New immunisation strategies via mucosal routes:
comparative analysis of sublingual, intravaginal and intranasal immunisation

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades
Doktorin der Naturwissenschaften
Dr. rer. nat.
genehmigte Dissertation
von
Dipl.-Biol. Kirsten Scholz
geboren am 22.04.1984 in Hannover

2012

Referent: Prof. Carlos A. Guzmán, MD, PhD

Korreferent: Prof. Dr. Roland Jacobs

Tag der Promotion: 20.08.2012

Table of contents

Table of contents **4**
Abbreviation index **10**
Ia Kurzzusammenfassung **15**
Ib Abstract **16**
1. Introduction **18**
1.1. Vaccination as effective strategy for disease prevention 18
1.1.1. Impact of infection and vaccination 18
1.1.2. Types of vaccines 18
1.1.3. Adjuvants 20
1.1.3.1. Licensed adjuvants 20
1.1.3.2. Innovative adjuvants 23
1.1.4. Disadvantages of parenteral vaccination 26
1.1.5. Benefits of mucosal immunisation 27
1.2. The mucosal port of entry 28
1.3. Herpes-simplex-virus 30
1.4. Mucosal immunisation routes 32
1.4.1. Oral immunisation 32
1.4.2. Intranasal route 34
1.4.3. Intravaginal route 36
1.4.4. Sublingual route 38
1.5. Role of interleukin-17 in mucosal immune responses 41
1.6. Aim of the thesis 44
2. Material **46**
2.1. Chemicals and reagents 46
2.1.1. General 46
2.1.2. Enzyme-linked immunosorbant assay (ELISA) 47
2.1.3. Enzyme-linked immune spot technique (ELISPOT) 48
2.1.4. Proliferation 50
2.2. Media and buffer 50
2.2.1. General 50
2.3. FACS-analysis 51
2.3.1. Antibodies against murine antigens 51

2.3.2. Reagents and chemicals/-iterative chip based cytometry (ICBC) chips ... 51
2.3.3. Histological staining ... 52
2.3.4. Kits ... 53
2.4. Antigens and peptides ... 53
2.5. Adjuvants ... 53
2.6. Cell lines ... 54
2.7. Virus ... 54
2.7.1. Stock preparation reagents ... 55
2.7.2. Material and instruments for virus stock preparation ... 55
2.8. Mice ... 56
2.9. Chemicals ... 57
2.9.1. Chemicals for murine application ... 57
2.10. Consumables ... 58
2.11. Instruments ... 59
2.12. Software ... 61
3. Methods ... 63
3.1. Methods for cell culture/*in vitro* experiments ... 63
3.1.1. Isolation of mouse vaginal immune cells ... 63
3.1.2. Determination of cell number and vitality test ... 63
3.1.3. Lysis, freezing, and thawing of cells ... 64
3.1.4. *In-vivo*-CFSE-proliferation ... 65
3.1.5. HEK-Blue™ LPS detection ... 65
3.2. Flow Cytometry ... 66
3.2.1. Direct surface staining ... 66
3.2.2. Intracellular phenotyping and cytokine determination ... 67
3.2.3. Chipcytometry ... 67
3.2.3.1. Sample preparation for chipcytometry ... 68
3.2.4. Cytokine array (Mouse Th1/Th2 10-plex Flow Cytomix) ... 69
3.2.5. Acquisition of samples using FACS ... 69
3.3. Methods with laboratory animals and handling of mice ... 69
3.3.1. Marking of mice ... 69
3.3.2. Taking blood and serum preparation ... 70
3.3.3. Strategies to synchronise the murine oestrus cycle ... 70
3.3.3.1. DEPO-treatment ... 70

3.3.3.2. Whitten-effect for synchronisation of the oestrus cycle 70
3.3.4. Immunisation/challenge strategies 71
3.3.4.1. Intranasal immunisation 71
3.3.4.2. Sublingual immunisation 71
3.3.4.3. Oral immunisation 72
3.3.4.4. Intravaginal immunisation 72
3.3.4.5. Subcutaneous injection 73
3.3.4.6. Immunisation schedule 73
3.3.5. Health and constitution monitoring 73
3.3.5.1. Measurement of weight 73
3.3.5.2. Health monitoring expressed in scores 73
3.3.5.3. Killing 74
3.3.5.4. Narcosis 74
3.3.5.5. Anaesthesia 74
3.3.6. Collection of organs 74
3.3.6.1. Spleen resection 74
3.3.6.2. Resection of vagina 75
3.3.6.3. Resection of lymph nodes 75
3.3.7. Collection and processing of lavages 76
3.3.7.1. Nasal lavage (NL) 76
3.3.7.2. Saliva 76
3.3.7.3. Bronchoalveolar lavage (BAL) 77
3.3.7.4. Vaginal lavage (VL) 77
3.4. Vaginal smears 77
3.4.1. Vaginal smears with swabs 77
3.4.2. Vaginal treatment with cytobrush 78
3.5. HSV-1 78
3.5.1. 17SynIGR20Luc and 17SynIGR20GFPgJZ virus stock preparation 78
3.5.2. Plaque-assay 80
3.6. Technics of staining cells in liquid and in sections 81
3.6.1. Cryosections 81
3.6.2. Staining of vaginal smears 81
3.6.2.1. Diff-Quik staining 81
3.6.2.2. Pappenheim staining 81

3.7. Immunological methods 82
3.7.1. ELISA 82
3.7.1.1. Detection of antigen-specific IgG in sera 82
3.7.1.2. Detection of antigen-specific IgA in mucosal lavages 82
3.7.2. Enzyme-linked immune spot technique (ELISPOT) 84
3.7.3. Proliferation assay 84
3.7.4. Cytokine secretion 85
3.7.5. Statistical analysis 86
4. Results 87
4.1. Establishment of a synchronisation strategy for the murine oestrus cycle 87
4.1.1. Whitten-effect for oestrus cycle synchronisation 87
4.1.2. DEPO-treatment 88
4.1.3. Identification of the phase of the oestrus cycle allowing to obtain the most efficient immune responses following immunisation 89
4.2. Defining the optimal immunisation protocol 92
4.2.1. Identification of the most potent adjuvant stimulating the strongest immune response after i.n. and i.vag. immunisation 92
4.2.2. Identification of the most suitable model ag for stimulating immune responses after i.n. and i.vag. immunisation 95
4.2.2.1. Induction of local and systemic humoral immune responses in C57BL/6 mice after immunisation by i.n. and i.vag. route 95
4.2.2.2. Stimulation of cellular immune responses in C57BL/6 mice after i.n. and i.vag. immunisation 97
4.2.3. Comparative studies of ag-specific immune responses in C57BL/6 and BALB/c mouse strains 101
4.2.3.1. Induction of efficient local and systemic humoral immune responses in BALB/c mice after i.n. and i.vag. immunisation 102
4.2.3.2. Stimulation of ag-specific cellular responses in BALB/c mice immunised by i.n. or i.vag. route 105
4.2.4. Influence of mucosal adjuvants on the production of IL-17 109
4.2.4.1. Stimulation of efficient local and systemic humoral immune responses in BALB/c mice immunised with β-Gal co-administered with mucosal adjuvants by i.n. or i.vag. route 110
4.2.4.2. Induction of enhanced cellular proliferation and cytokine production in BALB/c mice immunised with β-Gal co-administered with adjuvants by i.n. or i.vag. route 114
4.3. Evaluation of the s.l. immunisation strategy as alternative approach for i.n. immunisation 119

4.3.1. Immunisation via s.l. route differs from oro-gastric application 119
4.3.1.1. Immune responses stimulated following s.l. and oral immunisation differ in strength and shape 119
4.3.1.2. Determination of the cellular immune responses elicited after s.l., oral and i.n. immunisation 122
4.3.2. Immunisation via s.l. route requires increased vaccine dosages as compared to the i.vag. and i.n. route 124
4.3.2.1. Humoral immune responses 124
4.3.2.2. Cellular immune responses 127
4.3.3. Side-by-side comparison of immune responses stimulated following immunisation by s.l. and i.vag. routes 128
4.3.3.1. Immunisation by the s.l. route elicits efficient mucosal, humoral immune responses 129
4.3.3.2. Effects resulting from s.l. immunisation on cellular immune responses 134
4.3.4. Combination of i.vag. and s.l. immunisation does not result in a further increase of humoral and cellular immune responses 139
4.3.4.1. Humoral immune responses stimulated following immunisation via i.vag. and s.l. route 139
4.3.4.2. Humoral local immune responses following combined immunisation via i.vag. and s.l. route 143
4.3.4.3. Systemic and local cellular immune responses stimulated following combined immunisation via i.vag. and s.l. route 145
4.4. Summary of the humoral and cellular responses obtained following different immunisation strategies 154
4.5. Immunisation strategies to protect against mucosal challenge with HSV-1 155
4.5.1. Protection against β-Gal expressing HSV-1 after s.l. vaccination with β-Gal 155
4.5.2. Protection against HSV-1 challenge after s.l. vaccination with virus co-administered with adjuvants 155
5. Discussion 159
5.1. Mucosal immunisation 159
5.1.1. Establishment of successful immunisation protocols 160
5.1.2. Benefits and drawbacks following i.vag. immunisation 165
5.2. Immunisation via s.l. route is a promising alternative to i.n. and i.vag. immunisation 167
5.3. Conclusions 172
6. References 174

7. Appendix 187
Figure index 187
Table index 190
Acknowledgement 191
Curriculum vitae 192
Declaration 193

Abbreviation index

%	Percent
℃	Degree Celsius
α	Alpha
β	Beta
β-Gal	Beta-galactosidase
γ	Gamma
δ	Delta
µm	Micrometre
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic)
ACK	Ammonium chloride-potassium hydrogen carbonate
AEC	3-amino-9-ethylcarbozole
ag	Antigen
α-GalCer, MPEG	Alpha-galactosyl-ceramide, pegylated
AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cell
APC	Allophycocyanin (section 4.3.1)
AS03	Alumosilicate 3
AS04	Alumosilicate 4
ASC	Antibody-secreting cell
BAL	Bronchoalveolar lavage
BALT	Bronchial-associated lymphoid tissue
BPPcysMPEG	S-[2,3-bispalmitoyiloxy-(2R)-prpoyl]-R-cysteinyl-amido-monomethoxyl polyethylene glycol
BSA	Bovine serum albumin
CARD	Caspase-recruitment domain
CCL-2, 3, 4	Chemokine ligand-2, 3, 4
CCR	Chemokine receptor
CD 3/4/8	Cluster of differentiation (*e.g.* 3,4,8)
c-di-AMP	cyclic-dimeric-adenosine 3',5'-monophosphate
c-di-GMP	cyclic-di-guanosine monophosphate
CEE	chronic environmental enteropathy
CFDA-SE	Carboxyfluorescein diacetate Succinimidyl ester
cLN	Cervical lymph nodes
CNS	Central nervous system
ConA	Concanavalin A
CPM	Counts per minute
CT	Cholera toxin
CTB	Cholera toxin subunit B
CTL	Cytotoxic T lymphocyte

CTLA-4	Cytotoxic T-cell antigen 4
C-type lectin	Calcium-dependent-type lectin
CXCL8	CXC-motif-chemokine 8
DC, plural DCs	Dendritic cell; pl. Dendritic cells
DEPO	(pregn-4-ene-3,20-dione, 17-(acetyloxy)-6-methyl-, (6α-))
DMEM	Dulbecco's modified eagle medium
DMF	Di-methylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DQ	Diff-Quik
E. coli	*Escherichia coli*
e.g.	Exempli gratia
E2	Estradiol
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immune spot technique
FACS	Fluorescence activated cell sorter
Fc	Fragment crystallisable
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box transcription factor protein 3
FSC-A/-H/-W	Forward scatter-area/-height/-width
g	Gram
GALT	Gut-associated lymphoid tissue
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage-colony stimulating factor
GFP	Green fluorescent protein
h	hour
HA	haemagglutinin
H_2O_2	Hydrogen peroxide
H5N1	Hemaglutinin5-Neuraminidase1
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIV	Human immunodeficiency virus
HLT	Heat-labile toxin
HPV	Human papilloma virus
HRP	Horseradish peroxidase
HSV-1/HSV-2	Herpes simplex virus 1/2
i.e.	Id est
iLN	Iliac lymph node
i.n.	Intranasal/ intranasally
i.p.	Intraperitoneal/ -ly
IRIV	Immunopotentiating reconstituted influenza virosomes
ITAM	Immunoreceptor signalling motif

i.v.	Intraveniously
i.vag.	Intravaginal/-ly
IBD	Inflammatory Bowel disease
ICBC	Iterative chip-based cytometry
IFN-γ	Interferon-gamma
IgA/E/G/M	Immunoglobulin A/E/G/M
IGR	Intergenetic region
IL	Interleukin
IL-17RA	Interleukin-17A-receptor
kbp	Kilo base-pairs
$KHCO_3$	Potassium bicarbonate
khZ	Kilo Hertz
KO	knockout
l	Litre
LD	Lethal dose
LL /BAL	Lung lavage/Broncheoalvear lavage
LPS	Lipopolysaccharide
LTI	Lymphoid-tissue inducer
LV	Large variant
m^2	Square metre
MALP-2	Mycoplasma macrophage activating lipopeptide-2
MALT	Mucosa-associated lymphoid tissue
M-cells	Microfold-cells
mg	Milligram
MHC	Major histocompability-complex
MIP	Macrophage-inflammatory protein
MIP-1	Macrophage-inflammatory protein-1
MLD	Mouse lethal dose
mM	Millimolar
MOI	Multiplicity of infection
MPL	Monophosphoryl-lipid A
MyD88	Myeloid differentiation primary response gene 88
NA	Neuraminidase
NALP3	nucleotide-binding site and leucine-rich repeats with N-terminal pyrin domain containing 3
NALT	Nasal-associated lymphoid tissue
NaOH	Sodium hydroxide
NFkB	Nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells
NH_4Cl	Ammonium chloride
NK	Natural killer
NKT	Natural killer T-cell
NL	Nasal lavage
NLR	nucleotide oligomerisation domain like receptors

nm	nanometres
NOD	nucleotide oligomerisation domain
OB	Olfactory bulb
OVA	ovalbumin
p-value	Probability value
P4	Progesterone
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PFU	Plaque-forming units
pg	Picogram
pH	Potential hydrogenii
p.i.	Post infection
PI3K	Phosphoinositide 3-kinase
PMSF	Phenylmethanesulfonyl- fluoride
PRR, plural PRRs	Pathogen-recognition receptor, -s
PYCARD	apoptosis-associated speck-like protein containing a caspase-recruitment domain
RA	Rheumatoid arthritis
RAR	Retinoic acid receptor
RIG	Retinoic acid-inducible gene
RNA	ribonucleic acid
RORC	RAR-related orphan receptor C
RORγt	RAR-related orphan receptor gamma
RPM	Rounds per minute
RPMI	Royal Park Memorial Institute
RT	Room temperature
s.c.	subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
SFU	Spot-forming units
s.l.	Sublingual-ly
SI	Stimulation index
sIgA	Secretory IgA
SLIT	Sublingual immunotherapy
SPF	Specific pathogen free
SSC-A/-H/-W	Side scatter-area/-height/-width
STAT	Signal transducer and activator of transcription protein 3
STD, plural STDs	Sexual transmitted disease,-s
STI, plural STIs	Sexual transmitted infection,-s
STING	stimulator of IFN genes
TCR (γδ)	T cell receptor (gamma, delta)
TGF-β	Transcription-growth-factor-β
Th1	T helper 1
Th2	T helper 2

TLR	Toll-like receptor
TNF	Tumour-necrosis factor
T_{regs}	Regulatory T cell
Trif	TIR (Toll/interleukin-1 receptor) domain-containing adaptor protein inducing interferon *β*
UL	Unique long
Us5	Unique short sequence locus 5
VALT	Vagina-associated mucosal tissue
VHS	Virion-host shut off
VL	Vaginal lavage
WHO	World health organisation

Ia Kurzzusammenfassung

Die meisten Pathogene dringen über mukosale Oberflächen in den Wirt ein. Vakzinierung über mukosale Routen ist daher ein sehr vielversprechender Ansatz, schützende Immunantworten dort zu initiieren, wo der erste Kontakt mit den Krankheitserregern stattfindet. Weiterhin wird die mukosale Immunisierung besser akzeptiert als der Nadelgebrauch beim parenteralen Impfen. Auch hat es die Vorteile, dass es nicht zur Kreuzkontamination durch horizontalen Transfer zwischen Individuen kommt, dass durch die einfache Handhabung eine Selbstapplikation möglich ist und dass es weniger Nebenwirkungen birgt. Bisher schien die Administration über die intranasale (i.n.) Route der vielversprechendste Ansatz für mukosale Impfungen, da die i.n. Mukosa ein Gewebe repräsentiert, das nach Infektion oder Vakzinierung sehr potent humorale und zelluläre Immunantworten hervorruft. Leider stellte sich heraus, dass Vakzine, die über diese Route appliziert werden aufgrund ihres enthaltenen Adjuvants, bestehend aus Toxinuntereinheiten, zum olfaktorischen Bulbus redirigiert werden und schwere Nebenwirkungen verursachen können. Auch die Mukosa des Genitaltrakts repräsentiert eine wichtige induktive Seite für schützende Immunantworten. Allerdings ist die Immunisierung über diese Route nur dann effizient, wenn die Frauen in einem bestimmten Zyklusabschnitt immunisiert werden. Die sublinguale (s.l.) Gabe von Vakzinformulierungen gilt als potentielle Alternative zur i.n. Route. Die s.l. Mukosa wird als etabliertes Verfahren zur Immuntherapie bei Allergiebehandlungen eingesetzt, es sind keine Nebenwirkungen bekannt. Wie bei i.n. Immunisierung ruft auch die s.l. Immunisierung starke lokale und systemische Immunantworten, sogar an entfernten Seiten wie der vaginalen Mukosa, hervor.

Hauptziel dieser Arbeit war, das Potential der s.l. Vakzinierung als Alternative zur etablierten i.n. Strategie zu untersuchen und zu prüfen, ob starke lokale und systemische Immunantworten, auch an distalen Geweben, hervorgerufen werden. Um die effizienteste Vakzinierungsstrategie zur Stimulation starker Immunantworten im Genitaltrakt zu bestimmen, wurden verschiedene mukosale Anwendungsrouten verglichen. Dazu wurden verschiedene Adjuvantien in Kombination mit Modelantigenen über i.n., intravaginale und s.l. Routen appliziert. In der mukosalen Wirtsabwehr scheinen IL-17 und Th17 Zellen eine wichtige Rolle zu spielen, dennoch ist nur wenig über ihre Funktion im Genitaltrakt bekannt. Daher wurde die Rolle von IL-17/Th17 bei der Immunität gegen Genitalinfektionen durch Modulation der IL-17/Th17 Antworten untersucht. Zu diesem Zweck wurden die Adjuvantien zyklisches di-Adenosin-3´,5´-Monophosphat und pegyliertes alpha-Galactosylceramid eingestzt, welche in der Lage sind, die Produktion von IL-17 zu steigern bzw. zu hemmen. Die erzielten Ergebnisse zeigen, dass die Immuisierung über die s.l. Route sowohl lokal als auch systemisch starke humorale und zelluläre Immunantworten hervorruft und damit einen vielversprechenden Ansatz für mukosale Vakzinierungen darstellt.

Ib Abstract

Most pathogens enter the host via mucosal surfaces. Thus, vaccination via mucosal routes is a very promising approach to elicit protective immune responses at the area of antigen contact. Furthermore, unlike the use of needles for parental immunisation, mucosal immunisation is more accepted. In addition, it offers several advantages, such as the lack of risk of cross-contamination, an easy administration logistic which would even allow self-administration and much lower risk for local side effects. Until now, administration via the intranasal route has seemed to be the most promising approach for mucosal vaccination, since the intranasal mucosa constitutes a very potent inductive site for humoral and cellular immune responses. Unfortunately, recent findings showed that vaccines applied by this route which contained A-B moiety toxins as adjuvants were redirected to the olfactory bulb, and lead to concerning side effects. The mucosa of the genital tract also constitutes an important inductive site for protective immune responses. However, only women in a specific stage of their oestrus cycle can be effectively immunised via this route. Sublingual (s.l.) administration of vaccine formulations represents a potential alternative to the intranasal (i.n.) route. Like i.n. immunisation, s.l. vaccination stimulates strong local and systemic immune responses, even at distant sites such as the vaginal mucosa.

The major aim of this thesis was to investigate the potential of s.l. vaccination as alternative approach to the well described i.n. immunisation strategy to promote local and systemic immune responses, as well as protection at local and distant mucosal sites. Different mucosal application routes were compared in order to identify the most efficient vaccination strategy to stimulate immune responses at the level of the genital tract. To this end, different adjuvants were used in combination with model-antigens administered via the i.n., intravaginal and s.l. routes. Despite that fact that IL-17 and Th17 cells seem to play a crucial role in mucosal host defence, little is known about these effectors and their function in the genital tract. To this end, the role of IL-17/Th17 in immunity to vaginal infections was investigated by modulating the IL-17/Th17 responses using the adjuvants cyclic-dimeric-adenosine 3',5'-monophosphate and pegylated alpha-galactosyl-ceramide, which were able to enhance or inhibit IL-17 production following vaccination. The obtained results indicate that the s.l. route constitutes a promising alternative for mucosal vaccination strategies aimed at stimulating strong humoral and cellular immune responses both at local and systemic level.

Schlagworte:

Mukosale Vakzinierungsstrategien
Adjuvantien
Th17-Zellen

Keywords:

Mucosal vaccination strategies
Adjuvants
Th17 cells

1. Introduction

1.1. Vaccination as effective strategy for disease prevention

1.1.1. Impact of infection and vaccination

Today, a quarter of deaths worldwide are due to infectious diseases. Among them, over 25% are caused by respiratory tract pathogens, whereas about 20% can be attributed to acquired immunodeficiency syndrome (AIDS). A significant number of people also die from diarrhoea (for example caused by *Salmonella)*, tuberculosis, malaria, measles, tetanus and others [1]. In addition, according to the World Health Organisation (WHO) Regional Office for Europe, more than 340 million new cases of sexually transmitted bacterial and viral infections occur every year worldwide. Thus, the spread of infectious diseases urgently needs to be reduced. Vaccination represents one of the most cost-effective interventions in the fight against infectious diseases. Today, millions of people are vaccinated every year to help prevent the spread of numerous diseases. As declared in 1980 by the WHO, the small-pox vaccine was even able to eradicate the small-pox at a worldwide scale. However, vaccine formulations are still not perfect. Numerous vaccines seem very promising in preclinical animal studies, but are not successful in large clinical trials [2]. A central challenge associated with the creation of new vaccines is to create a formulation that would not harm the vaccinee, but retain the capacity to be recognised by the host's immune system, thereby triggering the elicitation of protective immunity.

1.1.2. Types of vaccines

Most of the vaccines licensed for human use are live attenuated, whole cell inactivated, or subunit-vaccines. Live attenuated vaccines consist of a weakened version of the pathogen, which is unable to cause disease, but retains the capacity to persist and even to some extend replicate inside the host. Examples of live attenuated vaccines include: measles, mumps, rubella, yellow

fever, and small-pox. These vaccines induce a strong antibody and cellular immune response in the vaccines, which mimic the immunity that occurs after natural infection [3], [4]. These vaccines are able to promote both humoral and cellular immunity.

On the other hand, inactivated vaccines are made from killed pathogens, for example vaccines against hepatitis A, whooping cough and cholera. After vaccination with this type of vaccines, antigenic fragments of the protein components of the vaccine formulation will be presented via major histocompability-complex II (MHC class II)-molecules and specific co-receptors to T-cells, thereby stimulating $CD4^+$ T-helper-cells [5]. However, since the pathogen is dead, it would not be able to produce proteins in the cytosol of the host cell. This means viral peptides cannot be efficiently presented via MHC class I-molecules. As a result, efficient cytotoxic $CD8^+$ T-cell responses would not be stimulated [5]. In fact, for stimulation of cytotoxic $CD8^+$ T-cells, the antigens need to be processed by antigen-presenting cells (APCs) and presented via MHC class I-molecules. Then, antigen-specific activated $CD8^+$ T-cells would be able to recognise the antigens displayed by infected cells, which would be in turn killed. Since this process does not efficiently occur with inactivated vaccines, these vaccines provided limited protection against intracellular pathogens, such as many viruses [5].

Finally, subunit vaccines consist of purified fragments of the pathogen intended to elicit protective immunity in the host. Today these vaccines are the most common types of vaccines under development, but unfortunately purified antigens are weakly immunogenic. Consequently, the so-called adjuvants (lat. adiuvare = to help) should be incorporated into the formulation to strength and modulate antigen-specific immune responses [3]. In studies by Bowen and Simmons, it was shown that certain adjuvants, like cholera toxin (CT), can stimulate mucosal cytotoxic T-lymphocytes (CTL) responses after oral or i.n. administration of formulations containing protein and peptide subunits [6], [7]. Approximately half of the vaccines available on the market today include adjuvants needed to enhance and/or modulate the immune responses

stimulated after vaccination. Examples of subunit vaccines enhanced by adjuvants include: Cervarix® and Gardasil® for HPV infection, Boostrix® for diphtheria and tetanus, and Meningitec® for meningococcus.

1.1.3. Adjuvants

1.1.3.1. Licensed adjuvants

Globally, only five adjuvants are currently licensed for use in humans: alum, adjuvant system 03 (AS03), adjuvant system 04 (AS04), MF59 and liposomes [8].

Introduced in the 1930s, alum is an aluminium salt-based adjuvant that can be emulsified with the co-administered antigen [9]. It induces a T-helper 2 (Th2)-biased immune response and is able to induce Toll-like receptor (TLR)-independent antibody responses. Furthermore, it directly affects IL-4 producing $Gr1^+$ cells that prime B-cells to undergo clonal expansion leading to optimal antibody production [10], [4], [11]. According to Koll *et al.*, murine studies indicated that monocytes with the phenotype $CD11b^+Ly6C^{high}Ly6G^-F4/80^{int}$ are recruited to the injection site after alum vaccination [12]. Then, these cells take up the antigen and invade the draining lymph nodes where they undergo differentiation into inflammatory dendritic cells (DCs). In a recent publication it is also shown that alum binds to the lipid plasma membrane of DCs [13]. This triggers lipid sorting, aggregation of immunoreceptor signalling motif (ITAM)-containing receptors and an inflammatory phagocytic response mediated by phosphoinositide 3-kinase (PI3K). The antigen uptake is due to endocytosis. The endosomal antigen-processing therefore leads to CD4-responses, also humoral immune responses and B-cell activation [13]. In another publication, the nucleotide-binding site and leucine-rich repeats with N-terminal pyrin domain containing 3 (Nalp3) inflammasome was identified as the element on which alum is acting, resulting in an inflammasome pathway directing a humoral adaptive immune response [14]. The Nalp3 belongs to the cytosolic nucleotide oligomerisation domain (NOD) like receptors (NLR)-family and forms together with the apoptosis-associated speck-like protein containing a caspase-

recruitment domain (CARD) [Pycard] a caspase-1 activated inflammasome, which for example regulates the release of pro-inflammatory cytokines [15].

A disadvantage of alum is that it does not efficiently stimulate cell-mediated Th1 or cytotoxic T cell responses [16]. Consequently, it is not able to induce optimal protective immunity needed for viral pathogens or parasites, such as the human immunodeficiency virus (HIV) and plasmodium.

MF59 consists of a squalene based oil-in-water emulsion [17]. This adjuvant is co-administered with certain influenza vaccines and has been licensed in Europe since 1997 [4], [18]. MF59 promotes an early $CD4^+$ T cell response and has been shown to promote the elicitation of persistent protective antibodies against H5N1 influenza [8], [19]. According to Mosca *et al.*, MF59 strongly induces cytokines, their receptors, and certain adhesion molecules, triggering also the influx of $CD11b^+$ blood cells according to what observed in murine studies [20]. Furthermore, *in vitro* studies in human cells performed by Seubert *et al.* showed that the adjuvant MF59 (as well as alum) has the potential to induce chemokine secretion as well, for example chemokine-ligand 2 (CCL2), CCL3, CCL4 and CXC-motiv-chemokine 8 (CXCL8) [21]. These chemokines have a role in recruiting cells from blood into peripheral tissues. MF59 targets monocytes, macrophages and even granulocytes. MF59 seems to augment antigen uptake, promoting also upregulation of homing receptors enabling DCs to home and prime adaptive immune responses, leading to the recruitment of immune cells to the site of vaccination [21].

AS03 is an adjuvant based on a 10% oil-in-water emulsion and is very similar to MF59. It contains α-tocopherol and squalene as oil-in-water emulsion [22]. It was tested for the use in an influenza A vaccine and licensed in the vaccine Prepandrix® from GlaxoSmithKline [16], [23].

AS04 is a combination adjuvant composed of 3-O-desacyl-4'-monophosphyril lipid A (MPL), a TLR 4 ligand, adsorbed to alum [24]. This adjuvant is licensed for two vaccines, Cervarix®, a vaccine against HPV, and Fendix®, a vaccine against hepatitis B [24], [25], [26], [27]. MPL is a detoxified derivate from

lipopolysaccharide (LPS) isolated from *Salmonella minesota* R595 and acts as TLR 4 agonist. TLR 4 stimulation leads to nuclear-factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) transcriptional activation, which in turn results in the expression of pro-inflammatory cytokines, like IL-6 and tumour-necrosis factor-α (TNF-α), and enhanced activation and maturation of APCs [24], [28], [29], [30], [31], [32], [33]. The increased number of antigen (ag)-specific DCs and monocytes in the lymph nodes draining the injection site in turn leads to the activation of adaptive immune responses by increasing the number of ag-specific T-cells [33]. While alum alone promotes a Th2-biased immune response, the addition of MPL is able to drive a Th1-biased response.

Liposomes are vesicles containing an aqueous core enclosed in phospholipid layers [34],[35]. They can serve as vehicles for ags and fuse with the plasma membrane of APCs. Liposomes are able to stimulate humoral and cellular immune responses. Although $CD8^+$ T-cells can be also stimulated [36], CTL responses are usually not very strong. This is a bottleneck, since this type of response is critical to combat intracellular pathogens, such as viruses, *Mycobacterium tuberculosis* and *Listeria monocytogenes*. This shortcome can be solved by the use of virosomes, which are liposomes containing proteins from the influenza virus. This favours the release of the virosomes cargo into the cytoplasm, subsequent processing and presentation by MHC class I-molecules, and elicitation of CTL responses.

The virosomal influenza formulation [immunopotentiating reconstituted influenza virosomes (IRIV)] is one example. It is composed of reconstituted virus envelopes containing viral surface glycoproteins, but not genetic material [37]. This formulation retains cell binding and membrane fusion capabilities of the virus [38]. The main constituents of IRIVs are naturally occurring phospholipids and phosphatidylcholine [39]. Up to 30% of the membrane component is formed by envelope phospholipids from the influenza virus, providing the glycoproteins neuraminidase (NA) and haemagglutinin (HA). The NA, a tetramer composed of four equal, spherical hydrophobically subunits, intercalates into the phospholipids membrane and is centrally embedded in the IRIV membrane. The

influenza HA is intercalated into the phospholipid bilayers and stabilises the liposome, preventing fusion with other liposomes [40]. HA is also responsible for the fusion of the virus with the endosomal membrane [41], [42], [43]. The number of lipid layers and the electric charge can influence their adjuvant properties, affecting their capacity to promote humoral and cellular immune responses [44], [45]. Virosomes have been also exploited as delivery system for a current vaccine against the hepatitis A vaccine (Epaxal™).

1.1.3.2. Innovative adjuvants

The development of subunit vaccines has highlighted the limitations of the currently available adjuvants. Moreover, no licensed mucosal adjuvants exist so far. Thus, the generation of new innovative adjuvants is crucial to the development of new vaccines. In this context, stimulation of the innate immune responses via microbial proteins or pathogen-associated molecular patterns (PAMPs), like the LPS from *Escherichia coli* (*E. coli*), offers promising adjuvant candidates. The innate immune system recognises these PAMPs by conserved structures, the so-called pattern-recognition receptors (PRRs), which are expressed by numerous cells, *e.g.* DCs and macrophages [46]. PRRs, such as TLRs, C-type lectin receptors, NLRs and retinoic acid inducible gene-based-like receptors (RIG), recognise bacterial cell wall components, their deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), as well as conserved structures of viruses or fungi [16], [47], [48], [49], [50], [51], [52], [53]. Moreover, by stimulation of different TLRs it would be possible to modulate the elicited immune responses, since TLR agonists can activate a broad range of pathways. As an example, DCs primed by TLR 3, 7 or 9 induce a Th1-biased immune response, whereas activation of DCs by TLR 6 or 2 results in Th2 or regulatory T-cell (T_{reg})-dominated immune responses [16], [54]. On the one hand, Th17 responses can be induced via stimulation of dectin-1, a C-type lectin [55], [54]. Regarding the decision for lineage commitment, transcription factors control which kind of T-cell will develop. Each transcription factor is activated by a combination of cytokines that are produced after PRR stimulation via TLRs on

DCs. Then, naïve T cells are primed to differentiate into the correct phenotype. [54].

There are known adjuvants signalling through TLRs, which exert their activities when administered via the mucosal route. For example, the synthetic S-[2,3-bispalmitoyiloxy-(2R)-propyl]-R-cysteinyl-amido-monomethoxyl polyethylene glycol (BPPcysMPEG) is a TLR 2/6 agonist showing potent adjuvant properties for cross-priming against cellular ags [56]. This adjuvant is a synthetic derivate of the macrophage activating lipopeptide-2 (MALP-2) of *Mycoplasma fermentas*, which is able to signal via TLR 2 in a myeloid differentiation primary response gene 88 (MyD88)-dependent signalling pathway [57]. MALP-2 acts on macrophages, B-cells and DCs, stimulating their maturation and activation [58], [59]. MALP-2 effector functions on B-cells are exerted in a T-cell independent manner [58], [60]. This adjuvant is also promoting proteasome maturation [59].

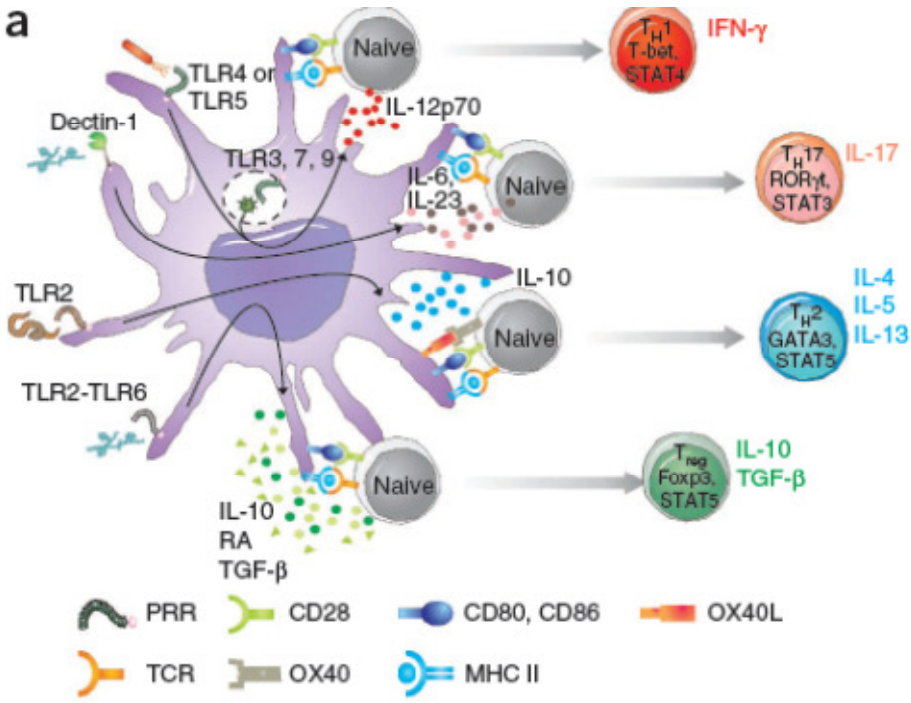

Figure 1: Stimulation of TLRs leads to induction of specific Th-phenotype development [54]

It has been suggested that the use of adjuvants might result in non targeted immune reactions, thereby contributing to chronic diseases. For example, in a

mouse model lacking TLR 4 or MyD88, it is shown that atherosclerosis can be reduced [61], [62]. TLR 9 was also suspected to participate in activation of autoreactive B-cells secreting autoantibodies, the so-called rheumatoid factor. These autoreactive B-cells are persistent in healthy individuals, but co-stimulation of the ag-receptor and TLR 9 could result in their activation with secretion of rheumatoid factor [63].

However, there were other molecules tested as adjuvants, which do not act via TLRs. Recently, cyclic-di-nucleotides were tested as mucosal adjuvants and were shown to elicit protective immune responses [64]. The cyclic-di-guanosine monophosphate (c-di-GMP) is not detected by TLRs that generally sense nucleic acids [65]. Studies were performed using cells in which the TIR (Toll/interleukin-1 receptor) domain-containing adaptor protein inducing interferon *β* (trif) and MyD88 were knocked-out. The *MyD88*$^{-/-}$*Trif*$^{-/-}$ double-knockout cells are not able to signal through known TLRs, but they are still able to respond to nucleic acid ligands via the cytosolic immunosurveillance pathways [65], [66], [67]. When c-di-GMP was tested in these cells, it was able to stimulate them. This indicates that there must be a distinct non- TLR pathway for the recognition of the c-di-GMP. Additional studies showed that also other c-di-nucleotides can exert adjuvant properties [64], [68]. Recently, the stimulator of IFN genes (STING) was identified as an innate immune sensor of cyclic dinucleotides (cited from [69]). It detects pathogen-derived cytosolic DNA-like c-di-nucleotides, leading to a type I interferon response (cited from [69]), [70], [65], [71], [72].

In this thesis, cyclic-dimeric-adenosine 3',5'-monophosphate (cyclic-di-AMP) was assessed as potential adjuvant for use in various mucosal immunisation routes (i.n., intravaginal (i.vag.) and s.l.). The c-di-AMP is a second messenger molecule in bacteria and archeae and is involved, for example, in the sporulation processes of *Bacillus subtilis* and signalling for DNA integrity [73], [74], [68]. The adjuvant cyclic-di-AMP was shown to induce strong Th1/Th2/Th17 responses when co-administered with the model-ag β-galactosidase (β-Gal) via the i.n., i.vag. or s.l. route [68].

