokines *in vitro* 1 x 10^5 Flt3-ligand cultured BMDC were incubated in duplicates with increasing amounts of free or conjugated ODN, OVA alone or no stimulus in 200 µl conditioned media for 6 h (TNFα) or in 200 µl complete RPMI 1640 supplemented with 20 ng/ml IL-3 for 18 h (IL-6). After incubation supernatant was recovered and cytokine concentrations were quantitated.

Cytokine concentrations were determined in triplicates by antibody-sandwich ELISA (figure 6) (Hornbeck et al., 2002). First, wells of microtiter plates were coated with 50 µl of specific capture Ab (table 2) over night at 4°C, washed (3 times with ELISA washing solution) and blocked with 200 µl complete RPMI 1640 for 45 min at RT to

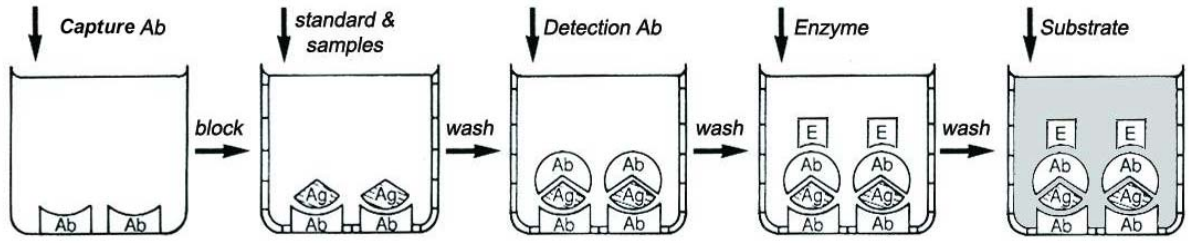


Figure 6: Principle of antibody-sandwich ELISA. Ab: antibody; Ag: antigen (cytokine); E: enzyme (modified after Hornbeck et al., 2002)

prevent unspecific binding of the evaluated cytokine. Plates were washed again 3 times before samples and standards diluted in complete RPMI 1640 were incubated for 2 h in these wells. Subsequently plates were washed 3 times, 50µl of biotinolated detection Ab in complete RPMI 1640 was added to each well for 1 h followed by 3 washes. 50 µl streptavidin-horseradish peroxidase (1:1000 diluted in complete RPMI 1640) was incubated in the plates for 30 min and plates were washed 6 times to remove unbound peroxidase. 50 µl OPD substrate solution (8 mg per 20 ml aqua bidest) was added and plates were incubated 10 to 30 min until highest standard dilutions were clearly visible. Reaction was stopped by adding 30µl 0.18 M sulfuric acid and hydrolysis of substrate – which is proportional to the amount of cytokine present – was measured in a microtiter plate reader at 490nm with 650nm as reference wavelength.

ELISA: Ab dilutions	**m IL-6**	**m IL-12**	**m TNFα**
Capture Ab			
Stock concentration	0.5 mg/ml	2.0 mg/ml	144 µg/ml
Working concentration	2.0 µg/ml	2.0 µg/ml	0.8 µg/ml
Standard			
Stock concentration	1.0 µg/ml	200 ng/ml	100 ng/ml
Working concentration	20 ng/ml	10 ng/ml	5 ng/ml
Detection Ab			
Stock concentration	0.5 mg/ml	0.5 mg/ml	50 µg/ml
Working concentration	2.0 µg/ml	1.0 µg/ml	0.3 ng/ml

Table 2: ELISA: Antibody and standard dilutions; m: murine

2.11.3 Induction of cytotoxic T lymphocytes *in vivo*

To assess the potency of conjugated OVA-ODN compared to OVA in mixture with ODN to prime cellular immunity, the induction of OVA-specific CTL *in vivo* was examined using the cytotoxicity assay (figure 7) (Vabulas et al., 2000).

Performing this assay mice were injected with OVA and ODN mixed or conjugated diluted in DPBS to a final volume of 50 µl into both hind footpads. 4 days later draining popliteal LN were removed and transferred into test-tubes containing 2% FCS-DPBS buffer. Thereafter LN were smashed through a screen and a single-cell suspension was prepared. LN cells were cultured at 3 x 10^6 cells per ml for 4 days in media conditioned with 10 IU/ml rIL-2.

Chromium release assay

After 4 days of culture a ^{51}Chromium release assay was performed with these cultivated LN cells. For this, 2 x 10^6 EL-4 target cells were labelled with 150 µCi of the γ-emitter $Na_2{}^{51}CrO_4$ for 1 h at 37°C. Half of the cells were subsequently incubated with SIINFEKL (Lipford et al., 1995) peptide working solution for additional 30 min. Peptide-untreated EL-4 cells served as specificity control. After washing 1 x 10^3 target cells were incubated with replicate serial dilutions of cultured LN cells. After 4 h of culture 100 µl supernatant from each well was used to detect γ-irradiation. Supernatant for determination of spontaneous and maximal lysis was obtained by

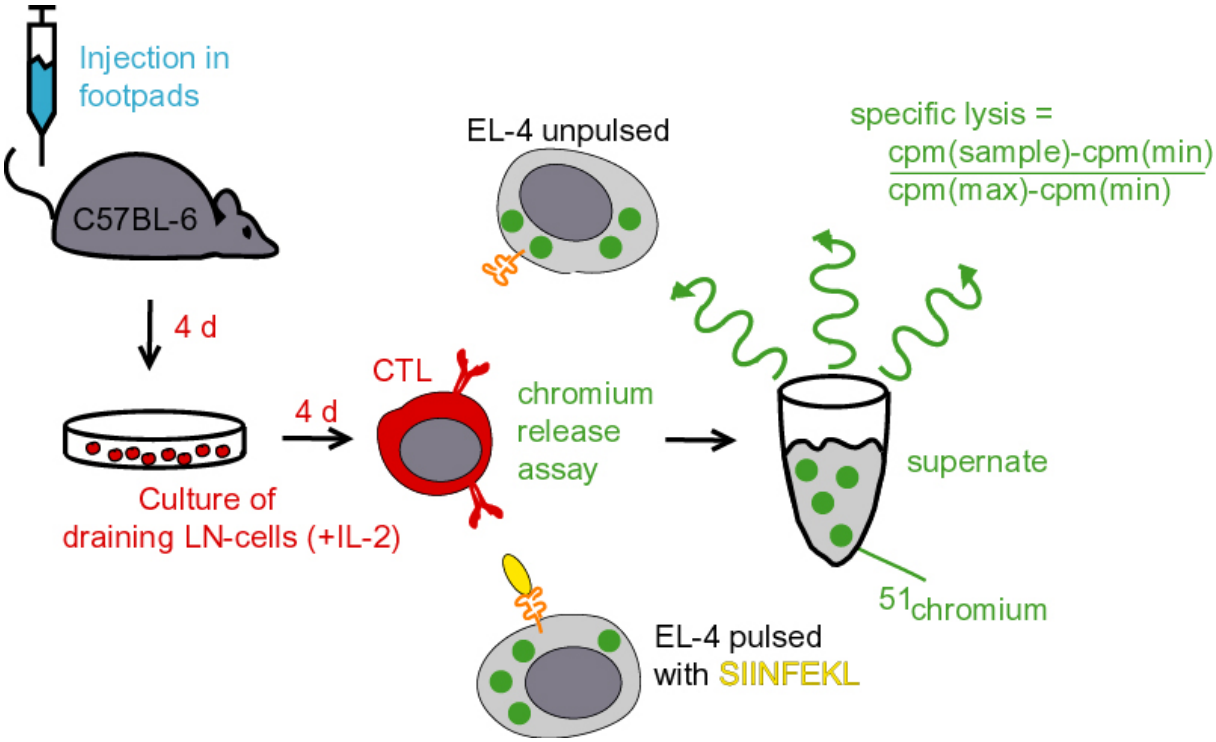


Figure 7: Cytotoxicity assay

taking the supernatant of chromium-labelled EL-4 cells carefully respectively after vigorously mixing. Specific lysis was calculated according to the formula: % specific lysis = [cpm (sample) – cpm (spontaneous release) / cpm (maximum release) – cpm (spontaneous release)] × 100. Specific lysis correlates with OVA-specific CTL activity, which in turn correlates with the induction of specific CTL by immunization.

2.11.4 Uptake of FITC-labelled conjugates into APC

To evaluate the uptake of fluorescent OVA in APC flow cytometry and confocal microscopy were applied.

Fluorescent probes are able to absorb light of a certain wavelength and emit light of a slightly longer wavelength (Stokes shift). In fluorescence microscopy cells are therefore illuminated with light of the absorbing wavelength and viewed using a barrier filter which only allows transmission of the longer emitted light (Coling and Kachar, 1998). This method can be optimized using a laser-scanning confocal microscope that selectively collects light from thin (~1 µm) optical sections of cells. This optical sectioning is accomplished by scanning the specimen with a focused beam of laser light and collecting fluorescence signals via a pinhole aperture selectively from the focused plane. Since essentially no light from out-of-focus areas is detected, structures appear more sharply defined than they would with a conventional microscope (Smith, 1998).

Uptake analysis by fluorescence activated cellsorter (FACS)

To study the uptake of antigen *in vitro* the cell lines ANA-1, J774A.1 and RAW 264.7 or BMDC, either cultured with human Flt3-ligand or GM-CSF, were used. 2.5 x 10^5 cells per 250µl media were exposed to FITC-labelled OVA alone, mixed or conjugated with ODN. Concentration of protein used ranged from 0.025 µg/ml to 20 µg/ml, corresponding to 18 nM to 0,9 µM ODN. Cells were incubated at 37°C in a water bath or at 37°C and 5% CO_2 in gas incubators for up to 5 h. Subsequently cells were centrifuged at 5 x 10^3 g for 5 min, supernatant was removed and cells were washed with ice-cold FACS staining buffer. BMDC were stained with CD11c mAb for 45 min and washed with ice-cold FACS staining buffer. All cells were fixed in 250 µl 1% PFA solution.

To examine the ability of “third party” ODN to block uptake of FITC-labelled conjugates, 2.5 x 10^5 cells of the macrophage-line ANA-1 per 250µl media were incubated with 4 µg/ml FITC-labelled OVA alone, mixed or conjugated with 180 nM

ODN for up to 2 h at 37°C in a water bath. Increasing concentrations of free CpG-ODN (1668), GpC-ODN (1720) or CpG-ODN modified with a poly-guanosine tail (1668pG) were added. Cells were treated as described above.
In some experiments possible surface staining with FITC-labelled OVA was quenched by fixing cells in 250 µl 1% PFA-trypanblue solution (Hed et al., 1987).
Cells were analysed on a FACSCalibur flow cytometer acquiring at least 2 x 10^4 events per sample. FACS data was analysed using CellQuest software.

Uptake analysis by confocal microscopy

To investigate intracellular compartmentation of antigen *in vitro* the cell-line J774A.1 was used because of its out-spread shape. 2.5 x 10^5 cells per 250µl media were incubated with 4 µg/ml FITC-labelled OVA alone, mixed or conjugated with 180 nM ODN for 1 to 2 h at 37°C in a water bath. Subsequently cells were treated as described above. After fixation in 250 µl 1% PFA solution 100 µl (1 x 10^5 cells) were cytospun on objective slides for 5 min at 30 g. Slides were covered by mounting fluid (Labsystems Oy) and cells were examined with a Plan-Neofluar 40 1.3 oil lens under a laser-scanning confocal microscope. Pinhole settings were adjusted to get 1.2 µm sections of the cell at a resolution of 1024 × 1024 pixels per cm.