The α-GalCerMPEG is a pegylated synthetic derivate of the alpha-galactosylceramide, which was originally isolated from an extract of the marine sponge *Agelas mauritianus* and is known to induce natural killer T-cells (NKT-cell) activity via stimulation of the T-cell receptor (TCR) of NKT cells by CD1d molecules of APCs [75] [76]. NKT cells are categorised into 3 different kinds: (i) the classic invariant NKT cell that possesses invariant Vα14-Jα18 TCR α-chains and reacts to α-GalCer in a CD1d-dependent manner, (ii) non-classic NKT-cells that do not react to α-GalCer and have various TCR α-chains and are CD1d-dependent, and finally (iii) CD1d-independent NKT cells, which do not react to α-GalCer and have various TCR α-chains [77]. Once NKT cells receive an activation signal, they produce Th1 and Th2 cytokines that up-regulate humoral and cellular immune responses [78]. It exist also $CD44^{high}$ $NK1.1^{neg}CD4^{neg}ROR\gamma t^{pos}$ iNKT-cells which produce large amounts of IL-17 after stimulation with *e.g.* α-GalCer [79], [80]. These cells secrete only very low amounts of IFN-γ and IL-4 [80].

1.1.4. Disadvantages of parenteral vaccination

Nearly all vaccines currently available are administered via the parenteral route, either intramuscularly or subcutaneously. In general, parenteral vaccines do not efficiently stimulate primary immune responses in the mucosal tissues. This is a major disadvantage because most pathogens enter via mucosal surfaces. In addition, parenteral vaccine formulations mainly induce Th2-biased responses, which are able to neutralise bacterial toxins and can protect the host against extracellular infections. However, in order to clear intracellular and viral infections, a Th1-biased immune response is required [23]. Another practical drawback is that acceptance of parenteral vaccines is low, since they are applied with needles, being associated with pain and stress.

1.1.5. Benefits of mucosal immunisation

Most pathogens enter the host through mucosal routes. Because the most powerful immune responses can be elicited at the pathogen's site of invasion, mucosal immunisation is ideal. According to Czerkinsky, the immune system of a healthy adult comprises about 400m^2 mucosal surfaces and contains about 80% of all the body's lymphocytes [81], [82]. These membranes defend against pathogens at this large portal of entry using several mechanical and chemical cleansing mechanisms. They also have a huge potential as site of vaccination. Possible routes for mucosal immunisation are conjunctival, i.n., oral, s.l., i.vag. and intrarectal. Unlike parenteral immunisation, local mucosal immunisation through these routes elicits secretory immunoglobulin A (sIgA) and CTL responses at the mucosal site [83]. In addition, it has the possibility to stimulate systemic responses. With a vaccine formulation administered via the mucosal route, many infectious diseases, *e.g.* sexually transmitted diseases (STDs), could be curtailed. With parenteral immunisation, mostly Th2-biased immune responses are stimulated, for example alum, which is one of the few approved human adjuvants, elicits no Th-1 response [84]. These Th1-biased immune responses can be enhanced through mucosal immunisation, which is critical for protection against HIV infection [85], [86].

Mucosal immunisation also offers other societal and logistical advantages. For example, there is no risk of cross contamination through the use of needles, which helps to increase their safety profile [87]. Moreover, medical staff would not be required for administration, allowing for the possibility of self-vaccination. This is especially important in the context of resource limited settings. The development of effective mucosal vaccines would therefore provide a more cost effective and safer alternative to existing vaccines. Thus, the establishment of approaches to develop mucosal vaccines able to confer maximal protection without adverse side effects is a major aim in vaccinology.

1.2. The mucosal port of entry

The portal of entry for most pathogens is the mucosa. For example, the nasal mucosa for the influenza virus, the oral or eye mucosa for the herpes-simplex-virus type 1 (HSV-1), and the vaginal, urethral or rectal mucosa for HIV. Since these mucosal surfaces are covered by a protective mucous, the risk of invading pathogens rises with surface dehydration or the presence of lesions.

There are two major subtypes of mucosal surfaces. Type I mucosal surfaces have a simple columnar epithelium, like that of the gut and lung. Type II mucosal surfaces possess protective stratified squamous epithelia, like that covering the eye, mouth and vagina. To be precise, the endocervix and uterus consists of type I mucosa and the outer area (close to vulva) and inner areas of the vagina and the ectocervix consist of type II. The type I mucosa has IgA transport mechanisms and mucosa associated lymphoid tissue (MALT), whereas the type II mucosa is not linked to MALT [88] .

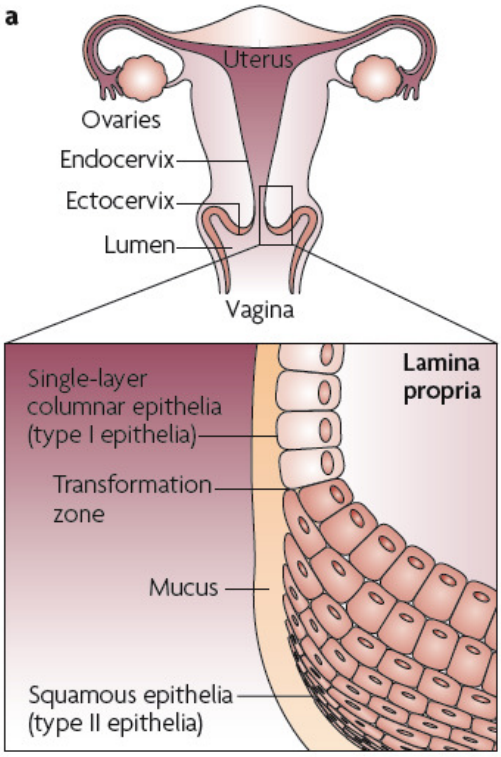

Figure 2: Anatomy of the genital mucosa [88].
In the picture from Iwasaki *et al*. it is shown where the type I and II mucosal surfaces are located in female genital tract.

The MALT can be further divided into subclasses based on location, *e.g.* the nasal-associated lymphoid tissue (NALT), the broncheoalvelar-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT) and the vagina-associated lymphoid tissue (VALT). These act as the induction sites for immune responses [89]. MALT also contains accumulations of lymphoid cells in the exocrine glands and the parenchyma of mucosal organs, which are the areas of effector immune responses. The priming of the mucosa-associated cells occurs in the draining lymph nodes.

In the type II mucosa, the mucus is locally produced by secreting epithelial cells. This mucus contains mucins, antimicrobial peptides, immunoglobulins (Ig) and the complement system. These components play specific roles: [88] (i) mucins contain glycoproteins that are able to cover pathogen structures and prevent penetration of the mucosal tissue; (ii) antimicrobial peptides are a component of the innate immune system that non-specifically kill invading pathogens; and (iii) the Igs exhibit an important role in the immune response, since they can (a) neutralise bacterial toxins, (b) mediate phagocytosis by opsonisation of bacteria in the extracellular space, and (c) activate the classical complement pathway [5]. The complement system is another component of the innate immune system that produces a cascade of proteolytic reactions on the surface of microorganisms, resulting in either their death by lysis or uptake by professional phagocytes.

MALT exhibits a high degree of compartmentalisation and is independent of the systemic immune system. This high degree of compartmentalisation allows activated lymphocytes to migrate and act at distant mucosal sites within these compartments [81]. Therefore, immunisation at one site can also lead to immune responses at distant sites. For example, activated s.l. lymphocytes are able to migrate to the vaginal mucosa. As reviewed in Hervouet *et al.*, s.l. immunisation with an HIV subunit vaccine induces antibodies and CTLs in the genital tract of female mice [90]. Here, antibody-secreting cells (ASC) originated from extra-genital lymphoid organs, the cervical lymph nodes, home to the genital tract after s.l. immunisation. This process is facilitated by the selective

recruitment of mucosal lymphocytes to specific tissues using homing- and chemokine-receptors linked to tissue-specific adressins and epithelial cell-derived chemokines that are differentially expressed at the various effector tissues. Additionally, for the nasal route, studies have shown that i.n. immunisation elicits immune responses at the genital tract of mice and humans. In an experiment conducted in Sweden, women were immunised either i.n. or i.vag. with cholera toxin B subunit (CTB) and CTB-specific IgA responses were found in the vaginal secretions of the i.n. vaccinated women [91].

In addition to fighting infection throughout the body, vaginal immunisation also has the potential to fight a wide variety of infectious diseases. At the genital tract, an infection with human papilloma virus (HPV) can promote the development of cervical cancer. According to Connie Celum, Bulletin WHO 2004, pre-existing genital HSV-2 infection double the risk of acquiring HIV through sexual transmission [92]. This is due to ulcerations and micro trauma of the genital epithelium caused by the HSV-2, which facilitates the entry of sexually transmitted pathogens, like HIV. Moreover, these lesions are invaded by $CD4^+$ T-cells which are the target of HIV. Consequently, vaccines against STDs could help to eradicate and reduce the spread of a number of different diseases, which represent a heavy burden in the context of human public health.

1.3. Herpes-simplex-virus

Two types of Herpes-viruses exist in the subfamily *Alphaherpesvirinae*, type I (HSV-1) and II (HSV-2). These double-stranded DNA viruses measure between 150 and 200 nm in diameter and were first classified in 1962 [93]. Their DNA genomes encode a viral core, an icosaedric capsid consisting of 162 capsomeres, and surrounding tegument proteins that are partially involved in important regulatory tasks, such as the transport of viral DNA out of the capsid during the early stages of infection [94]. The capsid and tegument proteins are enclosed by a lipid membrane, bearing several glycoproteins (gB-gN), these

proteins can bind to certain cellular receptors and allow the virus to enter host cells. For example, gD binds to nectin-1, a critical receptor for viral entry into vaginal mucosal cells [95]. These gylcoproteins are also important because they can induce neutralising antibody-responses in infected individuals supporting viral clearance [96].
The genome of HSV-1 is 152 kilo base pairs (kbp) and shows 83% DNA homology to the protein coding regions of HSV-2 [97], [98]. The genes of HSV can be categorised into immediate early (α), early (β), early late (γ1) and late (γ) genes, based on when they are expressed during infection. The α-genes encode transcription inducers, the β-genes encode viral enzymes like DNA-polymerase and γ-genes encode structural proteins [99].

HSV exists in a couple of different life stages. For example, the virus can exist in an active stage, called lytic phase, where replication into host cells occurs and leads to death of host cells. But the virus can also persist in a latent stage without reproduction and host cells are able to survive. In the latent stage, the virus persists in the sensory nerve ganglia and clearance by the immune system is avoided [100]. Depending on the health status of the host and other factors, such as secondary infections, sun-exposure and/or stress reactivation can be promoted. Otherwise, the latent stage can be maintained for long periods, since herpes- viruses have co-evolved with humans and are well adapted to the human immune system [100]. In fact, many viral components are directly involved in immune escape, thereby promoting viral persistent infection [101], [102], [103].

Both types of HSV are able to infect either the oral mucosa (preferentially HSV-1) or the genital mucosa (preferentially HSV-2) of humans [104]. Typically, they are transmitted to the host by direct contact, during sexual intercourse or through oral/genital contact. Once infected, the virus causes painful blisters at the mucosal surfaces and in certain situations can even lead to death (*e.g.* due to encephalitis, neonatal infections are often deadly) [105], [106]. Approximately 95% of humans suffer from HSV-1 and 30% from HSV-2 infection [1]. In this study, the HSV-1 strain LV was used to perform immunisation and challenge

studies to assess the comparative efficacy of the i.vag and s.l. routes of immunisation, since this virus has the capacity to naturally infect the host via these routes. As shown by Manservigi *et al*., the LV strain is highly pathogenic "causing stromal keratitis with neurological involvement, resulting in encephalitis and death of animals" [107].

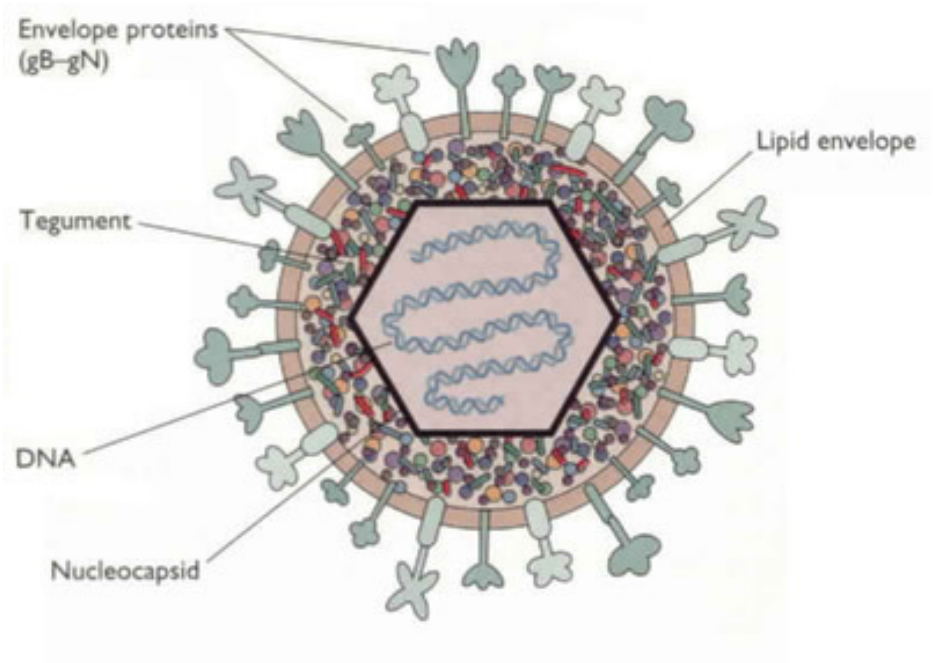

Figure 3: Structure of the HSV-1 virus. [108]

1.4. Mucosal immunisation routes

1.4.1. Oral immunisation

The oral mucosa is an immune-privileged site able to promote tolerance against specific ags, which are in continuous contact with the host. Thus, it is in principle possible to promote both tolerance and effector adaptive responses following immunisation by this route. Therefore, a major challenge for the host immune system is to be able to discriminate between self and non-self, as well as between harmful and non-harmful non-self-structures. A high amount of non-harmful ags are ingested or inhaled and the immune system does not respond to them. When the host immune system is able to suppress reaction to a foreign

body, such as an allergen, this is called tolerance [109]. Both DCs and T-cells are involved in tolerance.

Tolerance can be induced by low- or high-dose ag administration, but they involve different mechanisms [110]. With low doses of ag, DCs are stimulated which induce T_{regs}. These T_{regs} express cytotoxic T-cell ag-4 (CTLA-4) and cytokines like interleukin-10 (IL-10) or interleukin-6 (IL-6) and promote active suppression [111], [110]. High ag dosages are mostly presented by non-professional APCs, here enterocytes, which are able to present ag without co-stimulatory molecules like CD80/CD86. This results in the promotion of ag specific T-cell anergy or deletion [111].

Oral vaccine formulations require careful consideration due to the strong acidic and enzymatic environment at the gastrointestinal tract. If a stable formulation is administered via the oral route, the ag is captured and processed by specialised microfold (M-) cells located in the Peyer's patches of the intestine and also by DCs (reviewed in [112]). Consequently, T-cells are stimulated to develop a protective memory immune response.
The advantages of oral immunisation include ease of administration and the potential to stimulate local and systemic immune responses occurring at the small intestine, proximal colon, as well as in the salivary and mammary glands [113], [112], [114], [115], [91], [116], [117]. The local production of IgA is often important for conferring protective immunity against enteric pathogens [111], [112]. However, a major disadvantage of this route is the high amount of ag needed, which can increase the incidence of adverse side effects. Another concern associated with oral immunisation is the development of chronic environmental enteropathy (CEE). This is a condition in which defects in digestive tract affect the absorptive properties of the gut, a serious problem common in developing countries [81]. Furthermore, the overgrowth of intestinal flora and lack of vitamin A and zinc might also contribute to limited efficacy of oral vaccines in developing countries. Nevertheless, it is important to highlight that there are several examples of successfully implemented oral vaccines. In

fact, one of the first vaccines administered by mucosal route was the oral polio vaccine, which nearly eradiated polio worldwide.
Given the above-mentioned concerns associated with oral vaccines, it is important to investigate alternative mucosal routes. In this context, it would be critical to perform head- to- tail- comparative studies of other routes (*e.g.* i.n., i.vag. and s.l.). Moreover, the compartmentalisation of the mucosal immune system makes that cells induced after oral vaccination will not necessarily home with equal efficiency to any given mucosal territory. For example, oral immunisation results in efficient local responses at the gut and mammary glands, but not in vagina [112], [118]. In contrast, protective immune responses can be elicited in the genital tract after immunisation by the i.n., i.vag. or s.l. route. Therefore, other mucosal routes of vaccination seem to be more promising in order to elicit protection against STDs.

1.4.2. Intranasal route

The i.n. route is of interest since the administered ag dose can be significantly reduced compared to oral administration, whereas preserving the easiness of administration [119]. The nasal mucosa is in constant contact with respiratory particles and offers the first line of defence against inhaled pathogens. It contains ciliated membranes and produces mucous that helps to clear foreign particles. The nasal lumen is lined by an epithelium containing M-cells, which are involved in the uptake of ag. The submucosal tissue underlies the M-cells and contains a layer are B-cell follicles and T-cell areas followed by the lamina propria [120].

Immunisation by the i.n. route stimulates humoral and cellular immune responses [121]. In murine studies, it has been shown that i.n. vaccination induces the NALT [122]. Humans have similar lymphoid follicles enriched mucosal area associated to the nose, which can be compared to the NALT [123]. In the submucosal tissue, ags are processed and presented to T- and B-cells, which are then activated [121], [122], [124], [125]. After priming, the ag-specific T- and B-cells drift from the NALT to the draining cervical lymph nodes

(cLN) and begin circulation in the lymphatic system. At this point, they are able to home to distant effector sites and exert their effector function [121]. For example, ag-specific IgA is produced, which then bind and neutralise the ag and/or enable their binding to receptors on phagocytes recognising the fragment-crystallisable (Fc) region of the Ig, thereby leading to ag or pathogen clearance. After i.n. vaccination, the compartmentalisation of the mucosal immune system results in the elicitation of IgA antibody responses in saliva, nasal secretions and in airway mucosa, as well as in the genital tract [115], [117], [112], [113], [114], [116]. Human studies have supported these finding by showing that after nasal vaccination with CTB, CTB-specific IgA can be found in the serum, vaginal secretions and the upper respiratory tract [91], [115].

Given these robust and distant immune responses, the i.n. mucosa was long considered as a prime target site to induce mucosal immunity. In recent years, however, it has been shown that certain adjuvants included into the formulation (*i.e.* bacterial A-B moiety toxins and their derivates) can be redirected to the central nervous system (CNS) via retrograde axonal transport and become harmful to the host. Murine studies, for example, have shown that CT and CTB, when delivered i.n., are redirected to the olfactory epithelium with subsequent retrograde transport into the olfactory bulb (OB) of the CNS [126]. Furthermore, in 2000 Nasalflu, a very promising inactivated virosomal-subunit vaccine against influenza, was formulated into a spray and clinically tested in Switzerland. Like most subunit vaccines, an adjuvant was required to elicit protective immune responses; in this case *Escherichia coli* heat-labile toxin (HLT) was used. Alarmingly, however, the vaccine was shown to cause reversible Bell's palsy (paralysis of the trigeminal ganglia). This can be explained by different potential mechanisms, such as toxicity resulting from the retrograde axonal transport of *E. coli* HLT caused by application via the i.n. route, or mechanic compression of the nerve as a result of the ensuing local inflammatory reaction, or inflammatory stress resulting in reactivation of persistent viral infection or increased susceptibility to infection by neurotropic viruses [127]. Subsequent clinical trials with genetically inactivated derivatives of the HLT further confirm the safety concerns associated with the use of A-B moiety toxins and/or their derivatives

[128]. Since these severe safety problems were discovered, the i.n. route is no longer considered a favourable mucosal vaccination route, at least for this class of adjuvants. Nevertheless, the immune responses and protective efficacy induced after i.n. can also serve as a gold standard when evaluating other mucosal routes.

1.4.3. Intravaginal route

Especially for STDs the delivery to the vaginal mucosa constitutes another possible mucosal application route. The i.vag. immunisation in female mice elicits mucosal humoral and systemic immune responses. After vaccination ags are processed by Langerhans cells and presented to T-cells, which migrate to the draining lymph nodes [the iliac lymph nodes (iLN)], where they differentiate into effector-T-cells [129]. When ags are co-administered with certain adjuvants, protective immune responses can be initiated, including IgA and CTL responses [90]. Unfortunately, studies have shown only marginal systemic immune responses after immunisation via this route, in contrast to what observed after i.n. vaccination [112], [113], [114], [115], [91, 116]. On the other hand, vaginal cell composition changes according to the hormonal status. For example, in rodents four stages can be distinguished in the oestrus cycle: diestrus, prooestrus, oestrus, and metoestrus. In this context, the success of i.vag. vaccination seems to depend on which stage of the oestrus cycle is implemented, being more efficient in the dioestrus. This can be explained, at least in part, by differences in the movement of particles across the epithelium according to the oestrus cycle [130]. Movement is most frequent in the dioestrus, less frequent in prooestrus and metoestrus, and almost negligible in the oestrus. This cycle dependent change in protein movement might be related to the change in the predominant cell populations and the thickness of the epithelium at the various stages of the oestrus cycle [131].

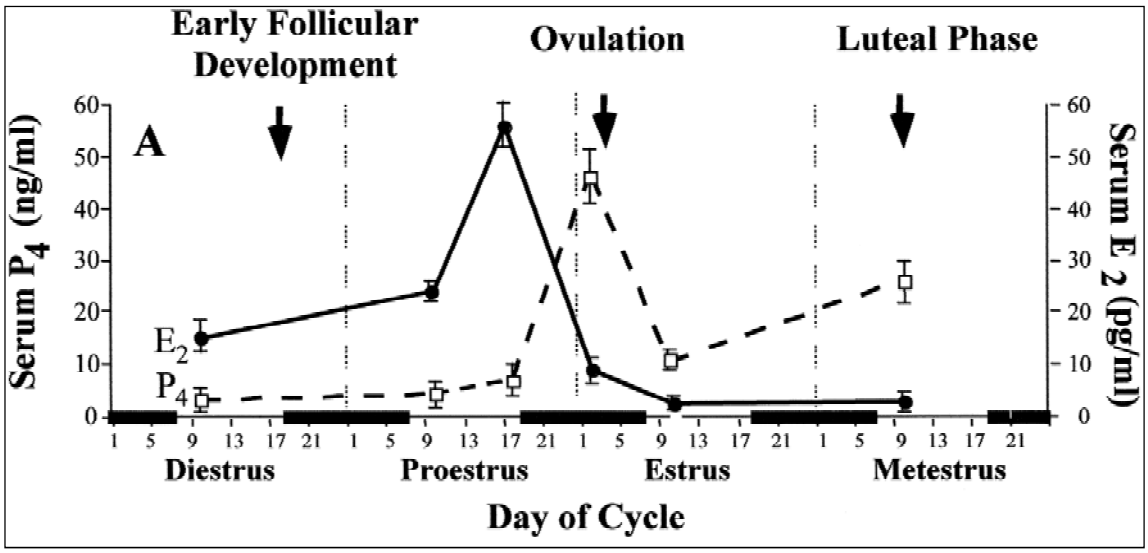

Figure 4: Hormonal changes in the female oestrus cycle in rats [132].
Graphical representation of the changes of serum levels of oestrogen (E_2) and progesterone (P_4) during the four stages of the oestrus cycle.

In the figure from Edwards *et al*., the distribution of serum levels of progesterone (P_4) and oestrogen (E_2) is shown for rats, but the effects are comparable in mice [132], [133]. The prooestrus stage is characterised by increasing oestrogen levels that peak in this stage and then begin to decline. During prooestrus, the number of cell layers in the vagina increases. In vaginal smears, various nucleic epithelial cells are detected. The decline of oestrogen corresponds with increasing progesterone that peaks at the beginning of ovulation. This marks the beginning of the oestrus stage, characterised by proliferation of the basal layer resulting in a very thick epithelium and a large number of anucleated cells. The luteal phase occurs in metoestrus. Here, the oestrogen concentration in sera shows the same basal level as in the oestrus stage and serum concentration of progesterone continues to increase. This stage of the cycle is characterised by a thinner epithelium, anucleated epithelia cells, invading neutrophils and mucus production. Toward the end of dioestrus follicular development starts. Here, the serum levels of progesterone are very low, but the oestrogen concentration is increasing [132]. During dioestrus the epithelium is the thinnest, cornified epithelial cells as well as a high number of neutrophils are present, and mucus is produced. It is during this stage of the cycle, dioestrus, that successful immunisation can occur.

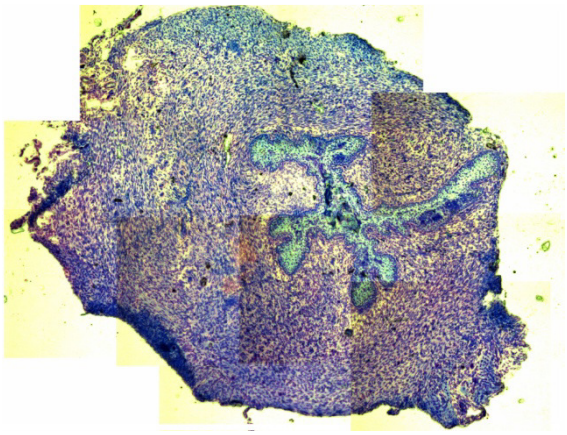

Figure 5: Histological section of vagina, cross section.
The organ was removed from the genital tract and cryosections of ~10 µm thickness were performed. Finally, the section was stained with Diff Quik. In the centre of the picture, the lumen (blue) is visible, containing cornified epithelial cells indicating the oestrus stage of the female cycle. The morphology of the cells is visible, as well as the presence of mucus in the lumen and immune cells, like neutrophils. Merge of pictures, 100x magnification, microscope used: Axioplan, Zeiss.

An obvious and probably the most important limitation of the vaginal immunisation route is that only females can be vaccinated through this route. However, i.vag. immunisation might still represent a valid vaccination route, particularly in the prevention of STDs caused by agents like HSV, HIV, *Chlamydia spp*. or HPV [134], [91], [88]. Thus, it is important to identify other vaccination strategies able to stimulate a similar response pattern to that achieve using the i.vag. route.

1.4.4. Sublingual route

Until recently, the s.l. route has rarely been investigated in vaccine formulations. However, this route is widely accepted for the treatment of allergy disorders [135]. A clinical technique used to induce tolerance is sublingual immunotherapy (SLIT), which has been of special interest for treating patients with certain allergic disorders. SLIT is used to re-develop tolerance to specific allergens. This therapy seems to induce T_{regs} that suppress T-cell responses in the early phase of treatment and anergy and clonal deletion of allergen-specific T-cells in the late phase of treatment [136]. It also seems that prior to SLIT treatment patients have more Th2 than Th1 circulating T-lymphocytes, but SLIT treatment also reverses this ratio of Th2 and Th1 in these patients [137], [135].

Furthermore, no anaphylactic shock or other adverse side effects have been observed in human studies [138], [139]. Given these findings, the s.l. route might be a promising alternative for vaccine administration.

Although s.l. immunisation has similarities to both SLIT and oral immunisation, these processes have crucial differences that must be outlined. On the one hand, both SLIT and s.l. immunisation are used for delivery of a vaccine formulation resulting in the elicitation of protective immune responses. SLIT, however, is used for treating certain allergies with the aim of blocking hyper-responsiveness and promoting suppression, thereby shifting the existing immune response pattern. This is not the case for s.l. immunisation, which is aimed at stimulating efficient adaptive immune responses able to protect against subsequent challenge. To achieve this aim, the ag should be co-formulated with an adjuvant.
Tolerance induction can be prevented by selecting a correct dosage and time schedule. The s.l. immunisation results in the elicitation of high IgA titres locally as well as at distant mucosal compartments, like the genital tract [140].

Histologically, the s.l. mucosa consists of keratinised stratified epithelium covering a lamina propria composed by capillary vessels, fibroblasts and mononuclear cells [141]. The oral mucosa is an immune privileged site with a compact immunological network containing local DCs. According to Cuburu *et al*. [119], the s.l. epithelium contains numerous DCs expressing CCR6, CD80, CD86 and CD40. These surface markers are similar to those of epidermal Langerhans cells. At cellular level, CCR7-CCL19/CCL21-regulated DCs activate T- and B-cells [141]. In the steady state, the epithelium and the lamina propria bears MHC class II^+ and/or $CD11b^+$ cells, which have been shown to increase after s.l. immunisation with CT [141]. The squamous stratified epithelium lines the oral cavity, which is characterised by a large degree of vascularisation and permeability. This in turn facilitates fast ag absorption and direct entry into the bloodstream, thereby circumventing the intestine [109], [142], [143], [144], [145].

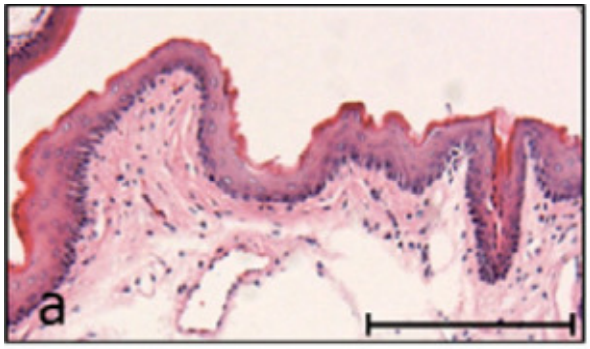

Figure 6: Morphology of sublingual mucosa [138].
Longitudinal cut at the length of the tongue (paraffin-embedded) and stained with haematoxylin-eosin.

The immune response after s.l. immunisation is characterised by the production of sIgA at the port of entry of pathogens, namely the oral cavity. Additionally, due to the compartmentalisation and the "common mucosal immune system" antibody production can also be obtained at distant mucosal sites, such as the vagina [146], [118], [147]. The draining lymph nodes for the s.l. immunisation route are the cLN, where ag-specific T-cell proliferation occurs after vaccination [141]. It has also been reported that in addition to systemic and mucosal antibody responses, s.l. immunisation can also induce cytotoxic $CD8^+$ T-cells in systemic lymphoid organs after vaccination with OVA + CTB [119]. Functional CTL responses have also been observed in the lungs after immunisation via the s.l. route [148].

Vaccination by the s.l. route prevents many of the problems/limitations associated with other mucosal routes, therefore arising considerable interest. For example, s.l. immunisation does not result in retrograde axonal transport, as tested by Song *et al.*, who worked with inactivated and live A/PR/8 virus combined with mCTA/LTB, a chimeric adjuvant resulting from an inactive mutant of the CT A subunit (E112K) and a pentameric B subunit derived from LT [148]. It is important to mention that the receptor for CT, GM-1, is on the surface of cells like axons, which in turn promote the redirection to the CNS [149], [150]. Furthermore, vaccines delivered through this route are not transported across the intestine or liver. Therefore, it is of special interest in the vaccination against infectious diseases because the administered ag is well tolerated and enters directly into the blood stream [142], [143], [144], [145]. Taken together, s.l.

vaccination appears as a very promising vaccination route to elicit efficient protection after mucosal immunisation.

1.5. Role of interleukin-17 in mucosal immune responses

Park *et al.* were the first to describe the Th17 lineage as a T-helper line distinct from Th1 or Th2 cells, which is characterised by IL-17 production. Furthermore, Th17 cell development is independent of cytokines and transcription factors which normally regulate Th1/Th2 cells [151]. However, IL-17 was first characterised in 2005 by Harrington *et al*., who found that IL-23 induces IL-17 secreting $CD4^+$ T-cells [152]. Th17 cells are the primary producers of IL-17, but they are not the only cells [153]. The cytokines IL-17A and IL-17F are produced by various cell types, such as $CD4^+$ T-cells, $CD8^+$ T-cells, Th17 cells, NKT-cells, natural killer (NK)-cells, neutrophils, lymphoid-tissue inducer (LTI)-cells and γδ T-cells [154], [155]. The receptor for IL-17A and IL-17F is IL-17 RA, which is predominantly expressed on hematopoietic cells, endothelial cells, epithelial cells, fibroblasts and osteoblasts [156], [157], [158]. After stimulation of IL-17 RA, these cells begin to produce granulocyte macrophage colony-stimulating factor (GM-CSF), IL-6, IL-8 and mediate granulopoiesis [159]. Th17 cells need three to five days to become induced, NK-cells produce IL-17 after several hours [160]. IL-17 is therefore able to link the innate and adaptive immune responses. IL-17 has pro-inflammatory properties and can act in immunity against extracellular pathogens, some intracellular pathogens and fungi [161], [162]. For example, in mice IL-17 can promote B-cell isotype class switching from IgG to IgG2a and IgG3, which further supports B-cell differentiation [163]. IL-17 also stimulates recruitment of neutrophils to the site of inflammation [159]. In humans, IL-17 leads to secretion of IL-6, IL-8 and granulocyte colony-stimulating factor (G-CSF) in epithelial, endothelial and fibroblastic cells [164]. For example, during early infection (4-8 hours), IL-17 secretion is activated in cells from the innate immune system [160]. Generally, Th17 cells are characterised by expression of CD4 and they bear the transcription factor RAR-related orphan receptor gamma (ROR-γt). Ivanov *et al*. described the orphan

nuclear receptor ROR-γt as the transcription factor for development of Th17 cells from naïve precursor T-cells, which is encoded by the gene RAR-related orphan receptor C (RORC) [165]. The transcription factor RORα has a synergistic relationship, being also required for Th17 differentiation [166], [167]. ROR-γt transcribes the IL-17-encoding genes and RORα further supports Th17 differentiation by promoting IL-17 expression on Th17 cells. The transcription factor RORα is controlled in a signal transducer and activator of transcription protein 3 (STAT-3)- dependent manner [168], [165], [167], [167]. STAT-3 is a protein regulating T-helper cell differentiation, its activation is necessary for induction of ROR-γt [159]. The anti-inflammatory cytokine transcription-growth-factor-β (TGF-β) and IL-6 are responsible for successful stimulation of naïve T-cells leading to Th-17 development by induction of ROR-γt expression and it also controls IL-17 gene expression [169], [170], [166]. In the absence of IL-6, the cytokine IL-21 (secreted by NK- and NKT- cells) can replace IL-6 in Th17 development, *i.e.* IL-21 together with TGF-β is able to activate ROR-γt in cells that differentiate into Th17 cells [171]. IL-23, which is secreted by various APCs, supports Th17 lineage commitment and promotes further IL-17 secretion [166]. The receptor for IL-23, IL-23R, is only expressed by activated or memory $CD4^+$ T-cells.

T_{regs} are the antagonists of Th17 cells and keep the balance of the immune system. They are phenotypically characterised by $CD4^+$ and $CD25^+$ expression and the transcription factor forkhead-box-protein 3 (FoxP3). T_{regs} play a role in self-tolerance, autoimmunity and dampening immune responses [172]. Similar to Th17 cells, the differentiation of naïve T cells into T_{regs} depends on the presence of TGF-β. However, the main difference is that T_{reg} differentiation is independent of the cytokine IL-6. In the initial stages of immune response, immune cells begin to secrete IL-6. This shifts the development of naïve T cells to Th17 cells in order to combat the infection. In the later phase, the production of IL-17 is reduced, thereby facilitating the differentiation of T_{regs}.
These cells are also stimulated to differentiate after TGF-β contact, but only in the absence to IL-6. In the presence of IL-6 naïve T cells develop into Th17,

whereas in the absence of IL-6 naïve T cells develop into T_{regs} [173]. Bettelli *et al*. describes this as the reciprocal relationship of Th17 cells and T_{regs} [173].

The main role of Th17 cells is the clearance of pathogens, particularly in regards to pathogens resistant to the effector functions of Th1/Th2 cells [159]. Th17 are able to induce potent inflammation responses via secretion of cytokines like IL-17 and IL-22. For example, IL-22 acting together with IL-17 (A or F) can induce antimicrobial peptides like β-defensins or several S100A proteins [174]. IL-22 production by Th17 cells can be enhanced by IL-23. Liang *et al*. showed that these cytokines (IL-22 and IL-17A, F) are involved in gene regulation associated with the innate immunity of the skin [174]. IL-17A is also involved in host defence against *Candida albicans* infection [175]. This response is mainly mediated by neutrophil recruitment and by the antimicrobial peptides stimulated by IL-17 [176]. It has been shown that IL-17 also stimulates protective properties during lung infection, as it has been demonstrated in a murine model for *Klebsiella pneumoniae* infection [177]. Here, IL-17 contributes in enhancing secretion of G-CSF and chemokine production, resulting in improved clearance. The pro-inflammatory cytokine IL-17 also plays important roles in mucosal immune responses. It is known that it has an influence on providing defence through innate immune cells until adaptive immune cells are able to control infection [178]. In fact, previous studies indicate that IL-17 is involved in protection against extracellular bacterial infections (*e.g.* in the lung) by recruiting neutrophils and chemokines to the target site [179], [177].
Furthermore, IL-17 seemed also to play a role in HSV infection by promoting Th-responses. Studies performed in a HSV-1 mouse corneal infection model it was observed high local amounts of IL-17 24 hours post-infection [180]. The main-producers were γδ T-cells, being the effect transient. Interestingly, interferon-γ-knockout (IFN-γ–KO) mice showed elevated IL-17 production and a faster rate of corneal opacity [180]. A likely explanation is that IFN-γ regulates the production of IL-17 in an inhibitory manner due to suppression of IL-23 [152]. Mouse corneal fibroblasts express IL-17R on their surface, which when stimulated by IL-17 induces the production of chemokines, such as IL-6 and

macrophage-inflammatory protein 2 (MIP-2), thereby leading to an influx of neutrophils [180].

Additionally, IL-17 is often associated with autoimmune diseases like rheumatoid arthritis (RA) or inflammatory bowel disease (IBD) [181]. Patients suffering from RA show increased IL-17A levels in sera and synovial fluids [182], [183]. This in turn leads to increased neutrophil recruitment and increases inflammation by stimulating the secretion of pro-inflammatory cytokines [181]. In IBD increased amounts of IL-17 are also detected, though the mechanism is not completely understood [184].

1.6. Aim of the thesis

The major aim of this thesis was to investigate the potential of s.l. vaccination as alternative approach to the well described i.n. immunisation strategy to promote local and systemic immune responses, as well as protection at local and distant mucosal sites. In this context, IL-17 as well as Th17 cells seems to play a crucial role in mucosal host defence. However, little is known about these effectors and their function in the genital tract. Thus, a secondary aim was to asses the role of IL-17/Th-17 in protective immunity to vaginal infections.

To this end, it was performed a head-to-head comparison of the immune responses stimulated following i.n., i.vag. and s.l. vaccination using different ags and adjuvants, as well as challenge studies using HSV as a mouse experimental infection model. By selecting the appropriate adjuvants, a strong humoral and cellular immune response can be induced. Vaccination via i.n. route using the potent adjuvant c-di-AMP stimulates mixed Th1/Th2/Th17 humoral and cellular response, which seems to be inefficient in order to protect mice against HSV infection. However, responses can be tailored by using adjuvants which optimise the required responses according to the route of application. In this context, co-administration of α-GalCerMPEG allowed to tailor IL-17 responses following s.l. vaccination, thereby promoting protection against i.vag. challenge with HSV-1. The obtained results demonstrated that s.l.

vaccination in combination with the appropriate adjuvant is very promising approach to promote the elicitation of protective immunity.

2. Material

2.1. Chemicals and reagents

2.1.1. General

2-Mercaptoethanol (50 mM)	Gibco, Invitrogen GmbH, Darmstadt, Germany
2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic) (ABTS)	Sigma Aldrich, Chemie GmbH, Steinheim, Germany
Albumine fraction V, 200 g (BSA)	Carl Roth GmbH, Karslruhe, Germany
Brefeldin A	Sigma Aldrich, Chemie GmbH, Steinheim, Germany
Citric acid	0.1 M (21 g added to 1l aqua dest)
Collagenase D (from *Chlostridium histolyticum*)	100 mg, Cat. No: 11088858001, Roche Diagnostics GmbH, Penzberg, Germany
Concanavalin A (ConA)	Sigma Aldrich, Chemie GmbH, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	100 ml, Euroclone, Euroclone S.p.A. Life Science Division, Pero, Italy
Dispase II (from *Bacillus polymyxa*)	Roche Diagnostics GmbH, Penzberg, Germany
DNase I	100 mg, Cat. No: 11284932001, Roche Diagnostics GmbH, Penzberg, Germany
Ethylendiamintetraacetat (EDTA)	Fluka, Switzerland
Foetal calf serum (FCS)	Greiner Bio-One, USA
L-Glutamine	Gibco, Invitrogen GmbH, Darmstadt, Germany
Paraformaldehyde	Merck, Germany

Penicillin /Streptomycin	Gibco, Invitrogen GmbH, Darmstadt, Germany
Phenylmethanesulfony fluoride (PMSF)	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Saponin	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Trypsin-EDTA	100 ml, PAA, The cell culture company, PAA Laboratories GmbH, Pasching, Austria

2.1.2. Enzyme-linked immunosorbant assay (ELISA)

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic) (ABTS)	Sigma Aldrich Chemie GmbH, Seinheim, Germany; for stock preparation: 300 mg ABTS added to 1 l citric acid [0.1 M], pH= 4.35
ABTS working solution	ABTS (stock) + H_20_2 (30%) (1:1000 v/v)
Blocking solution	PBS + 3% BSA
Carbonate buffer	1.59 g/l Na_2CO_3; 2.93 g/l $NaHCO_3$; pH 9.6
Coating with antigen	2 µg/ml antigen (OVA or β-Gal) in carbonate buffer
Detection antibody	IgG 1 biotin conjugated, Cat. No: 1072-08, Southern Biotech, USA; used dilution 1:5000
	IgG 2a biotin conjugated, Cat. No: 1082-08, Southern Biotech; used dilution 1:10.000
	IgG 2b biotin conjugated, Cat. No: 1092-08, Southern Biotech, USA; used

	dilution 1:10.000
	IgG 3 biotin conjugated, Cat. No: 1102-08, Southern Biotech USA; used dilution 1:5000
	IgA biotin conjugated, Cat. No: 1040-08 Southern Biotech USA; used dilution 1:5000
	IgM biotin conjugate, Cat. No: B-9265, Sigma Aldrich; used dilution 1:5000
Hydrogen peroxide, 30%	Fluka, Switzerland
Standard curve and total IgA coating IgA	Anti-mouse IgA (2 µg/ml working solution in carbonate buffer), Cat. No: M-8769, Sigma Aldrich Chemie GmbH; Steinheim, Germany
Standard curve IgA	Standard mouse IgA, kappa, Cat. No: M-1412, Sigma, beginning with 0.2 µg/ml in PBS + 3% BSA
Streptavidin-horseradish peroxidase (HRP)	Cat. No: 554060, BD Pharmingen, USA, 1:1000 in PBS + 0.1% Tween 20 + 1% BSA
ELISA plate, Microlon 96 well, flat bottomed, high binding	Greiner bio one, Frickenhausen, Germany
PS-coverplate for microplate	Greiner bio one, Frickenhausen, Germany
Tween 20	Greiner bio one, Frickenhausen, Germany

2.1.3. Enzyme-linked immune spot technique (ELISPOT)

Acetate solution (0.1 mM)	148 ml of 0.2 mM acetic acid to 352 ml of 0.2 mM sodium acetate; adjust volume to 1 l with water; pH 5.0

3-amino-9-ethylcarbozole (AEC)	For stock solution: 150 mg AEC + 10 ml DMF
Blocking solution	Royal Park Memorial Institute (RPMI) complete
Capture antibody	IFN-γ (Cat. No: 51-2525KZ) IL-2 (Cat. No: 51-1816KZ) IL-4 (Cat. No: 51-1819KZ), all BD Biosciences IL-17 A (Cat. No: 16-7175-68), ebioscience
Coating buffer	1x PBS
Detection antibody	IFN-γ (Cat. No: 51-1818KA) IL-2 (Cat. No: 51-1817KZ) IL-4 (Cat. No: 51-1804KZ), all BD Biosciences IL-17 A (Cat. No: 13-7177-68), ebioscience
Di-methylformamide (DMF)	Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
Hydrogen peroxide, 30%	Fluka, Switzerland
MultiScreen ^{HTS} filter plates, 96 well	Millipore, USA
Substrate solution	333.3 ml AEC stock solution + 10 ml 0.1M Acetat solution, sterile filtrated, 5 µl H_20_2 (30%) are added immediately before use.
Streptavidin-HRP	BD Biosciences Pharmingen, USA
Wash buffer I	1 x PBS containing 0.05% Tween 20
Wash buffer II	1 x PBS
Wash buffer III	1 x PBS + 10% FCS

2.1.4. Proliferation

[methyl-3H]thymidine solution (5.0 Ci/mmo	Amersham, Germany
Plate paper	Perkin Elmer, USA
Melt-on scintillation wax, Meltilex A	Wallac, Finland
Cassette Filtermat	Perkin Elmer, USA
Filter cell harvester, Filtermat A	PerkinElmer, USA

2.2. Media and buffer

2.2.1. General

Ammonium chloride-potassium hydrogen carbonate (ACK)-buffer	155 mM NH_4CL 8.3 g/l (1 M= 53.49 g/l) 10 mM $KHCO_3$ 1 g/l (1 M= 100.1 g/l) 1 mM ETDA 0.37 g/l (1 M 372.2 4 g/l) add 1l aqua dest. pH 7.3 with 1 M NaOH autoclave or sterile filtrate
Ampuwa, 1000 ml	Fresenius Kabi, Bad Homburg, Germany
Coulter®Isoton® II Dilutent	Beckman Coulter
Dulbecco's modified eagle medium (DMEM)	Cat. No: 419657039, Gibco /Invitrogen GmbH, Darmstadt, Germany
Freezing media	FCS + 10% DMSO
Ficoll Lympholyte®- M	Cedarlane, USA
MilliQ water filter	Millipore, Germany
Phosphate buffered saline (PBS)	Dulbeccos, 1x /10x, PAA, Pasching, Austria
PBS/BSA	1x PBS + 3% BSA

PBS/FCS (Lavage buffer)	1x PBS + 5% FCS
PBS + paraformaldehyde (PFA)	1x PBS + 2% PFA
Permeabilisation buffer	1 x PBS + 0.5% BSA + 0.5% Saponin
RPMI 1640, with L-Glutamine	Cat. No: E15-840, PAA, Pasching, Austria; also used: Cat. No: 31870, Gibco Invitrogen; L-glutamin, penicilline/streptomycine and 10% FCS are added; called then RPMI complete

2.3. FACS-analysis

2.3.1. Antibodies against murine antigens

Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)	Carboxyfluorescein diacetate succinimidyl ester, stock: 10 mM in DMSO, Invitrogen, Darmstadt, Germany
Fc-Block	Purified rat anti-mouse CD16/CD32, 0.5 mg/ml, Cat. No: 553142, BD Biosciences, USA
CD3	Clone: 145-2C11, BD Biosciences, USA
CD4	Clone: L3T4, RM4-5, BD Biosciences, USA
CD8	Clone: 53-6.7, BD Biosciences, USA

2.3.2. Reagents and chemicals/-iterative chip based cytometry (ICBC) chips

Histofix	Carl Roth, Karlsruhe, Germany

Axioimager M1, motorised	Carl Zeiss, Goettingen, Germany
Permeabilisation buffer	Ebioscience, USA
Cell adhesive microfluidic chips	Special preparation technique by Dr Christian Hennig, Department of Pneumology, Allergy and Neonatology, Hannover Medical School, Germany
Parafilm	Laboratory film, American National Can™, Neenah, USA
Glass slides for sections	18 x 18 mm, Menzel-glasses, Menzel GmbH & Co KG, Braunschweig, Germany
CD4	Clone: RM4-5, BD Biosciences, USA
CD8a	Clone: Ly-2, BD Biosciences, USA
CD11b	Clone: M1/70, BD Biosciences, USA
CD11c	Clone: HL3, BD Biosciences, USA
CD19	Clone: 1D3, BD Biosciences, USA
CD45	Clone: (LCA Ly5) (30-F11), BD Biosciences, USA
TCR-γδ	Clone: GL3, BD Biosciences, USA
F4/80	Clone: BM8, BD Biosciences, USA
FoxP3	Clone: FJK-16F, BD Biosciences, USA
Gr-1	Clone: LyC/G RB6-8C5, BD Biosciences, USA
IL-17	EBIO17B7, ebiosciences, USA
RORγt/RORC2/NR1F3	Clone: 600380, R&D Systems, USA

2.3.3. Histological staining

Diff-Quik staining kit	Cat. No: 130832, Medion Diagnostics, Duedingen, Switzerland
Giemsa	Fluka, Switzerland
May-Grünwald	Merck, Darmstadt, Germany

2.3.4. Kits

HEK-BLUE™ LPS Detection Kit	InvivoGen, USA
Mouse Th1/Th2 10 plex FLOW Cytokine kit	Bender Med Systems, USA

2.4. Antigens and peptides

β-D-Galactosidase galactohydrolase	from *Escherichia coli*, Roche Diagnostics GmbH, Mannheim, Germany
β-Gal peptide	Synthesised by Dr Tegge, Helmholtz-Zentrum für Infektionsforschung, Braunschweig, Germany
OVA	Endograde, lypophylised, Hyglos, Bernried am Starnberger See, Germany
OVA peptide	Synthesised by Dr Tegge, Helmholtz-Zentrum für Infektionsforschung, Braunschweig, Germany

2.5. Adjuvants

α-Galactosylceramide MPEG	Synthesised at Helmholtz-Zentrum für Infektionsforschung, Braunschweig, Germany
BPPcysMPEG	Synthesised at Helmholtz-Zentrum für Infektionsforschung, Braunschweig, Germany
c-di-AMP	Synthesised at Helmholtz-Zentrum für Infektionsforschung, Braunschweig, Germany

CTB	CTB subunit from *Vibrio cholerae*, Cat. No: C9903-1MG, Sigma-Aldrich
Lipopolysaccharide from *E.coli*	*E. coli* 026:B6, L2651-1MG, 107K4046, Sigma-Aldrich

2.6. Cell lines

Vero cells are a cell line were originally isolated from kidneys of green monkeys [185].

2.7. Virus

The Herpes simplex virus 1 strains LV (large variant) LacZ and LV wild type were obtained from our cooperation partner, Dr Peggy Marconi, University of Ferrara, Italy. The LV wild type is a HSV-1 strain Kos without mutations and was originally isolated of a patient [186]. The LVLacZ is strain LV with lacZ integrated into the unique long (UL) 41 that codify for virion-host shut off protein (VHS), this gene is consequently interrupted. Also the stains 17SynIGR20HE/gJZ and 17SynIGR20Luc were provided from the cooperation partner. The 17SynIGR20HE/gJZ is a HSV-1 strain 17 with luciferase into the intergenetic region 20 (IGR20) and green fluorescent protein (GFP) into unique short 5 sequence locus (Us5) that codify for glycoprotein J. The 17SynIGR20Luc has luciferase into IGR20.

2.7.1. Stock preparation reagents

Solutions:	
Optiprep™	Cat. No: 1030061, Axis-Shield, Heidelberg, Germany
Solution B	2.8 ml 5 M NaCl; 6 ml HEPES 1 M, pH 7; 1.2 ml EDTA 0.5 M, pH 8.0; bring to 100 ml and sterile filtrate, keep at 4°C
Solution C	2.8 ml 5 M NaCl; 1 ml HEPES 1 M, pH 7; 200 µl EDTA 0.5 M, pH 8.0; bring to 100 ml and sterile filtrate, keep at 4°C
Solution D	5 volume Optiprep + 1 volume solution B; fresh preparation before use
Solution E	Virus (4 ml) + solution C, total 4.8 ml
Solution F	Top-up solution; 1.27 ml solution C + 1 ml solution D. Equals the final concentration of 22% Optiprep that the gradient will have; fresh preparation before use
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Hepes)	Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
NaCl	Merck, Darmstadt, Germany
EDTA	Sigma-Aldrich, Chemie GmbH, Steinheim, Germany

2.7.2. Material and instruments for virus stock preparation

Cell scraper 30 cm	TPP, Switzerland
Centrifugation tubes	Oak Ridge tubes; Nalgene, USA
Ultracentrifugation tubes	Cat. No: 344059, Beckman Coulter, USA

Crystal violet-methanol	crystal violet, Serva, Heidelberg, Germany; solution was used as: 5% crystal violet in 100% ethanol + 25ml H_2O + 25 ml methanol
Methanol	Sigma-Aldrich, Chemie GmbH, Steinheim, Germany
Carboxymethylcellulose	Sigma-Aldrich, Chemie GmbH, Steinheim, Germany Germany
Rotor SS34, T875, Th-641	Sorvall; Thermo Scientific
Ultracentrifuge OTD combi	Sorvall; Thermo Scientific

2.8. Mice

The female BALB/c H-2^d and C57BL/6 H-2^b mice, 6-8 weeks of age, were purchased from Harlan (Harlan-Winkelmann GmbH, Horst, Netherlands). The mice were housed under specific pathogen free (SPF)-conditions and according to local and European community guidelines. The permission numbers for animal experiments are 33.42502-084/06 and 33.11.42502-04-017/08/2007. A maximum of five mice were kept in each cage with water and food *ad libitum*.
Also BALB/c from the company Janvier were used and kept as described for BALB/c mice from Harlan.

C57BL/6J-IL-17a $IL17^{ftm1Prnz}$ knock-out-mice on C57BL/6 background were kindly provided by PD Dr Immo Prinz, Institute for Immunology, Hannover Medical School. These mice are not able to produce IL17A and IL17F.
The mice were then breed in the animal facility of the Helmholtz-Zentrum für Infektionsforschung, Braunschweig.

OT II transgenic mice were bred and maintained at the animal facility of the HZI, Braunschweig. They have a transgenic TCR on $CD4^+$ T cells recognising OVA-peptide (323-339) [187].

2.9. Chemicals

2.9.1. Chemicals for murine application

Depo-Clinovir	Depo-Clinovir, 150 mg, medroxyprogesteronacetat (pregn-4-ene-3,20-dione, 17-(acetyloxy)-6-methyl-, (6α-)), 1 single-use syringe to 1 ml, N3, sterile aqueous suspension for intramusculary injection, PHARMACIA GmbH a company of the Pfizer-Gruppe, Karlsruhe, Germany
Isofluorane	Baxter Deutschland GmbH; Unterschleißheim, Germany
Ketamine	Ketamin Inresa, 50 mg/ml injection solution, 10 ml, Inresa Arzneimittelwerk GmbH, Freiburg, Germany
Ketamine/Rompun	Used for i.p. application as: 1 ml Ketamine + 0.5 ml Rompun + 8.5 ml NaCl, 100 µl working solution per 10 mg bodyweight
Pilocarpine hydrochloride	Sigma-Aldrich, Chemie GmbH, Steinheim, Germany, used at a concentration of 500 µg/kg mouse; 100 µl/mouse were injected i.p.
Rompun	2% injection solution Xylazin (as Xylazinhydrochloride), 25 ml, Bayer Health Care, Bayer Vital Gmbh Geschäftsbereich Tiergesundheit, Leverkusen, Germany

2.10. Consumables

15 ml Cellstar® tubes	Greiner bio-one, Frickenhausen, Germany
50 ml Cellstar® tubes	Greiner bio-one, Frickenhausen, Germany
6-well flat bottomed plates	TPP, Switzerland
96-well flat bottomed plates	TPP, Switzerland
96-well round bottomed plates	TPP, Switzerland
Buttoned cannula	Laboratory equipment
Cell strainer 100 µm Nylon BD Falcon	BD Bioscience, USA
Combitips, 2.5 ml, 5 ml, 10 ml	Eppendorf, Germany
Container for cryo-cooling	Nalgene® Mr.Frosty™ Cryo 1°C Freezing Container, USA
Cotton swab	For taking vaginal smears; Cat. No: 1032648, Heinz Herenz, Medizinalbedarf GmbH, Hamburg, Germany
CryoTube™ Vials 1.8 ml	Nunc, Denmark
Cytobrush medex	Medesign I.C. GmbH, Dietramszell, Germany
FACS-PE 5 ml tubes	BD Biosciences, USA
Glass slide	Menzel-Gläser, Super-Frost, geschliffen, 76x26 mm, Menzel GmbH & Co KG, Braunschweig, Germany (for smears)
Injection needle 1ml 30G x ½”	Omnican F, B Braun, Melsungen, Germany
Injection needle 1 ml, 5 ml, 10 ml	B Braun, Melsungen, Germany
Micro haematocrit tubes for sera collection, 75 µl	Brand GmbH+ Co. KG, Wertheim, Germany
Multistepper Matrix	Thermo Scientific, Karlsruhe, Germany

Parafilm	Laboratory film, American National Can™, Neenah, USA
Pasteur pipettes (glass)	Brand GmbH + Co. KG, Wertheim, Germany
Petri dishes	Greiner bio-one, Frickenhausen, Germany
Pipet tips 200 µl	Easy load, Greiner bio-one, Frickenhausen, Germany
Pipet tips 10 µl, 100 µl, 200 µl, 1000 µl	Thermo Scientific, Karlsruhe, Germany
Pipettes Eppendorf	Eppendorf, Hamburg, Germany
Pipettes Fintip	Thermo Scientific, Karlsruhe, Germany
Plastic pipettes, 5 ml, 10 ml, 25 ml	Serological pipette, Roth, Karlsruhe, Germany
Safe lock reaction tube 0.5 ml, 1 ml, 2 ml	Eppendorf, Hamburg, Germany
Scissors	Aesculap, B Braun, Melsungen, Germany
Serological pipette	Roth, Karlsruhe, Germany
Syringe for lung lavage	Winged needle infusion, 18G/ 1 ¾ “ Interocan, Luer Lock, B Braun, Melsungen, Germany
Syringe for spleen	2 ml Luer Solo, Inject®, B Braun, Melsungen, Germany
Tissue culture flask 75 cm^2, 150 cm^2	TPP, Switzerland
Cytobrush medex	Medesign I.C. GmbH, Dietramszell, Germany

2.11. Instruments

Biostack	Bio-Tek, BioTek Laboratories GmbH, Bad Friedrichshall, Germany

Cell counter Z2 Coulter Particle Count and Size analyser	Beckman Coulter, USA
Ultracentrifuge OTD combi	Sorvall
Centrifuge Biofuge pico	Heraeus Instruments, Osterode, Germany
Centrifuge Megafuge R 40	Heraeus Instruments, Osterode, Germany
Centrifuge Multifuge 3 S-r	Heraeus Instruments, Osterode, Germany
Cryotom	Rotation microtom, Thermo Scientific
CTL Immunospot® analyser	CTL-Europe GmbH
ELISA Reader	Synergy 2, Bio-Tek, BioTek Laboratories GmbH, Bad Friedrichshall, Germany
ELISPOT Reader, ImmunoSpot, Series 3A	Cellular Technology Ldt., USA
Flow Cytometer FACSCanto	BD Biosciences, USA
Freezer	-20℃, Liebherr
Freezer	-80℃, Thermo Scientific Karlsruhe, Germany
Fridge	4℃, Liebherr
Cell harvester, ICH-110	INOTECH, Switzerland
Handystep	Brand, Germany
Heating stirrer	IKA Labortechnik, Braunschweiger Laborbedarf
Incubator	Heraeus Instruments, Osterode, Germany
Micropipettor	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Microscope, Axioplan + Axiocam	Zeiss, Jena, Germany
Microscope Axioimager M1	Zeiss, Jena, Germany
Microscope Motic AE 20	Motic Instruments, Germany

Microscope Nicon Eclipse E200	Nicon, Duesseldorf, Germany
Microwave	Sharp, Germany
pH-meter	Hannah Instruments Deutschland GmbH, Kehl am Rhein, Germany
Pipetboy	Thermo Scientific, Karlsruhe, Germany
Plate pipetting system	Precision 2000, 96/384 well automated microplate pipetting system, Bio-Tek, BioTek Laboratories GmbH, Bad Friedrichshall, Germany
Plate washer	ELx 405, Bio-Tek, BioTek Laboratories GmbH, Bad Friedrichshall, Germany
Scale TE 1502S	Satorius, Goettingen, Germany
Scintillation Counter Wallac 1450 MicroBeta TriLux	PerkinElmer, Finland
Shaker	Janke & Kunkel, IKA Labbortechnik, Staufen, Germany
Sonicator	Elmasonic one, 35kHz, Elma GmbH & Co. KG, Singen, Germany
Sterile hood Hera Safe	Thermo Scientific, Karlsruhe, Deutschland
Vortex	Vortex Genie-2, Scientific Industries, Omnilab
Water bath	Köttermann, Braunschweiger Laborbedarf, Braunschweig, Germany

2.12. Software

CellhopperX, IR-engine 3.1	Software of Dr. Christian Hennig, Hannover Medical School, Germany
FACSDIVA	Becton Dickinson, USA
Endnote X4	EndNote X4, Thomson Reuters
FlowCytomix Pro software	Bender MedSystems, Vienna, Austria

Gen5 version 1.06	Bio-Tek Instruments, Inc., Bad Friedrichshall, Germany
Graph Pad Prism version 5.04	Prism 5 for Windows, Version 5.04, GraphPad Software, Inc., La Jolla, USA
ImmunoSpot image analyzer v3.2	Bio-Tek Instruments, Inc., Bad Friedrichshall, Germany
Multi32 Coulter Z2 Accu Comp Version 3.01, 2000	Beckman-Coulter, USA

3. Methods

3.1. Methods for cell culture/*in vitro* experiments

3.1.1. Isolation of mouse vaginal immune cells

First, mice were killed by either CO_2 asphyxiation or cervical dislocation. Then, with the mouse on its back and using a tiny pair of scissors, deep vertical cuts were made around the vagina. The vagina was then removed by pulling with rounded tweezers. It was important that the uterus was visible, thereby indicating that the vagina had been completely removed. The organ was then placed in a 1.5 ml Eppendorf tube containing 500 µl of dispase (4 mg/ml) in PBS and incubated for one hour at 37°C. The organ was then cut into 0.5-2 mm fragments using scissors and incubated in 1 ml of a solution containing DNAse I (30 µg/ml), collagenase D (0.425 mg/ml), and dispase (4 mg/ml) in PBS for 45 minutes, shaking at 300 rounds per minute (rpm). After incubation, the digested organ was pushed through a 100 µm mesh screen and into a 50 ml reaction tube containing 10 ml RPMI complete (containing additives, see 2.2.1), which was then centrifuged for eight minutes at 314 g. The supernatant was further discarded and the cells were washed a second time in 1 ml PBS. The final pellet was ready for analysis (*e.g.* FACS staining or Cytospot).

3.1.2. Determination of cell number and vitality test

Cell number was determined by an automated cell counter (BC). First, a counting cuvette was filled with 10 ml Coulter®Isoton® II Dilutent, then 10 µl of cells were added (~1:1000 dilution). Before analysis, the cuvette was carefully inverted three times and the counting parameters were entered into the system's software (dilution factor, cell size). After counting, a histogram of counted cells appears.

From the histogram, cell number was determined by gating based on its size, which excludes cell debris from the cell count. Cell vitality could be further determined using trypan blue. For this, a Neubauer cell chamber was used and a cell solution containing 10 µl (10 µl cell solution + 10 µl of trypan blue) was added to the chamber. Trypan blue is an acidic staining substance and its anion is binding to cell proteins. It can only enter through destroyed membranes and therefore only dead cells become blue. The percentage of dead cells is determined by counting the number of dead cells per 100 cells.

3.1.3. Lysis, freezing, and thawing of cells

Erythrocytes (*e.g.* spleen erythrocytes) in samples were lysed by adding 3 ml of ACK-buffer and pipetting up and down with a 5 ml pipette constantly for one minute. During lysis, the solution changed from dark red to cherry-red. Lysis was stopped by adding 30 ml RPMI complete and centrifuging at 4°C for ten minutes at 314 g. The supernatant was then discarded and the cells were washed a second time in 10 ml PBS. The final pellet was then ready to be counted or frozen.

To freeze the cells, the pellets were resuspended in 900 µl of FCS with 20% DMSO and placed in cryo vials. The vials were then placed in special 1°C freezing containers, Nalgene® Mr.Frosty™ Cryo 1°C, which were stored at -80°C for 24-48 hours. After 24-48 hours, the vials were placed in liquid nitrogen (-196°C) and stored until processing.

To thaw the cells, the vials were first removed from liquid nitrogen and briefly placed on ice. They were then rapidly thawed by gentle agitation in a 37°C water bath. Once thawed, the toxic DMSO was quickly removed by washing the cells with 5 ml of RPMI complete and centrifugation for five minutes at 314 g, 4°C. The DMSO containing supernatant was then discarded and the cells were washed a second time before being added to the appropriate growth media.

3.1.4. *In-vivo*-CFSE-proliferation

The donor animals for the adoptive transfer are OT-II mice, which have a transgenic T-cell-receptor on $CD4^+$ T-cells recognising the $OVA_{323-339}$ epitope. The spleen and various lymph nodes were extracted from these transgenic animals and a single-cell suspension was prepared (see 3.3.6.1). Then, the cells resuspended at a concentration of $2x10^7$ cells/ml were stained with CFSE. To this end, an equal volume of CFDA-SE (20 µM) in PBS was added to the cell suspensions, which were then incubated for five minutes at room temperature (RT) in the dark. Next, an equal volume of heat-inactivated FCS was added to stop the reaction. After incubating for another five minutes at RT in the dark, cells were centrifuged at 314 g, washed with RPMI complete media, and resuspended in PBS at a concentration of $1x10^8$ cells/ml.

Before immunising, the stage of the cycle was determined in C57BL/6 recipient mice by vaginal smears stained with Pappenheim-staining. A total amount of $1x10^7$ cells in 100 µl were then injected intravenously (i.v.) to the recipients. After injecting the donor cells, the animals were immunised by i.vag. route using OVA-protein (50 µg) + BppCysMPEG (0.5 µg) according to each stage of the oestrus cycle. On day five after transfer, animals were sacrificed. The spleens were removed, single cell suspensions were prepared (see 3.3.6.1) and cells were stained for CD4 (see 3.2.1). Finally, cell suspensions of spleen cells were analysed on FACSCanto, gating on $CD4^+$ T-cells. Because CFSE stains plasma proteins in cells, the fluorescence intensity is reduced by 50% each time that cells are divided following proliferation in response to the OVA-protein.

3.1.5. HEK-Blue™ LPS detection

To determine if the adjuvants used in this study were contaminated with LPS, the HEK-BLUE™ LPS detection kit was used, according to manufacturer's instructions. The endotoxin LPS is the major cell wall component of Gram-negative bacteria which is sensed by PRRs and can induce the activation of NF-κB and the production of pro-inflammatory cytokines (*i.e.* IL-1 or TNFα) by binding to the TLR 4 receptor. The HEK-Blue™ LPS Detection Kit enables the

detection of the contamination level of LPS in the used stock solutions. HEK-BlueTM-4 cells are engineered HEK293 (Human Embryonic Kidney cells) cells stably transfected with multiple genes involved in TLR 4 recognition that include TLR 4 and the co-receptors MD2 and CD14. The kit is very sensitive for the detection of very low LPS concentrations (0.3 ng/ml). As positive control, LPS derived from *E.coli K12* strain was used in different concentrations in the range of 0.1 ng/ml up to 100 µg/ml. The ag stock solutions (*e.g.* β-Gal, OVA) were tested at three different concentrations (2.5, 0.25, and 0.025 µg/µl) in triplicates and had a LPS contamination of 5 ng/ml at a concentration of 50 µg/ml. Also the synthetic adjuvant stock solutions were analysed, α-GalCerMPEG or c-di-nucleotides (*i.e.* c-di-AMP), which were used for our *in vivo* experiments and showed that even in 5 to 10-fold higher concentrated samples (50 µg) no LPS contamination was observed, respectively.

3.2. Flow Cytometry

3.2.1. Direct surface staining

The cells to be stained were initially resuspended at a concentration of $5x10^6$ cells/ml in PBS. Then, 100 µl of this cell suspension were added to a 5 ml-FACS-tube, to which 1 µl of Fc-block was added, being the solution further incubated for five minutes at 4°C. The antibody was then added according to the required dilution and incubated for 20-30 minutes at 4°C in the dark. A small volume of cells were left unstained (~ 200 µl) and used as a negative control for setting the fluorescence activated cell sorter (FACS) gating parameters. After staining, the cells were washed by adding 3 ml PBS and subsequent centrifugation for five minutes at 314 g (4°C). The washing step was then repeated and the supernatant decanted. The sample was ready for analysis on FACSCanto.

3.2.2. Intracellular phenotyping and cytokine determination

Restimulated cells were used for intracellular phenotyping. The restimulation of cells was carried out as it is described for the proliferation test (see 3.7.3) in 96-U-bottom plates. In the case that surface markers must also be stained, direct surface staining was performed in advance. After washing restimulated cells in PBS (see 3.2.1), cells were fixed with 200 µl of PBS + PFA (2%) per well. Cells were then incubated for 30 minutes to 24 hours at 4°C, followed by centrifugation for five minutes at 314 g. The supernatants were then decanted and the cells were washed by adding 200 µl of permeabilisation buffer (PBS + 0.5% BSA + 0.5% Saponin) to each well. This was followed by incubation for 45 minutes at 4°C. The cells were then centrifuged again and the supernatant was decanted. Next, 50 µl of the titrated antibody was added according to the required dilution in permeabilisation buffer and incubated for 30 minutes at 4°C. After incubation, 200 µl of perm buffer were added to the cells, which were then centrifuged for five minutes at 314 g (4°C) and the supernatant was decanted. This step was then repeated two more times: once again with permeabilisation buffer and a second time with PBS. Finally, 100 µl of PBS were added to each well and the cells were transferred to FACS tubes. The samples were then ready for FACS analysis on FACSCanto.

3.2.3. Chipcytometry

An iterative chip-based cytometry (ICBC) method was used that allows analysis at single cell level with a virtually unlimited set of markers per cell. This concept was developed by Dr Christian Hennig, Department of Pneumology, Allergology and Neonatology (laboratory of Professor Gesine Hansen, Hannover Medical School), who also analysed the chips used in this study. The method is described by Hennig *et al.* in 2009 [188]. Briefly, cells were resuspended in 5 µl PBS and applied to cell-adhesive microfluidic chips. Cells were allowed to self-immobilise for five minutes on the chip surface, followed by fixation of the cells using Histofix. Thereafter, markers were analysed in a cyclic staining-imaging-bleaching process by automated quantitative microscopy on a motorised

Axioimager M1 (Zeiss). After analysis of the surface markers, cells were permeabilised using permeabilisation buffer for measuring intracellular markers. For surface markers, antibodies were diluted 1:10 in PBS and incubated on the cells inside the chip for five minutes at RT, followed by washing the channels with 200 µl PBS. For intracellular markers, antibodies were diluted in permeabilisation buffer and incubated for one h at RT, again followed by a washing step using 200 µl of permeabilisation buffer. The imaging process, image-recognition and image-processing, as well as data visualization were controlled by an in-house developed software (CellhopperX, IR-engine 3.1).
To visualise the complexity of the resulting marker-cell combinations and cell-populations, as generated by chipcytometry, hierarchical clustering of cytometric data was used. Before clustering, the fluorescence values of different markers were normalised to each other in order to minimise the influence of different antibody labellings and stains. Agglomerative average-linkage clustering was used as clustering algorithm.

3.2.3.1. Sample preparation for chipcytometry

Chipcytometry was used to analyse spleen cells of immunised mice. In brief, the spleens were extracted and a single cell suspension was prepared with lysis of erythrocytes (as described in 3.1.3 and 3.3.6.1). Cells were seeded in 96-well-U-bottom plates at a concentration of $5x10^5$ cells/well for restimulation. The cells were then restimulated with the appropriate ag (*e.g.* β-Gal) at a concentration of 40 µg/ml for 48 hours at 37°C, 5% CO_2. After 44 hours, Brefeldin A (1 µg/ml) was added to prevent further cytokine secretion. This step is necessary to detect cytokines at the intracellular level of the cell. Finally, the cells were washed with PBS (10 min, 300 g, 4°C) and filtered through a 35 µm cell-strainer and $2x10^6$ cells contained in 10 µl were loaded on the chip. A staining procedure was then conducted, as described by Hennig *et al*. [188].
Chipcytometry is also valid for histological sections of tissues. Here, the method was applied to cryo-sections of vaginas isolated from immunised mice. The sections were 4 to 6 µm thick and they were placed onto 18 mm x 18 mm glass slides with two 2 mm parallel bars of parafilm affixed to the backside of the slide.

This slide was then placed on a larger (26 mm x 76 mm) glass slide with a chip-specific channel, sandwiching the vagina in a flow chamber between the glass slides.
The markers investigated were listed in 2.3.2.

3.2.4. Cytokine array (Mouse Th1/Th2 10-plex Flow Cytomix)

The cytokine array was performed according to the manufacturer's instructions. The system is comparable to a sandwich-ELISA. The kit contains beads coated with antibodies binding to certain cytokines and also beads which are coated with a biotinylated detection antibody. These beads bind to each other and the cytokines from the sample is bound to the antibody-coated bead, consequently the sample is sandwiched. Then the complexes are stained with streptavidin-phycoerythrin (PE) and can be analysed in the FACS. A standard is inserted in the kit allowing quantitatively determination of cytokines.

3.2.5. Acquisition of samples using FACS

The FACS allows analysis of single cell suspensions stained with fluorescent colours. The cells are aspirated by the nozzle and in fluid sheath covered. In this flow, they pass through different lasers. The forward- and side scatter discriminate cells according to their size and granularity. With the staining of cells by antibodies conjugated with fluorescent dyes, the molecules of interest according to cells can be analysed.

3.3. Methods with laboratory animals and handling of mice

3.3.1. Marking of mice

Mice were marked by cutting a fragment of their earlobe(s). Generally, no marking indicated "Mouse 1", a marking on the right ear indicated "Mouse 2", a

marking on the left ear indicated “Mouse 3”, a marking on both ears indicated “Mouse 4”, and a triangular notch on one ear indicated “Mouse 5”.

3.3.2. Taking blood and serum preparation

To collect blood, mice were tightly held behind their neck causing an accumulation blood in this region. The retro-orbital venous plexus was then punctured using a glass capillary. Bleeding was stopped by removing the capillary and releasing the mouse. Using a micropipetter, the blood was transferred to an Eppendorf tube and allowed to stand first for 60 minutes at 37°C and then for 60 minutes at 4°C for agglutination. To ensure proper sera separation, the tube was centrifuged for five minutes at 3000 g, 4°C. The serum was then collected and stored at -20°C until it was processed.

3.3.3. Strategies to synchronise the murine oestrus cycle

3.3.3.1. DEPO-treatment

For synchronising the oestrus cycle of mice, 2 mg of DEPO-Clinovir was injected subcutaneously (s.c.). After five days, the mice’s hormone levels were synchronised. Their cycles were then arrested for a period of about two weeks in the dioestrus stage, which is characterised by a high amount of mucus, neutrophil granulocytes, and keratinised epithelia cells in the vagina. To make sure that cycles had been successfully arrested, vaginal smears were stained with Diff-Quik and then viewed under a light microscope (20x, Nicon Eclipse).

3.3.3.2. Whitten-effect for synchronisation of the oestrus cycle

To mimic the most natural synchronisation process of their hormonal cycles, female mice were housed in cages originally used by male mice. This type of synchronisation via the odour of hormones, however, was not successful.

3.3.4. Immunisation/challenge strategies

3.3.4.1. Intranasal immunisation

For intranasal immunisation, mice were anesthetised by inhalation of Isoflurane to prevent antigen loss by sneezing. After being anesthetised, mice were gently held at the neck with their body hanging vertically and 10 µl of solution were then pipetted into each nostril.

3.3.4.2. Sublingual immunisation

For sublingual immunisation, mice were first anesthetized with Ketamine/Rompun (i.p.; 100 µl per 10 mg of bodyweight). Then, the vaccine formulation was gently delivered under the tongue using a 10 µl pipette. During administration, the mouse was fixed in a vertical position with its head at a 90° angle to its body, which helped to ensure that the formulation was delivered sublingually and not oro-gastrically. To further ensure that the vaccine was not ingested, the mice were returned to their cages in a sitting position with their head lying onto their stomach (see photos below).