2.11.5 Presentation of OVA-derived peptides on MHC I

To evaluate the presentation of OVA-derived peptides on MHC I *in vitro* after incubation with conjugated or unconjugated OVA, respectively, the OVA-specific cytotoxic T cell hybridoma B3Z was used (Karttunen et al., 1992). B3Z cells represent an OVA/K^b-specific cytotoxic T cell-clone transfected with the lacZ-gene under the transcriptional activity of IL-2 promoter elements (figure 8). Recognition of the on MHC I presented OVA peptide 257-264 (SIINFEKL) by its T cell receptor leads to transcriptional activation of these promoter elements resulting in production of the enzyme β-galactosidase. Activity of β-galactosidase, which correlates with presentation of OVA-derived peptides on MHC I, is measured by absorbance of wells after cell-lysis and addition of the substrate chlorophenolred-β-D-galactopyranoside.
In this study 2 x 10^5 Flt3-ligand cultured BMDC were incubated in triplicates with increasing amounts of OVA alone, mixed or conjugated with ODN in 200 µl conditioned media for 5 h in round-bottom 96-well plates. Plates were centrifuged at 300 g, washed with media and 5 x 10^4 B3Z cells in 200 µl conditioned media were added to each well. After additional incubation over night cells were lysed by addition

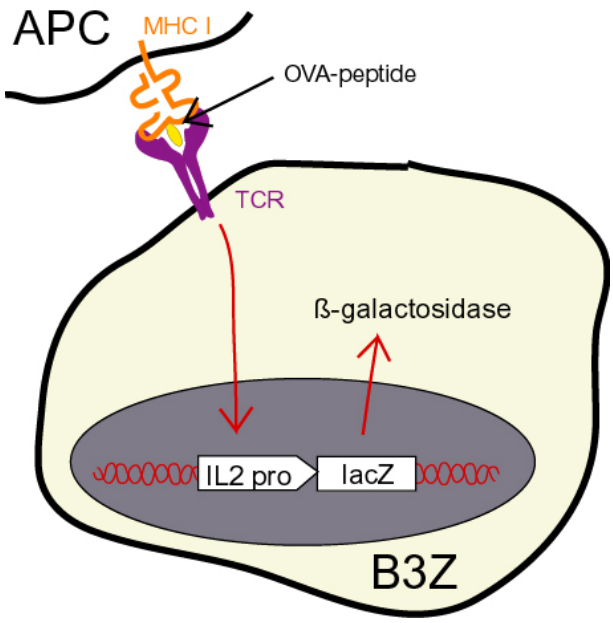


Figure 8: Activation of the T cell hybridoma B3Z. TCR: T cell receptor; IL2 pro: IL-2 promotor elements

of 150µl Z-buffer containing β-galactosidase substrate. After 24 h absorption of individual wells was read in a microplate reader at 570nm, with 650nm as reference wavelength.

2.12 Statistics

Where applicable statistical calculations were performed. In most experiments the mean and standard deviation are shown. For analysis of SIINFEKL presentation and cytokine induction – *in vivo* as well as *in vitro* – the unpaired Student´s t-test was used with "*" indicating $p<0,05$, "**" indicating $p<0,01$ and "***" indicating $p<0,001$.

3 Results

3.1 Establishing a protocol to covalently link ODN to antigen

Since we were interested if CpG-DNA-antigen conjugates are superior compared to a mixture of antigen and immunostimulatory DNA regarding their immunostimulatory potency, we first established a procedure to covalenty link ODN and protein. This procedure was based on a protocol previously described (Cho et al., 2000), yet several modifications were made.

Proteins conjugated included the well-studied model antigen OVA, the bacterial enzyme β-galactosidase and the pollen allergen Phleum p 5 from timothy grass. The stimulatory CpG-B DNA 1668, its non-stimulatory GpC-DNA control 1720 (Krieg et al., 1995), 1668 modified with a poly-guanosine tail at the 5´-end as well as the CpG-A DNA 2216 were used for conjugation. Conjugates produced are shown in Table 3.

Conjugates	ODN : protein
OVA-1668	2.5 : 1
OVA-1668pG	1 : 1
OVA-1720	2.5 : 1
OVA-2216	2 : 1
OVA-FITC-1668	2 : 1
OVA-FITC-1668pG	2 : 1
OVA-FITC-1720	2 : 1
Phlp5a-1668	2.5 : 1
Phlp5a-1720	2.5 : 1
β-gal-1668	4 : 1
β-gal-1720	4 : 1

Table 3: Conjugates and estimated ratio of bound ODN to each molecule of protein

The established protocol differs from the original one in several aspects:

Instead of the crosslinker S-SMCC (sulfosuccinimidyl-4-N-maleimidomethyl) the crosslinker S-MBS which possesses essentially the same chemical qualities was used for conjugation.

For the removal of the reducing agent DTT as well as for the removal of unbound crosslinker by gel chromatography a different matrix was applicated to maximize recovery. Gel chromatography relies on molecular weight differences of the substances desired to separate. While the proteins OVA and β-galactosidase with a molecular weight of 45 kD and 116 kD, respectively, represent rather large molecules, Phleum p 5 (MW: 28 kD) and the ODN (MW: ~6.5kD) are medium-sized and therefore removal of free crosslinker (S-MBS: 416 D) or reducing agent (DTT: 154 D) requires a suitable gel matrix for efficient purification. Two different gel matrixes were tested. Figure 9 shows a typical elution profile of reduced sulfhydryl-

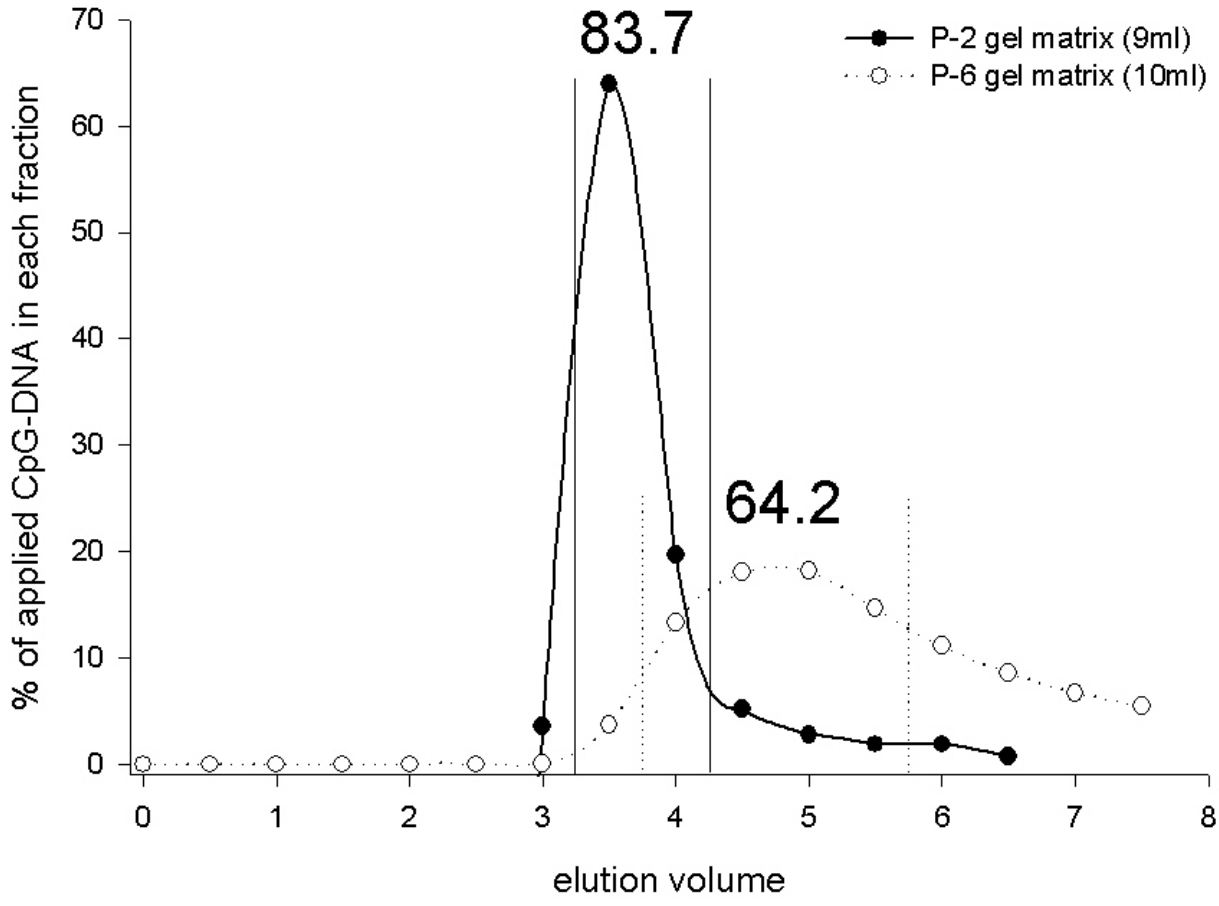


Figure 9: Elution of activated CpG-ODN over gel chromatography columns (determined by absorption of eluent at 280 nm): elution using P-2 gel matrix (column with 9 ml bed volume) yielded 83.7% of activated CpG-DNA in 1 ml, while by elution via P-6 gel matrix (10 ml bed volumne) only 64.2% in 2 ml could be recovered.

modified CpG-DNA (1668) on a self-prepared Biorad P-2 gel column with a separation range of 0.1 – 1.8 kD versus elution on a prepacked Biorad P-6 gel column (Econo-Pac 10DG Columns) with a separation range of 1 – 6 kD comparable to the G-25 column used by Cho et al. As can be seen in figure 9 purification of CpG-ODN over a P-2 gel chromatography column yields concentrated fractions with minimal dilutional effects. Usually over 80 % of ODN added on the column could be recovered in 1ml compared to about 65 % in 2 ml when using the P-6 gel matrix. Since separation from the reducing agent and – in the case of protein – from unbound crosslinker is still achieved, the P-2 gel matrix was used routinely. Of note, the elution of the pollen allergen Phleum behaves in similar ways.

Following purification over the P-2 gel chromatography columns only fractions with highest concentrations of CL-modified protein and reduced ODN were co-incubated at RT for 2.5 h. Longer incubation did not translate into more efficient crosslinking of ODN to protein. This may be due to hydrolysis and thus inactivation of the crosslinker. Considering this, the addition of cysteine to quench any remaining CL and to prevent formation of aggregated protein complexes at the end of the incubation period might be of no importance. Incubation of S-MBS modified protein and activated ODN at 4°C, though, led to precipitation of the protein showing the importance of temperature during the conjugational process.

After conjugation a purification step is required to remove free ODN. At the same time minimal dilution and loss of sample is desired. To achieve this, several methods were evaluated: While dialyzing with membranes (MW cutoff: 8 kD and 20 kD) and centrifugation with specialized filter units (Biomax-10, Millipore; MW cutoff: 10 kD) proved inefficient, only chromatography over a Sephadex G-75 superfine gel matrix (Amersham Biosciences, separation range: 3 kD – 70 kD) showed promising results in initial experiments. For this reason purification over a Superdex 75 HR column with the same separation range using FPLC was chosen. This method achieved complete removal of the CpG-DNA ODN 1668 (in monomeric or dimeric form) with minimal sample dilution (30 – 40 % of initial concentration) as can be seen in figure 10. Furthermore a 0.2 x diluted DPBS buffer was used for elution giving the possibility for future concentration of the sample without increasing salt content exceedingly.

Following this protocol generally about 40 % of the protein initially employed can be recovered conjugated to ODN.

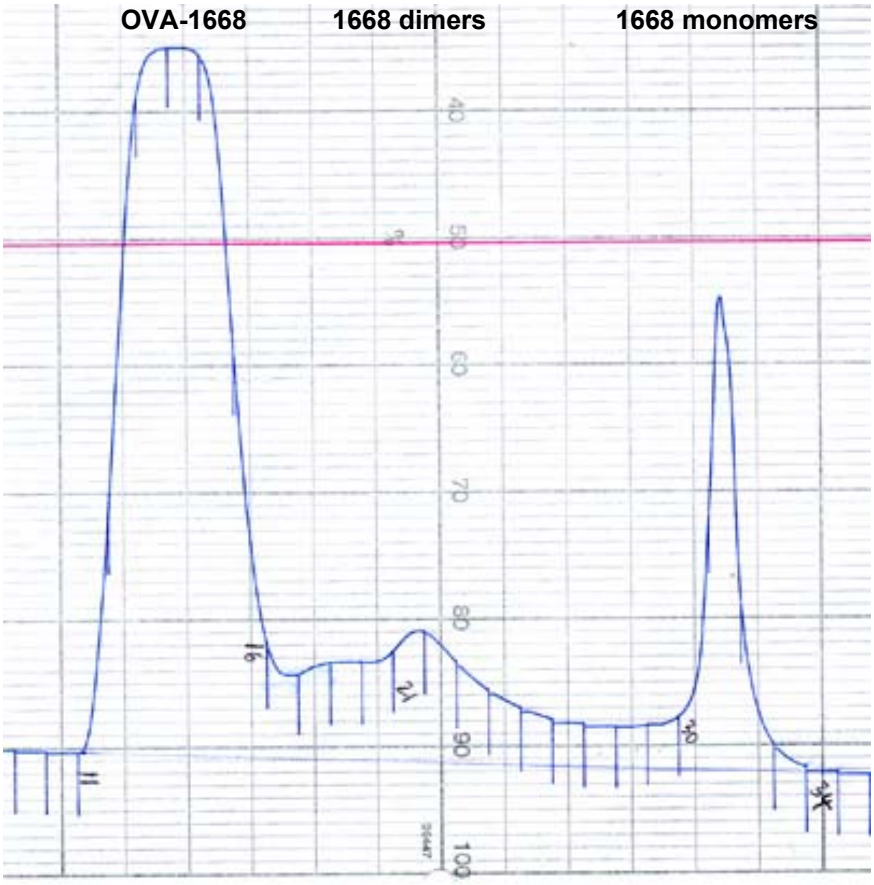


Figure 10: Efficient removal of unbound ODN from conjugates by FPLC: OVA-1668 conjugates (eluted in the first fractions) are completely separated from 1668 monomers as well as dimers (blue line; red line: reference)

3.2 Analysis of conjugates

Ratio of bound ODN on protein is determined after running a 6-20% SDS-PAGE and performing a silverstain. Figure 11 shows a typical SDS-PAGE silverstain obtained from an OVA-1668 conjugate.