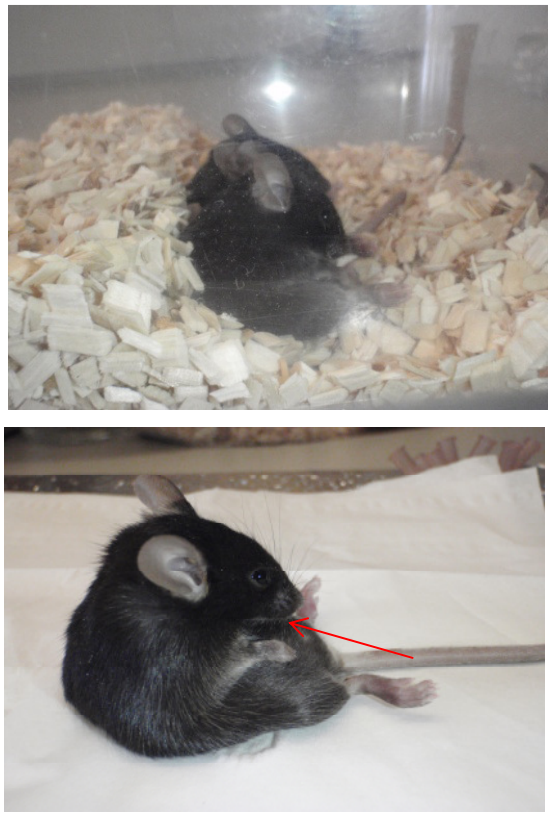

Figure 7: Mice were under narcosis and placed in this position, sitting head in ante flexion as long as narcosis is lasting.
The top picture represents how the animals are placed in their cage. In bottom picture, the arrow indicated where the formulation is administered, under the tongue.

3.3.4.3. Oral immunisation

For administration of formulations into the gastrointestinal tract, a feeding needle was used. Mice were held in a position which allows a head tilt-chin lift manoeuvre, so that the feeding needle can be easily inserted into the stomach. Then, the formulation was released with the help of a syringe.

3.3.4.4. Intravaginal immunisation

The i.vag. immunisation is a non-invasive manoeuvre. During this procedure, mice were held with their tail in the air. The palmar reflex kept the mice immobilised. A pipette with a 200 µl tip was used to gently pipette 10 µl of solution into the outer part of the vagina. The position was maintained for several minutes to ensure that the drop was absorbed and not pressed out by the mouse's muscles.

3.3.4.5. Subcutaneous injection

For administration of formulations under the skin, the mice were first shaved on their backs close to the tail. Then, using a needle of 30Gx1/2", 100 µl of solution were injected into this area.

3.3.4.6. Immunisation schedule

Immunisations were given on day 0, 14 and 28, and the sampling (animals were sacrificed and the organs processed) was done at day 49. For the i.vag. immunisation DEPO-treatment occurred 5 days prior to the immunisation given at day 0. Consequently, the immunisation protocol for these mice starts at day -five respect to the immunisation.

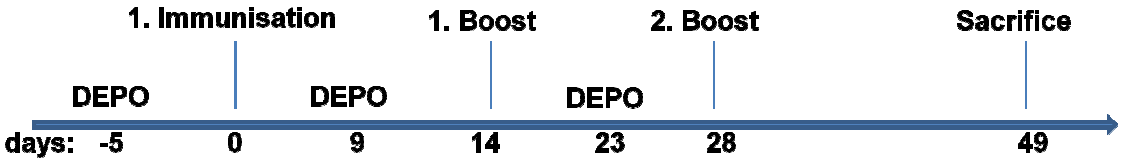

Figure 8: Immunisation strategy under DEPO treatment.
Diagram illustrating the experimental design for identifying the optimal vaccine formulation for further studies. DEPO is included for i.vag. immunisation.

3.3.5. Health and constitution monitoring

3.3.5.1. Measurement of weight

Using a balance, the weight of each mouse was monitored during the immunisation protocol at days 0, 2, 4, 9, 14, 16, 18, 23, 28, 30, 32, 37, 42 and 49. The mice were placed in a plastic container while they are weighed.

3.3.5.2. Health monitoring expressed in scores

Mice infected with HSV were monitored and a daily health score was assigned.
Score = 0: very healthy, no sign of illness

Score = 1: minimal weight loss
Score = 2: minimal weight loss and ruffled hair
Score = 3: mouse less vital, hair loss and weight loss
Score = 4: mouse lost 20% of its weight, eat rarely, possible hind limb paralysis, ruffled hair, signs of tissue inflammation
Score = 5: mouse is dead

3.3.5.3. Killing

Mice were either killed by CO_2 asphyxiation or cervical dislocation. Death was confirmed by pinching between the toes of both feet to test for reflexes and by feeling for a heartbeat.

3.3.5.4. Narcosis

Isofluorane narcotises mice for around 30 seconds. For this method, 1 ml of Isofluorane was pipetted into a plastic container with a closable lid containing paper tissues. The mouse was then placed into the container and removed once its breathing and movement slowed.

3.3.5.5. Anaesthesia

For deep anaesthesia, Ketamine/Rompun was used. The mouse to be anesthetised was fixed on its back and 100 µl of working solution per 10 mg bodyweight were administered intraperitoneal (i.p.). After a few minutes, the mouse was anesthetised and remained this way for about 30 minutes.

3.3.6. Collection of organs

3.3.6.1. Spleen resection

After removing the spleens from each mouse, they were pooled based on the vaccine group and placed in Petri dishes containing RPMI. Then, they were

mechanically pushed through 100 µm sterile filters using a syringe plunger. The resulting cell suspension was then centrifuged for 10 min at 314 g, 4°C. Next, the erythrocytes were lysed in ACK-buffer by pipetting up and down continuously for 1-2 minutes. Lysis was stopped by adding 15 ml of RPMI. After another centrifugation step, the pellet was again mechanically pushed into a 100 µm mesh filter. At this point, the cell number could be determined and analysis could begin.

3.3.6.2. Resection of vagina

With the mouse on its back, deep vertical cuts were made around the vagina, using a tiny pair of scissors. The vagina was removed by pulling with rounded tweezers. The organ was completely removed once the uterus could be seen. The vagina was then cut into 1-5 mm fragments and digested with enzymes.

3.3.6.3. Resection of lymph nodes

For lymph node extraction, the mouse was completely opened. The sublingual lymph nodes are positioned in the fatty tissue surrounding the cervix and below the mandible and they are the draining lymph nodes for sublingual immunisation. The iliac lymph nodes are located next to the vena cava near the hind legs and they are the draining lymph nodes for i.vag. immunisation. These lymph nodes were extracted with tweezers and placed into a 100 µm cell-strainer in a Petri dish containing RPMI complete media. A single cell suspension was prepared using a method similar to the one described for spleen cells (see 3.3.6.1) without lysing the erythrocytes.
These lymph nodes can be used to determine cell proliferation via thymidine incorporation (3.7.3).

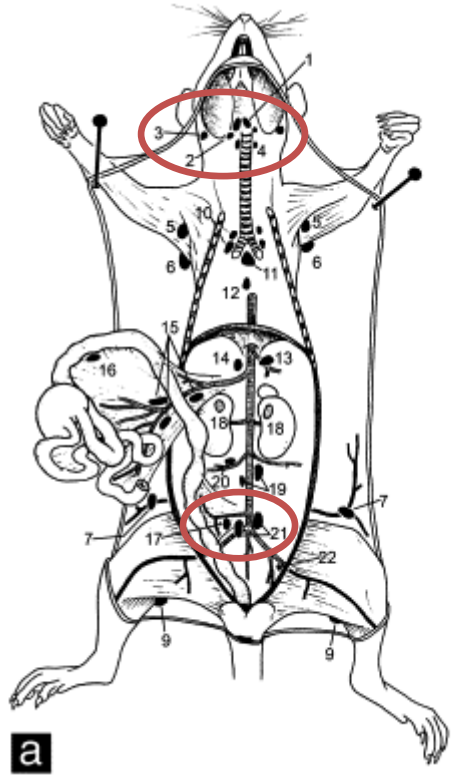

Figure 9: Lymph nodes of a mouse. Lymph nodes 1-3 are here named sublingual lymph nodes and lymph nodes no. 21 are the iliac lymph nodes extracted in this study [189]
Fig. 4a.

3.3.7. Collection and processing of lavages

3.3.7.1. Nasal lavage (NL)

With the mouse lying on its back and its head down, the nasal lavage was performed by first opening the mouth and disconnecting the upper and lower parts of the jaw. Then, from inside of the jaw, the *choanae* was flushed out through the nostrils and the droplets were collected in a 1.5 ml Eppendorf reaction tube containing 10 µl PMSF (40 µM). Centrifugation and storage were performed as described for vaginal lavage.

3.3.7.2. Saliva

For saliva collection, mice were first narcotised with Ketamine/Rompun i.p. and then injected with pilocarpine (500 µg/kg mouse; i.p.). After the animal began to

salivate, the sample was collected using a pipette and transferred to a vial containing 10 µl PMSF (40 µM). Centrifugation and storage were performed as described for vaginal lavage.

3.3.7.3. Bronchoalveolar lavage (BAL)

The bronchoalveolar lavage (also called lung lavage (LL)) was conducted by opening and flaying the mice. With the thoracic and abdominal wall completely open, a small incision was made between two rings of the trachea in order to insert a long pipette. The pipette was connected to a syringe containing 500 µl lavage buffer. The lavage was flushed twice and directly transferred to a 1.5 ml Eppendorf reaction tube containing 10 µl PMSF (40 µM). Centrifugation and storage were carried out as described for vaginal lavage.

3.3.7.4. Vaginal lavage (VL)

The vagina was flushed twice with 250 µl of lavage buffer (PBS + 5% FCS) and the lavage was transferred to a vial containing 10 µl PMSF (40 µM). The vial was then centrifuged for five minutes at 3000 g, 4°C. After centrifugation, the supernatant was collected and placed into a new vial containing 10 µl PMSF (40 µM) and stored at -20°C until it was processed.

3.4. Vaginal smears

3.4.1. Vaginal smears with swabs

The upper part of the vagina was gently swabbed using a thin cotton swab (150x0.9-1.0 mm, Heinz Herenz, Germany). The swab was then rolled onto an object slide and allowed to air dry. At this point, it was possible to stain the slide.

3.4.2. Vaginal treatment with cytobrush

Before the mice could be infected with HSV-1 virus by i.vag. route, a cytobrush (medex, Medesign) had to be used to cause microtrauma to the vaginal epithelium. This trauma prepared the tissue to fully resorb the virus. Therefore, the cytobrush was inserted into the vagina and rotated five times to the right side and five times to the other side.

3.5. HSV-1

3.5.1. 17SynIGR20Luc and 17SynIGR20GFPgJZ virus stock preparation

The 17SynIGR20GFPgJZ and 17SynIGR20 Luc stocks were prepared at University of Ferrara, Italy. One additional stock of 17SynIGR20GFPgJZ and stocks of LVwt and LVLacZ were prepared at HZI, Braunschweig.

To propagate the virus, Vero cells were infected. In brief, Vero cells were seeded into 20x150 cm^2 tissue culture flasks at a concentration of 8-10x10^6 cells for each flask in 20-25 ml high glucose DMEM growth media (completed with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin). The cells were then incubated at 37°C, 5% CO_2 for 24 hours and reached confluence. Next, the media was removed from the flasks and the adherent Vero cells (about 24x10^6 cells/flask) were infected with the virus at a multiplicity of infection (MOI) of 0.01. Calculating the MOI from the virus stock concentration, 96 µl of virus (*e.g.* 17SynIGR20GFPgJZ) were used and diluted in 100 ml DMEM (without FCS). For a homogenous distribution of the virus, the virus was sonicated for several seconds and then cooled on ice. 5 ml of this virus dilution was then added to each flask and incubated for 1 hour at 37°C, 5% CO_2 and gently shaken every 10 minutes. This ensured viral contact for each cell and guaranteed the highest rate of infection of the Vero cells monolayer. After incubation, the supernatant containing excess virus was removed and 25 ml of DMEM complete media was added to each flask. Incubation followed for

the next 24 hours. During this time the virus was replicated inside Vero cells. Under the microscope, it was observed that the cells change their appearance by becoming rounded and begin to come off the flasks. This indicated successful infection. With a cell scraper, adherent cells were disconnected from each flask and transferred into 50 ml tubes with lids. These suspensions were then centrifuged at 1251 g for 15 minutes, 4°C. The supernatants were then decanted and saved on ice. The pellets were resuspended in the small remaining volume of supernatant in each tube, then combined into one tube and centrifuged again. The final pellet was resuspended in 3 ml supernatant, transferred to a 15 ml tube and shortly frozen in liquid nitrogen and stored over night at -80°C. The decanted supernatants were also stored over night at -80°C. The next day the pellet was thawed and re-frozen three times by cycling between liquid nitrogen and a 37°C water bath, vortexing between each cycle for a few seconds. After the final thaw, the virus was sonicated three times for 10-20 seconds with a 10 seconds pause in ice between each sonication cycle (35 kHz). This ensured that the cell membranes of the Vero cells were destroyed and all viral particles were released. The cell debris was centrifuged two times at 1501 g for 15 minutes, 4°C. The pellet was discarded and the virus containing supernatant was put into Oak Ridge tubes for ultracentrifugation along with the original virus supernatant which was still frozen at -80°C from the day before. With a Sorvall centrifuge and a SS34 rotor, the supernatants were centrifuged at 43667 g for 30 minutes at 4°C. Next, the supernatant was discarded and the pellets were resuspended by adding 1 ml growth media (without FCS). All resuspended pellets were combined and centrifuged again at 43667 g for 30 minutes at 4°C. The final pellet was then kept on ice for one hour before the purification.

For purification, solutions B-F were prepared and kept at 4°C (see Material section). Next, 2.6 ml of solution D was pipetted into ultracentrifugation tubes and 2.1 ml of solution E was slowly added. Finally, 1 ml of solution F was added almost completely filling each tube. To ensure the tubes are exactly balanced with each other, they were weighed and solution F was added where necessary. Each tube was then sealed with a cap and loaded into a Sorvall-ultracentrifuge using a T 875 rotor or TH-641 swinging rotor and centrifuged for 9-16 hours at

63580 g at 4°C in vacuum at maximum acceleration and no brake in deceleration. After centrifugation, the tubes were placed on ice. The virus band appeared as a result of density gradient formation. This band was extracted by puncturing the tube with a syringe placed 2 mm under the band and carefully collecting only the virus band. To concentrate the virus, the collected virus band was put into ultracentrifugation tubes and diluted (1:2) with PBS and centrifuged again using a Sorvall-ultracentrifuge, this time using a SS34 rotor at 43667 g for 30 minutes at 4°C. The final pellet was resuspended in 1 ml of PBS and sonicated for 15 seconds three times. The viral solution was placed on ice between each sonication step. Finally, the purified virus was aliquoted into 20 µl aliquots and stored at -80°C until usage. In addition, the viral stocks were titrated by determining the cell number of plaque-forming units (PFU)/ml, using a Plaque-assay.

3.5.2. Plaque-assay

The plaque-assay was used to determine the PFU/ml of the viral stock. First, Vero cells were seeded into a 6-well-plate and grown to confluence. Next, the virus was diluted in RPMI media to reach 10^{-4} to 10^{-9}. The media was then aspirated from the Vero cells and 200 µl of the dilutions were added to each well of Vero cells. This was followed by incubation for one hour at 37°C and 5% CO_2 with gently shaking every 10 minutes. Finally, the viral solution was removed and each well was covered with 2 ml of methylcellulose and incubated for four days. The methylcellulose was then removed and each well was washed with 2 ml of PBS, and then stained with 2 ml of crystal violet–methanol solution and air dried. Finally, the titre of the viral stock was determined by counting the plaques. Here, 21 plaques at a dilution of 10^{-7} were counted. The titre of the prepared stock 17SynIGR20GFPgJZ was therefore determined to be $1.05x10^{9}$ PFU/ml.

3.6. Technics of staining cells in liquid and in sections

3.6.1. Cryosections

To create cryosections of the vaginal tissue the organ was directly extracted, put into cryosection tubes, frozen in liquid nitrogen and stored at -80°C until it was processed. The organ was cut using a cryotome into 4-6 µm slices at -25°C. The slices were then placed onto glass slides and air dried. At this point the slides could be stained. For ICBC, the sections were frozen again at -80°C, and then stained and scanned by Dr Christian Hennig at Hannover Medical School.

3.6.2. Staining of vaginal smears

3.6.2.1. Diff-Quik staining

Using the Diff-Quik staining solution, it was possible to differentiate between cell subsets quickly. After the sample to be stained had air dried, it was dipped three times, for five seconds each time, into Diff Quik fixing solution. This procedure was performed identically when using DQ Solution I and Solution II. The slides were then rinsed carefully with distilled water and allowed to air dry.

3.6.2.2. Pappenheim staining

First, the slides were kept in May-Grünwald staining solution for five minutes. Then, they were washed with PBS for two minutes and subsequently stained for 20 minutes with Giemsa staining solution, diluted 1:20 in distilled water. Finally, the slides were washed with normal water and air-dried. The entire staining procedure took place at RT.

3.7. Immunological methods

3.7.1. ELISA

3.7.1.1. Detection of antigen-specific IgG in sera

The determination of antigen-specific IgG or IgG subclasses in sera was done by substrate reaction measured with ELISA reader Synergy 2 (Bio-Tek) by detection of optical density at 405 nm. The 96-well-plates were coated with 2 µg/ml antigen diluted in coating buffer and left overnight at 4°C or for one hour at 37°C. Plates were then washed with PBS (+ 0.1% Tween 20) using an ELISA washer (ELx 405, Bio-Tek) on a 6-cycle program. To avoid unspecific binding, the plates were blocked with PBS/BSA (3%) for 1-2 hours at 37°C. The serum was diluted 1:1000 in PBS /BSA. Next, the liquid from the plates was discharged and the diluted serum was pipetted into the first well (200 µl) of each row, 100 µl PBS/BSA had already been pipetted into the other wells for performing a serial dilution (1:2) using a plate pipetting system robot (Precision 2000, Bio-Tek). The last well of each row was left empty. Then, the plates were incubated for one hour at 37°C, followed by washing. The detection antibodies were given at an appropriated dilution, and the plates were further incubated for 1 hour at 37°C. After additional washing, streptavidin-HRP solution (1:1000 in PBS+ Tween + BSA) was added to the plates, which were incubated for 30 minutes. After washing, the substrate solution ABTS plus H_2O_2 (30%) (1:1000 v/v) was pipetted on the plates (100 µl/well). Finally, the plates were read using an ELISA reader after 5, 15 and 30 minutes at 405 nm. For final results, always the value measured after 30 minutes was regarded. Sera of single animals were used for analysis.

3.7.1.2. Detection of antigen-specific IgA in mucosal lavages

To assess the amount of IgA in the sera and lavages, an ELISA for IgA detection was performed. As in the IgG ELISA, the plates were coated over night at 4°C. For the standard and the total IgA, the plates were coated with 100 µl of anti-mouse IgA antibody (2 µg/ml) diluted in carbonate buffer. For specific

detection of IgA, the plates were coated with antigen (2 µg/µl) diluted in carbonate buffer. Following incubation, plates were washed as described for IgG ELISA and blocked for two hours by pipetting 200 µl of blocking solution into the wells. After incubation for 1-2 hours at 37°C, the solution was discharged and the samples were transferred to the plate. For a standard curve, standardised mouse IgA antibody was diluted in PBS + BSA (3%) starting with a concentration of 0.2 µg/ml. The serial dilution (1:2) was completed by a plate pipetting system robot (Precision 2000, Bio-Tek). For this, 200 µl of the starting dilution were pipetted into the first well of each row and 100 µl of the dilution buffer were pipetted into all other wells, which were then diluted 1:2 by the robot. The first half of the plate was filled with a 1:20 dilution from the vaginal, nasal and lung lavages, and a 1:250 dilution for saliva, respectively. Similar to the standards, serial 1:2 dilution was completed by the robot, up to a 1:640 dilution for VL, NL and BAL; and 1:8000 for saliva. The other half of the plate was used for antigen-specific IgA detection. For this, the VL, NL and BAL samples were diluted 1:4 and the saliva samples 1:50. Here, a 1:2 serial dilution was completed by the robot, which continued to a dilution of 1:128 for the VL, NL, and BAL samples; and up to 1:1600 for the saliva samples. The samples were then incubated for 1-2 hours at 37°C and then washed using an ELISA washer (ELx 405, Bio-Tek) on a 6-cycle program. The IgA detection antibody was diluted 1:5000 in PBS + 0.1% Tween 20 + 1% BSA, and 100 µl of this dilution was placed in each well and allowed to incubate for another hour. This was followed by a washing step and the plates were then filled with Streptavidin-HRP at a 1:1000 dilution in PBS + 0.1% Tween 20 + 1% BSA. After a 30 minutes incubation and a washing step, ABTS plus H_2O_2 (30%) (1:1000 v/v) was added to the plates. Using the ELISA reader, the colour reaction of enzyme-substrate-solution was analysed after 5, 15 and 30 minutes at 405 nm. For final calculation of results, always the value measured after 30 minutes was regarded. Lavages of single animals were used for analysis.

3.7.2. Enzyme-linked immune spot technique (ELISPOT)

The ELISPOT is done to detect individual cytokine secreting cells. The multiscreen plates were coated with 100 µl capture antibody diluted in coating buffer. The plates were stored at 4°C over-night. Next, the coating solution was discarded and the plates filled in with blocking solution (200 µl/well) and further incubated for two hours at RT. After that, the solution was discarded and the plates were loaded with protein or peptide, media, ConA and spleen cells. The final concentration of antigen or ConA was 5 mg/ml. After preparing the plates with the antigen, mitogen and media, cell solutions were prepared. Pooled spleen cells were given at a concentration of $5x10^5$ cells/well and $2x10^5$ cells/well. The plates for detection of IFN-γ were incubated for 24 hours, whereas those for IL-2, IL-4 and IL-17 detection were incubated for 48 hours (37°C, 5% CO_2). After incubation, the cell suspension was removed and the plates were washed twice with deionised water. The wells were soaked for 3-5 minutes between the washing steps. The wells were washed three times with 200 µl/well of wash buffer I and they were then loaded with 100 µl of detection antibody (1:250 in dilution buffer). The plates were incubated for two hours at room temperature. After removing the solution, plates were washed three times with wash buffer I. Then, 100 µl of avidin-HRP-solution was added to the plates, which were incubated for one hour at room temperature. Further, the plates were washed four times with wash buffer I and two times with wash buffer II. Then, 100 µl of the substrate solution was added to each well and spot development was observed and stopped by adding deionised water. The plates were air dried over-night and the next day they were automatically read using the ELISPOT reader (program Image Acquisition 4). This information was necessary for the program Immunospot 3, which counted the number of spots in each well, with each spot being created by a distinct cytokine secreting cell.

3.7.3. Proliferation assay

Splenocytes or lymph node cells were used for the proliferation assays. The organs from each group were pooled to perform the assay. Single cell

suspensions were prepared on the sampling days and given to the wells at a final concentration of $5x10^5$ cells/well. Before adding the cells to a particular well, either media, ConA or the antigen at concentrations of 40 µg/ml, 20 µg/ml, 10 µg/ml or 5 µg/ml were added. The final volume of each well was 200 µl. After 72 hours of incubation in 5% CO_2 at 37°C, 1 µCi ^{3}H-thymidine in 50 µl media was added to each well and incubation continued for an additional 16 hours. Cells were harvested on glass fibre filters by using the Inotech® cell harvester. The principle underlying this method is that proliferating cells will incorporate ^{3}H-thymidine into their DNA and be immobilised on the filter plate, with unused ^{3}H-thymidine simply washing away. The filter plate was marked with a pencil and positioned into the machine and humidified with autoclaved water. The harvester was also rinsed. Then, the samples were put into position and a vacuum was used to remove the cell suspension. The cells remained on the filter and everything else was discarded into the radioactive waste tray. The wells were washed twice with sterile water and vacuum filtered once again, allowing the vacuum to run 30 seconds longer than necessary to dry the filter. The 96-well plate was then discarded and the filter was dried for 30 seconds in the microwave. Next, the filter was placed on a heater using tweezers and covered with scintillation wax. The wax was allowed to melt completely and then to air dry. To assess the radioactivity, the filter was placed into the cassette 1450-104 Filtermat of the scintillation counter. The counts appeared as a cell number in an excel file. These counts per minute were used to determine the amount of proliferating cells. First, the mean of the quadruplicates for each group and antigen or mitogen concentration were calculated. Then, the background (media alone) was subtracted and the standard deviation calculated. The stimulation index was calculated by dividing the mean of the quadruplicate of each sample by the mean of the media.

3.7.4. Cytokine secretion

As during the proliferation, the single cell suspensions of spleen or lymph node cells were restimulated with antigen concentrations of 40 µg/ml, 20 µg/ml, 10 µg/ml or 5 µg/ml, media or ConA. The cells had a concentration of $5x10^5$ in each

well of the 96-flat bottomed plate. After incubation for 90 hours, plates were centrifuged for five minutes at 314 g and the supernatant, which contained the released cytokines, was collected in 1.5 ml Eppendorf tubes. The samples were stored at -80℃ until they were processed using a Th1 /Th2 10-plex Flow cytokine kit (Bender Med Systems).

3.7.5. Statistical analysis

For analysing statistical significant differences occurring between the different groups, was analysed with Graph Pad Prism software using analysis of variance (One-way Bonferronis multiple comparison test and Two-way Anova). Probability (p) values of $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.00001$ (****) indicating differences were considered significant.

4. Results

4.1. Establishment of a synchronisation strategy for the murine oestrus cycle

The female oestrus cycle is defined by four different stages regulated by varying hormone profiles (see 1.3.3). The prooestrus stage is dominated oestrogens, whereas in the oestrus progesterone is the main hormone. Changes in the concentrations of these hormones are responsible for the restructuration of cell layers and mucus production, which result in a cycle of about four days for female mice. The dioestrus is the stage of the cycle in which the epithelial layers are the thinnest and ags and vaccines can successfully penetrate the mucosa. Thus, to immunise animals the oestrus cycle must be synchronised in order to obtain meaningful and comparable data. It is essential that all animals receive the vaccination on the same day in dioestrus stage to avoid huge variations in the obtained responses. One of the common methods to synchronise the oestrus of female mice is based on hormone treatment. However, since the amount given to the animals is usually above physiological values, the immune responses stimulated following vaccination might not reflect the natural situation. Thus, it was first tested if the cycle could be also regulated by a natural method.

4.1.1. Whitten-effect for oestrus cycle synchronisation

In an attempt to synchronise the oestrus cycle of female mice using a natural method, female mice were housed in cages which had previously been used by male mice. This could be effective because male mice leave the odour of their urine and hormones, and female mice are responsive to them. This results in the so-called Whitten-effect [190], [191]. However, in the present study this method did not provide robust and reproducible results. When the stage of the oestrus cycle was accessed by Diff-Quik staining of vaginal smears, the obtained results showed that both the period of time until the mice reached

synchronisation and the length of time during which synchronisation was maintained significantly varied. This strategy was therefore excluded for subsequent studies, shifting to hormone-based synchronisation methods.

4.1.2. DEPO-treatment

To synchronise the oestrus cycle of female mice a s.c. treatment with DEPO, a medroxyprogesterone acetate (pregn-4-ene-3,20-dione, 17-(acetyloxy)-6-methyl-, (6α-)) solution, was carried out (Fig. 10). This hormone is normally used as a three-month treatment to prevent pregnancy in humans. In mice, the administration of 2 mg of DEPO five days before i.vag. immunisation leads to a successful synchronisation at the dioestrus stage. Accordingly to our observations, this effect lasts for about ten days and must be repeated prior to each immunisation/boost. Figure 10 gives an overview of the oestrogen and progesterone levels during the oestrus cycle and the corresponding phenotype of the vaginal smears. It shows pictures of vaginal smears taken with a cotton swab in the vagina at 400x magnification using a Nikon Eclipse microscope. The staining makes cells and mucus clearly distinguishable. The prooestrus is characterised by cornified epithelial cells with nuclei in the centre and by small quantities of mucus in the vagina (Fig. 10B). During the oestrus, cornified epithelial cells are still visible, but they are already anucleated, the mucus disappeared and cell layers are in the process of conversion (Fig. 10C). Figure 10D shows the metoestrus, which is characterised by mucus production and infiltration of granulocytes. There are still cornified epithelial cells in the smears and the tissue degradation continues. In the dioestrus, cornified epithelial cells are regenerating, as indicated by the presence of a nucleus in these cells, and neutrophils are still infiltrating the tissue (Fig. 10A). This is the point of the cycle at which the cell layers are the thinnest. Therefore, animals are immunised at this stage of the oestrus cycle when the penetration of the vaccine ag through the skin barrier is most efficient. Figure 10A, lower picture, shows the dioestrus stage as it appeared when induced by DEPO-treatment, which is in turn indistinguishable from naturally-occurring dioestrus. Taken together our results, the hormone treatment represents the only successful synchronisation strategy.

However, one needs to keep in mind that the non-physiological hormone values might have a direct or indirect impact on the stimulated immune responses. There is a clear integration of the neuro-endocrine-immune axes [192]. For example, it has been shown that progesterone has immune modulatory effects, making also mice more susceptible to HSV-infection as a result of an impaired immune response [193]. On the other hand, NK-cell and CTL activity is decreased following progesterone treatment [194].

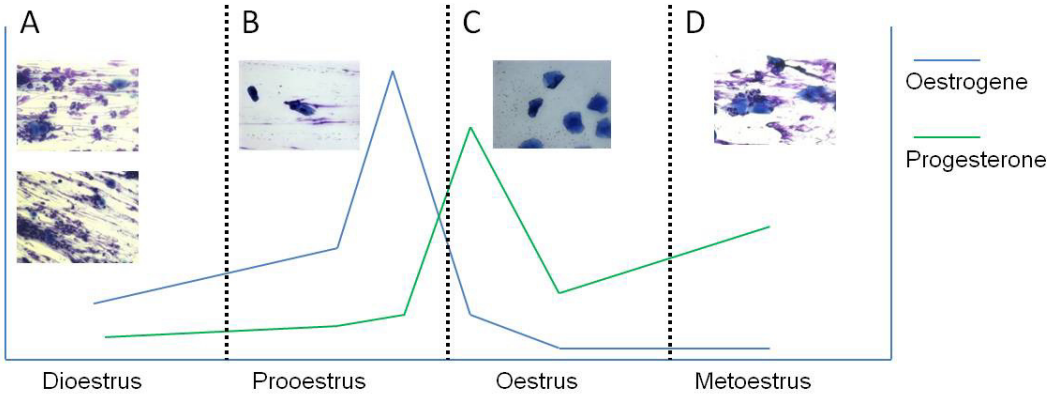

Figure 10: Stages of the murine oestrus cycle.
Overview of oestrogen and progesterone levels during the oestrus cycle and the corresponding phenotype of the vaginal smears, as modified from the original [132]. (A) Dioestrus with many leukocytes and few nucleated epithelial cells (lower panel shows dioestrus after synchronisation with DEPO), (B) prooestrus with few cornified but many nucleated epithelia cells, (C) oestrus with many cornified cells with degenerated nuclei, (D) metoestrus with many leukocytes and few cornified cells. Vaginal smears of female mice stained with Diff-Quik staining observed at a magnification of 400x.

4.1.3. Identification of the phase of the oestrus cycle allowing to obtain the most efficient immune responses following immunisation

Using an adoptive transfer model, proliferation of spleen cells was used as read-out to conclude in which stage of the oestrus cycle cells are maximally stimulated following i.vag. immunisation. For this experiment, OVA-protein co-administered with the adjuvant BPPcysMPEG was used for immunisation. Then, the transferred cells were CFSE stained to assess their proliferation after specific stimulation following the i.vag. administration of the ag. Consequently, each peak in a histogram (or cloud in dot plot) represents a successive

generation in which the intensity of the labelling was reduced by 50% (see Fig. 11 and 12). In figure 11, the gating strategy and the proliferated cells from animals immunised in dioestrus are shown (n=4).

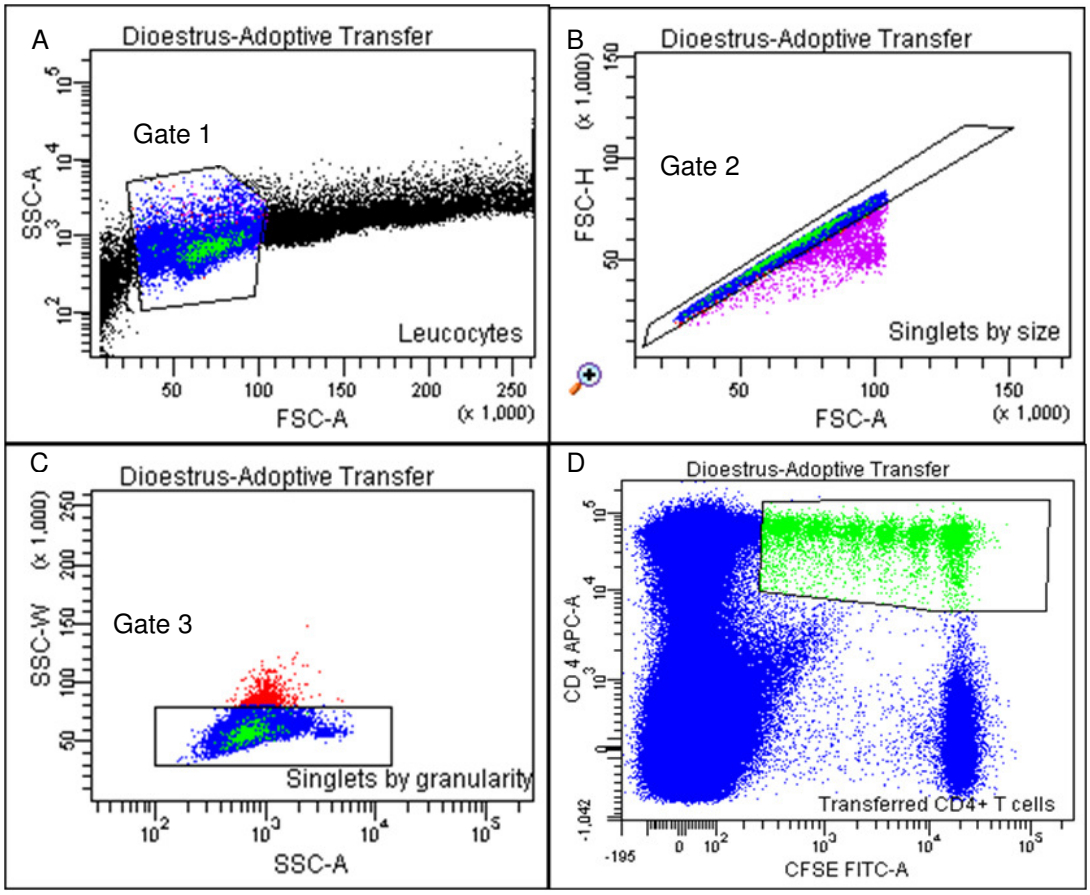

Figure 11: *In vivo* proliferation of spleen and lymph node cells following i.vag. immunisation.
C57BL/6 female mice which received CFSE-stained cells recovered from spleens and LN from OT-II animals were then immunised by i.vag. route during the dioestrus with OVA-protein co-administered with BPPcysMPEG. (A) All cells detected in the forward scatter–area (FSC-A) against side scatter–area (SSC-A) were gated on leukocytes, (B) doublet discrimination by gating on singlets in FSC-A against FSC-H gated on leukocytes, (C) doublet discrimination by gating on singlets in SSC-A against SSC-W gated on leukocytes,(D) CFSE-stained cells in FITC-channel against CD4-cells in the APC-channel.

In figure 11A, the total amount of cells detected in the forward-and side scatter are shown, the gate one is set on the leukocyte population. In figure 11B and 11C, singlets are chosen by doublet discrimination. In figure 11D, the CFSE-stained transferred cells are detected in the fluorescein isothiocyanate (FITC)-channel and CD4-cells are detected in the APC-channel, including only the cells from gate three. The $CFSE^{low}$ $CD4^{+}$ cells represent the cells that have proliferated due to the immunisation stimulus, whereas $CFSE^{high}$ cells are

undivided. The proliferation of these cells is plotted in a histogram and percentages of the divided, parent cells are indicated (Fig. 12).