OVA by itself shows up as a sharp band at around 45 kD. The fraction of OVA-CL used for conjugation can be visualized as broadening of the OVA band, sometimes even revealing two distinct bands. These two bands do not correspond to free OVA versus OVA with bound CL since both band disappear when ODN are added during the conjugation procedure. They might more likely be expression of SDS- and DTT-stable changes in some of the OVA introduced by the CL, which are resistant to denaturing and reducing conditions. After the incubation with the CpG-ODN 1668 a shifting of the OVA-CL band can be observed and bands corresponding to OVA-bound 1668 at different ratios are visible. These complexes are heat-stable (samples

are heated for 5 min at 95°C) as well as stable to reducing conditions (DTT) indicating that the crosslinker has formed a covalent bound between OVA and the ODN. Also dimers of OVA-1668 conjugate at around 120 kD and, to a much lesser extent, multimers can be observed. This formation of protein aggregates generally represents only a minor fraction of conjugated protein. When calculated in this case, an average of 2.5 CpG-ODN were conjugated per molecule OVA.

To prove that the shifting of bands observed on the SDS-PAGE which are equivalent to molecular weight changes of protein are due to protein-bound ODN and to show efficient removal of unbound ODN from conjugates non-denaturing, non-reducing PAGE were run, silverstained or visualized under UV-light after incubation in ethidium bromide. As can be seen in figure 11 the ethidium bromide stain selectively visualizes CpG-DNA but neither OVA by itself nor the crosslinker-modified OVA appear. In the silverstain, though, the running characteristics of OVA, OVA-CL and OVA-1668 conjugates can be studied. Binding of the CL to OVA leads to a completely different band pattern on the gel – another indication that all OVA is

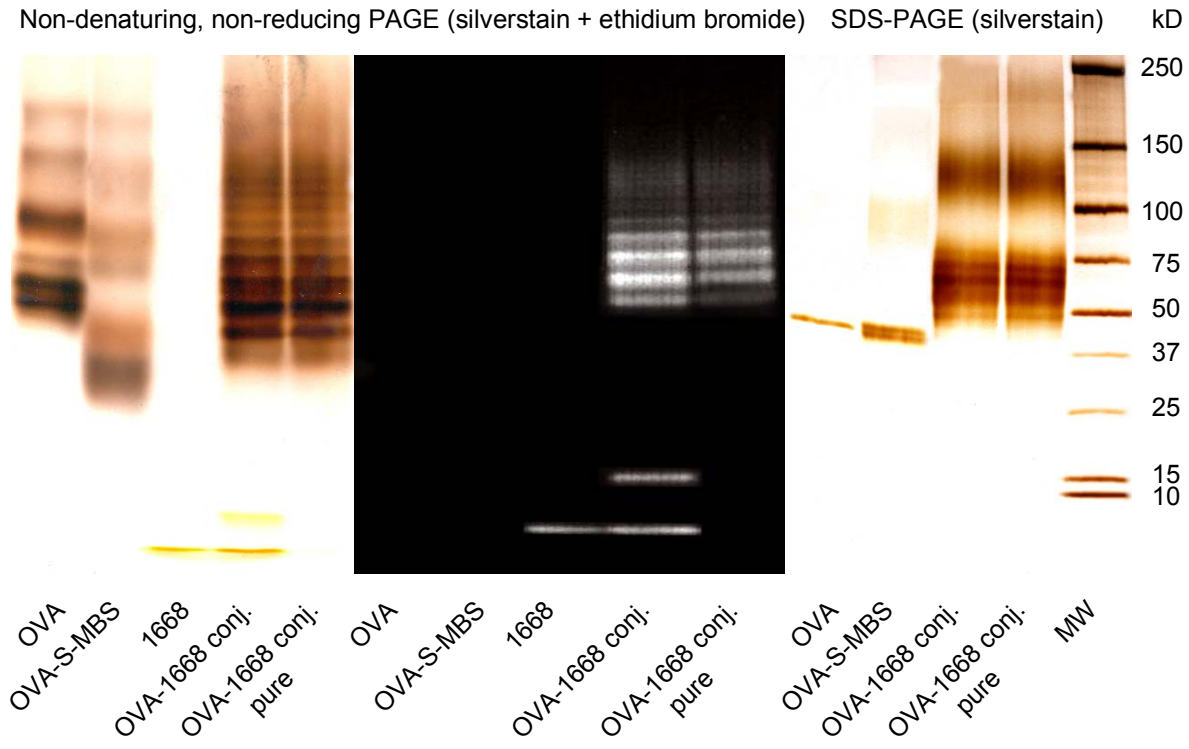


Figure 11: Gel analysis of OVA-1668 conjugates using non-denaturing, non-reducing 4-15% gradient PAGE (left and middle) or 6-20% gradient SDS-PAGE (right) which were consecutively silverstained (left and right) or visualized under UV-light after incubation with ethidium bromide (middle). Values of the weight marker (right lane in the SDS-PAGE) are given on the right of the SDS-PAGE.

modified by CL. Conjugation to CpG-DNA results again in a complete shifting of bands. Corresponding bands to this pattern can be observed in the ethidium bromide stain pointing to the fact that these OVA molecules indeed have bound CpG-DNA. After purification by FPLC the conjugates are free from unbound ODN monomers or dimers proven by the absence of bands below the OVA-bound CpG-DNA in the ethidium bromide stain.

Similar results by gel analysis could be obtained when analysing the other conjugates.

For determination of protein concentration a commercially available protein assay was used. Since both – CL-modified protein and protein-ODN conjugates –strongly interfere with the BCA and Bradford protein assay the modified Lowry method was applied. While the Coomassie dye (Bradford assay) primarily interacts with and binds to basic and aromatic side chains of proteins (e.g. residues of arginine, lysine, histidine) resulting in a shift in absorbance (Bradford, 1976), the BCA (bicinchoninic acid) protein assay (Smith et al., 1985) relies on the capacity of proteins to reduce Cu^{2+} to Cu^{+}. Subsequently Cu^{+} complexed with bicinchoninic acid leads to detectable colorimetric changes. It has been described (Wiechelman et al., 1988) that the protein structure, the number of peptide bonds and the amino acids cysteine, cystine, tryptophan and tyrosine can be hold responsible for the capacity of protein to reduce Cu. In the modified Lowry method, a reagent containing alkaline cupric sulfate reacts first in the presence of tartrate with peptide bonds of protein. This complex reduces a substrate (folin-ciocalteu phenol reagent) and the reaction can be monitored in an ELISA reader (Peterson, 1977). As mentioned above proteins modified with S-MBS as well as conjugates strongly interfere with the Bradford and the BCA method for protein quantification yielding higher results than the initially used amount of protein. The Lowry method on the contrary, seemed to measure protein content more accurately giving about the same results as if protein content of conjugates was calculated with a 30 – 40 % loss. Therefore the Lowry method was used to measure protein content of conjugates.

Fluorescence activity of FITC labelled OVA conjugates was compared to unconjugated FITC labelled OVA by measurement in a fluorescence reader. In general fluorescence activity of conjugates paralleled their estimated protein amount, so protein amount instead of arbitrary fluorescence units of OVA-FITC is given in the following sections.

3.3 Conjugated CpG-DNA upregulates costimulatory molecule expression

First we addressed the question whether CpG-DNA still possesses immunostimulatory potency after linking to protein. For this, the ability of OVA-conjugated CpG-DNA to upregulate co-stimulatory molecule expression was examined.

After incubation with OVA conjugated to stimulatory CpG-DNA, a mixture of OVA plus CpG-DNA or OVA alone for 24 h, BMDC were stained with CD11c mAb, CD86 mAb and CD40 mAb (figure 12). It was found that covalently linked CpG-DNA triggered upregulation of CD40 and CD86, co-stimulatory molecules necessary for priming CTL responses, almost as efficiently as unconjugated CpG-DNA in $CD11c^+$ BMDC.

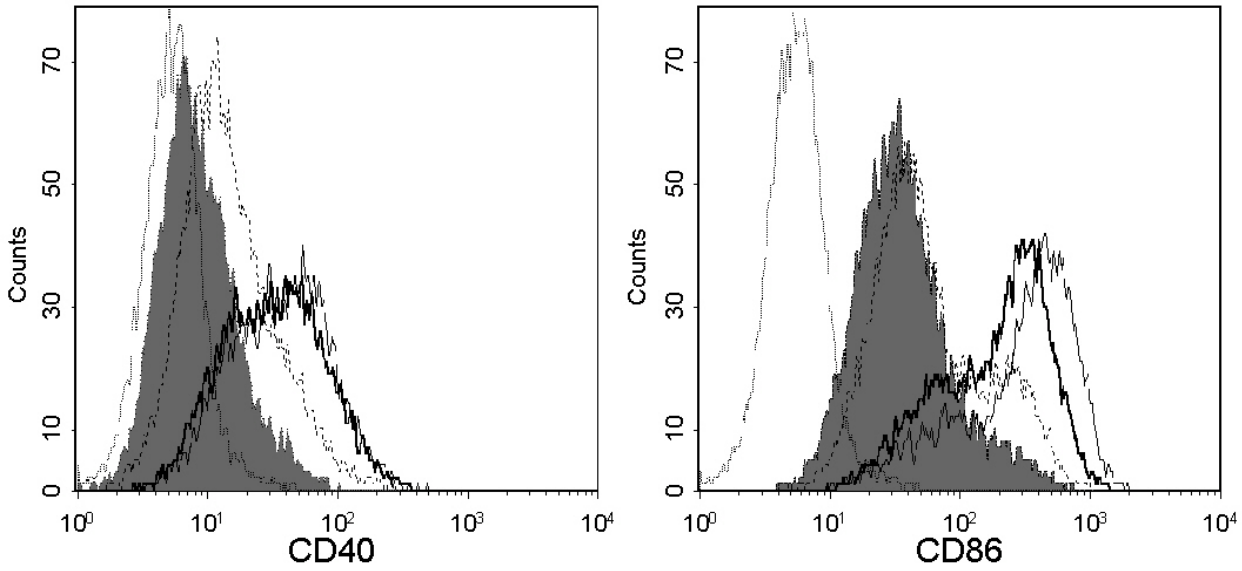


Figure 12: Conjugated CpG-DNA is still able to activate BMDC: Flt3-Ligand cultured DC were incubated for 16 h with 17.6 µg/ml OVA alone, mixed or conjugated with 1 µM CpG-DNA (1668). Cells were washed and stained for CD11c, CD40 and CD86 expression. Only live and $CD11c^+$ cells are shown. Thick line: OVA-1668 conjugate; thin line: OVA + 1668; broken line: OVA; shadowed area: no stimulus; dotted line: isotype control. A representative experiment is shown.

3.4 Covalently linked CpG-DNA evokes only moderate cytokine production

Next we analysed the ability of CpG-OVA conjugate to induce Th1-associated inflammatory cytokines *in vitro* and *in vivo*.

In vitro protein-linked CpG-DNA obviously loses stimulatory potency. BMDC incubated for 6 h with conjugated stimulatory 1668 produced significantly less TNFα than free 1668 ODN. Only when used in high concentrations (µM range) CpG-DNA conjugates provoke BMDC to release TNFα into the supernate (figure 13a). For IL-6 the same effect can be observed: only in high concentrations conjugates induce IL-6 secretion into the media in a CpG-specific manner while free CpG-DNA proved to be a lot more potent (figure 13b).

These results could also be confirmed in an *in vivo* setting. 2 h after i.p. injection mice challenged with 1668 conjugates showed only moderately elevated levels of TNFα and IL-12 compared to mice treated only with OVA alone (table 4). The mixture of stimulatory DNA and OVA, however, increased the systemic levels of these cytokines dramatically.

Thus, we concluded that stimulatory CpG-DNA loses stimulatory potency and evokes only moderate cytokine production after its conjugation to protein.

Antigen used for immunization	IL-12 [pg/mL]	TNFα [pg/mL]
OVA	8.1 $\pm$ 1	13.7 $\pm$ 2.1
OVA + 1668	1443 $\pm$ 37 ***[1]	233 $\pm$ 48 **[2]
OVA-1668 conjugate	48.6 $\pm$ 6	48.4 $\pm$ 30.6

Table 4: CpG-DNA conjugates cause only weak induction of proinflammatory cytokines *in vivo*: 3 mice per group were injected i.p. with 5nmol/mouse 1668 CpG-ODN either mixed or conjugated with 107.5µg OVA. One mouse injected with 107.5µg OVA served as control. 2 h later serum of mice was collected. Serum concentrations of IL-12 and TNFα were determined in triplicates by ELISA. Mean, standard deviation as well as the results of the unpaired t-test comparing the cytokine induction by CpG-DNA in free versus conjugated form are shown. 1: p<0,0003, 2: p<0,006.