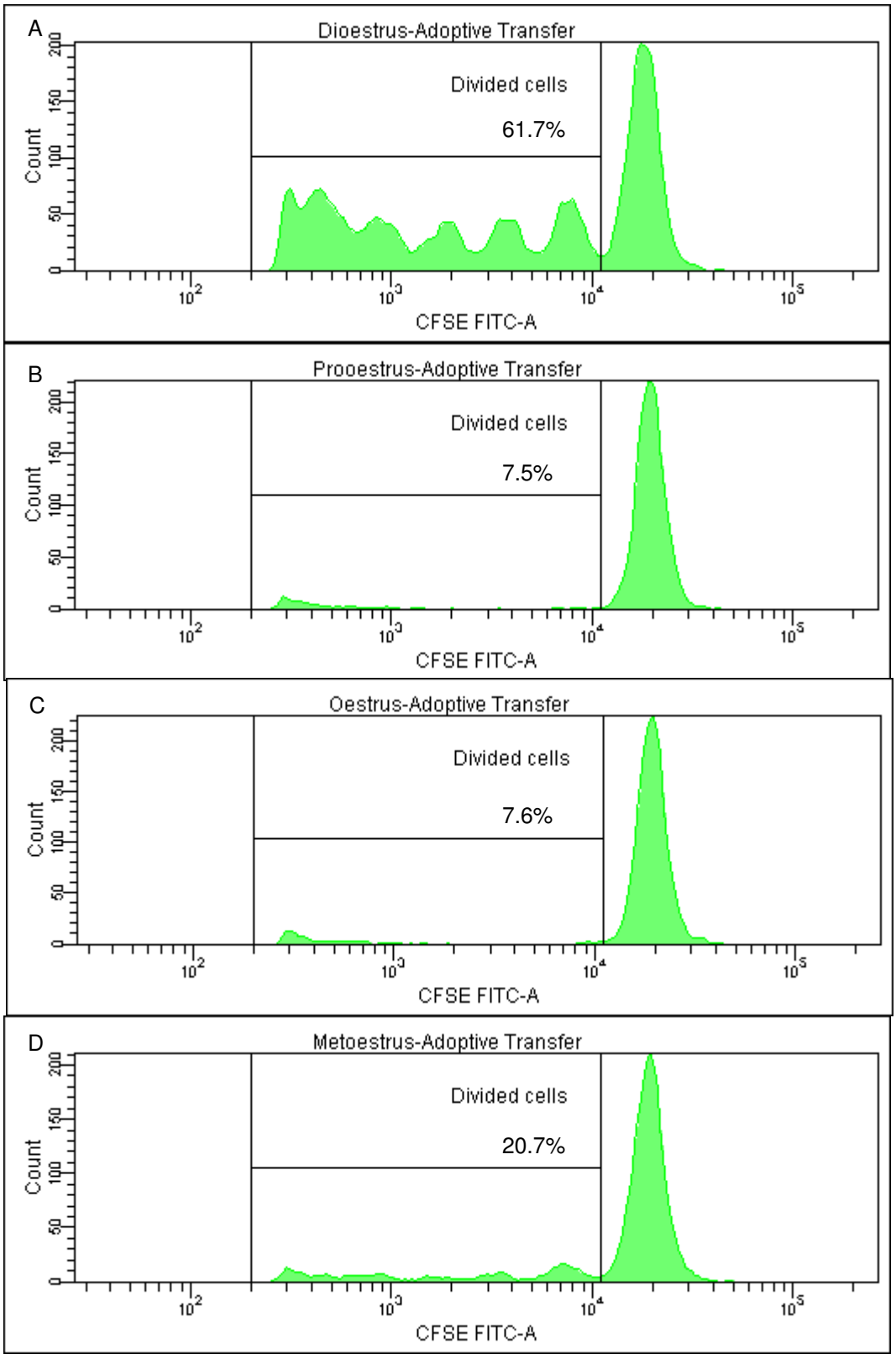

Figure 12: Proliferation of CD4$^+$ spleen cells after adoptive transfer.
CFSE-stained transgenic OT-II-cells were transferred i.v. to C57BL/6 wild type mice which were afterwards immunised by i.vag. route during different phases of the oestrus cycle. Five days

after immunisation, spleens were removed and analysed. Immunisation during the (A) dioestrus (n=4), (B) prooestrus (n=2), (C) oestrus (n=4) and (D) metoestrus (n=2).

As shown in figure 12, stimulation of strong proliferative responses (*i.e.* activation of ag-specific T cells) following immunisation by i.vag. route depends on the stage of the oestrus cycle. While i.vag. immunisation during prooestrus, oestrus or metoestrus is not resulting in proliferation of $CD4^+$ OT-II-spleen cells, strong proliferative responses have been obtained when mice were immunised during the dioestrus stage (Fig. 12A). This can be explained by improved ag uptake, as a result of high number of neutrophils and thinner vaginal cell layers in dioestrus, as compared to the other stages of the oestrus cycle. In contrast, the increased thickness of the vaginal cell layer and specific cellular composition in other stages of the cycle might not allow efficient uptake of the vaccine formulation, thereby resulting in very weak cell activation. To conclude, these results confirm that successful i.vag. immunisation requires ag administration during the dioestrus as it is mostly described in the literature.

4.2. Defining the optimal immunisation protocol

4.2.1. Identification of the most potent adjuvant stimulating the strongest immune response after i.n. and i.vag. immunisation

After identification of the most efficient strategy for obtaining the synchronisation of the cycle, it was critical to find the most suitable i.vag. immunisation protocol determining the most promising model ag and adjuvant. To this end, comparative immunisation studies were performed in which C57BL/6 female mice (n=5/group) received a model ag co-administered with different adjuvants by i.n. and i.vag. routes. The groups that received the formulation via the i.vag. route were DEPO-treated five days prior to each immunisation to synchronise their cycles (see Methods, Fig. 8).

OVA was given at 50 µg/dose, whereas the dosage for the adjuvants was as follows: c-di-AMP 1.5 µg/dose, LPS 10 µg/dose, α-GalCerMPEG 5 µg/dose and

BPPcysMPEG 5 µg/dose. (C-di-AMP and α-GalCerMPEG were tested to be LPS free with HEK-blue test, levels were below the detection level [195].) At day 49, animals were sacrificed, blood and VL were taken and the spleens were removed.

Immunisation of OVA co-administered with c-di-AMP (OVA + c-di-AMP) by i.vag. route resulted in the elicitation of the highest serum IgG titres (Fig. 13A). Similar results were obtained when OVA was co-administered with either LPS or c-di-AMP as adjuvant by the i.n. route. Cellular responses were investigated by assessing the production of cytokines by spleen cells. To this end, we evaluated the expression of the Th1-cytokines IFN-γ and IL-2, as well as the Th2 cytokine IL-4 and the Th17 cytokine IL-17. The Th17- cytokine was strongly produced by spleen cells of mice immunised with OVA + c-di-AMP by i.n. route, whereas Th1 responses were comparatively weaker (Fig. 13B). IL-4 was mainly induced in the groups where α-GalCerMPEG or c-di-AMP was co-administered as adjuvants. Based on these preliminary data, c-di-AMP was selected as most suitable mucosal adjuvant for further comparative studies following vaccination by different mucosal routes.

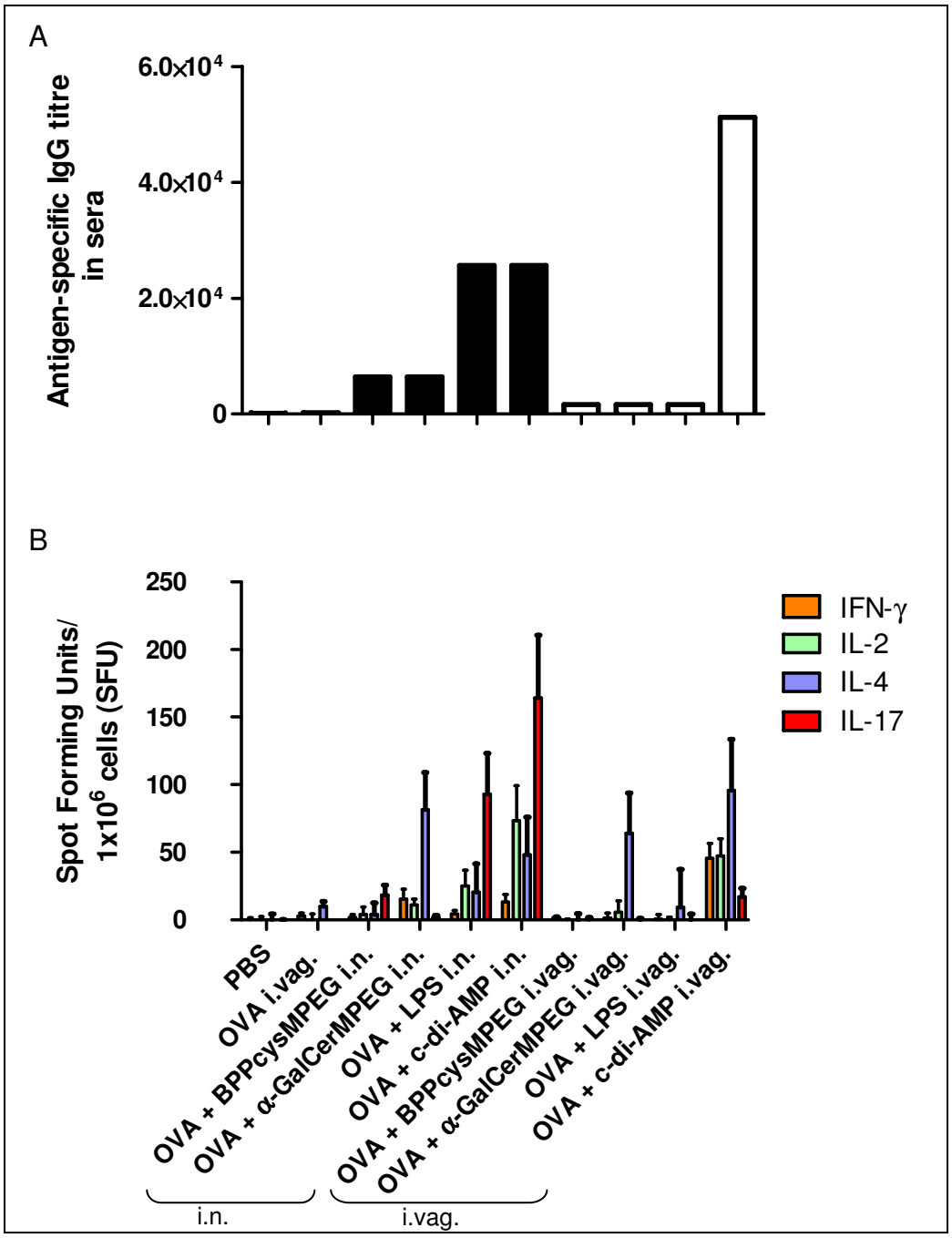

Figure 13: Identification of the optimal formulation for further studies.
C57BL/6 (n=5 per group) were immunised by i.n. or i.vag. route with OVA co-administered with different adjuvants. (A) Black and white bars indicate groups immunised by i.n. and i.vag. route, respectively. The displayed results correspond to OVA-specific antibody titres obtained using pooled sera. (B) Cellular response measured in spleen cells.

4.2.2. Identification of the most suitable model ag for stimulating immune responses after i.n. and i.vag. immunisation

In order to identify the optimal ag able to stimulate the strongest immune responses following immunisation by the two selected mucosal routes (i.n. and i.vag.), mice were immunised with either OVA (50 µg/dose) + c-di-AMP (5 µg/dose) or β-Gal (15 µg/dose) + c-di-AMP (5 µg/dose). (β-Gal was tested with HEK-blue test for LPS, at a concentration of 2.5 µg/µl β-Gal, 5 ng/ml LPS were detected [195].)

4.2.2.1. Induction of local and systemic humoral immune responses in C57BL/6 mice after immunisation by i.n. and i.vag. route

In general, i.n. immunisation resulted in the elicitation of higher IgG titres in sera of vaccinated mice, as compared to the responses triggered after i.vag. immunisation (Fig. 14A).

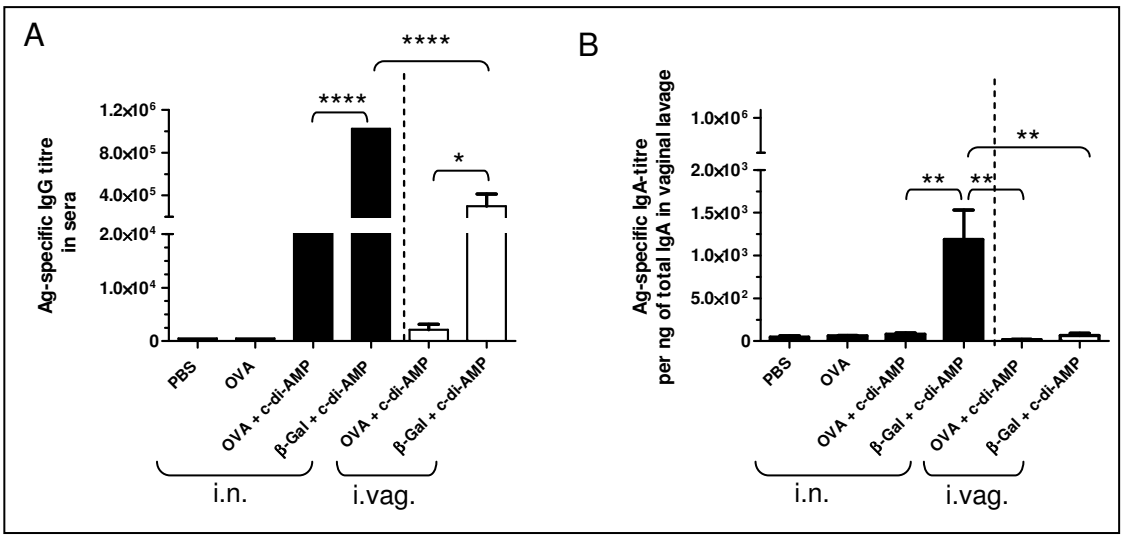

Figure 14: Humoral immune response elicited in C57BL/6 mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route.
(A) Analysis of ag-specific IgG titre in sera and (B) IgA titre in VL of C57BL/6 mice (n=3) immunised on days 0, 14 and 28. The black bars (left part) indicate the Ig titres of i.n. immunised animals, whereas the white bars (right part) represent the titres of i.vag. immunised mice. Standard error of the mean (SEM) is indicated by vertical lines. Differences were statistically significant at $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****) using the One-way ANOVA.

Moreover, formulations including β-Gal raised statistically significant higher humoral responses compared to OVA, as demonstrated by the levels of Igs in sera and VL (Fig. 14). Immunisation of mice using OVA + c-di-AMP induced

only marginal IgA production in the genital tract (Fig. 14B). On the other hand, immunisation of animals with β-Gal + c-di-AMP by i.n. route resulted in significant higher IgA titres in VLs, as compared to controls or groups receiving OVA + c-di-AMP ($p<0.01$), whereas i.vag. vaccination failed to induce high titres of ag-specific IgA in the vaginal mucosa. Taken together, in our experimental model β-Gal + c-di-AMP was the formulation inducing more robust immune responses.

The IgG subclass pattern analysed in these studies indicates if a Th1 or Th2-biased immune response is favoured. Thus, higher titres of IgG2a, IgG2b and IgG2c indicate Th1-dominated immune response, whereas IgG1 is specific for Th2-biased responses. Analysis of the IgG subclass pattern stimulated after i.n. immunisation with OVA alone showed a Th2 dominated response (IgG1), whereas a mixed Th1-Th2 response (increased levels of IgG1 and IgG2b) was observed in animals receiving OVA + c-di-AMP (Fig. 15). Immunisation via the i.vag. route favours the IgG2c rather than the IgG2b subclass (Th1 response) (Fig. 15). Immunisation via the i.vag. route seems to favour the stimulation of IgG2c rather than the IgG2b subclass (Th1 response) (Fig. 15). On the other hand, immunisation with β-Gal + c-di-AMP via the i.n. route resulted in the elicitation of similar IgG1, 2a and 2b titres, whereas administration of this formulation via i.vag. route stimulated stronger IgG2c titres followed by IgG2a and IgG1 (Fig. 15). However, mice immunised with β-Gal + c-di-AMP showed significantly higher ag-specific Ig titres in sera as compared to the titres induced in control groups receiving either PBS or OVA alone. This formulation also elicited significantly higher titres as compared to OVA + c-di-AMP given by either i.n. or i.vag. route ($p<0.0001$) (Fig. 15). Moreover, β-Gal + c-di-AMP elicited significant higher levels of IgG1 and IgG2b in sera after immunisation by i.n. route, as compared to the i.vag. route. Again the i.vag. delivered formulation induced significantly higher titres of IgG2b and 2c, as compared to control groups and groups receiving OVA as ag (IgG2a $p<0.001$, IgG2b $p<0.0001$) (Fig.15).

Taken together, i.n. administration of β-Gal + c-di-AMP induced strongest IgG subclass titres, characterised by a mixed Th1/Th2 response pattern.

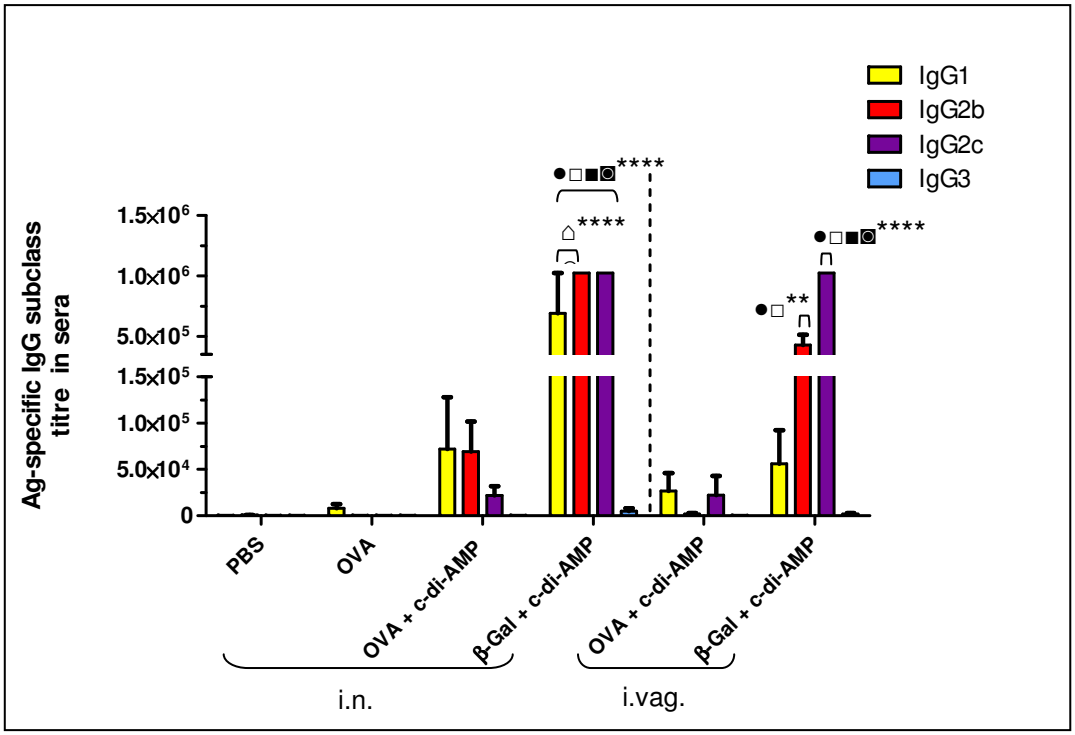

Figure 15: Pattern of IgG subclass titres induced in mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route.
Induction of ag-specific IgG subclass titres in sera of C57BL/6 mice (n=3) immunised on days 0, 14 and 28 by i.n. (left part) or i.vag. route (right part). SEM is indicated by vertical lines. The observed differences were statistically significant at $p<0.01$ (**), $p<0.0001$ (****), calculated using Two-way ANOVA, in comparison to the PBS group (●), the group receiving OVA alone (□) or the groups receiving OVA + c-di-AMP (■ i.n., ◙ i.vag) or β-Gal + c-di-AMP (◊ i.n., ⌂ i.vag.)

4.2.2.2. Stimulation of cellular immune responses in C57BL/6 mice after i.n. and i.vag. immunisation

The stimulation of efficient immune responses results also in the induction of cellular immunity, such as Th and cytotoxic T cell responses. Thus, proliferation of spleen derived immune cells was assessed by determination of ^{3}H-thymidine-incorporation (Fig. 16). For this assay, splenocytes were restimulated with OVA or β-Gal at concentrations of 0 µg/ml, 5 µg/ml, 20 µg/ml, 40 µg/ml. ConA (10 µg/ml) served as positive control.

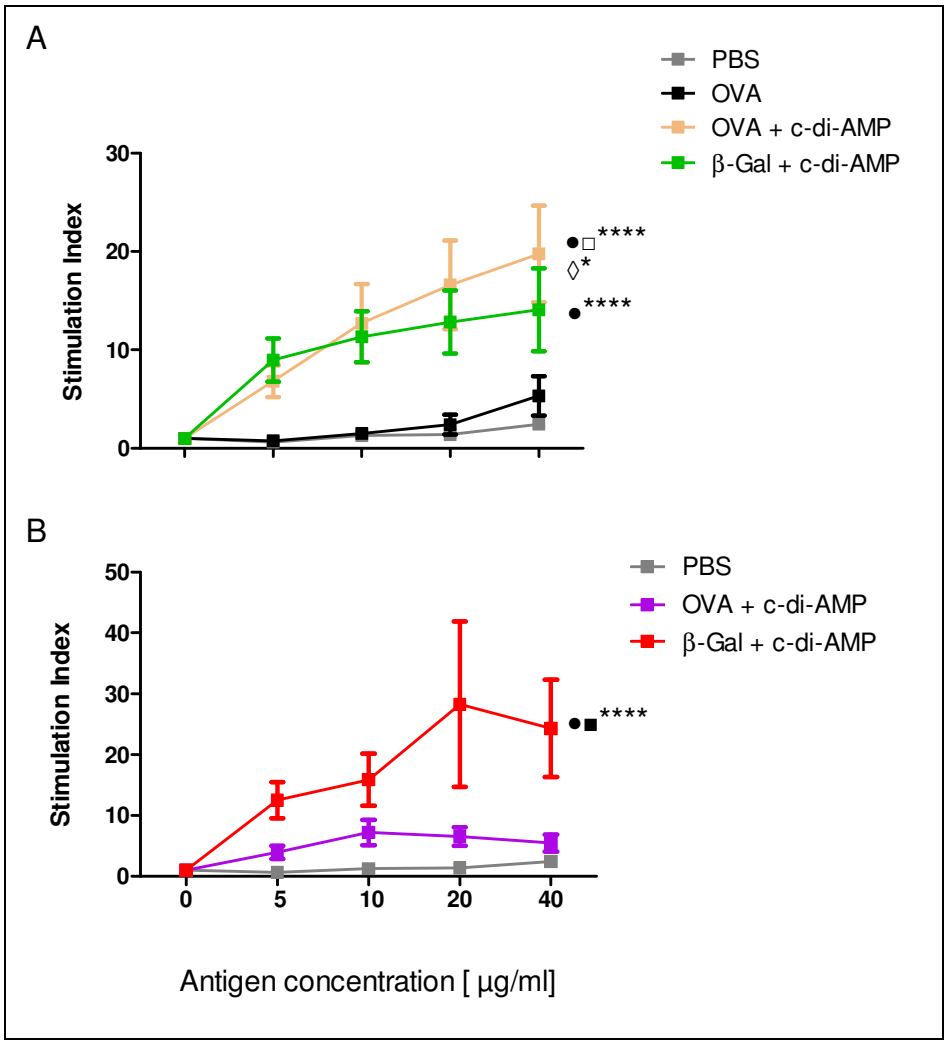

Figure 16: Evaluation of cellular immune responses in C57BL/6 mice immunised with OVA or β-Gal + c-di-AMP by mucosal routes.
Splenocytes derived from mice immunised by (A) i.n. or (B) i.vag. route were analysed for their proliferation. Spleen cells were *in vitro* restimulated with different concentrations of the corresponding ag. Results are expressed as the ratio between values (average of quadruplicates) from stimulated and non-stimulated samples (stimulation index). The standard deviation (SD) is indicated by vertical lines. The observed differences were statistically significant in comparison with the PBS group (●), the groups receiving ag alone (□) or the groups receiving OVA + c-di-AMP (■) or β-Gal + c-di-AMP (◊) at $p<0.05$ (*) and $p<0.0001$ (****) using the Two-way ANOVA.

The strongest spleen cell proliferation was stimulated in mice immunised with β-Gal + c-di-AMP via the i.vag. route. Here, the stimulation index of 28 was statistically different to that of splenocytes derived from mice receiving OVA + c-di-AMP or PBS alone ($p<0.0001$ (****)) (Fig. 16B). Lower differences in the proliferation rates of spleen cells were observed in mice immunised with OVA or β-Gal + adjuvant by i.n. route ($p<0.005$ (*)) (Fig. 16A). Nevertheless, for these groups stimulation was significantly higher as compared to the PBS group or

mice receiving ag alone with $p<0.0001$ (****). Mice immunised with OVA + c-di-AMP via i.vag. route showed marginal proliferative responses of spleen cells (Fig. 16B).

Cytokine secretion after restimulation of spleen cells with the corresponding ag was then assessed by an ELISPOT assay (section 3.7.4). Restimulation of spleen cells derived from mice receiving β-Gal + c-di-AMP resulted in increased numbers of cytokine secreting cells independently of the immunisation route, as compared to the results observed in mice receiving ag alone (Fig. 17). The highest levels of cytokine producing cells were obtained in mice immunised with β-Gal + c-di-AMP by the i.vag. route. Here, the higher numbers of cells produced IFN-γ, followed by those secreting IL-17 and IL-2. In all cases the secretion levels were significantly higher as compared to those observed in the control groups ($p<0.05$). Few IL-4 producing cells were detected (Fig. 17B). IFN-γ production was similar in spleen cells of mice immunised with β-Gal + c-di-AMP by i.vag. route as compared to that of mice immunised by the i.n. route (Fig. 17). IL-17 was significantly strongly produced after i.vag. immunisation using β-Gal + c-di-AMP than OVA + c-di-AMP ($p<0.05$). A similar profile, although with lower values, was observed in splenocytes derived from mice immunised with β-Gal + c-di-AMP and OVA + c-di-AMP by the i.n. route (Fig. 17A). In contrast, administration of OVA + c-di-AMP by the i.vag. route also elicited increased numbers of IFN-γ, IL-2 and IL-4 producing cells, but did not stimulate IL-17 production (Fig. 17B). Taken together, β-Gal was the most potent ag for stimulating strong cellular immune responses by either the i.n. or, more pronounced, the i.vag. route. OVA and β-Gal induced a mixed Th1/Th2/Th17 immune response after i.n. immunisation in combination with c-di-AMP. However, the cytokine profiles detected after vaccination seems to depend not only on the adjuvant but also on the ag used, since OVA + c-di-AMP was not able to stimulate strong IL-17 production when delivered by the i.vag. route.

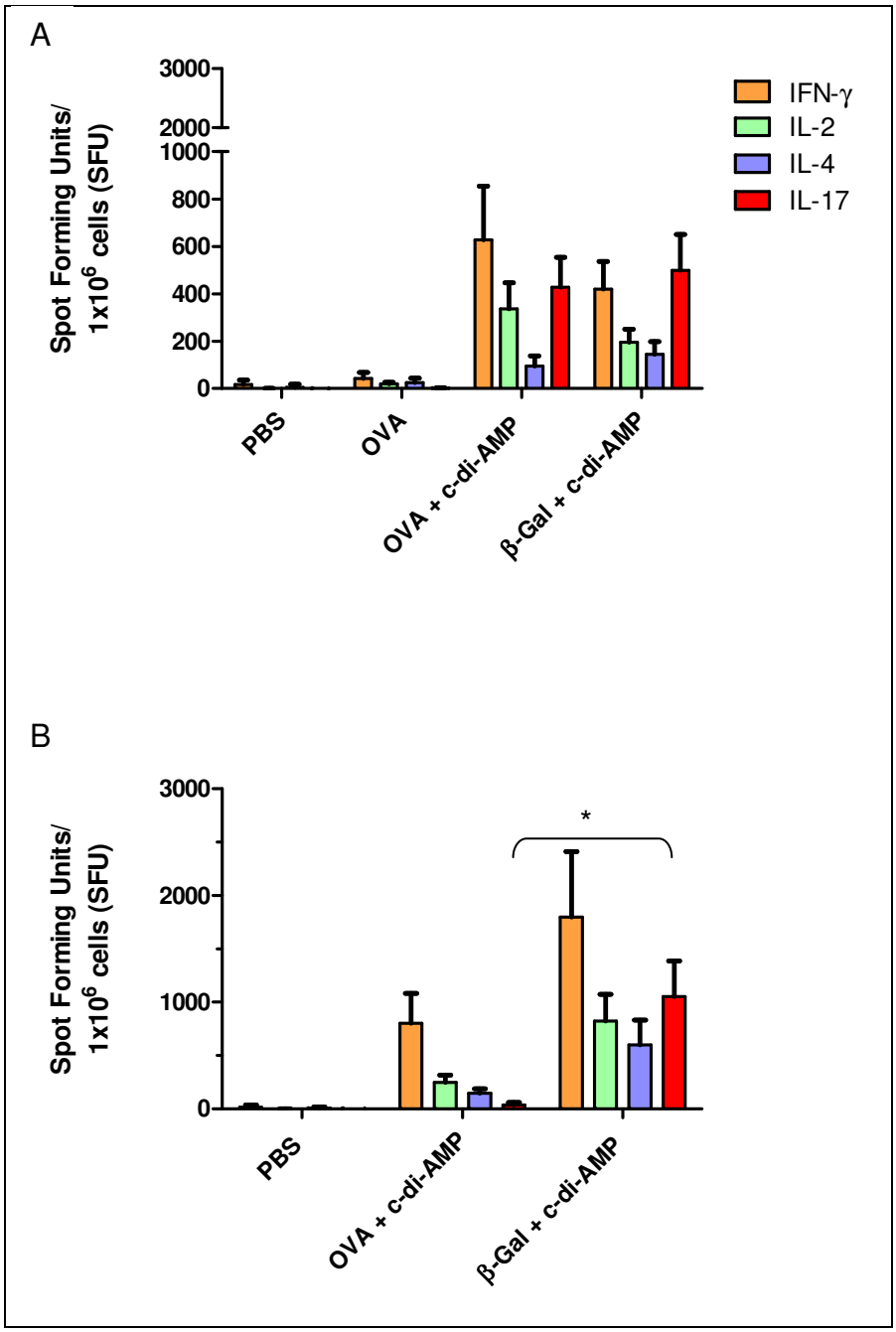

Figure 17: Secretion profile of cytokines of spleen cells after ag-restimulation.
Spleen cells from C57BL/6 mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by (A) i.n or (B) i.vag route were analysed for their cytokine profile. Splenocytes were cultured for 24 h (IFN-γ) or 48 h (IL-2, IL-4, IL-17) with the corresponding ag. Cytokine production was determined by ELISPOT. The SEM is indicated by vertical lines. Results are expressed as spot forming units per 10^6 cells with subtracted background. Differences were statistically significant at $p<0.05$ (*) analysed by the Two-way ANOVA test with respect to values in C57BL/6 mice receiving OVA + c-di-AMP.

4.2.3. Comparative studies of ag-specific immune responses in C57BL/6 and BALB/c mouse strains

As described in section 4.2.2, β-Gal + c-di-AMP induced stronger humoral and cellular responses at systemic and mucosal levels in C57BL/6 mice, as compared to OVA + c-di-AMP. However, it has been shown that the genetic background of the immunised animal can influence the polarisation of the stimulated immune responses. For example, infection of C57BL/6 mice with *Leishmania* induces a protective Th1 response, whereas in BALB/c mice an ineffective Th2 response is elicited [196]. Furthermore, even independently of the stimulated Th response pattern, genetic differences influence the susceptibility of the animals to certain pathogens, as shown before for the HSV-1 [197]. Another aspect is that C57BL/6 mice are able to develop a fast IFN type I response and therefore are less susceptible than BALB/c mice [198], [199]. Hence, studies have been performed to investigate the potential of the studied immunisation approaches in mice strains of different genetic background.

Most experiments in BALB/c mice were performed with β-Gal as ag. Here it was tested if and to which extent immune response of BALB/c mice immunised with β-Gal + c-di-AMP would vary from that of C57BL/6 mice. The i.n. route served as a golden standard and was compared to i.vag. administration. Animals received the formulations according to the usual immunisation protocol. At day 49 post priming the stimulated immune responses were evaluated. In this experiment the influence of DEPO-treatment on the immune responses stimulated after i.n. immunisation was also analysed. Therefore, all animals were DEPO-treated five days before each immunisation. From the literature, the levels of a-specific serum IgG, proliferation and cytokine secretion were in the same range in DEPO-treated and untreat mice following a similar i.n. immunisation protocol [68].

4.2.3.1. Induction of efficient local and systemic humoral immune responses in BALB/c mice after i.n. and i.vag. immunisation

Immunisation with β-Gal + c-di-AMP via the i.n. route induced the highest IgG titre in sera of BALB/c mice, followed by titre observed in mice receiving the same vaccine formulation by i.vag. route ($p<0.05$) (Fig. 18). In both experimental settings β-Gal + c-di-AMP induced statistically significant stronger IgG production as compared to OVA + c-di-AMP via the same route ($p<0.001$) (Fig.18). Concluding, β-Gal constitutes the most immunogenic ag to elicit humoral immune responses when co-administered with c-di-AMP. In contrast, in mice receiving PBS or ag alone only weak IgG titres have been stimulated if any at all.

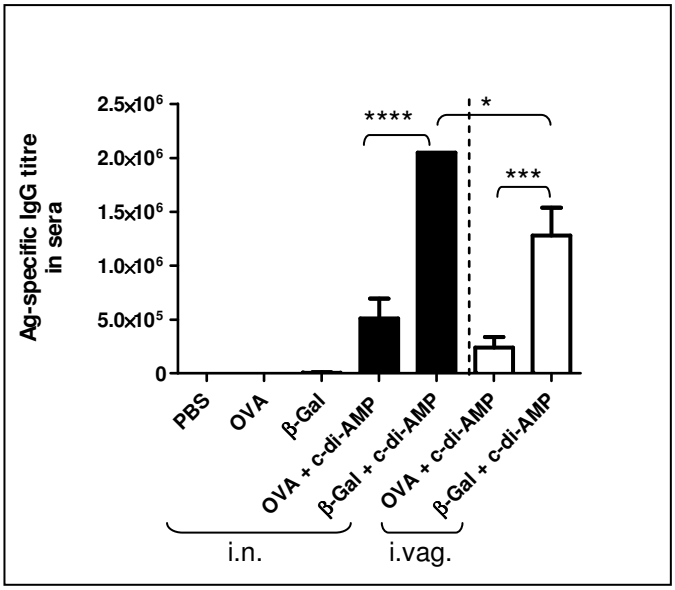

Figure 18: Induction of efficient systemic humoral immune responses in sera of BALB/c mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route.
Analysis of ag-specific IgG titres in sera of BALB/c mice (n=4/group) immunised by i.n. (black bars) or i.vag. (white bars) route. SEM is indicated by vertical lines. Differences were statistically significant at $p<0.005$ (*), $p<0.001$ (***) and $p<0.0001$ (****) shown by analysis with One-way ANOVA test.

When analysing the IgG subclass pattern stimulated in the different mouse strains, immunisation with ag + c-di-AMP via the i.n. or i.vag. route elicited similar profiles in BALB/c and C57BL/6 mice (Fig.15 and 19), respectively.

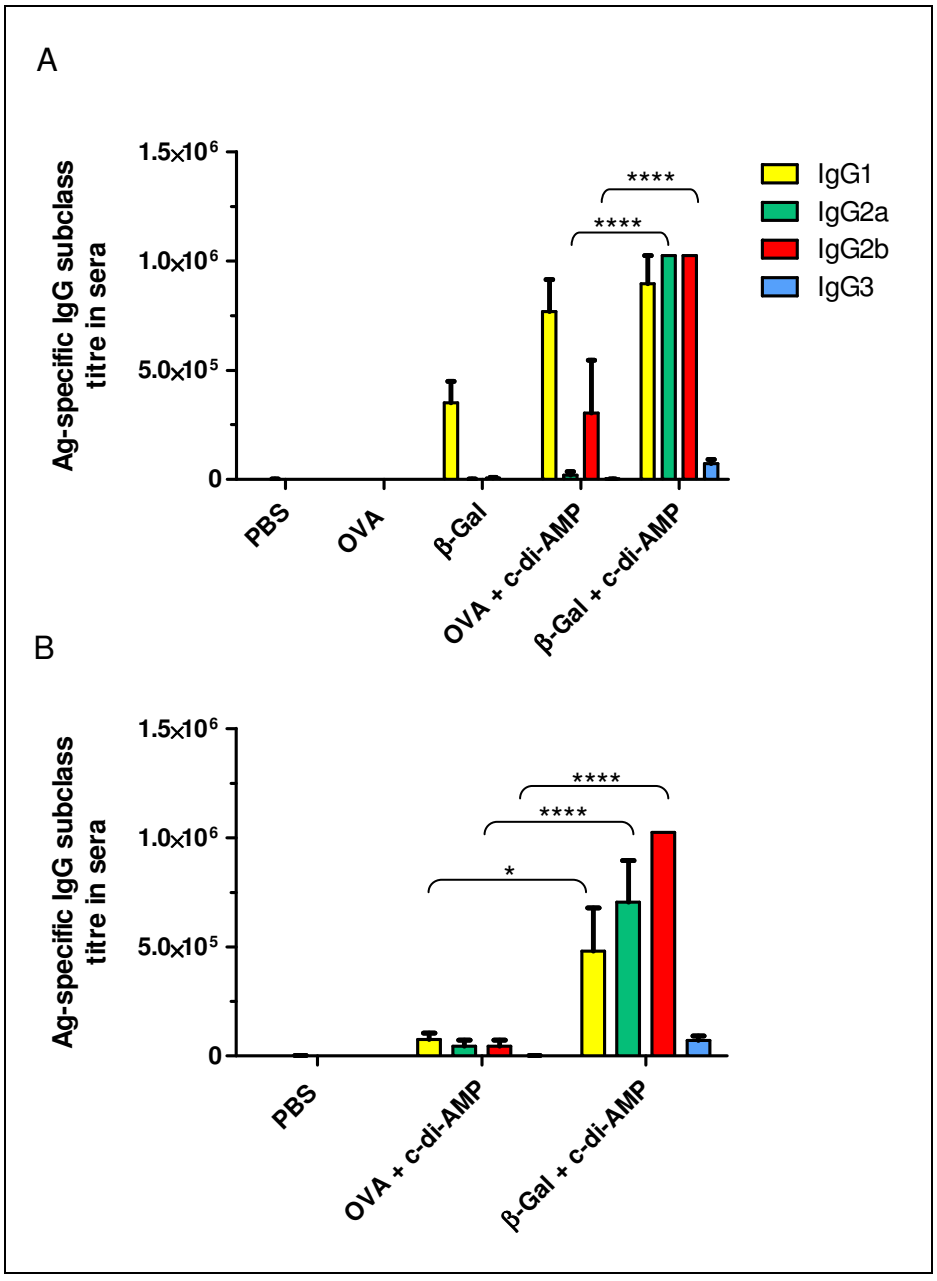

Figure 19: Distribution of IgG subclass titres in sera of BALB/c mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route.
Analysis of IgG subclass titres in sera stimulated in BALB/c mice after two booster immunisations (n=4/group) given by (A) i.n. or (B) i.vag. route. The SEM is indicated by vertical lines. Differences were statistically significant at $p<0.05$ (*), $p<0.0001$ (****) shown by Two-way ANOVA.

The IgG subclass distribution indicates similarities in groups immunised with β-Gal + c-di-AMP via i.n. and i.vag. route (Fig. 19). Significant higher IgG2a and 2b titres ($p<0.0001$) were observed in mice after immunisation with β-Gal with respect to animals receiving OVA + c-di-AMP. In animals immunised via the i.vag. route using β-Gal + c-di-AMP (Fig. 19), also IgG1 production was induced

significantly stronger, as compared to what observed using OVA + c-di-AMP by the same route ($p<0.05$). In all cases the levels of IgG3 were very low. Interestingly, while OVA co-administered with adjuvant delivered via the i.n. route stimulated increased levels of IgG1 and 2b but only weak IgG2a titres, immunisation via the i.vag. route stimulated similar levels of IgG1, 2a and 2b (Fig. 19). In none of the control groups β-Gal-specific IgG titres have been detected. Only i.n. immunisation with β-Gal alone stimulated an IgG1 titre of 1:352000. To sum up, immunisation with ag + c-di-AMP stimulated mixed Th1/Th2 responses, as indicated by the mixed IgG subclass pattern observed. However, stronger humoral responses have been elicited in BALB/c mice using β-Gal as model ag (Fig.15 and 19).

Beside the stimulation of antibody production at systemic level, we also analysed the differences in the level of IgA production in BALB/c and C57BL/6 mice. Again, β-Gal + c-di-AMP administered via i.n. route elicited the strongest titres of ag-specific IgA in VL, which were statistically significant different to titres obtained following OVA + adjuvant administration via i.n. or i.vag. route ($p<0.001$) (Fig. 20). Immunisation through the i.vag. route using β-Gal + adjuvant also induced strong IgA responses. However, these titres were not significantly higher, as compared to those obtained using OVA as ag. Comparing the levels of ag-specific IgA elicited after i.n. and i.vag. immunisation, i.n. delivery of β-Gal + c-di-AMP raised similar titres in VL of BALB/c and C57BL/6 mice, whereas i.vag. administration stimulated stronger IgA production in BALB/c mice (Fig.14 and Fig. 20). Groups receiving OVA alone showed only poor induction of IgA production in VL.

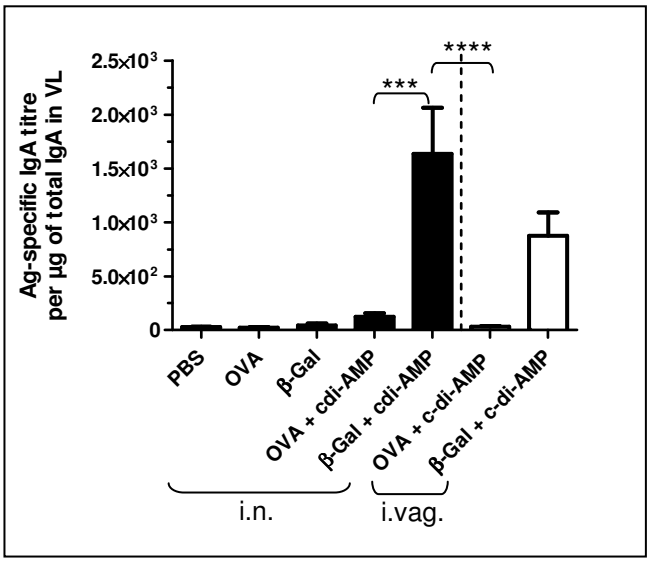

Figure 20: Local humoral IgA immune response of immunised BALB/c mice in VL.
Analysis of ag-specific IgA titres in lavage samples of BALB/c mice (n=4) after i.n. (black bars) or i.vag. immunisation (white bars). SEM is indicated by vertical lines. Differences were statistically significant at $p<0.001$ (***), $p<0.0001$ (****) and analysed by One-way ANOVA.

Thus, in order to achieve strong humoral immune responses at local as well as at systemic level, immunisation of BALB/c mice using β-Gal + c-di-AMP is the most efficient vaccination protocol tested here.

4.2.3.2. Stimulation of ag-specific cellular responses in BALB/c mice immunised by i.n. or i.vag. route

The proliferation of spleen cells after *ex vivo* restimulation was measured by ^{3}H-thymidine incorporation, which was evaluated as CPM and expressed as stimulation index. The strongest lymphocyte proliferation was observed in cells of mice immunised with β-Gal + c-di-AMP by either i.vag. (SI of 100) or i.n. (SI of 70) route (Fig. 21). However, the values obtained in mice immunised with β-Gal + c-di-AMP were also significantly different as those observed in groups receiving OVA + c-di-AMP following restimulation with 20 µg/ml (i.n.) or 40 µg/ml (i.vag.) of the corresponding ag ($p<0.001$ for i.n. and $p<0.01$ for i.vag. route, Fig. 21).

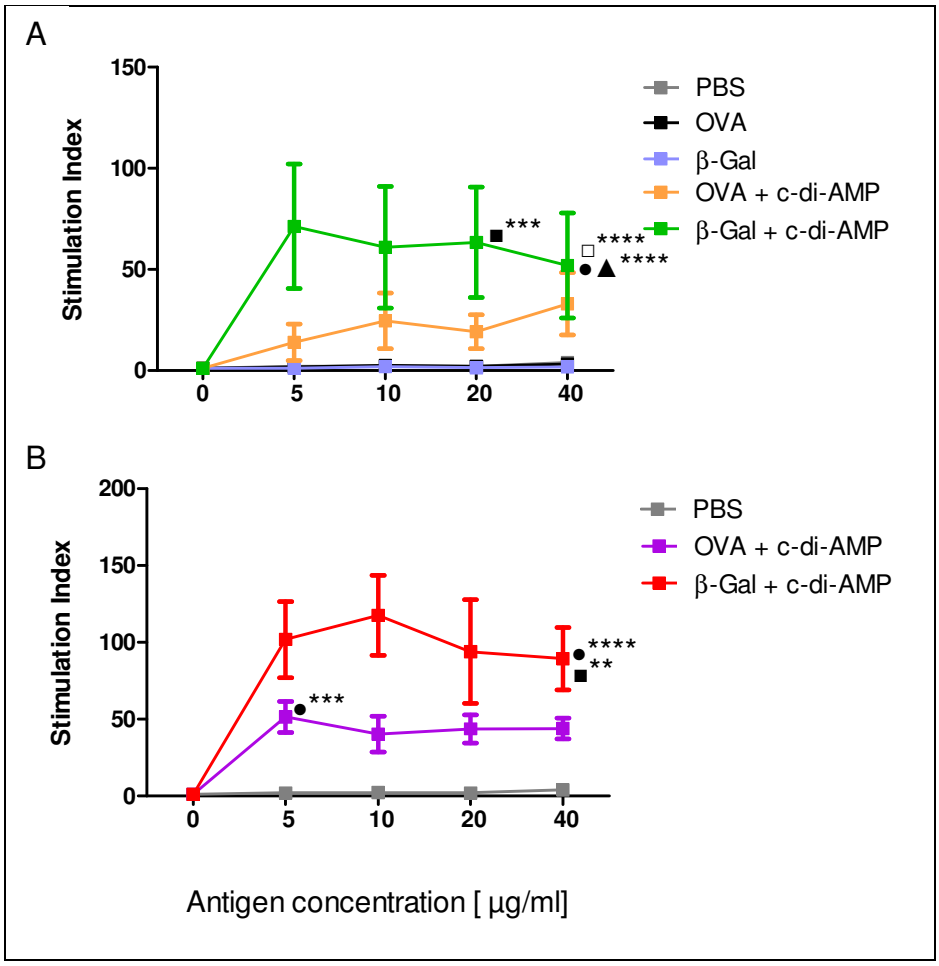

Figure 21: Proliferation of restimulated spleen cells of mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route.
Analysis of the proliferative capacity of spleen cells derived from BALB/c mice (n=4) immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by either (A) i.n. or (B) i.vag. route. SD is indicated by vertical lines. For the statistical analysis Two-way ANOVA was used and showed that differences were statistically significant at $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****), in comparison with the PBS control (●), the groups receiving OVA (□) or β-Gal alone (▲) or mice receiving OVA + c-di-AMP (■).

Splenocytes derived from BALB/c mice showed up to four times increased proliferative capacities as compared to those of C57BL/6 mice (Fig. 16 and 21). Interestingly, comparing the proliferative capacities of spleen cells from mice immunised with β-Gal + c-di-AMP via i.n. by i.vag. route, i.vag. application stimulated significantly higher proliferation rates than i.n. application ($p<0.0001$) (Fig.21). However, maximal proliferation rate of splenocytes was already reached after restimulation of splenocytes with 5 µg/ml of the corresponding ag (Fig. 21).

The strong immune-stimulatory capacity of β-Gal + c-di-AMP indicated by high stimulation index was further confirmed by the enhanced number of cytokine producing spleen cells.

Again, administration of this vaccine formulation promoted stronger cytokine production than the formulation containing OVA as ag (Fig. 22). In addition, as in the case of the immunisation studies using C57BL/6 mice, i.vag. immunisation stimulated higher numbers of cytokine secreting cells with respect to the i.n. application strategy (Fig. 22). This was also consistent with the results obtained immunising C57BL/6 mice (Fig. 17). However, the distribution pattern of the produced cytokines was similar when comparing values obtained following the i.n. and i.vag. immunisation protocols (Fig. 22).

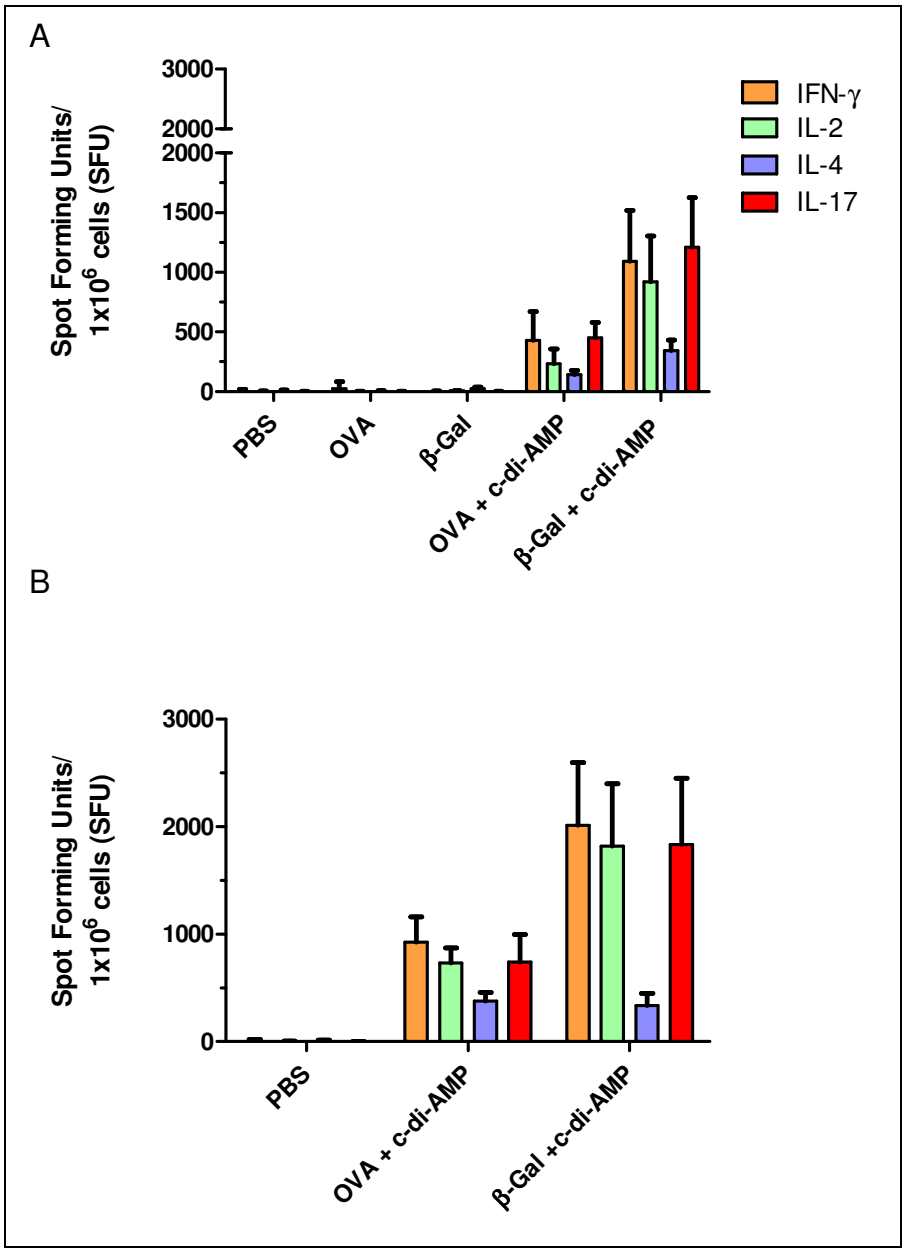

Figure 22: Cytokine secretion profile of splenocytes derived from BALB/c mice immunised with OVA/c-di-AMP or β-Gal/c-di-AMP by the i.n. or i.vag. route.
Cytokine production after (A) i.n. or (B) i.vag. immunisation. SEM is indicated by vertical lines.

Thus, the numbers of IFN-γ, IL-2 and IL-17 secreting cells were significantly different to those obtained in all control groups ($p<0.05$). Nevertheless, similar to what was observed immunising C57BL/6 mice, the results obtained using β-Gal were not significantly different as compared to those using OVA as model ag (Fig. 22). Remarkably, in contrast to the results obtained after immunising C57BL/6 mice, vaccination of BALB/c mice with β-Gal + c-di-AMP stimulated increased numbers of IL-2 producing splenocytes. In addition, vaccination via

the i.vag. route using OVA + c-di-AMP resulted in increased numbers of IL-17 producing spleen cells, which was also in contrast to what observed in C57BL/6 mice (Fig. 17B and 22B).

As in the case of C57BL/6 mice, i.n. immunisation of BALB/c mice with β-Gal + c-di-AMP stimulated the same pattern of cytokine secretion as immunisation via the i.vag. route. Here, only 65% of the spleen cells isolated after i.vag. immunisation have been detected to be IL-17 producing cells, followed by IFN-γ (50%), IL-2 (50%) and IL-4 (42%) producing cells (Fig. 22A). The same was true after immunisation of mice with OVA + c-di-AMP, with similar cytokine secretion profiles observed following i.n. and i.vag. vaccination. Moreover, higher numbers of cytokine producing cells were elicited after i.vag. immunisation, as compared to those obtained following i.n. immunisation.

In conclusion, β-Gal + c-di-AMP stimulated spleen cells to a stronger cytokine production than OVA did, with i.vag. immunisation triggering higher cytokine levels as compared to i.n. immunisation. The observed Th response pattern was similar, with ag + c-di-AMP promoting a mixed Th1/Th2/Th17 response.

4.2.4. Influence of mucosal adjuvants on the production of IL-17

Th17 cells are a pivotal component for mucosal immunity. In this context, the cytokine IL-17 seems to be important for protection against pathogens such as *Klebsiella pneumonia* or *Candida spp.* and can be induced by certain microbial peptides [200], [201], [202]. Since IL-17 is known to play a role in mucosal immune responses, it was of special interest in the context of this work. Therefore, the following experiments were conducted to characterise the influence of different adjuvants on the outcome of the stimulated immune responses. To this end, animals were immunised following the vaccination protocol which was identified to stimulate the strongest immune responses by the above-described studies.

BALB/c mice were immunised with β-Gal (15 µg/dose) co-administered with adjuvant c-di-AMP (5 µg/dose) by the i.n. route. In addition, another vaccine formulation was implemented based on the use of α-GalCerMPEG (5 µg/dose)

as adjuvant. The adjuvant α-GalCerMPEG has been chosen for the investigation of the role of IL-17 in host defence since it was shown to inhibit the IL-17 production in later phases of the immune response [203]. In contrast, c-di-AMP enhances IL-17 production at late phase of the immune response (three weeks after the last boost) [68].

Furthermore, a side-by-side comparison of the responses stimulated after i.n. and i.vag. immunisation of BALB/c mice has been performed, in order to investigate the impact of the immunisation route on the resulting immune responses. Vaccination followed the usual immunisation protocol, as described in 3.3.4.6. Animals immunised via the i.vag. route were DEPO-treated five days before each immunisation. As expected, immunisation with β-Gal + c-di-AMP stimulated high numbers of IL-17-producing splenocytes, whereas no IL-17 secreting cells have been observed following vaccination with β-Gal + α-GalCerMPEG (Fig. 23).

4.2.4.1. Stimulation of efficient local and systemic humoral immune responses in BALB/c mice immunised with β-Gal co-administered with mucosal adjuvants by i.n. or i.vag. route

Both tested adjuvants stimulated increased ag-specific IgG titres in sera of immunised mice, as compared to those observed in mice receiving the ag alone, independent of the administration route ($p<0.0001$) (Fig. 23). Furthermore, immunisation with β-Gal + c-di-AMP resulted in significantly higher IgG titres in sera of mice (n=5/group) than those observed in mice immunised with β-Gal + α-GalCerMPEG ($p<0.0001$) (Fig. 23).

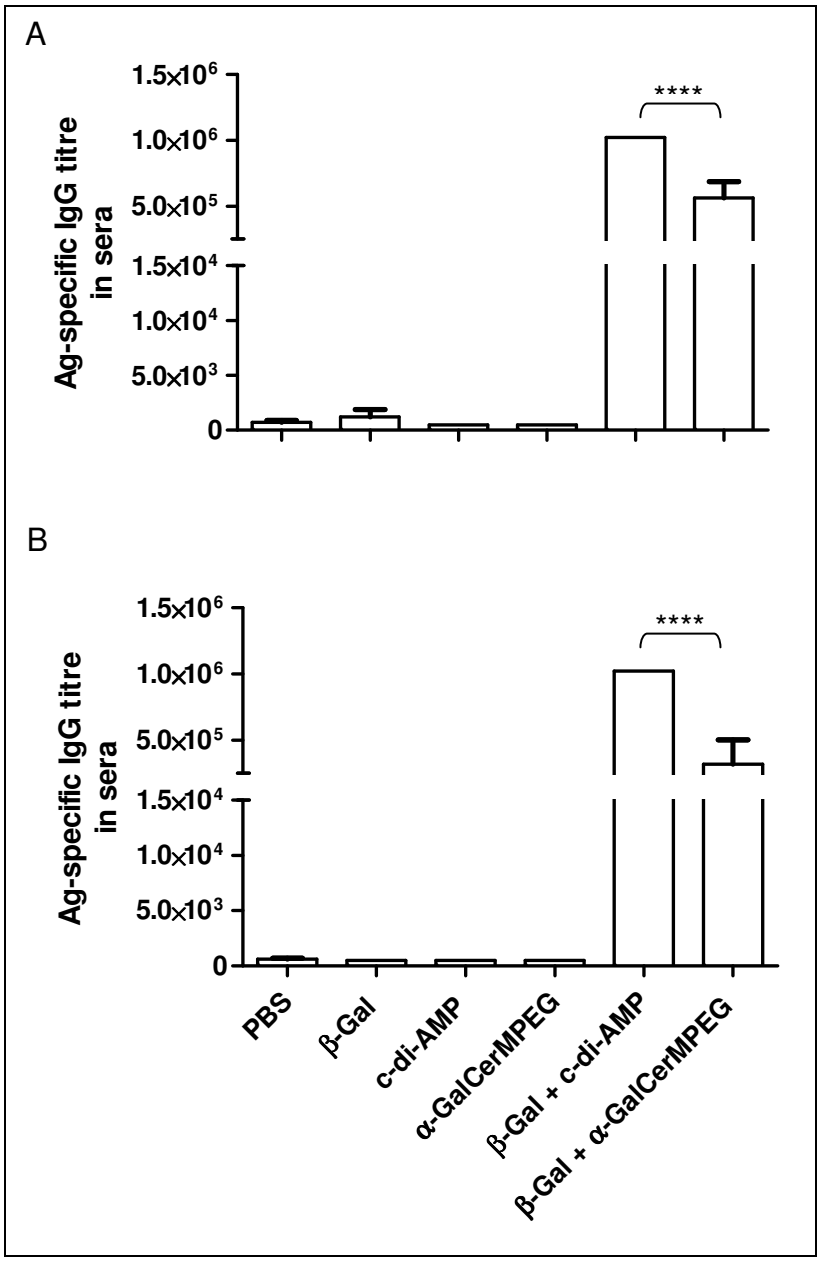

Figure 23: Induction of efficient systemic humoral immune responses in sera of BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG.
Ag-specific IgG titres in sera of BALB/c mice (n=5/group) stimulated after three immunisations through (A) i.n. or (B) i.vag. route. SEM is indicated by vertical lines. Differences were statistically significant at $p<0.0001$ (****) analysed by One-way ANOVA test.

Consequently, the adjuvant α-GalCerMPEG seems to be less potent than c-di-AMP at eliciting IgG responses in sera, independently of the route of immunisation.

Beside the strength of the humoral immune responses stimulated by α-GalCerMPEG also its immunomodulatory properties have been investigated. To

this end, the IgG subclass profiles stimulated after i.n. and i.vag. vaccination were evaluated. While c-di-AMP co-administered with ag elicited a mixed Th1/Th2 IgG response, as indicated by similar levels of IgG1, 2a and 2b, α-GalCerMPEG stimulated a IgG1 dominated Th2 immune response (Fig. 24). In fact, significantly lower IgG2a and 2b titres were elicited, as compared to those obtained using c-di-AMP as adjuvant ($p<0.0001$) (Fig. 24). In fact, significantly lower IgG2a and 2b titres were elicited, as compared to those obtained using c-di-AMP as adjuvant ($p<0.0001$) (Fig. 24). Interestingly, while administration of β-Gal + α-GalCerMPEG via i.n. route also stimulated increased levels of IgG2a and IgG2b, almost no production of these IgG subtypes have been observed when β-Gal + α-GalCerMPEG was administered via i.vag. route (Fig. 24).

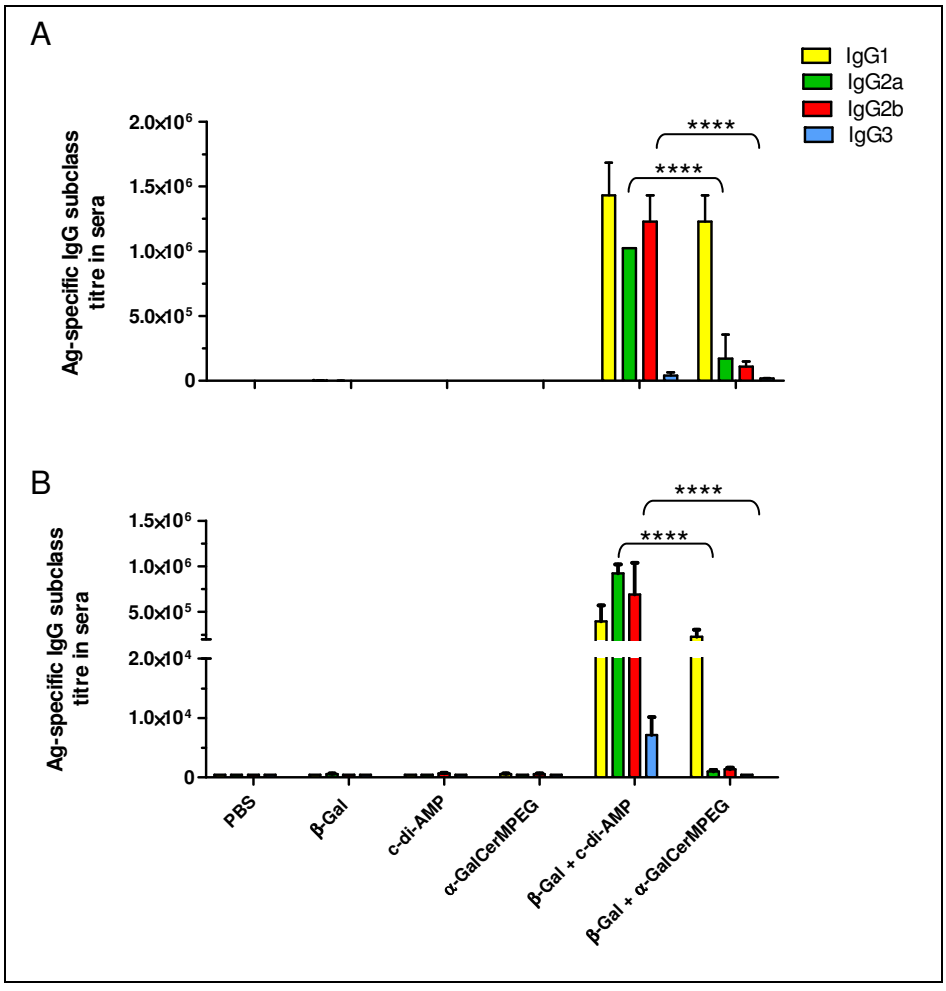

Figure 24: Distribution of IgG subclass titres in sera of BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG.
Analysis of the different IgG subclass titres in sera derived from BALB/c mice (n=5/group) immunised on days 0, 14 and 28 with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG by (A) i.n. or

(B) i.vag. route. SEM is indicated by vertical lines. Differences were statistically significant at p<0.0001 (****) analysed by Two-way ANOVA test.

It is known that i.n. vaccination stimulates immune responses not only in the respiratory tract but also at distant mucosal territories, such as the genital tract [204], [205], [206]. The stimulation of mucosal immune responses at areas distant to the site of infection or vaccine application is based on the compartmentalisation of the mucosal immune system. Furthermore, recent studies showed, that i.n. immunisation of mice with ag + c-di-AMP is able to stimulate increased IgA titres in different mucosal areas [68]. However, unlike to i.n. vaccination, immunisation via i.vag. route has been reported to stimulate immune responses mainly at the site of vaccine application [112]. Thus, the stimulation of efficient mucosal immune responses at local and distant territories using c-di-AMP and α-GalCerMPEG as adjuvant has been investigated in a side-by-side comparison.

When animals were immunised by the i.n. route, administration of β-Gal + c-di-AMP stimulated significantly increased levels of IgA in VL, as compared to those observed in animals receiving β-Gal + α-GalCerMPEG or in the control groups (p<0.001) (Fig.25).

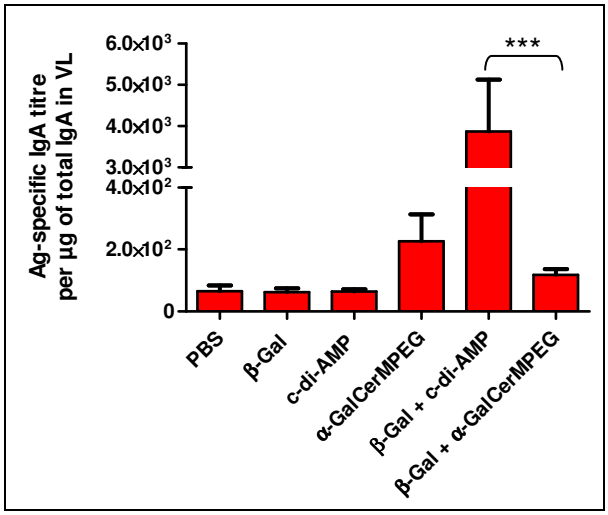

Figure 25: Induction of local humoral immune response in VL of BALB/c mice.
Ag-specific IgA titre in VL induced in BALB/c mice immunised three times with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG by i.n. route. SEM is indicated by vertical lines. Differences were statistically significant at p<0.001 (***) analysed by One-way ANOVA test.

Furthermore, i.vag. immunisation with β-Gal + c-di-AMP elicited significantly higher IgA titres in VL, as compared to immune responses stimulated after

administration of β-Gal + α-GalCerMPEG and in all control groups ($p<0.0001$) (Fig. 26A). As expected, no differences in the levels of ag-specific IgA were observed in saliva, NL and LL, when samples obtained from animals in the PBS control group and groups receiving ag + adjuvant were compared (Fig. 26B). This confirms that there is not efficient homing to distant mucosal territories following i.vag. vaccination [112].

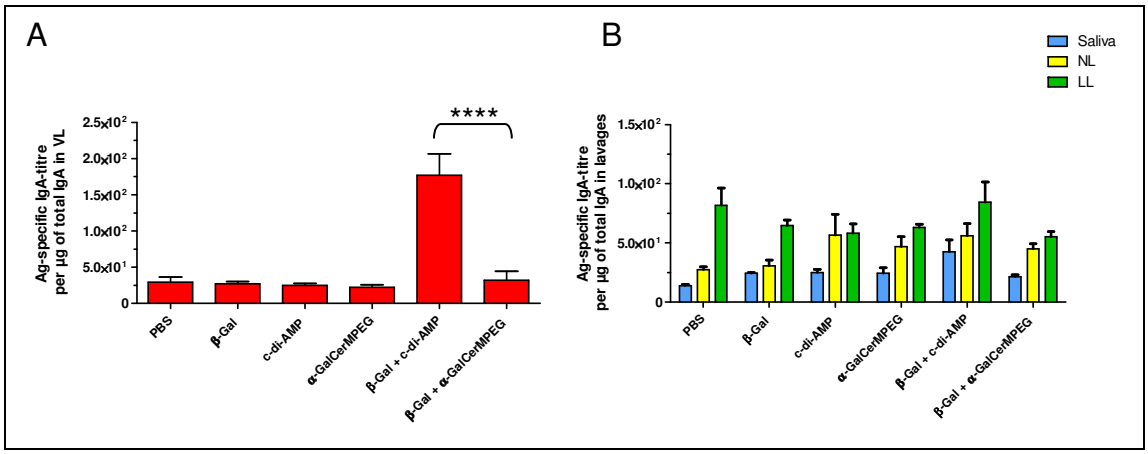

Figure 26: Induction of mucosal immune responses in VL samples of BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG by i.vag. route.
(A) The ag-specific IgA titre elicited was tested in VL derived from immunised mice. (B) Ag-specific IgA titre detected in saliva, NL and LL of immunised mice. SEM is indicated by vertical lines. Differences were statistically significant at $p<0.0001$ (****) and analysed by Two-way ANOVA test.

4.2.4.2. Induction of enhanced cellular proliferation and cytokine production in BALB/c mice immunised with β-Gal co-administered with adjuvants by i.n. or i.vag. route

Besides the stimulation of humoral immune responses, α-GalCerMPEG is also known to stimulate strong cellular responses when administered via the i.n. route [207]. Here, co-administration of β-Gal + α-GalCerMPEG also stimulated increased cellular responses, as compared to those of mice receiving β-Gal alone ($p<0.0001$). However, the proliferative capacity of spleen cells derived from mice immunised i.n. with β-Gal + α-GalCerMPEG was significantly weaker, as compared to that observed using c-di-AMP as adjuvant ($p<0.01$) (Fig. 27A). Similar results have been obtained following i.vag. immunisation (Fig. 27B). Again, vaccination with β-Gal + c-di-AMP stimulated significantly stronger

cellular proliferation (stimulation index of about 22) as compared to all other groups immunised by i.vag. route ($p<0.0001$) (Fig. 27B). Interestingly, while c-di-AMP seemed to stimulate stronger responses in mice immunised via the i.vag. than the i.n. route, the route of application seems to play a minor role when using α-GalCerMPEG as adjuvant (Fig. 27).

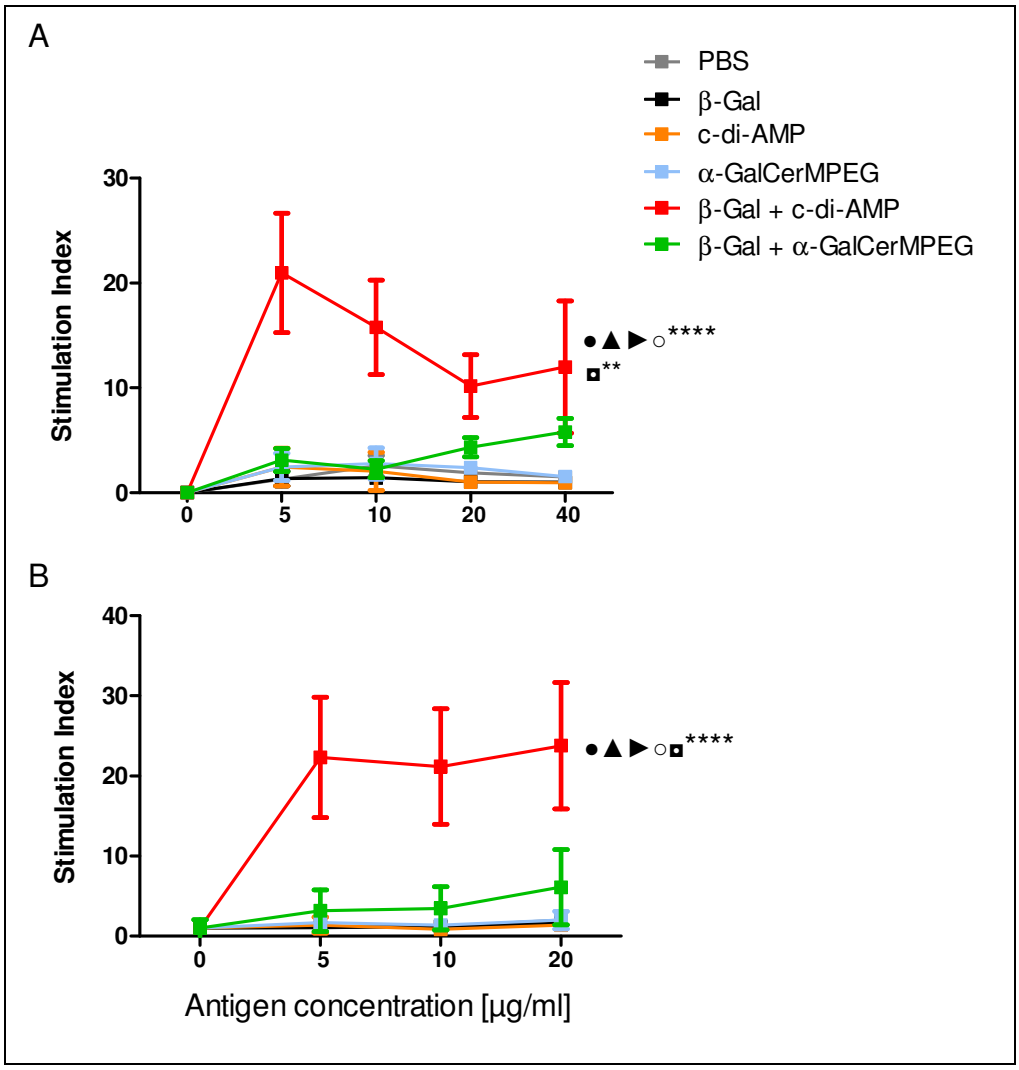

Figure 27: Cellular proliferation of *ex vivo* ag-restimulated spleen cells.
The proliferation of spleen cells derived from BALB/c mice immunised by (A) i.n. or (B) i.vag. route was tested. SD is indicated by vertical lines. Differences were statistically significant at $p<0.01$ (**) and $p<0.0001$ (****) and analysed by the Two-way ANOVA test with respect to the PBS control group (●), the groups receiving β-Gal (▲), c-di-AMP alone (►), α-GalCerMPEG alone (○) or β-Gal + α-GalCerMPEG (◘).

Then, the cytokine profiles stimulated in mice following vaccination with β-Gal + α-GalCerMPEG via the i.n. and i.vag. routes were investigated and compared with those stimulated using c-di-AMP as adjuvant (Fig. 28).

As expected, immunisation with β-Gal + α-GalCerMPEG stimulated significant lower levels of cytokine secreting cells, as compared to those observed in the group immunised with β-Gal + c-di-AMP, independently of the route of application (Fig. 28). However, the main sub-population stimulated by c-di-AMP was IL-17 secreting cells, followed by IFN-γ, IL-2 and IL-4 expressing splenocytes (Fig. 28). In contrast, groups immunised with the β-Gal + α-GalCerMPEG showed no IL-17 producing cells and only marginal numbers of IFN-γ and IL-2 secreting cells. Here, mainly IL-4 secreting cells were induced (Fig. 28). However, despite the clear trend, the detected numbers of cytokine secreting cells did not differ significantly from those observed in control groups. As seen for the proliferative capacities of the splenocytes derived from immunised mice, also the levels of cytokine producing cells were higher after i.vag. immunisation than following vaccination by i.n. route (Fig. 28). Thus, the number of IL-2 producing cells stimulated after i.vag. immunisation with β-Gal + c-di-AMP was three-fold higher than in the i.n. group ($p<0.01$) (Fig. 28). The same was true in case of the number of IL-17 producing cells. Again, the values observed following i.vag. immunisation with β-Gal + c-di-AMP were significantly higher as compared to those following i.n. vaccination ($p<0.05$) (Fig. 28).

In parallel experiments, it was tested if i.n. vaccination using β-Gal + c-di-AMP induced $CD8^+$ IFN-γ secreting cells in spleens after restimulation with the CD8-peptide (Fig. 28C left bar). Interestingly, i.vag. immunisation does not resulted in the stimulation of $CD8^+$ IFN-γ secreting cells in spleens (Fig. 28C right bar). In conclusion, the i.n. immunisation using the above-mentioned formulation stimulates $CD4^+$- and $CD8^+$-cells to secrete IFN-γ, which was tested by restimulation of spleen cells with ag-peptide (Fig. 28C) and ag-protein (Fig. 28A), whereas vaginal immunisation mainly stimulates $CD4^+$-cells to secrete IFN-γ after restimulation with the ag-protein (Fig. 28B).