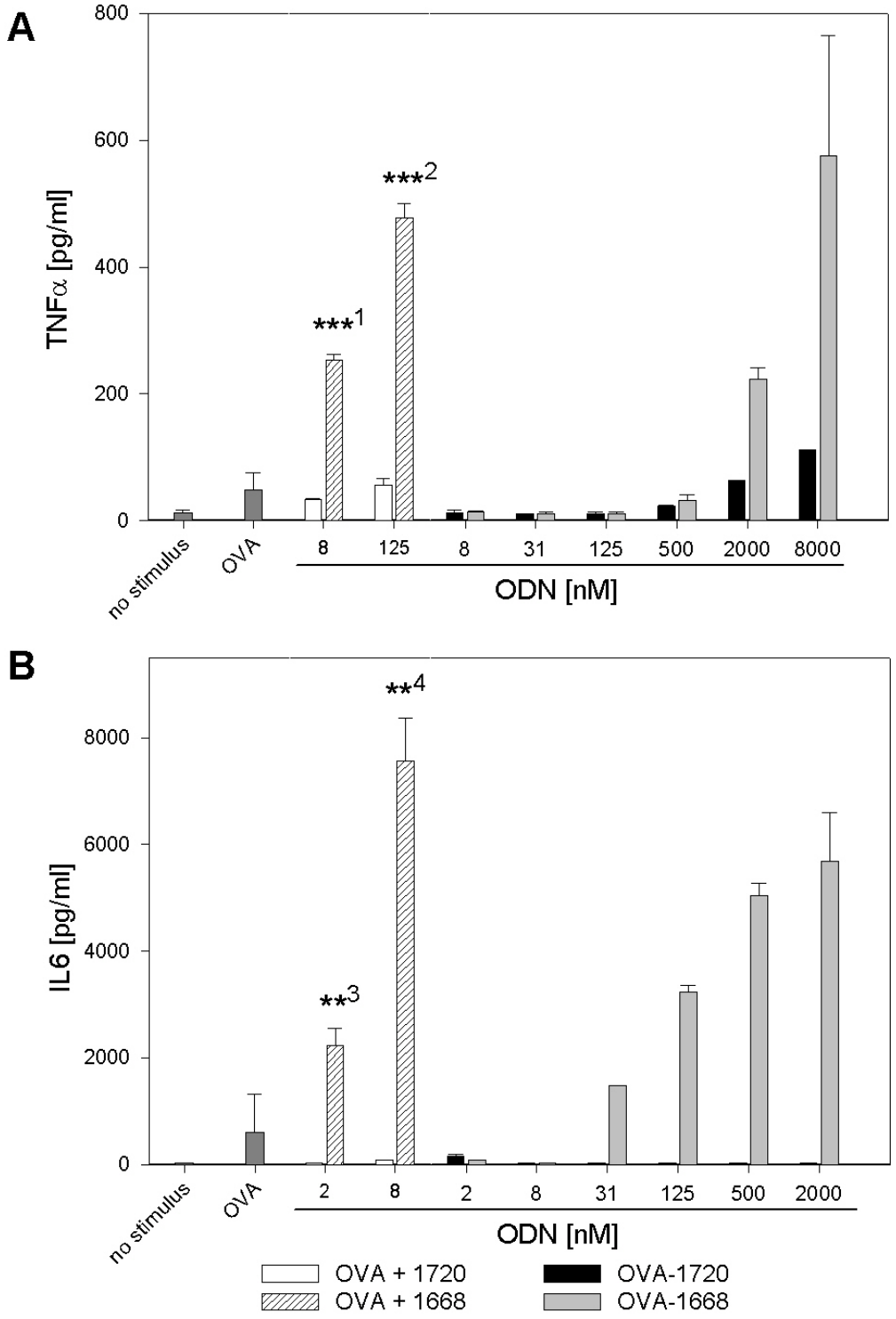


Figure 13: CpG-DNA conjugates cause only weak induction of proinflammatory cytokines *in vitro*: BMDC were incubated with indicated substances and media concentration of TNFα after 6 h (A) and IL-6 after 18 h (B) were determined by ELISA in tripilicates. A representative experiment is shown. Mean and standard deviation as well as results of the unpaired t-test comparing the cytokine induction by CpG-DNA in free and conjugated form at the same concentration are shown. 1: p<0,0006, 2: p<0,0009, 3: p<0,008, 4: p<0,004.

3.5 CpG-DNA conjugates enhance induction of antigen-specific CTL *in vivo*

A hallmark of a Th1 based immune response is the generation of CTL. Therefore we investigated the capacity of CpG-DNA conjugates to induce antigen-specific CTL *in vivo*.

Usually 300 µg of OVA and 10 nmol stimulatory CpG-DNA (1668) are used for priming OVA-specific CTL in draining LN of mice after injection into footpads (Sparwasser et al., 2000). Therefore co-injected OVA and CpG-DNA was titrated and amount still eliciting significant CTL responses was determined. Thereafter groups of

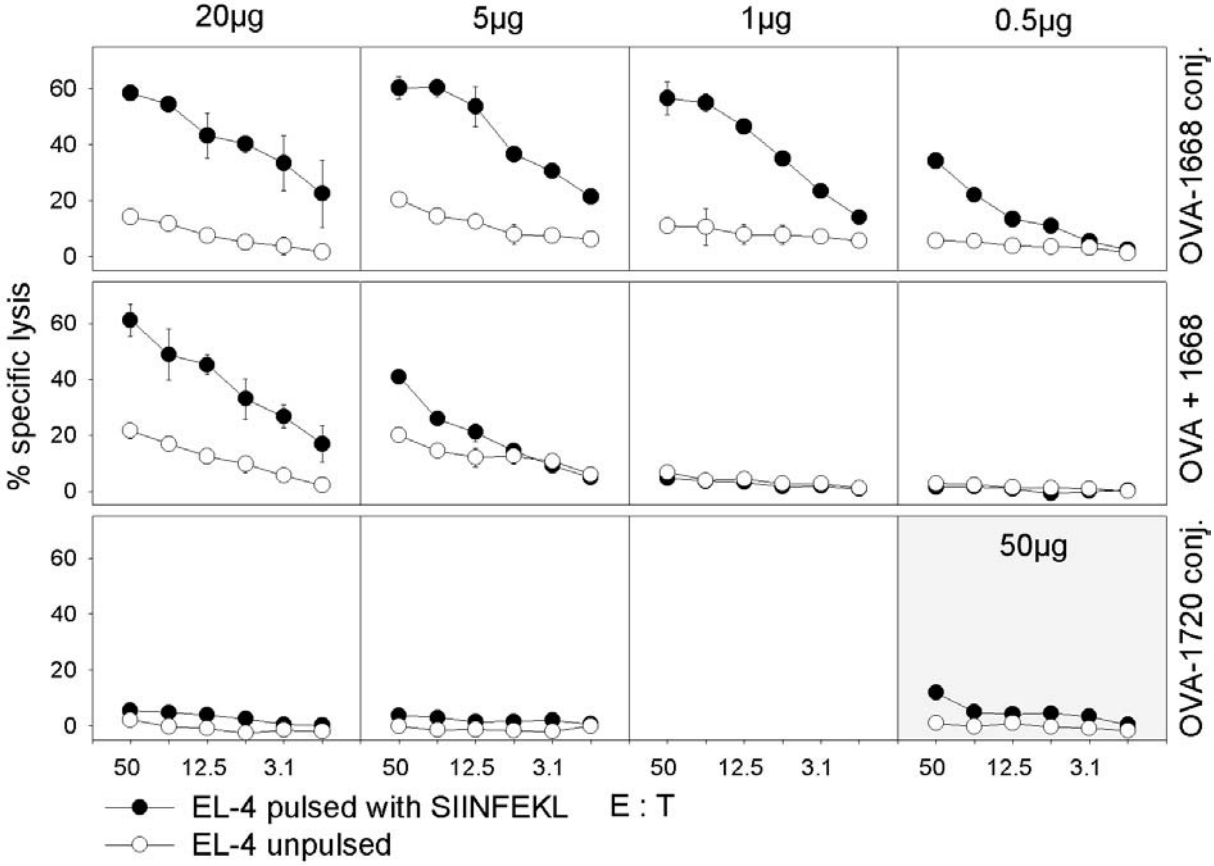


Figure 14: Enhanced immunogenicity of OVA-CpG conjugates: C57BL/6 mice were challenged into hind footpads with OVA mixed or conjugated with the CpG-DNA 1668 with protein amounts ranging from 20 to 0.5 µg (equaling 1120 to 28 pmol ODN) per mouse. After 4 days draining LN were harvested and cultured for additional 4 days before a chromium release assay (in triplicates) was performed. Cultured LN cells from mice immunized with OVA only (not shown) did not elicit CTL responses. Mean and standard deviation are shown. Solid symbols: EL-4 target cells pulsed with the OVA peptide SIINFEKL; open symbols: unpulsed EL-4 cells; E : T: ratio of effector cells (cultured LN cells) to target cells (EL-4 cells). A representative experiment is shown.

mice were challenged subcutaneously in the range determined in the first set of experiments with graded quantities of either OVA plus CpG-DNA in mixture or with CpG-DNA-OVA conjugates, respectively. Typically, as shown in figure 14, the amount of antigen injected could be as low as 0.5 µg provided it was conjugated to stimulatory CpG-DNA, whereas at least 5 µg of unconjugated OVA and CpG-DNA was necessary to evoke primary CTL responses. Thus CpG-DNA-OVA conjugates seem to be 10-fold more immunogenic compared to a mixture of OVA plus CpG-DNA. OVA mixed or conjugated to non-stimulatory GpC-DNA (1720) failed to induce significant CTL responses even at high antigen concentrations (50 µg protein equaling 2.8 nmol ODN), emphasizing the importance of the CpG-motif for immune stimulation.

3.6 Augmented sampling of conjugated protein by APC

APC are specially equipped cells which sample and process antigen and present derived peptides e.g. to $CD8^+$ T cells, thus enabling expansion of antigen-specific CTL clones. Since conjugates – compared to a mixture – demonstrate an increased efficacy to induce specific CTL although cytokine production was decreased, we

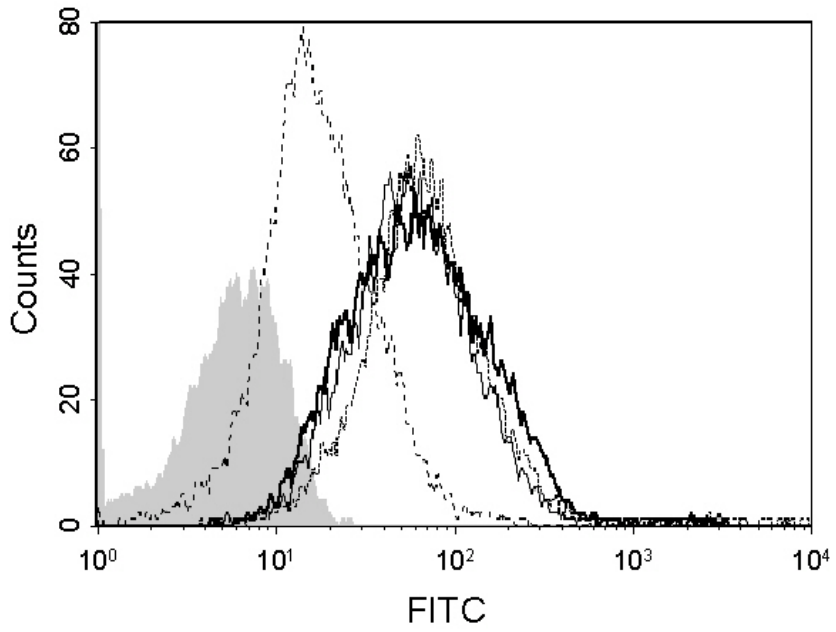


Figure 15: CpG-independent uptake of fluorescent conjugates in macrophages: ANA-1 cells were incubated with for 1 h with no stimulus, 5 µg/ml OVA-FITC or OVA-FITC conjugate, fixed and subsequently analysed by FACS. Thick line: OVA-FITC-1668; thinn line: OVA-FITC-1720; dotted line: OVA-FITC-1668pG; broken line: OVA only; shadowed area: untreated cells. A representative experiment is shown.

were interested, if APC show improved uptake of conjugated antigen. To achieve this, we examined the uptake of FITC-labelled OVA by FACS into cell lines or BMDC generated with human Flt3-ligand or GM-CSF.

3.6.1 Enhanced CpG-sequence independent uptake of conjugate

As can be seen in figure 15 the macrophage cell line ANA-1 consecutively samples FITC-labelled OVA resulting in a rightward shift of the whole population in the FACS analysis. This uptake is increased about 4 fold (as calculated by mean fluorescence intensity of the FITC signal) when conjugated to ODN. Since both – CpG-DNA or

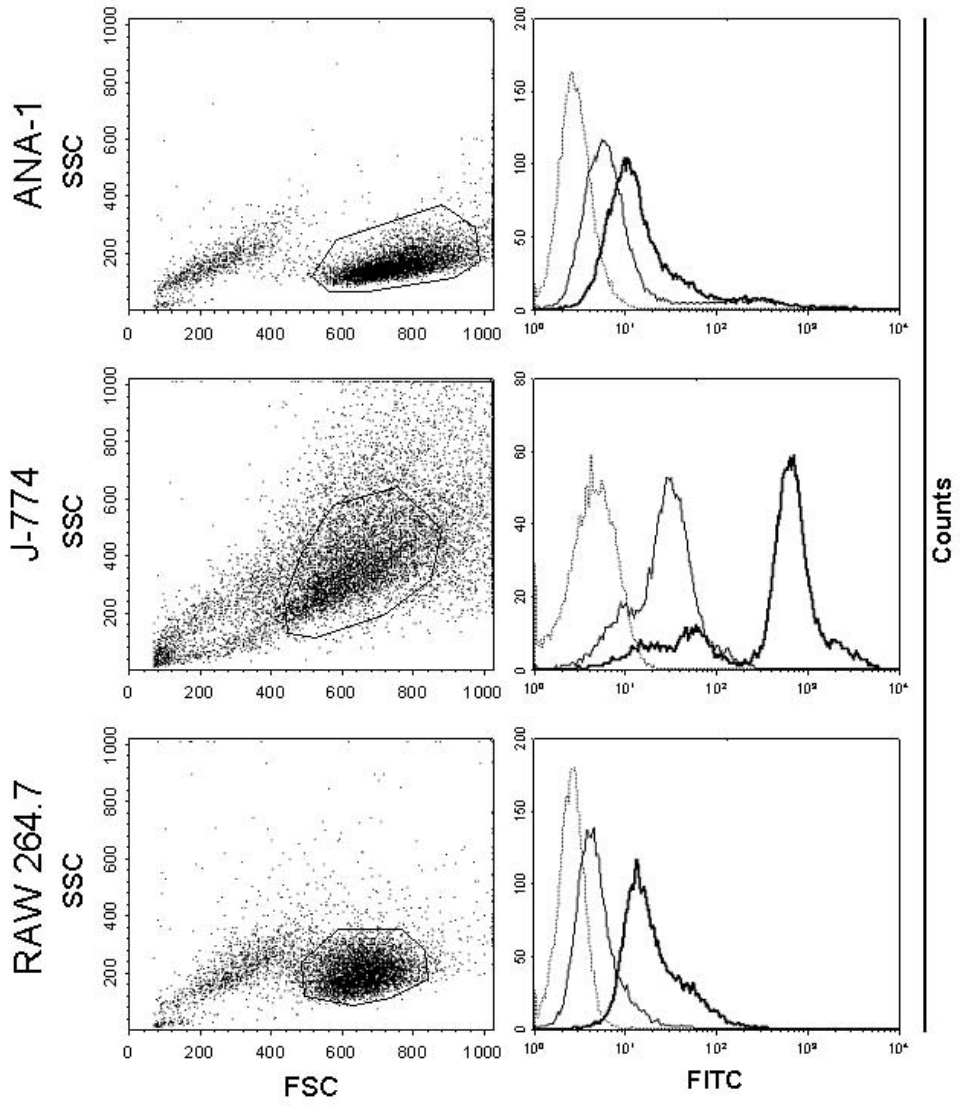


Figure 16: Uptake of fluorescent conjugates in ANA-1, J-774 and RAW 264.7 macrophages: cells were incubated for 45 min with no stimulus (dotted lines), 4 µg/ml OVA-FITC alone (thin lines) or conjugated to CpG-DNA (thick lines). Subsequently cells were fixed and analysed by FACS. Only gated cells (as shown in the forward/sideward scatter) were used for analysis. A representative experiment is shown.

non-stimulatory GpC-control conjugated FITC-labelled OVA – equally well enhance uptake of fluorescent protein, we concluded that uptake is CpG-sequence independent. 1668 modified with a poly-guanosine tail, even if conjugated at a lower ratio compared to CpG- and control-ODN conjugates, also increases fluorescence signal to a comparable level.

These observations can be made in different cell lines derived from C57BL-6 as well as Balb/c mice indicating that those findings are not mouse strain specific – although quantitative differences in uptake can be noted in different cell lines (figure 16). While the C57BL-6 derived macrophage cell line ANA-1 and RAW 264.7 macrophage cells from Balb/c mice show moderate levels of uptake (also of FITC-labelled OVA not conjugated to ODN), J774A.1 macrophages cultured from Balb/c mice seem to be more potent.

To further elucidate the nature of the receptor involved we tried to compete the uptake of conjugated FITC-OVA by excess “third party” ODN. The results obtained with the macrophage cell line ANA-1 prove that free ODN dose-dependently inhibit uptake of fluorescent OVA conjugated to CpG-ODN in a CpG-sequence independent

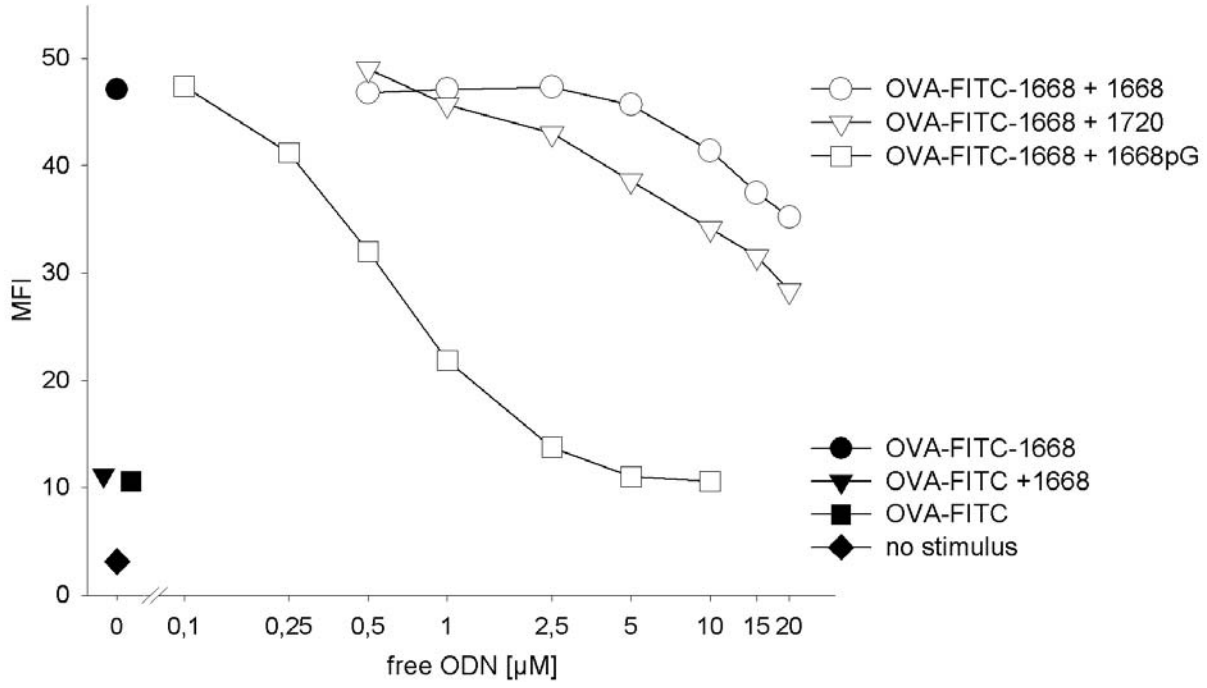


Figure 17: Free ODN dose dependently block uptake of OVA-FITC-1668 conjugate: ANA-1 cells were incubated for 1 h with 4 µg/ml OVA-FITC-1668 conjugate and increasing amounts of free CpG-ODN (1668), GpC-ODN (1720) or CpG-ODN modified with a poly-guanosine tail (1668pG) (open symbols). The solid symbols show data obtained from ANA-1 cells treated 4 µg/ml OVA-FITC alone, mixed or conjugated with CpG-DNA (180nM) or no stimulus. FITC staining of cells was analysed by FACS. Mean fluorescence intensity (MFI) of the FITC-signal is shown. A representative experiment is shown.

manner (figure 17). Of note, in these blocking experiments ODN modified with a poly-guanosine tail at the 3´ end inhibit uptake of fluorescent conjugate at much lower concentrations. This phenomenon can also be observed in cultures of BMDC.

3.6.2 Intracellular compartmentation of conjugate

To verify uptake into intracellular compartments and to exclude mere cell surface adherence of conjugates ANA-1 were incubated with fluorescent OVA conjugated or mixed with ODN for different time periods and subsequently fixed either in 1% PFA solution or in 1% PFA solution supplemented with trypanblue (figure 18). Trypanblue quenches FITC cell surface staining not affecting FITC signals from within the cell

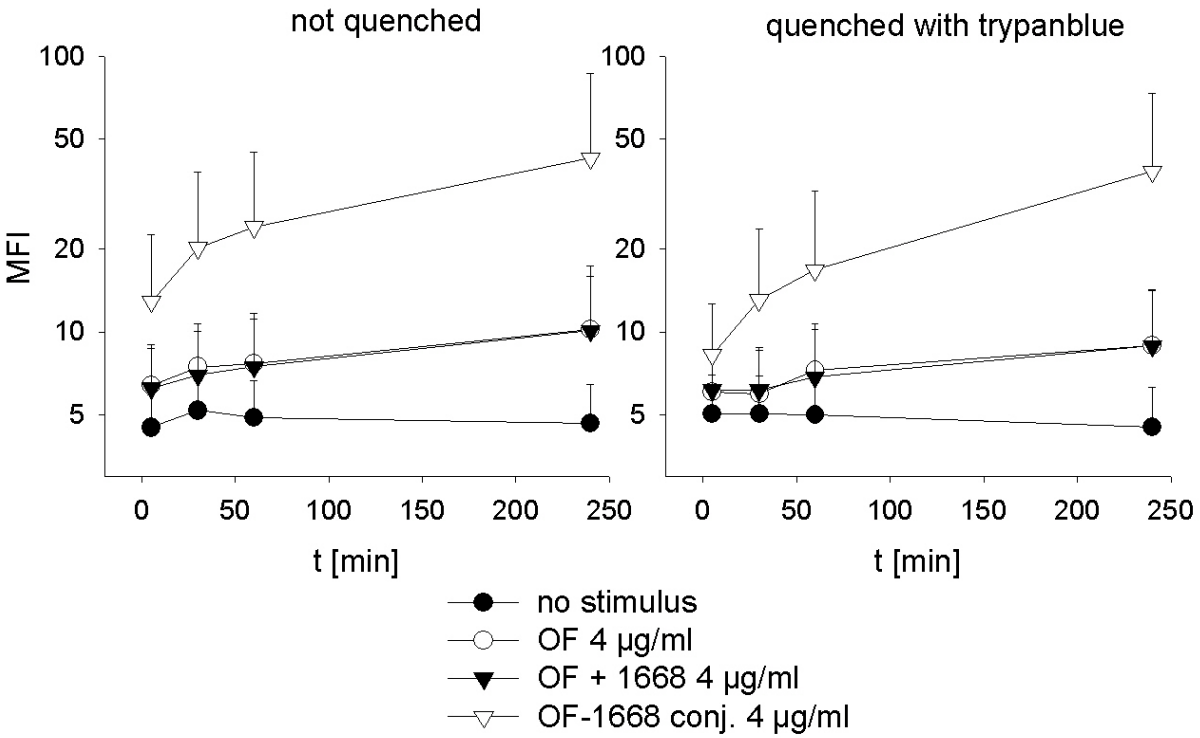
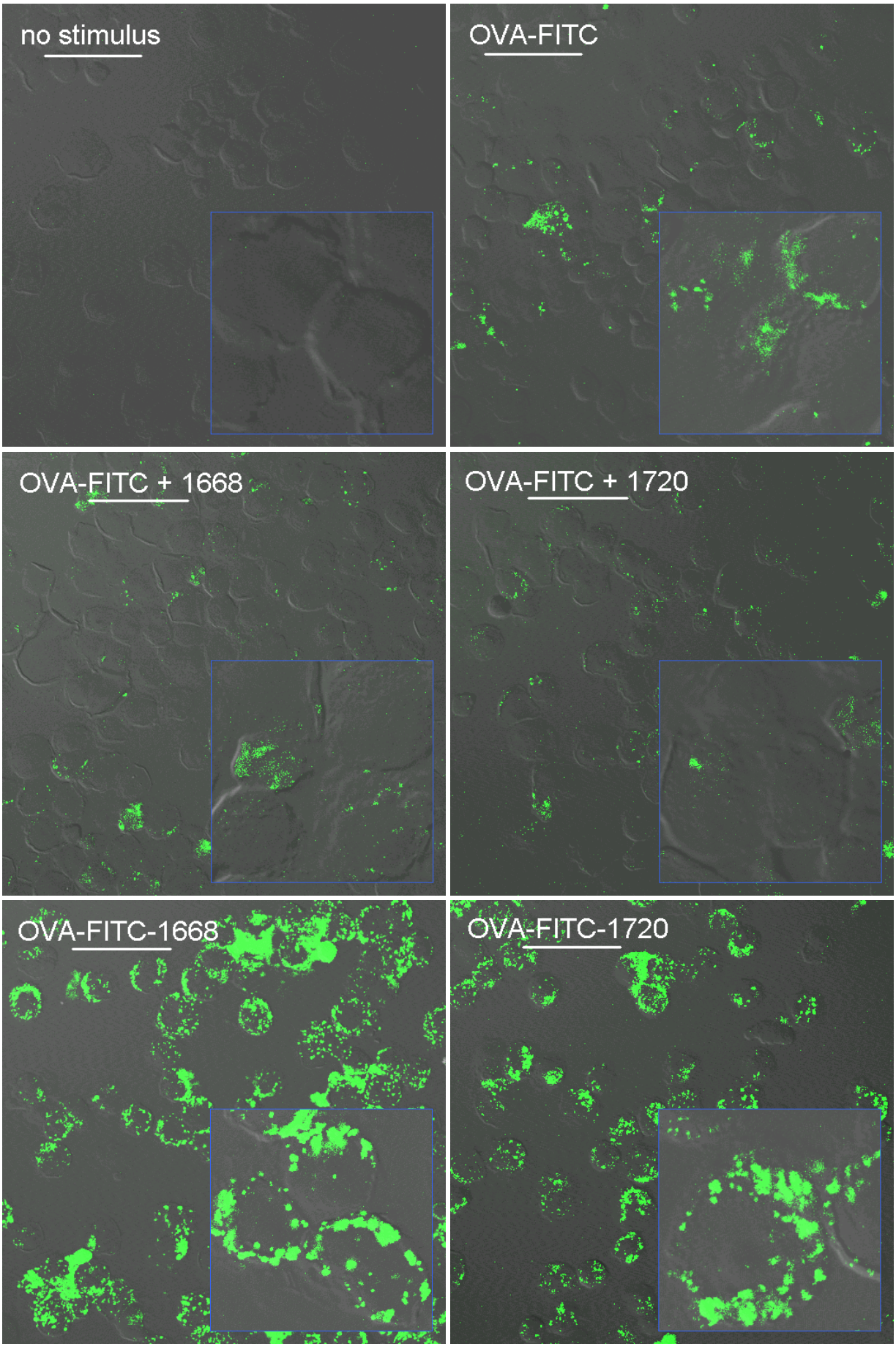