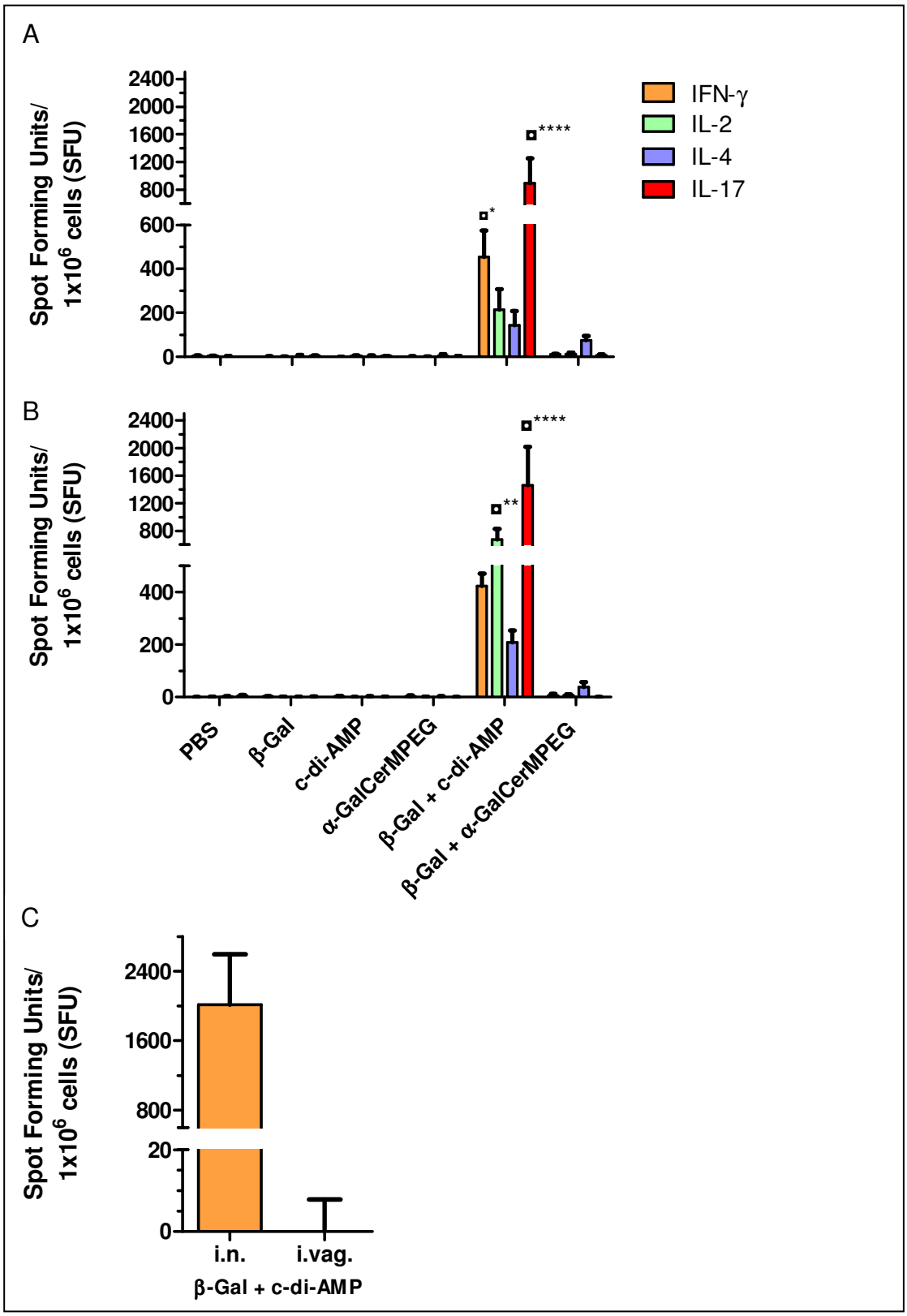

Figure 28: Cytokine profile of ag-restimulated spleen cells derived from BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG.
Cytokines secreted by *ex vivo* ag-restimulated spleen cells from BALB/c mice immunised three times by (A, C left bar) i.n or (B, C right bar) i.vag. route. Cells were incubated for 24 or 48 h in the presence of a peptide encompassing the immunodominant Ld-restricted epitope of β-Gal (TPHPARIGL), which is specific for MHC class I presentation (for IFN-γ i.n. and i.vag. Fig 28C), or the β-Gal protein for IFN-γ, IL-2, IL-4 and IL-17 (Fig. 28 A + B). SEM is indicated by vertical

lines. The differences were statistically significant tested with the Two-way ANOVA at p<0.05 (*), p<0.01 (**), p<0.0001 (****) with respect to the group receiving β-Gal + α-GalCerMPEG (◘).

To test if the compartmentalisation of the mucosal immune system allows also IL-17 secretion in the distant vaginal draining LN (iliac LN (iLN)) following i.n. vaccination, the stimulation of local cellular responses has been analysed. As shown in figure 29, β-Gal + c-di-AMP elicited also high numbers of IL-17 secreting cells in iLN, as compared to all other groups (p<0.05) (Fig. 29). In contrast, ag + α-GalCerMPEG was not able to stimulate IL-17 producing cells in the iLN (Fig. 29).

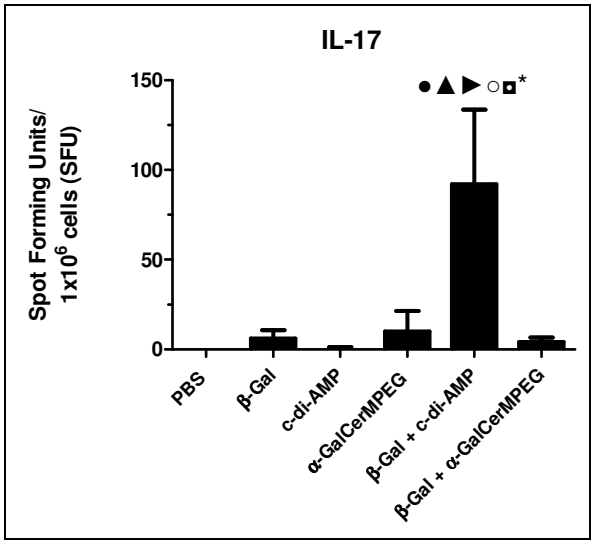

Figure 29: Cytokine secretion of IL-17 produced by iLN cells following i.n. immunisation. Cellular responses stimulated after ag-specific restimulation following three i.n. immunisations using β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG. SD is indicated by vertical lines. Differences were statistically significant tested with the One-way ANOVA at p<0.0001 (****) with respect to the PBS control group (●), and the groups receiving β-Gal (▲), c-di-AMP (►), α-GalCerMPEG (○), or β-Gal + α-GalCerMPEG (◘).

The cytokine IL-17 and Th17 cells seem to play an important role in mucosal host defence. Thus, due to their capacity to specifically induce or block Th17 polarisation, the two adjuvants c-di-AMP and α-GalCerMPEG constitute powerful tools to gain a more in depth knowledge of the underlying processes.

4.3. Evaluation of the s.l. immunisation strategy as alternative approach for i.n. immunisation

Immunisation via the i.n. route is a very prominent and powerful vaccination strategy. As shown above, vaccine formulations applied by this route stimulate strong humoral and cellular immune responses not only at systemic but also at local mucosal areas [121]. However, it has been shown that there is the risk of vaccine redirection to the central nervous system (CNS) via retrograde axonal transport, as well as an association to neurological side effects, such as Bell's palsy, after i.n. vaccination with formulations based in A-B moiety toxins and their derivatives [127]. Thus, alternative strategies need to be developed combining the advantages of i.n. vaccination with an improved safety profile. Immunisation via the s.l. route seems to be one of such approaches. Therefore, comparative studies were performed among formulations administered by other traditional mucosal routes and sublingually.

4.3.1. Immunisation via s.l. route differs from oro-gastric application

In order to confirm that in our experimental setting s.l. administration did not result in deglutition and subsequent oral immunisation, a side-by-side comparison of both administration routes was performed. To this end, BALB/c mice were immunised with β-Gal (30 µg/dose) + c-di-AMP (10 µg/dose) via s.l., oral and, as a golden standard, i.n. routes (n=4/group). The oral immunisation was performed using a feeding needle. Parts of the results in chapter 4.3.1. were pre-published [195].

4.3.1.1. Immune responses stimulated following s.l. and oral immunisation differ in strength and shape

Immunisation via s.l. route stimulated significant higher IgG titres in sera compared to oral immunisation ($p<0.0001$) (Fig. 30). However, the ag-specific

IgG titres stimulated following s.l. immunisation were similar to those detected after i.n. immunisation (Fig. 30).

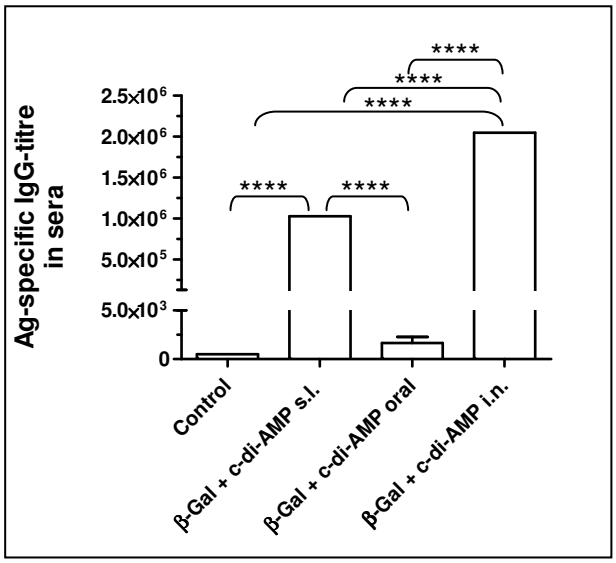

Figure 30: IgG titre in sera of BALB/c mice immunised by s.l., i.n. or oral route.
Analysis of ag-specific IgG titre in sera derived of BALB/c (n=4) immunised with β-Gal + c-di-AMP by s.l., oral or i.n route. SEM is indicated by vertical lines. Differences were statistically significant tested with One-way ANOVA at $p<0.0001$ (****).

Furthermore, as in the case of i.n. immunisation, vaccination via the s.l. route also stimulates a mixed Th1/Th2 response (Fig. 31). In both cases the observed IgG1, 2a and 2b titres were significantly higher, as compared to those occurring after oral immunisation ($p<0.05$). However, oral immunisation did not elicit significantly higher titres in sera, as compared to the titres induced in the control group (Fig. 31).

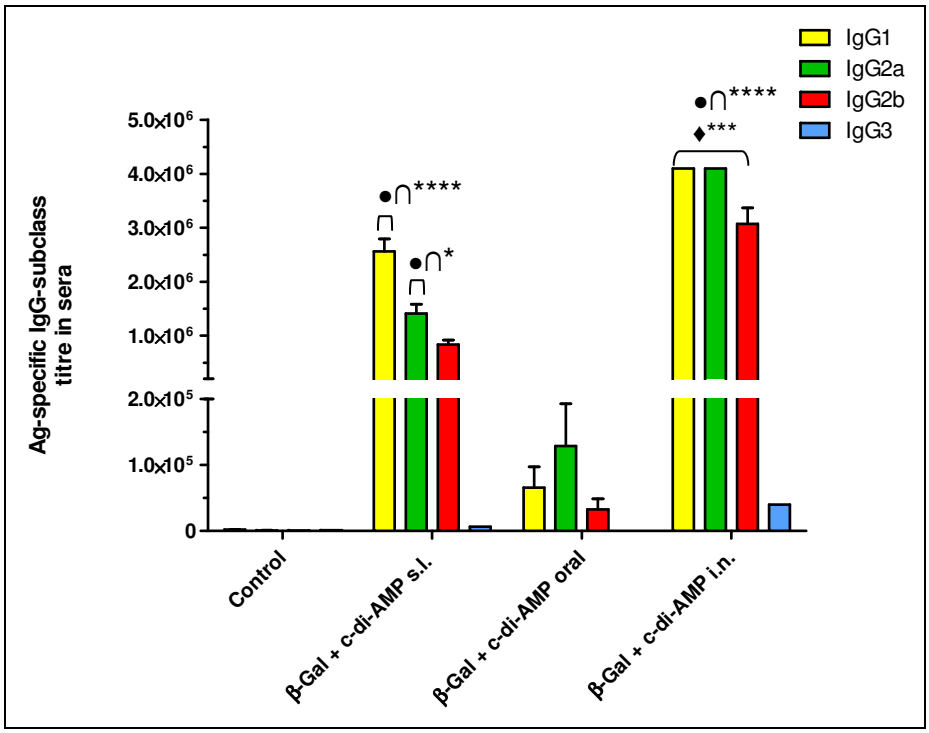

Figure 31: IgG subclass pattern in sera of BALB/c mice after immunisation via s.l. or i.n. or oral route.
Humoral immune responses of BALB/c immunised with β-Gal + c-di-AMP via the s.l., oral or i.n route. SEM is indicated by vertical lines. Differences were statistically significant, as tested by Two-way ANOVA, $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****), with respect to the PBS group (●), and the groups receiving β-Gal + c-di-AMP via s.l. (♦) or oral (∩) or i.n. (◊) route

These results confirmed that the responses obtained after s.l. vaccination are truly due to locally initiated process rather than by a trivial passage of ag and adjuvant to the gut. Furthermore, the obtained local humoral immune responses support the assumption that responses stimulated by s.l. administration differ qualitatively from those obtained after oral or i.n. immunisation. Nevertheless, in order to further validate this, the stimulated local immune responses were also analysed. Similar titres of ag-specific IgA were detected in mucosal samples of mice immunised via s.l. and i.n. route, respectively. While in both vaccination groups the highest titres were detected in saliva followed by VL, NL and LL, s.l. immunisation stimulated less IgA production in LL as compared to i.n. immunisation (Fig. 32). However, significant higher IgA titres were stimulated in saliva after s.l. and i.n. immunisation, as compared to the titres induced following immunisation by oral route ($p<0.001$) (Fig. 32).

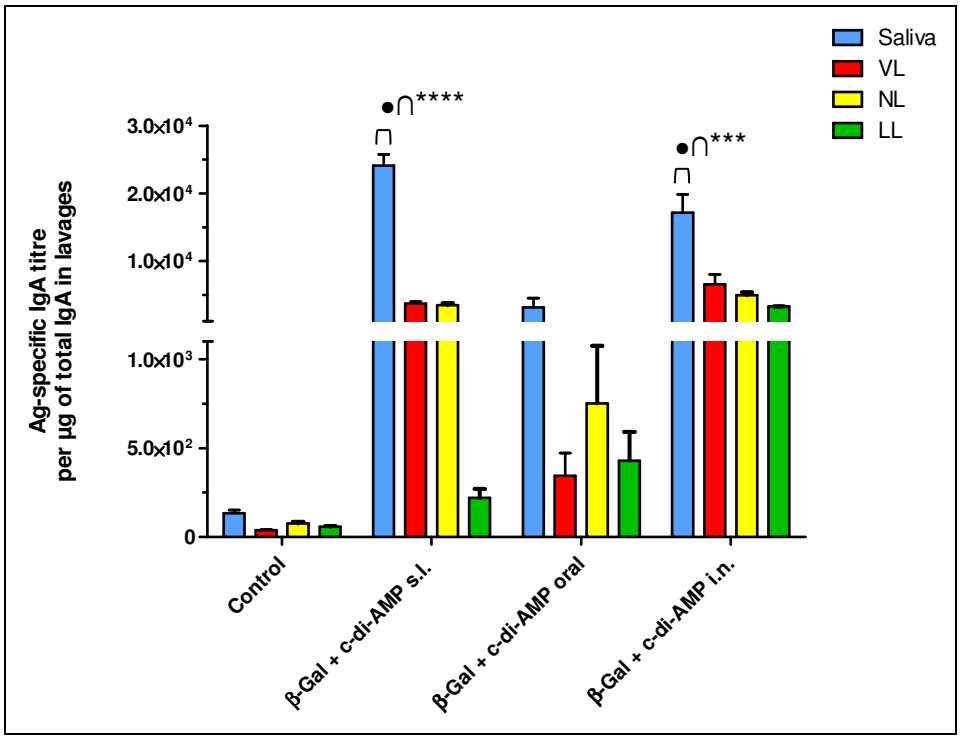

Figure 32: Elicitation of humoral immune responses at distinct mucosal sites following s.l., oral and i.n. immunisation.
BALB/c (n=4) were immunised with β-Gal + c-di-AMP via s.l., oral or i.n route. SEM is indicated by vertical lines. Differences were statistically significant as tested by Two-way ANOVA [$p<0.001$ (***), $p<0.0001$ (****)] with respect to the PBS control group (●) and the groups receiving β-Gal + c-di-AMP via oral (∩) or i.n. (◊) route.

4.3.1.2. Determination of the cellular immune responses elicited after s.l., oral and i.n. immunisation

After providing a clear proof that immunisation via s.l. route differs from oral administration of vaccines in terms of the capacity to stimulate humoral immunity, cellular responses were evaluated.

Immunisation with β-Gal + c-di-AMP via the s.l. route stimulated similar strong proliferative responses as i.n. immunisation (Fig. 33). Furthermore, both routes stimulate significantly higher spleen cell proliferation rates than oral immunisation ($p<0.0001$) (Fig. 33).

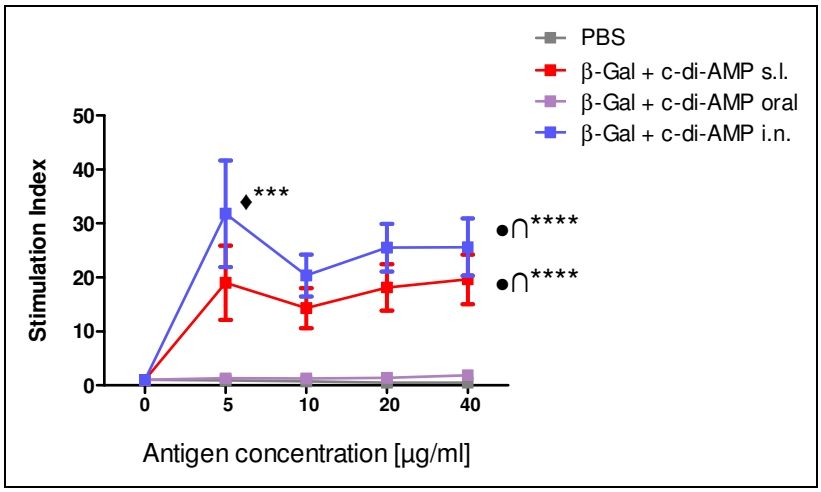

Figure 33: Cellular proliferation of *ex vivo* ag-restimulated spleen cells derived from BALB/c mice immunised by s.l., oral or i.n. route.
BALB/c mice were immunised three times with β-Gal + c-di-AMP. SD is indicated by vertical lines. Differences were statistically significant when tested with Two-way ANOVA at $p<0.001$ (***) and $p<0.0001$ (****) with respect to the PBS control group (●), and the groups receiving β-Gal + c-di-AMP via s.l. (♦) or oral (∩) route.

The same trend was observed analysing the stimulated cytokine response. Similar numbers of IL-2, IL-4 and IL-17 secreting spleen cells were stimulated following i.n. and s.l. immunisation (Fig. 34). Interestingly, while immunisation of mice with β-Gal + c-di-AMP via the i.n. route stimulated high numbers of IFN-γ producing cells, the same vaccine formulation administered via the s.l. route elicited only low numbers of IFN-γ secreting splenocytes (Fig. 34). However, both routes promoted significantly higher numbers of cytokine secreting cells, as compared to those observed following oral immunisation ($p<0.0001$). Again, oral immunisation elicited only very weak cellular immune responses (Fig. 34).

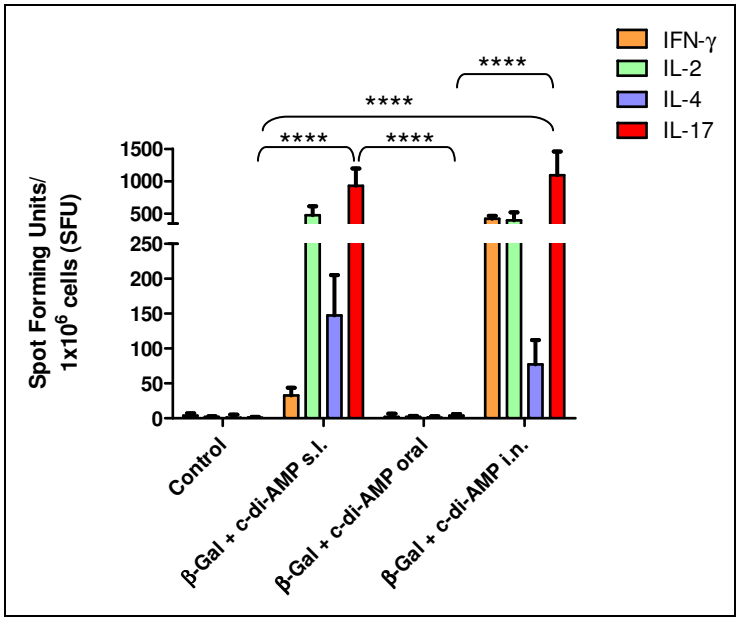

Figure 34: Effect of vaccination by different routes in the secretion of cytokines by *ex vivo* ag-restimulated spleen cells.
BALB/c mice were immunised three times with β-Gal + c-di-AMP by s.l., oral or i.n. route. Cells were incubated for 24 or 48 h in the presence of a peptide encompassing the immunodominant Ld-restricted epitope of β-Gal (TPHPARIGL), which is specific for MHC class I presentation (for IFN-γ), or the β-Gal protein (for IL-2, IL-4 and IL-17). SEM is indicated by vertical lines. Differences were statistically significant when tested with Two-way ANOVA at $p<0.0001$ (****).

4.3.2. Immunisation via s.l. route requires increased vaccine dosages as compared to the i.vag. and i.n. route

When comparing the i.n. and i.vag. immunisation routes 15 µg β-Gal and 5 µg c-di-AMP per dose were used. The optimal dose of β-Gal and c-di-AMP for s.l. immunisation turned out to be 30 µg and 10 µg per dose, respectively (data not shown). Thus, in order to allow a side-by-side comparison of the s.l. and i.vag. route studies were performed immunising BALB/c with the two different vaccine concentrations.

4.3.2.1. Humoral immune responses

Immunisation of mice via i.vag. route using the double amount of antigen and adjuvant, respectively, seemed to have only marginal effects on the stimulation of ag-specific IgG production if any at all (Fig. 35).

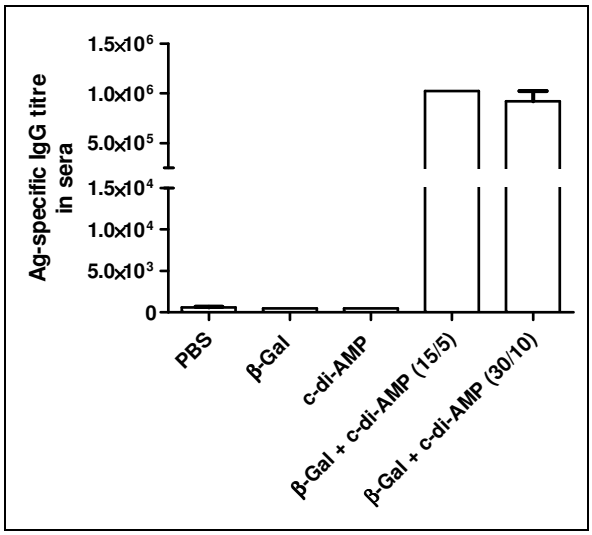

Figure 35: Humoral immune responses stimulated in vaccinated BALB/c mice using c-di-AMP as mucosal adjuvant.
The groups were immunised with dosages of 30 and 15 µg of ag, and 5 and 10 µg of adjuvant/dose. SEM is indicated by vertical lines.

Furthermore, no effect has been observed in terms of the stimulated IgG subclasses. Both dosages of ag co-administered with the adjuvant strongly elicited a mixed Th1/Th2 response characterised by the presence of ag-specific IgG1, IgG2a and IgG2b significantly higher to those observed for the control groups ($p<0.01$ to $p<0.001$).

However, when analysing the stimulated mucosal responses, administration of the high-dose vaccine formulation resulted in a similar stimulation of IgA responses in VL as compared to the low-dosage regimen (Fig. 36B).

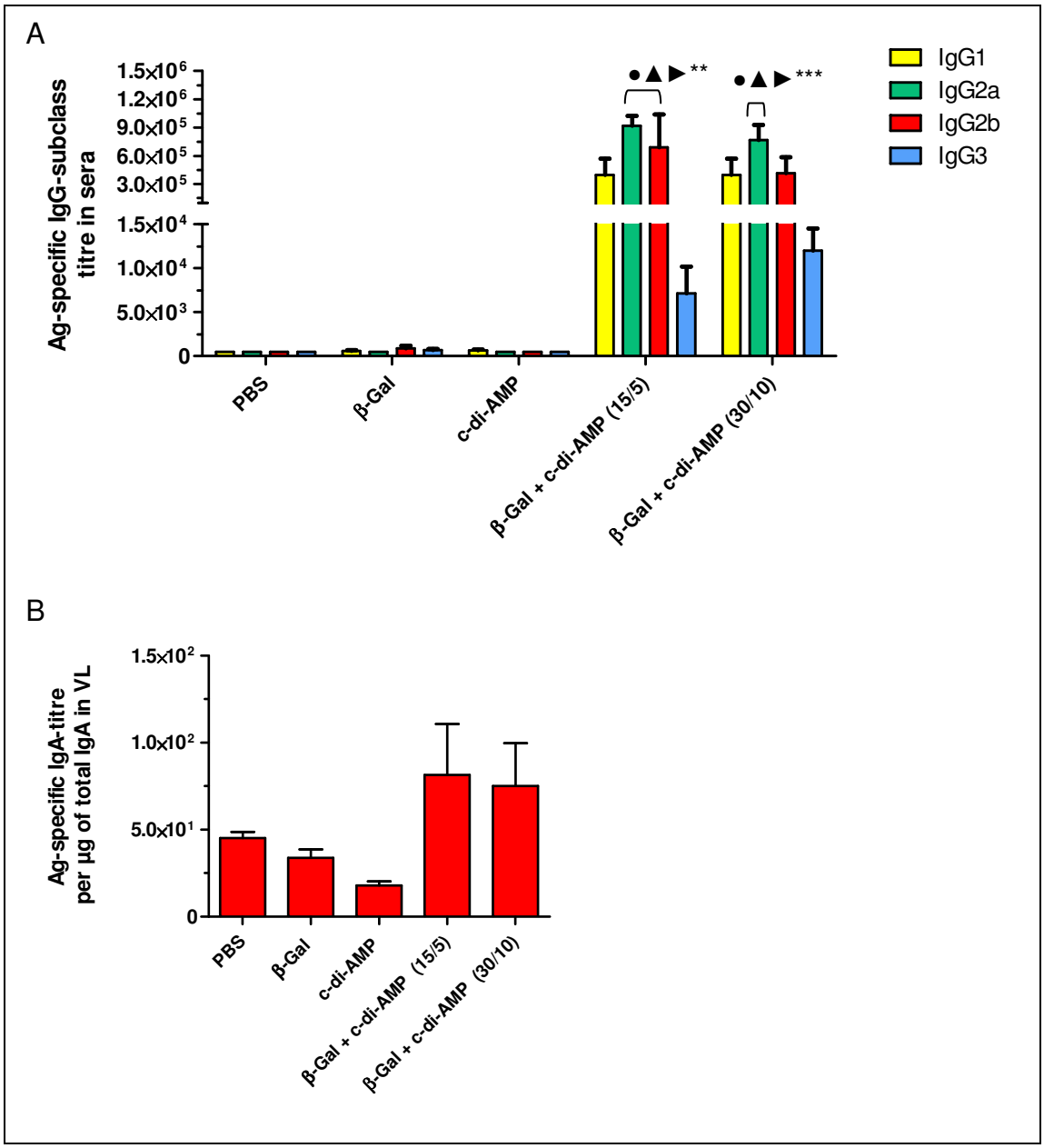

Figure 36: Humoral immune responses after i.vag. immunisation with low and high dose formulations of the ag and adjuvant.
(A) Systemic immune responses in BALB/c after three i.vag. immunisations, (B) Analysis of ag-specific IgA in lavage samples of vagina (VL) of immunised mice. Local immune response in BALB/c after three i.vag. immunisations. The animals were immunised using either 30 or 15 µg of ag and 10 or 5 µg of adjuvant per dose. SEM is indicated by vertical lines. The statistic analysis was performed using two-way ANOVA, $p<0.01$ (**), $p<0.001$ (***). The differences were statistically significant in comparison with the PBS control group (●), and the groups receiving β-Gal (▲), c-di-AMP (►) or β-Gal co-administered with c-di-AMP 15/5.

4.3.2.2. Cellular immune responses

At the cellular level, the high dose vaccine formulation using β-Gal + c-di-AMP seemed to stimulate stronger proliferative responses of splenocytes (Fig. 37). However, the obtained values only differ significantly as compared to the low dose formulation when splenocytes were restimulated *ex vivo* with high ag concentration [20 µg/ml] ($p<0.0001$) (Fig. 37).

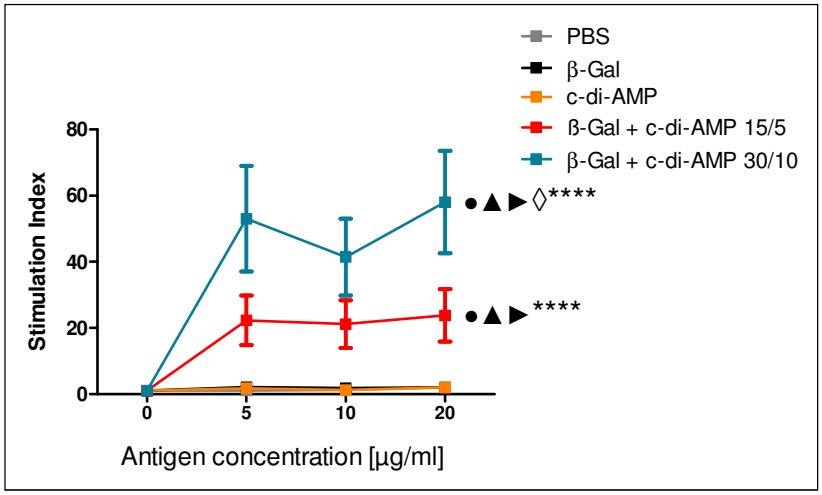

Figure 37: Cellular immune responses after i.vag. immunisation with low and high dose formulations of the ag and adjuvant.
SD is indicated by vertical lines. The statistical analysis was performed using Two-way ANOVA, $p<0.0001$ (****). The differences were statistically significant in comparison with the PBS control group (●), and the groups receiving β-Gal (▲), c-di-AMP (►) or β-Gal co-administered with c-di-AMP 15/5 (◊).

When analysing the stimulated cytokine profiles, no significant differences were observed comparing the low and high dose formulations administered by i.vag. route (Fig. 38). IL-17 producing splenocytes were stimulated by both vaccine formulations, followed by IL-2 and IL-4 secreting cells (Fig. 38). Interestingly, no IFN-γ producing cells were detected in both groups after stimulation with a peptide encompassing a MHC class I restricted epitope (Fig. 38).

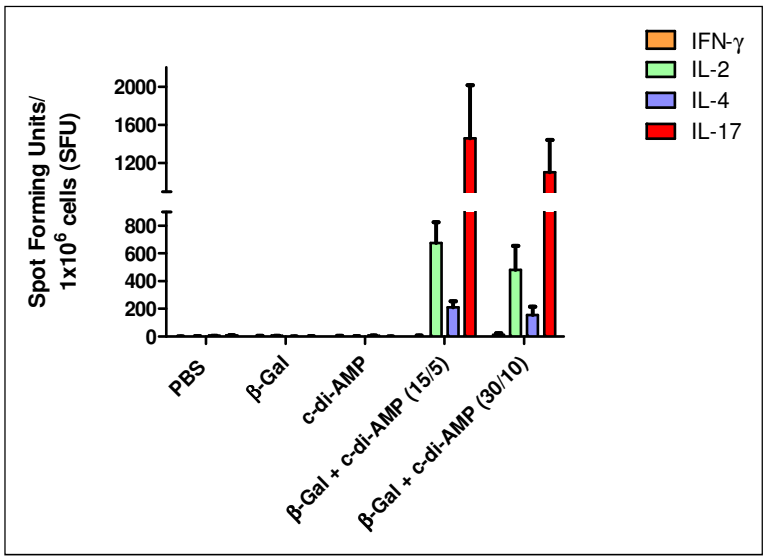

Figure 38: Cytokine secreting cells stimulated after i.vag. immunisation with low and high dose formulations.
Spleen cells were incubated for 24 or 48 h in the presence of a peptide encompassing the immunodominant Ld-restricted epitope of β-Gal (TPHPARIGL), which is specific for MHC class I presentation (for IFN-γ), or the β-Gal protein (for IL-2, IL-4 and IL-17). SEM is indicated by vertical lines.

Taken together, when mice were immunised by i.vag. route with the optimal ag-adjuvant dose for s.l. immunisation of 30 µg and 10 µg, respectively, no further increase in the stimulated immune responses was observed compared to those obtained using the optimal dose for i.n. immunisation of 15 µg and 5 µg.

4.3.3. Side-by-side comparison of immune responses stimulated following immunisation by s.l. and i.vag. routes

The formulation β-Gal + c-di-AMP stimulated most efficiently local and systemic immune responses in BALB/c mice. Thus, comparative studies for s.l. and i.vag. immunisations were performed using this vaccine formulation. However, in order to investigate the impact of the s.l. administration route on the stimulation of IL-17 production, α-GalCerMPEG was again used for down-modulation of the triggered IL-17 responses, as opposite to the IL-17 response obtained after immunisation using c-di-AMP.

To this end, BALB/c mice (n= 5/group) were vaccinated with β-Gal + c-di-AMP and β-Gal + α-GalCerMPEG, applied through the i.vag. or s.l. route. I.n. immunisation was used as a golden standard to ascertain if the s.l. route represents a valid alternative for mucosal responses at the level of the genital tract, which are similar to those obtained following i.vag. exposure to the ag.

4.3.3.1. Immunisation by the s.l. route elicits efficient mucosal, humoral immune responses

Administration of β-Gal + c-di-AMP through the s.l. route stimulated as strong IgG titres as application via either i.n. or i.vag. route (Fig. 39). The obtained values were significantly higher as compared to the control groups receiving PBS, ag or adjuvant alone ($p<0.0001$). Immunisation with β-Gal + α-GalCerMPEG also stimulated significant higher antibody titres in sera, as compared to those of animals in the control groups ($p<0.001$) (Fig. 39). However, α-GalCerMPEG was less efficient at stimulating humoral immune responses following vaccination via the s.l. and i.vag. routes than c-di-AMP (Fig. 39).

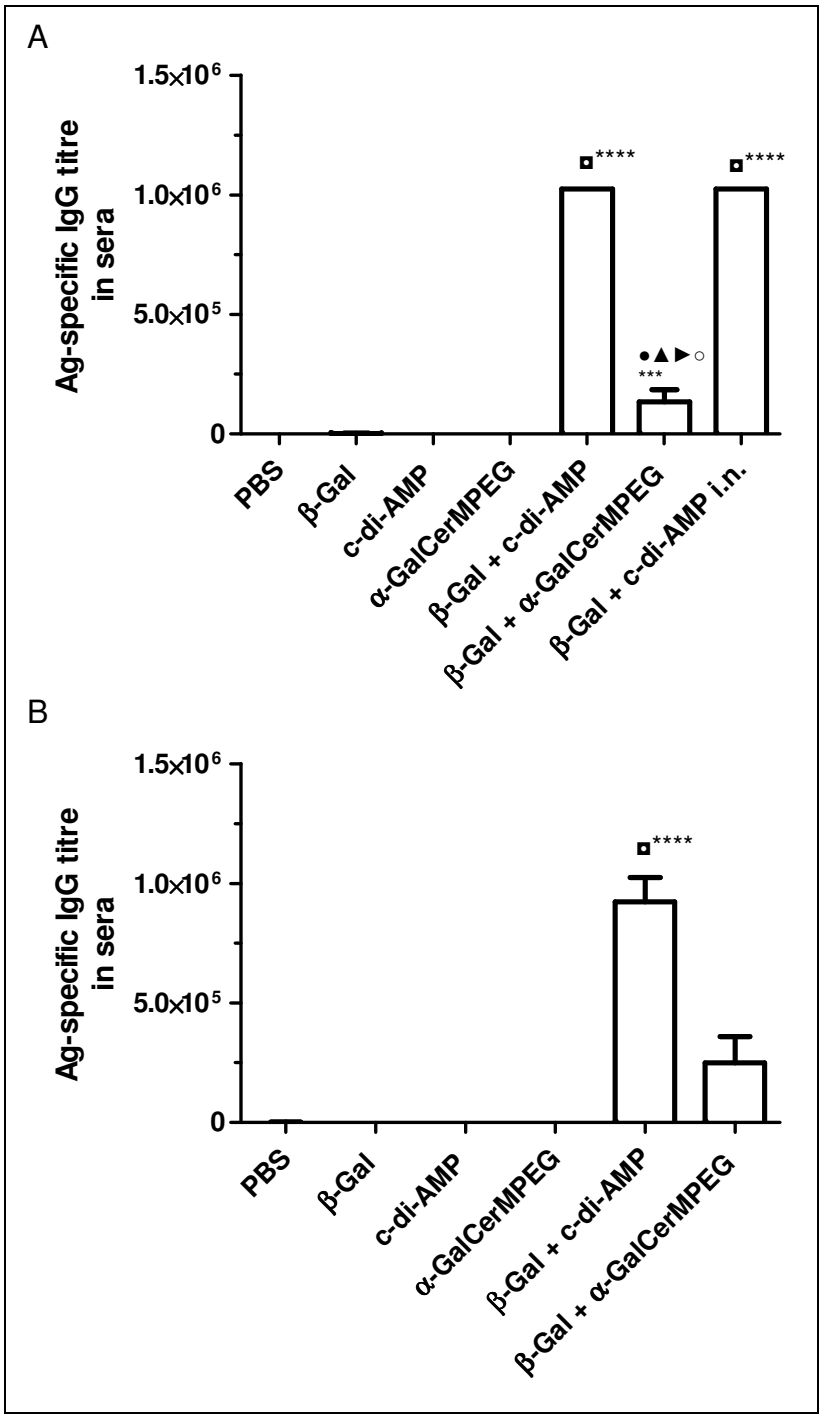

Figure 39: Ag-specific IgG titre in sera of BALB/c mice immunised by i.n., s.l. and i.vag. routes.
BALB/c mice were immunised three times with β-Gal alone or adjuvant by (A) s.l. or i.n. route, and (B) i.vag. route. The i.n. immunisation was used as golden standard. SEM is indicated by vertical lines. The differences were statistically significant when tested with One-way ANOVA at $p<0.001$ (***) and $p<0.0001$ (****) with respect to PBS group (●), the control groups receiving β-Gal (▲), c-di-AMP (►) or α-GalCerMPEG (○) alone, or the group receiving β-Gal + α-GalCerMPEG (◘) or β-Gal + c-di-AMP by i.n. route (◊).

To investigate the potential impact of s.l. immunisation the quality of the stimulated immune responses, the distribution of ag-specific IgG subclasses was analysed (Fig. 40).