Figure 18: Intracellular compartmentation of OVA-FITC conjugates: ANA-1 cells were incubated for different time periods with no stimulus, 4 µg/ml OVA-FITC alone, mixed or conjugated to CpG-DNA, fixed in 1% PFA with or without the addition of trypanblue and analysed by FACS. Mean fluorescence intensity (MFI) of the FITC-signal and standard deviation are shown. A representative experiment is shown.

Figure 19 (next page): Intracellular localization of fluorescent conjugates: J-774 cells were incubated for 2 h with no stimulus, 4 µg/ml OVA-FITC mixed or conjugated to 0.18 µM ODN. Cells were washed, fixed, cytospun on slides and examined under a confocal microscope. White bar: 50 µm. Magnification in lower right insert. A representative experiment is shown.


no stimulus
OVA-FITC
OVA-FITC + 1668
OVA-FITC + 1720
OVA-FITC-1668
OVA-FITC-1720

(Hed et al., 1987). Since the mean fluorescence signal intensity of cells incubated with fluorescent conjugates is reduced only to a minor degree when fixed with trypanblue, these findings indicate that most of the conjugates are localized intracellularly while the portion of FITC signal quenched by treatment with trypanblue corresponds to FITC-labelled conjugates adhering to the cell surface.

To reaffirm intracellular localization of labelled conjugates J774A.1 cells, macrophages derived from Balb/c mice, were examined under a confocal microscope after treatment with labelled OVA in mixed or conjugated form. Cells incubated with ODN-conjugated fluorescent protein show an increase in fluorescence compared to cells receiving the mixture (figure 19). Furthermore, the FITC signal in conjugate treated cells mainly originates from vesicular structures within the cell. Thus trypanblue quenching and examination of cells using confocal microscopy reveal intracellular compartmentation and therefore intracellular uptake of FITC-labelled DNA-conjugates.

3.6.3 Quantification of increased uptake of ODN-linked antigen

To quantitate by how much protein uptake can be increased when linked to ODN, Flt3-ligand cultured BMDC from C57BL-6 mice were exposed to OVA-FITC mixed or conjugated to the CpG-ODN 1668 in various concentrations (figure 20 A). Typically more than 90 % of gated cells from Flt3-ligand cultured BMDC express the surface antigen CD11c – a typical feature of dendritic cells (DC). While incubation with either OVA-FITC (0.5 µg/ml) or OVA-FITC (0.5 µg/ml) plus CpG-DNA resulted in about 5 - 7% FITC positive DC, FITC-labelled OVA-CpG-ODN conjugates (0.5 µg/ml) were taken up by approximately 32% of the DC. When incubated with graded dilutions of CpG-OVA-FITC conjugates it was found that uptake of ODN-conjugated OVA-FITC equaled that of OVA-FITC at about 20-fold lower concentration. Similar results could be obtained from GMCSF-cultured BMDC (data not shown).

3.6.4 TLR9-independent sampling of ODN-tagged antigen

Next we wanted to determine the role of TLR9, the pattern recognition receptor responsible for CpG-DNA mediated activation of APC (Hemmi et al., 2000), in the DNA-driven uptake of conjugates. At the beginning of this thesis the exact localization of TLR9 had not been elucidated and it had been speculated that TLR9, like other TLR, is expressed on the cell surface and might there already interact with

CpG-ODN. To address this issue, Flt3-ligand cultured BMDC from TLR9 -/- mice were treated in the same way as described above (figure 20 B). Exposure to fluorescence-labelled OVA (0.5 µg/ml) with or without CpG-DNA yielded about 4 – 6% positive DC. The percentage of positive DC increases to about 30%, if FITC-

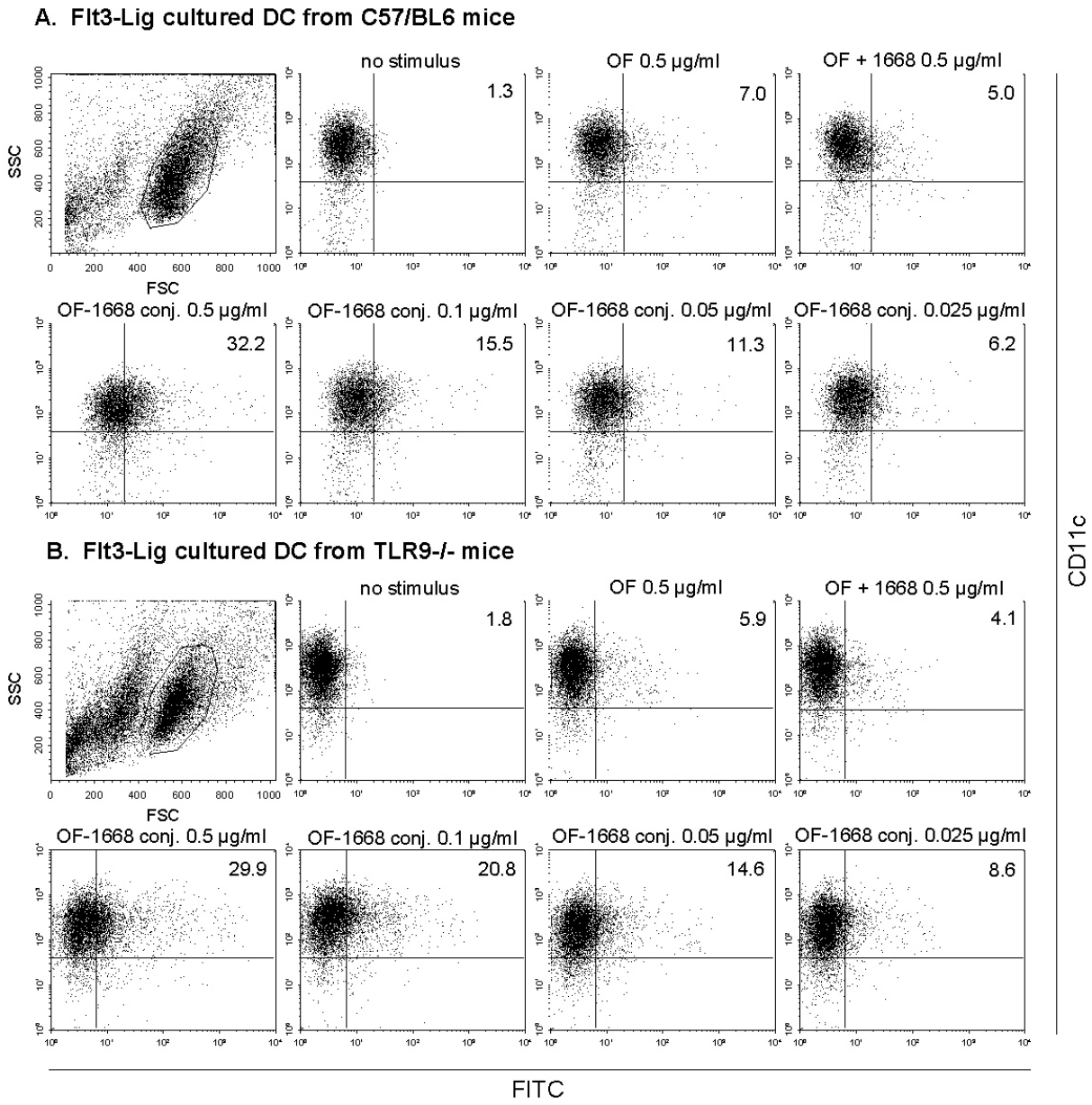


Figure 20: Uptake of OVA-FITC in BMDC is about 20 fold increased when conjugated to CpG-DNA in a TLR 9 independent manner: Flt3-Ligand cultured BMDC from C57BL/6 (A) or TLR9 -/- mice (B) were incubated for 1.5 h either with no stimulus, 0.5 µg/ml OVA-FITC alone, mixed or conjugated with 1668 or with dilutions of OVA-FITC conjugate. Cells were stained for CD11c expression and subsequently analysed by FACS. Only gated cells (as shown in the forward/sideward scatter) were used for analysis. Percentage of FITC-positive, antigen bearing $CD11c^+$ DC is shown in each right upper quadrant. A representative experiment is shown.

labelled OVA is presented in CpG-DNA conjugated form. Again, by titration of conjugate an at least 20-fold increase in uptake can be noted. These findings point to a TLR9-independent enhancement of DNA aided endocytosis.

We thus concluded that CpG-DNA, when conjugated to OVA, aids endocytosis of OVA, by routing the CpG-OVA complex to the receptor mediated endocytosis pathway, that internalizes DNA in a sequence non-specific way.

3.7 Increased uptake translates into enhanced MHC class I presentation

Naïve $CD8^+$ T cells require not only activation stimuli mediated by CD40-CD40L, CD80 and CD86 (signal 2) and cytokines (signal 3), but also presentation of the their

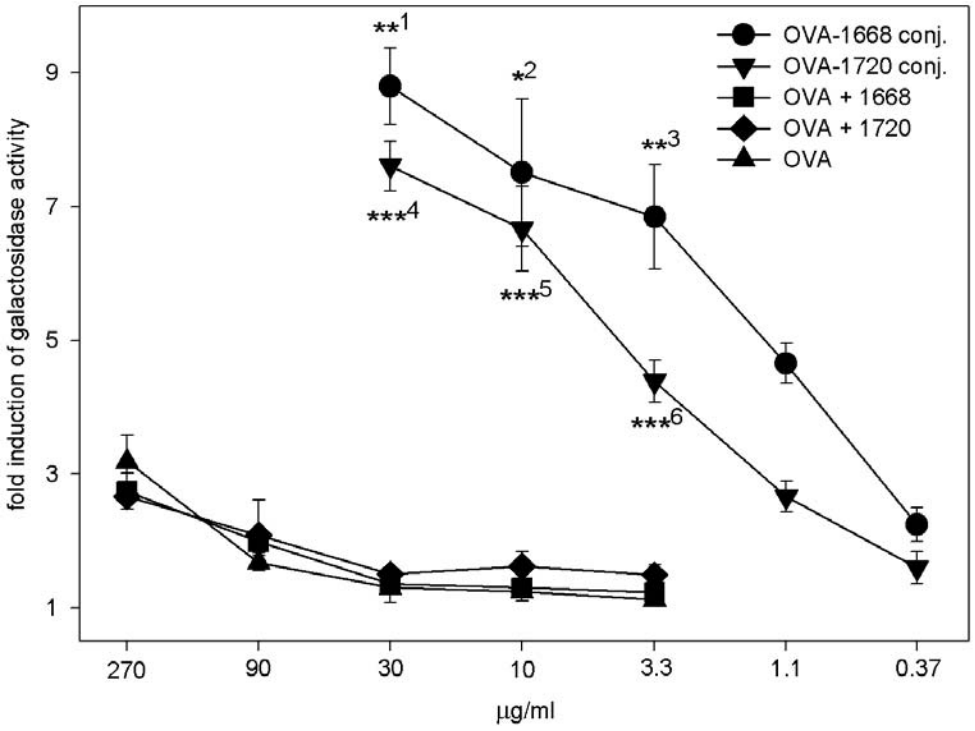


Figure 21: Sequence non-specific enhancement of SIINFEKL presentation by OVA-DNA conjugates: DC from Flt3-Ligand conditioned bone marrow cell cultures were incubated in triplicates with OVA alone, mixed or conjugated with either the CpG-DNA 1668 or the GpC-DNA 1720 for 5 h. Cells were washed and incubated overnight with B3Z cells and induced β-galactosidase activity was measured. Amount of protein (µg/ml) used is indicated. A representative experiment is shown. Fold induction of β-galactosidase activity (mean and standard deviation) compared to DC incubated with no stimulus is shown. Results of the unpaired t-test comparing the presentation of SIINFEKL in conjugate or with mixed substances treated B3Z cells at same concentrations are shown. 1: $p<0{,}003$, 2: $p<0{,}015$, 3: $p<0{,}007$, 4: $p<0{,}00002$, 5: $p<0{,}0003$, 6: $p<0{,}0002$.

specific epitope (signal 1) before expansion of antigen-specific CTL clones occurs. Therefore we were interested if the enhanced DNA mediated uptake of conjugates into APC also parallels with increased presentation of antigen-specific epitopes on MHC class I.

We thus examined presentation of the K^b-binding OVA peptide 257-264 (SIINFEKL) using B3Z, a lacZ-transfected SIINFEKL-specific CTL clone. Recognition of MHC I complexed OVA peptide leads to transcriptional activation of the lacZ gene resulting in production of the enzyme β-galactosidase. Enzyme activity correlates with amount of β-galactosidase synthesized by transcription of the lacZ gene and hence with peptide presentation. As shown in figure 21, both – incubation of BMDC with stimulatory as well as non-stimulatory DNA-OVA conjugates – lead to greatly enhanced presentation of SIINFEKL on MHC I compared to only poor presentation if uncoupled OVA or mixtures of OVA with DNA-ODN are used.

4 Discussion

4.1 Immunological qualities of CpG-DNA conjugates

CpG-DNA induces a Th1-based immune activation in mammals or vertebrate cell subsets. CpG-DNA linked to antigen evokes immunological responses that differ mainly in magnitude from responses brought about by a mere mixture of both, CpG-DNA and antigen. In this thesis several aspects of conjugate-driven immunological reactions and their underlying mechanisms are examined.

Effects on expression of co-stimulatory molecules

Upon recognition of free CpG-DNA APC upregulate co-stimulatory molecules like CD40, CD54, CD80 and CD86 and migrate to lymphoid organs, such as spleen and LN, where they liaise with and activate antigen-specific T cells (Banchereau and Steinman, 1998), (Hartmann et al., 1999), (Bauer et al., 1999).

Similarly, we and others found that covalently linked CpG-DNA trigger upregulation of co-stimulatory molecules like CD40, CD86 and MHC class II on the surface of $CD11c^+$ BMDC and even B cells – in a compareable manner to unbound immunostimulatory DNA-ODN (Shirota et al., 2001), (Maurer et al., 2002), (Shirota et al., 2002), (Heit et al., 2004). In TLR9 -/- mice, however, this CpG-DNA mediated upreagulation cannot be observed (Heit et al., 2003). Thus, conjugation of CpG-DNA to protein does not impair the essential interaction with TLR9 leading to sufficient stimulation of DC to upregulate their repertoire of co-stimulatory molecules.

Effects on cytokine production

Cytokines play a key role in regulating and mediating immune defence activation (Medzhitov and Janeway, Jr., 2000b). CpG-DNA ODN lead to induction of proinflammatory cytokines like TNFα, IL-1, IL-6, IL-12 and type I interferons which are associated with Th1-driven immune settings (Stacey et al., 1996), (Sparwasser et al., 1998), (Bauer et al., 2001), while Th2-like cytokines (e.g. IL-4, IL-5) are suppressed (Chu et al., 1997), (Lipford et al., 2000b).

CpG-DNA conjugates display basically the same qualities: upon stimulation with OVA-linked CpG-DNA we observed induction of IL-6, IL-12 and TNFα *in vitro* as well as *in vivo*. In our studies, though, stimulatory conjugates displayed a diminished capacity to evoke proinflammatory cytokines compared to free CpG-ODN (Maurer et al., 2002). Modifications in the conjugation protocol, especially using a different crosslinking agent, could eventually account for these phenomenons. Non-CpG-DNA conjugates did not ellicit significant levels of these cytokines.

In slight contrast to our findings stimulation of Th1- and Th2-enriched fractions of spleen cells from anti-OVA T cell receptor transgenic mice with OVA-conjugated CpG-DNA both produce high amounts of IFNγ – while mixtures of OVA with CpG-DNA do not lead to manifest levels of IFNγ (Shirota et al., 2001). Restimulation of spleen cells from mice immunized with β-gal or gp120 CpG-DNA conjugates enhances IFNγ generation compared to immunization regimes using unconjugated substances (Tighe et al., 2000a), (Horner et al., 2001a). DC, but also B cells, react towards CpG-DNA conjugates with increased synthesis and exocytosis of IL-12 (Shirota et al., 2001), (Shirota et al., 2002).

Thus, further research in this particular issue is necessary to elucidate the exact behaviour of CpG-DNA conjugates on cytokine induction. Interestingly, in some experiments also non-CpG-ODN conjugates show immunoenhancing characteristics without particular skewing toward Th1 responses (Sano et al., 2003).

Effects on humoral immunity

CpG-DNA conjugates also proved to be powerful stimulants to B cells leading to increased production of Th1-associated while decreasing Th2-typical antibody subsets.

Utilizing stimulatory OVA and Phleum conjugates in previously sensitized mice we observed a rise of specific IgG_{2a} and a reduction of antigen-specific and total IgE compared to treatment with allergen in mixture with CpG-DNA or alum (Ollert, unpublished data).

Consistently, challenge with the ragweed allergen Amba1 chemically linked to CpG-ODN induces a significantly stronger IgG_{2a} response than Amba1 and CpG-DNA co-immunization, and significantly weaker IgG_1 and IgE responses than Amba1/alum in mice, rabbits, and monkeys revealing its Th1-inducing capacity (Horner et al., 2001b), (Santeliz et al., 2002), (Tighe et al., 2000b). β-gal linked to

immunostimulatory DNA was shown to induce high levels of IgG_{2a} antibodies while lowering the induction of IgG_1. Gp120-CpG-DNA conjugates acted similarly and sera from immunized mice was able to neutralize HIV *in vitro* (Tighe et al., 2000a), (Horner et al., 2001a). Thus, CpG-DNA antigen conjugates potently induce a Th1-biased humoral immunity.

Effects on cellular immunity

The generation of antigen-specific CTL resembles a prominent feature of a Th1 immune response. Differentiation and activation of naïve CD8-positive T cells is mediated by the interaction of their antigen-specific T-cell receptor with MHC class I presented peptides on the cell surface of APC. Furthermore cytokines and signalling through the co-stimulatory molecule CD40 are prerequisites. Interaction with CD40 upreagulated on mature APC can replace the requirement of CD40 bearing Th cells for CTL activation (Bennett et al., 1998), (Schuurhuis et al., 2000), (Vabulas et al., 2000).

Here we demonstrate that antigen-CpG-DNA conjugates are superior to uncoupled antigen and CpG-DNA based vaccines in inducing CTL activity. In our experiments OVA-CpG-DNA conjugates proved to be about 10-fold more immunogenic compared to a mixture of OVA plus CpG-DNA in primary CTL assays (Maurer et al., 2002).

These findings are consistent with results others obtained. For example the highly immunogenic protein β-gal induced high levels of CTL in re-stimulation experiments not mattering if conjugated or mixed with CpG for vaccination. However, conjugates of gp120 – a less immunogenic antigen derived from the human immunodeficiency virus – induced significant levels of CTLs in contrast to mixtures (Tighe et al., 2000a), (Horner et al., 2001a). It also has been demonstrated that activation of CTL by protein linked to CpG-DNA is independent of T cell help (Cho et al., 2000).

Effects in models of allergy

Allergic diseases result from pathological Th2-driven immune responses towards certain allergens characterized by B cell activation, high IgE antibody titers and cytokines like IL-4, IL-5, IL-10 or IL-13 (Campbell et al., 2000), (Abbas et al., 1996). Therefore Th1-inducing adjuvants like CpG-DNA and CpG-DNA conjugates seem to be of therapeutical value especially in such disease entities (Horner et al., 2001b).

In murine asthma models OVA or Phleum and CpG-DNA, when conjugated together, reduced bronchial hyperresponsiveness severalfold compared to antigen and CpG-DNA in mixtures as determined by maximum tolerated methacholine concentration (Ollert, unpublished data).
Shirota and colleagues even report that stimulatory conjugates prevent airway eosinophilia and bronchial hyperresponsiveness 100-fold more efficiently than either OVA alone or OVA mixed with CpG-DNA (Shirota et al., 2000). Injection with the ragweed allergen Amba1 linked to stimulatory CpG-DNA induces a de novo Th1 response (increased IFNγ production, elevated IgG_{2a} and suppressed IgE, IgG_1 antibody formation) after challenge with Amba1 in mice primed for a Th2 response (Tighe et al., 2000b), (Santeliz et al., 2002). Interestingly, in anaphylaxis studies death rates can be reduced from 100% with native allergen to as low as 0% with high-ratio CpG-ODN-allergen challenge (Horner et al., 2002). Even in human basophils of patients with ragweed allergy Amba1 linked to CpG-DNA proved to be less allergenic than Amba1 alone, as shown by a 30-fold lower histamine release, whereas mixtures do not reduce histamine release significantly (Tighe et al., 2000b).

Taken together conjugation of antigen to CpG-DNA boosts its immunogenicity. CpG-DNA-antigen conjugates show improved potency to specifically activate the immune system against the conjugated antigen compared to free CpG-DNA and antigen in mixtures. At the same time unspecific activation of bystander cells that have not engulfed antigen is minimized when utilizing CpG-DNA linked antigen.

4.2 Cellular trafficking of conjugates

Prerequisites for this increased immunological potency are conjugate-specific phenomenons concerning cellular endocytosis, subcellular compartmentation and presentation of antigen derived peptides to cells of the immune system.

Endocytosis of DNA-ODN

APC possess several features that allow them to capture antigen. On one hand they can engulf particles by phagocytosis (Inaba et al., 1993), (Moll et al., 1993), (Reis e Sousa et al., 1993), (Svensson et al., 1997). Secondly, they can perform macropinocytosis by generating large pinocytotic vesicles in which extracellular fluid

and solutes are sampled (Sallusto et al., 1995). Besides these two mechanisms they are also able to selectively sample molecules by expressing cell surface receptors that mediate adsorptive endocytosis (Thery and Amigorena, 2001). Examples include C-type lectin receptors, which recognize glycosylated antigens, like the mannose receptor (Sallusto et al., 1995) and DEC-205 (Jiang et al., 1995), (Hart, 1997), as well as Fc receptors for IgE or IgG, which mediate internalization of immune complexes (Sallusto and Lanzavecchia, 1994), (Maurer et al., 1998), (Regnault et al., 1999) or specific receptors for heat-shock proteins such as gp96 and hsp70 (Castellino et al., 2000), (Singh-Jasuja et al., 2000).

For DNA, as well, such receptor-dependent endocytosis seem to exist (Bennett et al., 1985), (Yakubov et al., 1989). Five major phosphorothioate modified ODN binding protein groups have been described ranging in size from approximately 20 to 143 kD (Beltinger et al., 1995). Cell surface molecules like Mac 1 (CD11b/CD18) integrins engage singlestranded DNA while lacking sequence specificity (Benimetskaya et al., 1997).

Cellular uptake is a precondition for CpG-DNA to deploy its immunostimulatory properties. Pretreatment of cells with monodansylcadaverine, an agent that interferes with early steps in endocytosis, or competitive block by non-CpG-DNA abolishes signalling of CpG-DNA (Ahmad-Nejad et al., 2002), (Hacker et al., 1998). Furthermore CpG-ODN when immobilized on solid support cannot stimulate lymphocytes (Krieg et al., 1995), (Manzel and Macfarlane, 1999). Apart from cellular uptake into endosomes, acidification of these vesicles is of critical importance. Chloroquine, bafilomycin A, quinacrine or monensin – all inhibitors of acidification – hamper a process termed endosomal maturation of stimulatory DNA and therefore block CpG mediated effects (Yi et al., 1998), (Macfarlane and Manzel, 1998), (Hacker et al., 1998), (Yi et al., 1999), (Strekowski et al., 1999).

This receptor mediated sampling of DNA-ODN was found to be independent of the presence of an immunostimulatory CpG-motif: uptake of ODN can competitively be blocked by polynucleotides of any length (Loke et al., 1989), (Hacker et al., 1998). In addition stimulatory and nonstimulatory ODN bind equally well to cell membranes (Krieg et al., 1995), (Yamamoto et al., 1994) and are endocytosed in a compareable manner (Hacker et al., 1998) into distinct cellular compartments (Ahmad-Nejad et al., 2002). The only exeption of this nonsequence-specific binding is that ODN containing poly-guanosine runs added at the 3' end of the ODN show enhanced binding (Kimura

et al., 1994), (Hughes et al., 1994), (Yao et al., 1996), (Lipford et al., 2000a), (Dalpke et al., 2002).

Increased sampling of DNA-conjugated antigen

The same observations can be made with conjugated ODN. We and others show that both – CpG-DNA or non-stimulatory GpC-control ODN antigen conjugates – equally well enhance uptake of linked fluorescent protein compared to unconjugated protein and therefore constitute a efficient shuttle mechanism for antigen (Shirota et al., 2001), (Maurer et al., 2002), (Sano et al., 2003), (Shirota et al., 2002). These antigen conjugates are engulfed into vesicular structures within the cell, as can be noticed by confocal microscopy (Shirota et al., 2001), (Maurer et al., 2002). Free ODN dose-dependently inhibit uptake of DNA-linked fluorescent OVA in a CpG-independent manner. Moreover, in blocking experiments ODN modified with a poly-guanosine tail at the 3´ end proved to be a much more powerful inhibitor of conjugate-uptake corresponding to findings mentioned above. For some time TLR9 which is mediating CpG-triggered activation of immune cells had also been discussed as a possible receptor for DNA uptake until it was discovered that TLR9 is localized – contrary to other TLR – exclusively within the cytoplasm and not at the cell surface (Ahmad-Nejad et al., 2002). We could prove in TLR9 -/- mice that TLR9 is not involved in DNA aided endocytosis of conjugates – albeit TLR9 is essential for CpG-mediated activation (Heit et al., 2003).

Cross-presentation of extracellular antigens

Classical antigen-presentation studies revealed that endocytosed antigens are processed and presented on MHC class II while intracellular proteins, cut into peptides, are loaded onto MHC class I molecules in the endoplasmic reticulum before those complexes are transferred to the cell surface (Germain, 1994), (Banchereau and Steinman, 1998).

This strict dichotomy, though, has been challenged in recent years by several studies (Yewdell et al., 1999), (Heath and Carbone, 2001) that show that exogenous proteins can gain access to the cytosol by yet undefined pathways (cytosolic diversion) and can therefore be presented on MHC class I (Rock et al., 1990), (Kovacsovics-Bankowski and Rock, 1995), (Norbury et al., 1995), (York and Rock, 1996), (Rock, 1996). These processes, referred to as „cross-presentation“, explain why injection of

various types of exogenous antigens efficiently prime MHC class I restricted CTL responses (Schirmbeck et al., 1994), (Jondal et al., 1996), (Brossart and Bevan, 1997). Especially immature DC (Shen et al., 1997), (Norbury et al., 1997), (Albert et al., 1998), furthermore macrophages (Castellino et al., 2000), (Singh-Jasuja et al., 2000) and – just recently discovered – B cells (Shirota et al., 2002), (Heit et al., 2004) are particularily efficient in cross-presentation.

Increased cross-presentation of conjugated antigen

Cross-presentation can be improved by providing antigen linked to latex beads (Shen et al., 1997), complexed with IgG (Fc mediated uptake) (Regnault et al., 1999), (Schuurhuis et al., 2002), expressed on the surface of recombinant bacteria (Rescigno et al., 1998) or by administering antigen in apoptotic cells (Albert et al., 1998). In our studies we could show for the first time that also the use of stimulatory as well as non-stimulatory DNA-OVA conjugates leads to greatly enhanced presentation of the OVA-peptide SIINFEKL on MHC class I compared to only poor presentation if uncoupled OVA or mixtures of OVA with DNA-ODN are applied (Maurer et al., 2002), a finding that was confirmed by Shirota and colleagues (Shirota et al., 2002).

Thus, CpG-DNA conjugates effectively induce antigen specific effector mechanisms by dual means: firstly, they augment the uptake of antigen leading to increased presentation of antigen derived peptides on MHC class I. Secondly, these antigen conjugates carry an intrinisic activation signal – the CpG-motif – that stimulates APC after they have engulfed the CpG-DNA antigen complexes.

4.3 Toxicity of conjugates

Bacterial DNA or certain synthetic oligonucleotides displaying unmethylated CpG-motifs can trigger an enormous release of TNFα in D-Galactosamine-sensitized mice that can lead to the manifestation of a lethal toxic shock syndrome (Sparwasser et al., 1997b), (Sparwasser et al., 1997a). Moreover, in mice immunostimulatory DNA potentiates the sensitivity to the toxic effects of LPS (Cowdery et al., 1996).
However, in our experiments CpG-DNA linked to antigen evokes only a moderate induction of proinflammatory cytokines like TNFα, IL-6 or IL-12 (Maurer et al., 2002).

In addition, a long history of human therapy with crude forms of CpG-DNA supports its safety (Tokunaga et al., 1999). Millions fo human subjects have received vaccinations using bacterial extracts containing DNA with resulting transient local inflammatory responses like self-limited and not progressive arthritis (Deng et al., 1999) or flu-like symptoms (Krieg et al., 1995). Also, in some individuals increased levels of autoantibodies have been noted – without causing any signs of autoimmune disease (Tokunaga et al., 1999).

With the use of antigen-CpG-DNA conjugates, however, also the toxicity of the antigen has to be taken into account – particularly obvious in allergic diseases. In such cases antigen linked to stimulatory DNA might improve safety and decrease allergenicity compared to unconjugated allergen extracts (Horner et al., 2002). In mast cell degranulation studies conjugates were approximately 100-fold less allergenic than native allergen and in an murine anaphylaxis model death rates were reduced from 100% with native allergen challenge to as low as 0% with CpG-DNA-allergen challenge (Horner et al., 2002). Besides, by virtue of their increased efficacy lower amounts of conjugated antigen and CpG-DNA would have to be employed.

These results raise the hope that DNA-antigen conjugates might prove rather non-toxic and systemic side effects of both, uncoupled antigen and free CpG-DNA ODN, could be minimized or even avoided.

4.4 Future of DNA-antigen conjugates

Currently it is evaluated if the Th1-activating potency of CpG-DNA ODN can be harnessed for immune therapy of cancer, allergy and infectious diseases (Krieg, 2002).

Its effectiveness aganist cancer could be proven in several murine tumor models including B cell lymphoma, IE7 fibrosarcoma, B16 melanoma, and 3LL lung carcinoma (Weiner et al., 1997), (Kawarada et al., 2001). For example, mice receiving a combination of antitumor monoclonal antibody and CpG-ODN developed tumor in 20% compared with 90% of mice treated with antibody alone in an immunocompetent lymphoma model. In this study a single dose of CpG-ODN appeared to be as effective as multiple doses of IL-2 at inhibiting tumor growth when combined with antibody (Wooldridge et al., 1997). For conjugates, so far, experience is limited. In an preventive tumor model CpG-conjugates seemed to be the most

effective vaccine for stimulating resistance to tumor growth – even more powerful than the tumor antigen and CpG-DNA in mixture. Also, if mice were immunized after tumor challenge stimulatory DNA-conjugates could subsequently suppress tumor growth (Cho et al., 2000).

In models of allergy coadministration of antigen with CpG-DNA have been shown to modify and correct the underlying pathological Th2-biased immune response supporting the potential of CpG-ODN in the treatment of these diseases (Kline et al., 1998), (Campbell et al., 2000). CpG-DNA conjugated to allergen increased the efficiency compared to mixtures (Santeliz et al., 2002) while at the same time reducing allergenicity of these antigens in such murine asthma models (Tighe et al., 2000b). Since injection of allergen by itself – as it is performed in hyposensibilization therapy – is associated with the risk of allergic and sometimes even life-threatening anaphylactic reactions, formulations with a low risk-to-benefit ratio are desired. In this context CpG-DNA conjugates are auspicious (Spiegelberg and Raz, 2002).

Beyond the effectiveness of CpG-ODN in models of cancer or allergy they also support the immune system in the fight against infectious diseases. Utilized as adjuvant in DNA-based vaccines CpG-DNA is more potent than the gold standard, CFA, as measured by its ability to drive the differentiation of CTL and IFNγ secreting T cells (Krieg, 2002), (Krieg and Davis, 2001). In orangutans the addition of CpG-ODN to hepatitis B vaccine markedly increased the seroconversion rates and greatly enhanced protective humoral immunity from 8% with commercial vaccines to 100% after two doses (Davis et al., 2000). Likewise in humans: two weeks after the first vaccination of healthy volunteers 92% of subjects receiving CpG-ODN combined with vaccine produced antibodies compared to 0% of the subjects receiving vaccine alone. Two and four weeks after the second dose, antibody titers were more than 30 times higher in subjects receiving CpG-ODN plus vaccine when compared to vaccine alone (Weiner, 2000). Immunizations using CpG-DNA conjugates with the HIV-1 envelope protein gp120 or Escherichia coli β-galactosidase, again, augmented the immune response against those antigens compared to mixtures of antigen and immunostimulatory DNA concerning CTL induction, (neutralizing) Ab production, secretion of HIV-inhibitory chemokines and induction of Th1 cells secreting IFNγ (Tighe et al., 2000a), (Horner et al., 2001a).

In summary, conjugation of antigen to stimulatory CpG-DNA sequences greatly enhances vaccine efficacy in a variety of disease entities and may therefore prove a

valuable tool for immunotherapy. Despite the promising results of CpG-DNA-antigen conjugates in these fields further work under both – laboratory and clinical conditions – is needed before we can estimate their true potential as investigational or therapeutical agents.

5 Summary

Bacterial DNA activates the immune system of vertebrates. This is due to unmethylated CpG motifs flanked by palindromic sequences that occur much less frequent in mammalian genomes and can therefore be recognized by the host immune system. Synthetic singlestranded CpG-DNA ODN, as well, can mimic those immunostimulatory effects seen with bacterial DNA.

The pathogen-associated molecular pattern CpG-DNA is detected by TLR9 which excites cells of the innate immune system that direct the emanating anti-pathogen defence mechanisms. In mammals CpG-DNA ODN trigger robust CTL formation, production of certain cytokines and antibody subclasses. These so-called Th1-immune responses can be useful in the struggle against cancer, allergy or infectious diseases. Thus, CpG-DNA could yield therapeutic potential as adjuvant in vaccine strategies targeting these diseases. Refinement of such CpG-DNA based vaccination formulas, that is, reducing allergenicity and toxicity while at same time improving immunogenicity of antigens, especially in allergy, is of particular interest.

In this study we show that linking the model antigen OVA to CpG-DNA enhances vaccine efficacy by triggering peptide specific CTL responses *in vivo* at 10-fold lower antigen doses compared to mixtures of CpG-DNA plus OVA. This can be explained by the fact that conjugation of antigen to DNA-ODN causes an increase in uptake of antigen by immature DC compared to unconjugated antigen. Futhermore, sampling of those conjugates is not affected by the absence of TLR9 nor stimulatory DNA-sequences suggesting the involvement of a DNA-binding endocytosis receptor lacking any sequence specificity. The enhanced uptake of DNA-conjugated OVA translates into efficient generation and presentation of the OVA-derived CTL epitope SIINFEKL on MHC class I. However, only stimulatory DNA conjugates provide the DC maturation signal required to induce antigen-specific immune activation.

Thus, CpG-DNA ODN in conjugates fulfill two tasks: shutteling antigen-uptake and mediating activation. Stimulatory CpG-ODN linked to antigen lead to efficient antigen-specific activation of the immune system while preventing unspecific stimulation and reducing undesired side effects that can be seen with antigen and CpG-DNA in mixture. Further research in this field, however, is necessary to evaluate the true therapeutic value of CpG-DNA conjugates.

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