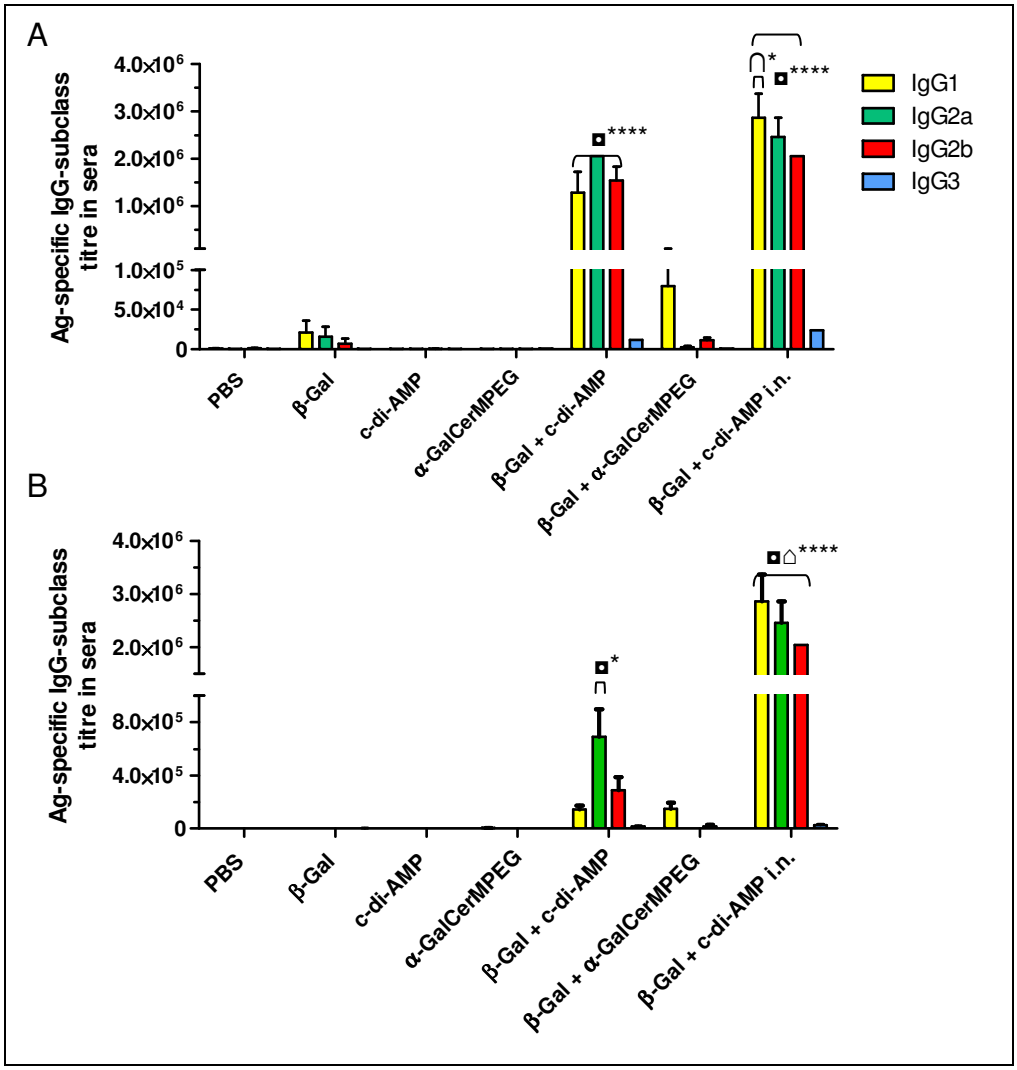

Figure 40: Distribution of IgG subclass titres in sera derived from BALB/c mice immunised by i.vag., s.l. and i.n. route.
BALB/c mice were immunised three times with PBS, β-Gal alone or co-administered with adjuvant by (A) s.l. or (B) i.vag. route. As positive control the i.n. administration strategy was used. SEM is indicated by vertical lines. The differences were statistically significant when tested with Two-way ANOVA at $p<0.05$ (*), $p<0.001$ (***) and $p<0.0001$ (****) with respect to to control groups receiving PBS (●), β-Gal (▲), c-di-AMP (►) or α-GalCerMPEG (○) alone, or groups receiving β-Gal co-administered with α-GalCerMPEG (◘) or with c-di-AMP by i.vag. (⌂) or s.l. (∩) route.

No significant differences in the distribution of IgG subclasses in sera have been observed, when comparing the values obtained following vaccination with β-Gal + c-di-AMP by the different immunisation routes (Fig. 40). Thus, in all of these

groups a mixed Th1/Th2 response has been stimulated as indicated by similar titres of IgG1, 2a and 2b subclasses. In addition, the obtained titres were significantly higher as compared to those observed in the control groups, and the groups receiving β-Gal + α-GalCerMPEG ($p<0.0001$) (Fig. 40). Nevertheless, statistically significant higher IgG1, 2a and 2b titres in sera were stimulated after i.n. immunisation, as compared to those observed after s.l. ($p<0.05$) and i.vag. immunisation ($p<0.0001$) (Fig. 40). The amount of IgG3 detected after vaccination via s.l. or i.vag. route was negligible (Fig. 40).

To investigate the potential of the s.l. immunisation strategy to stimulate mucosal immune responses not only at local level but also at distant mucosal areas, ag-specific IgA levels in different mucosal lavages were determined (Fig. 41). Comparing the IgA titres stimulated following vaccination of mice with β-Gal + c-di-AMP, s.l. immunisation strongly increased the IgA production in the mucosa, whereas immunisation via the i.vag. route elicited only weaker ag-specific IgA in VL (Fig. 41). Furthermore, co-administration of β-Gal + c-di-AMP was the most efficient approach for stimulating IgA production, whereby the highest titres were observed in saliva of immunised mice, followed by VL, LL and NL (Fig. 41A). However, only in saliva statistically significant differences have been observed when comparing the IgA titres obtained following immunisation of mice with β-Gal + c-di-AMP by either s.l. or i.n. route ($p<0.0001$) (Fig. 41). In any case, immunisation of mice with this vaccine formulation stimulated significantly higher IgA titres, as compared to the formulation including α-GalCerMPEG as adjuvant ($p<0.0001$) (Fig. 41).

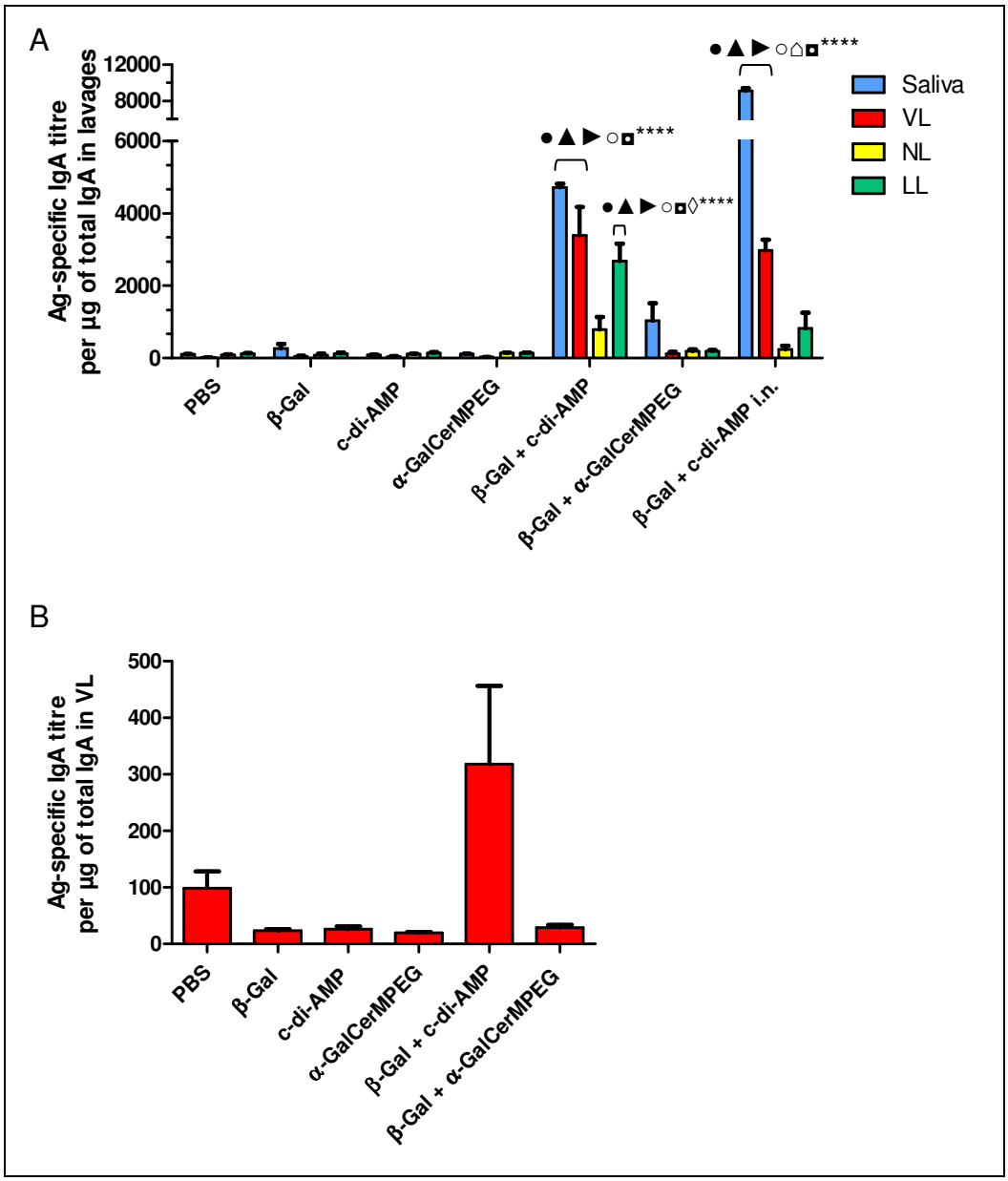

Figure 41: Ag-specific IgA titre in lavage samples derived from BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG by s.l., i.n. or i.vag. route.
The ag-specifiic IgA titres were tested in saliva, VL, NL and LL derived from mice immunised by (A) s.l. or (B) i.vag. route. As positive control mice were immunised by i.n. route. SEM is indicated by vertical lines. Differences were analysed by Two-way ANOVA test and statistically significant at $p<0.01$ (**) and $p<0.0001$ (****) with respect to groups receiving PBS (●), β-Gal (▲), c-di-AMP (►) or α-GalCerMPEG (○) alone, or groups vaccinated with β-Gal + α-GalCerMPEG (◘) or β-Gal + c-di-AMP by i.vag., s.l. (⌂) or i.n. (◊) route.

As indicated by this data, s.l. immunisation efficiently triggers high IgA levels in local as well as in distant mucosal territories, to a similar extent to those obtained after i.n. immunisation. Thus, vaccination via s.l. route constitutes indeed a valid alternative to the i.n. immunisation strategy, particularly when responses at the level of the genital tract.

4.3.3.2. Effects resulting from s.l. immunisation on cellular immune responses

After evaluating the capacity of s.l. immunisation to stimulate efficient humoral immune responses, the impact on cellular responses was investigated. The highest proliferation of spleen cells was induced in mice immunised with β-Gal + c-di-AMP via the i.vag. route (SI around 10) and the s.l. route (SI between 6-12) (Fig. 42). After i.n. immunisation, a stimulation index of about five was calculated. However, proliferation rates of splenocytes derived from mice immunised via s.l. route were not significantly different to those obtained after i.n. immunisation (Fig. 42A). Administration of β-Gal + c-di-AMP via the i.vag. route again stimulated significantly stronger proliferative capacities of spleen cells, as compared to the group receiving β-Gal + c-di-AMP by i.n. route ($p<0.0001$) (Fig. 42B). In contrast, the adjuvant α-GalCerMPEG stimulated only weak cellular responses after i.vag. or i.n. vaccination. The obtained indexes are rarely different compared to the control groups receiving PBS, ag or adjuvant alone.

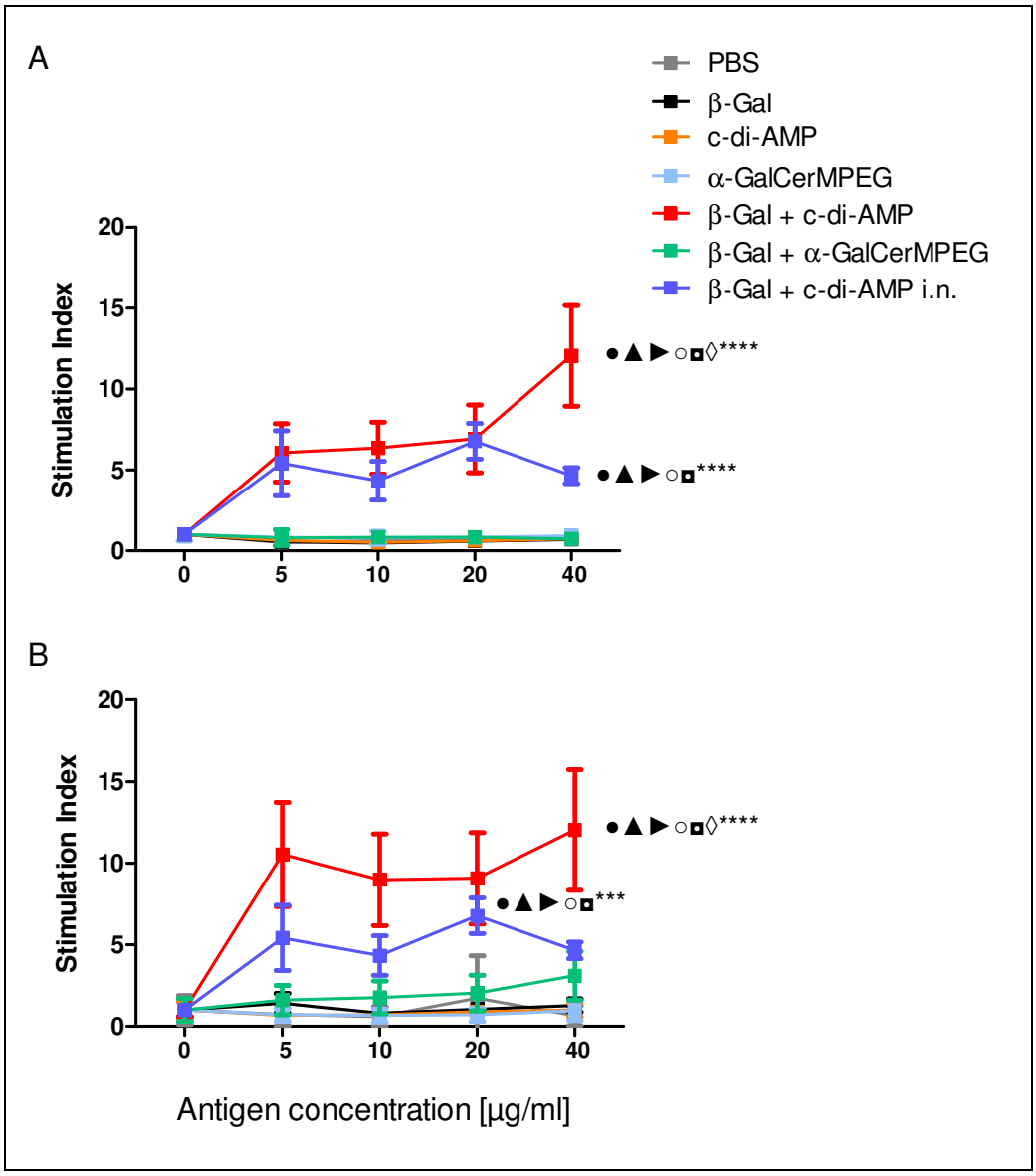

Figure 42: Evaluation of the cellular responses stimulated in mice vaccinated by different mucosal routes.
BALB/c mice were immunised three times with β-Gal + c-di-AMP via (A) s.l. or (B) i.vag. route. Results are averages of quadruplicates and they are expressed as stimulation index. SD is indicated by vertical lines. Differences were statistically significant when tested with Two-way ANOVA at $p<0.001$ (***) and $p<0.0001$ (****) with respect to the control groups receiving PBS (●), β-Gal (▲), c-di-AMP (▶) or α-GalCerMPEG (○) alone, and groups receiving β-Gal + α-GalCerMPEG (◘) or β-Gal + c-di-AMP (◊) by the i.n. route.

Then, the proliferative capacity of lymphocytes derived from the draining LN was evaluated, to assess the local cellular immune responses stimulated by different formulations and routes. To this end, iLN (draining the vagina) and cLN (draining the sublingual tissue and the nose) were collected from immunised mice and proliferation assays were performed (Fig. 43).

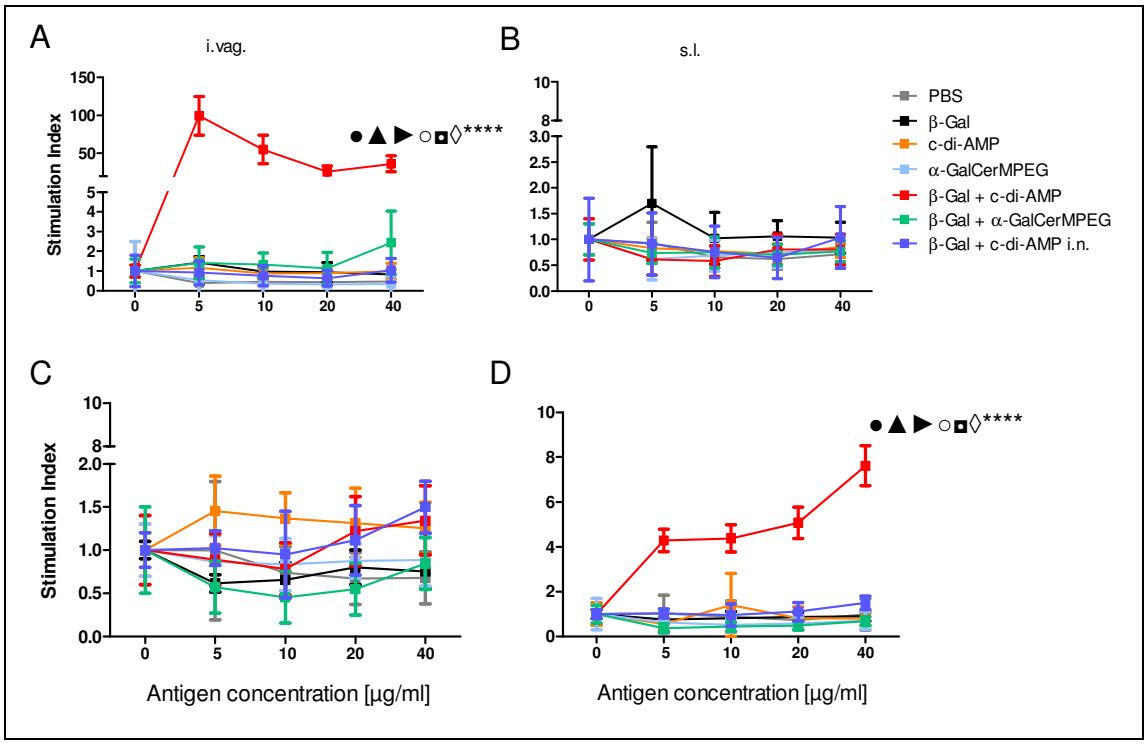

Figure 43: Evaluation of the cellular responses of LN cells derived from BALB/c mice vaccinated by different routes using c-di-AMP as adjuvant.
Spleen cells from vaccinated animals were restimulated with different concentrations of β-Gal for 96 h. Cellular proliferation was then assessed by determination of the [^{3}H] thymidine incorporated into the DNA of replicating cells. iLN and cLN cells were derived from mice immunised by (A+C) i.vag. or (B+D) s.l. route. Results are averages of quadruplicates and they are expressed as stimulation indexes. SD is indicated by vertical lines. The differences were statistically significant when tested by Two-way ANOVA at $p<0.0001$ (****) with respect to those from control groups receiving PBS (●), β-Gal (▲), c-di-AMP (▶) or α-GalCerMPEG (○) alone, or groups immunised with β-Gal co-administered with α-GalCerMPEG (◘) or with c-di-AMP by the i.n. (◊) route.

Cells isolated from iLN of mice immunised with β-Gal + c-di-AMP via i.vag. route showed the highest proliferation rates (SI between 25-99), which were significantly stronger as compared to those values observed in all other groups ($p<0.0001$) (Fig. 43 A). Efficient proliferative responses were also observed after s.l. immunisation with β-Gal + c-di-AMP (SI between 4-7; $p<0.0001$) (Fig. 43D). Only marginal proliferation rates were observed for cells derived from mice immunised with α-GalCerMPEG (Fig. 43). Thus, analogue to what was observed for the humoral immune responses, s.l. immunisation with β-Gal + c-di-AMP stimulated increased proliferative capacities at both systemic and local levels (Fig. 42 and 43).

These results were further validated by analysing the stimulated cytokine profiles (Fig. 44).

Again, co-administration of ag + c-di-AMP via i.vag. route stimulated most efficient cytokine production, as compared to those stimulated using α-GalCerMPEG as adjuvant (Fig. 44). The detected values of IL-2 and IL-17 producing cells were significantly higher than those observed in mice immunised via s.l. route ($p<0.001$) and all control groups ($p<0.0001$) (Fig. 44). Interestingly, while c-di-AMP was able to stimulate IFN-γ production by $CD8^+$ cells when administered via either s.l. or i.n. route, no IFN-γ secreting cells were observed in spleens of mice immunised via the i.vag. route (Fig. 44). Using α-GalCerMPEG as adjuvant, only low numbers of cytokine producing cells were stimulated following immunisation, with similar levels of IFN-γ and IL-4 secreting cells (Fig. 44).

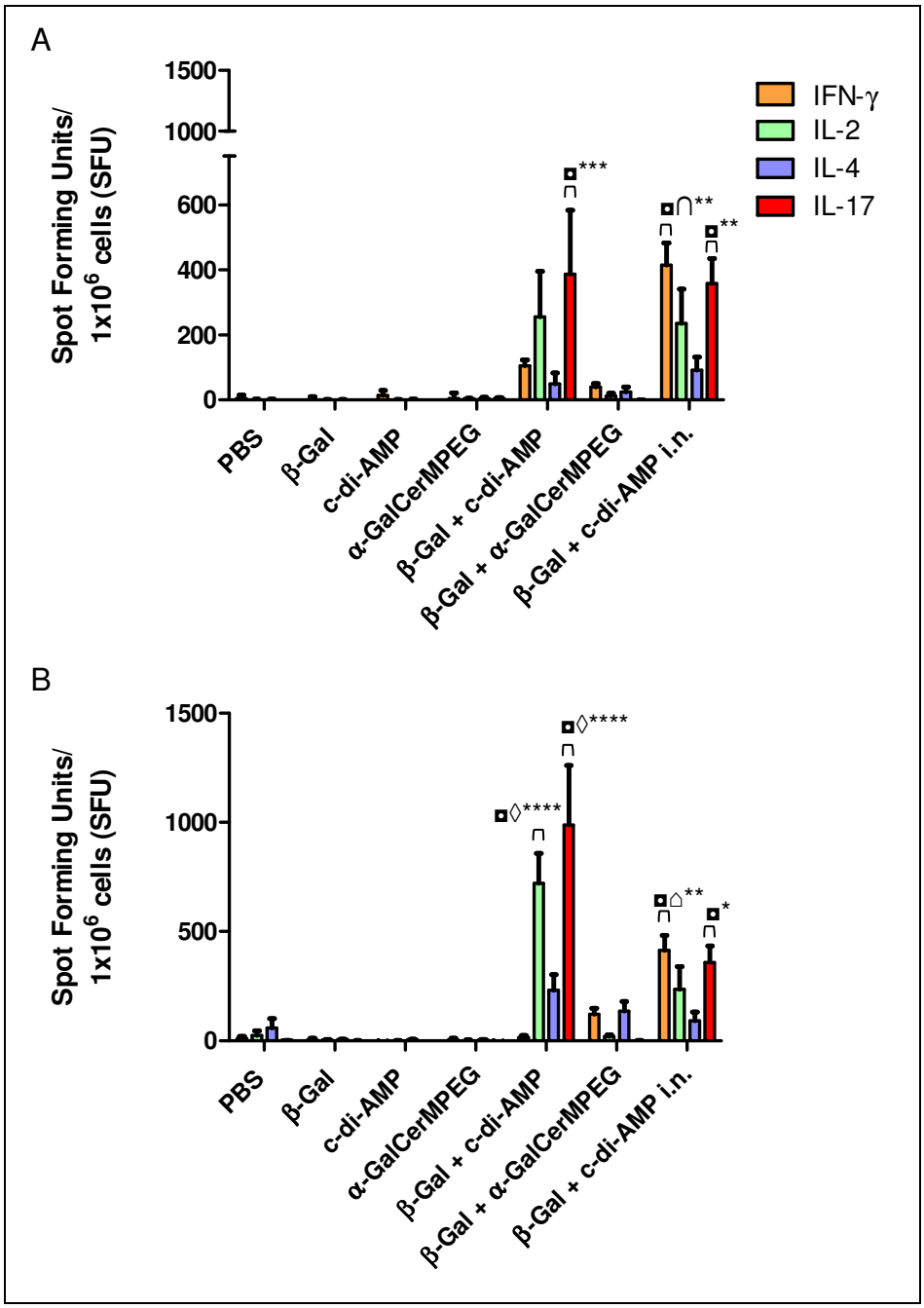

Figure 44: Analysis of the cytokine producing cells from vaccinated mice.
Spleen cells recovered from BALB/c mice immunised via (A) s.l. or (B) i.vag. route were incubated for 24 or 48 h in the presence of a peptide encompassing the immunodominant Ld-restricted epitope of β-Gal (TPHPARIGL), which is specific for MHC class I presentation (for IFN-γ), or the β-Gal protein (for IL-2, IL-4 and IL-17). Then, the numbers of cytokine producing cells were determined by ELISPOT. As golden standard, responses stimulated after i.n. administration were analysed. SEM is indicated by vertical lines. The differences were statistically significant when tested with Two-way ANOVA at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) with respect to values from control groups receiving PBS (●), β-Gal (▲), c-di-AMP (►) or α-GalCerMPEG (○), or vaccinated with β-Gal co-administered with α-GalCerMPEG (◘) or c-di-AMP by i.n. (◊), s.l. (∩) or i.vag. (⌂) route.

In conclusion, similar patterns of cytokine secreting cells have been observed when the different immunisation routes were compared. Moreover, the highest levels of cytokine producing splenocytes were stimulated by i.vag. immunisation, followed by s.l. and i.n. immunisation.
Interestingly, while s.l. immunisation seems to elicit superior immune responses at humoral level as compared to i.vag. immunisation, exactly the opposite was observed when comparing the stimulated cellular responses. Here, i.vag. immunisation led to the stimulation of stronger cellular responses than s.l. vaccination.

4.3.4. Combination of i.vag. and s.l. immunisation does not result in a further increase of humoral and cellular immune responses

As mentioned above, i.vag. immunisation with β-Gal + c-di-AMP elicits stronger cellular immune responses than s.l. immunisation, which in turn promotes stronger humoral responses. Thus, it was investigated if the combination of both immunisation strategies would lead to optimal humoral and cellular immune responses.

4.3.4.1. Humoral immune responses stimulated following immunisation via i.vag. and s.l. route

The analysis of the ag-specific IgG titres in sera of mice immunised following a combined vaccination strategy (*i.e.* simultaneous administration of formulations via s.l. and i.vag. route; 30 µg of ag and 10 µg of adjuvant divided in two aliquots) showed that there is no further increase in antibody production observed (Fig. 45). In fact, the combination regime stimulated similar ag-specific IgG titres to the conventional approach when using c-di-AMP as adjuvant. In the groups in which α-GalCerMPEG was used as adjuvant, even less efficient ag-specific IgG responses were observed following the combined strategy (Fig. 45). However, in all cases animals immunised with β-Gal + c-di-AMP showed

significant higher IgG titre in sera compared to groups immunised with β-Gal + α-GalCerMPEG ($p<0.0001$ for i.vag. and i.vag. + s.l., $p<0.001$ for s.l.).

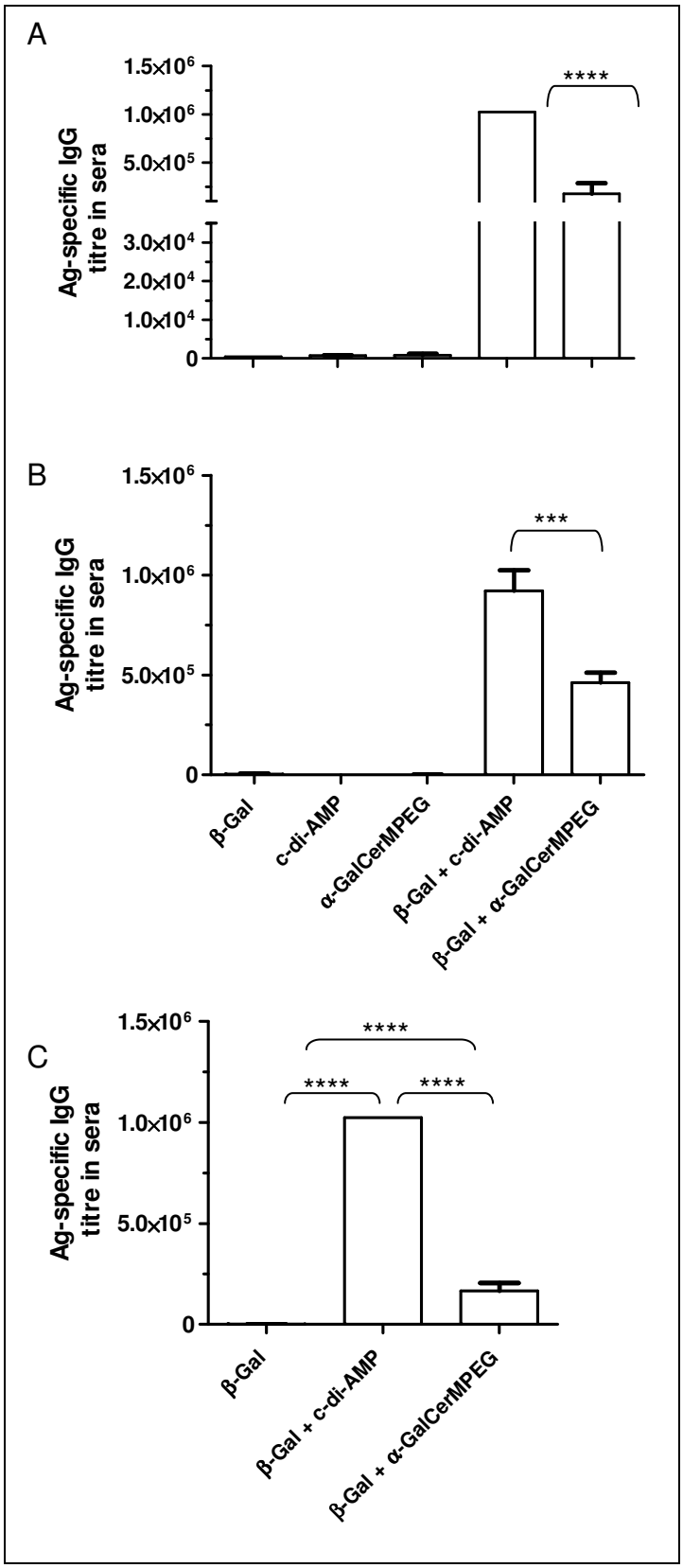

Figure 45: Ag-specific IgG in sera of BALB/c stimulated following single and combined vaccination strategies.

Humoral immune responses of BALB/c immunised three times with β-Gal + c-di-AMP via the (A) i.vag., (B) s.l or (C) combination (simultaneous administration of formulation to both routes) route. SEM is indicated by vertical lines. The statistic significance of the observed differences was evaluated using Two-way ANOVA, $p<0.001$ (***), $p<0.0001$ (****).

When the IgG subclass pattern stimulated by the combined vaccine formulation encompassing β-Gal + c-di-AMP was analysed, a mix of the subclass pattern stimulated by the individual regimens was detected. Thus, while β-Gal + c-di-AMP administered via the i.vag. route stimulated similar titres of IgG1, 2a and 2b, and administration of the same formulation via s.l. route stimulated high titres of IgG2b followed by IgG2a and IgG1, simultaneous immunisation by both routes stimulated high titres of IgG2a followed to IgG2b and IgG1 (Fig. 46). In contrast, no effect on the stimulated IgG subclass profiles has been observed when α-GalCerMPEG was used as adjuvant (Fig. 46). However, the IgG2a and IgG2b titres stimulated following vaccination with β-Gal + c-di-AMP were significantly higher with respect to those of mice receiving β-Gal + α-GalCerMPEG ($p<0.0001$), independently of the vaccination strategy (Fig. 46). No differences in the values of IgG1 have been observed comparing the titres stimulated by c-di-AMP and α-GalCerMPEG when administered by i.vag. route alone or by both routes together (Fig. 46A + C). In fact, s.l. immunisation of mice with β-Gal + α-GalCerMPEG following the combined vaccination protocol stimulated significantly higher IgG1 titres, as compared to those obtained using c-di-AMP as adjuvant ($p<0.001$) (Fig. 46B). However, while β-Gal + c-di-AMP stimulated a mixed Th1/Th2 response, as indicated by similar values of IgG1, 2a and 2b, β-Gal + α-GalCerMPEG stimulated a Th2 dominated response, as indicated by high levels of IgG1 (Fig. 46).

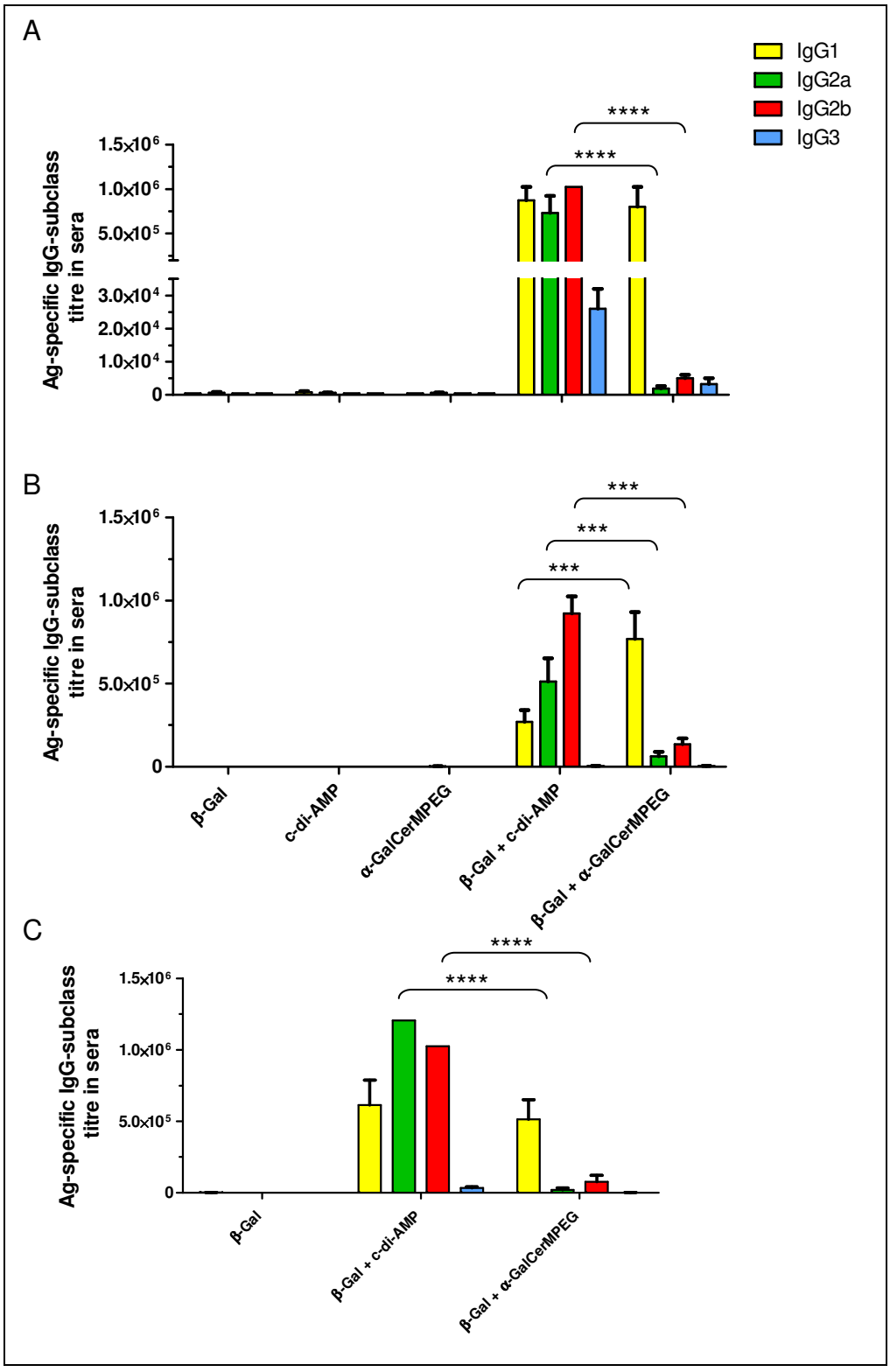

Figure 46: Humoral immune responses in sera after i.vag and s.l. immunisation and a combination of these routes.
Humoral immune responses of BALB/c immunised three times with β-Gal + c-di-AMP via the (A) i.vag., (B) s.l or (C) combination of the routes. SD is indicated by vertical lines. The statistic significance of the observed differences was evaluated by Two-way ANOVA, $p<0.001$ (***), $p<0.0001$ (****).

4.3.4.2. Humoral local immune responses following combined immunisation via i.vag. and s.l. route

As in the case for the observed IgG titres in sera of vaccinated mice, by and large the combination of both immunisation routes did not result in a further increase of the amount of ag-specific IgA in the different mucosal secretions of vaccinated animals (Fig. 47). Only in VL of mice immunised with β-Gal + c-di-AMP following the combined vaccination strategy, significantly higher IgA titres were observed, as compared to those obtained following the individual immunisation protocol (Fig. 47A + B). Interestingly, when α-GalCerMPEG was used as adjuvant for the combined strategy, even a decreased IgA titre was detected (Fig. 47).

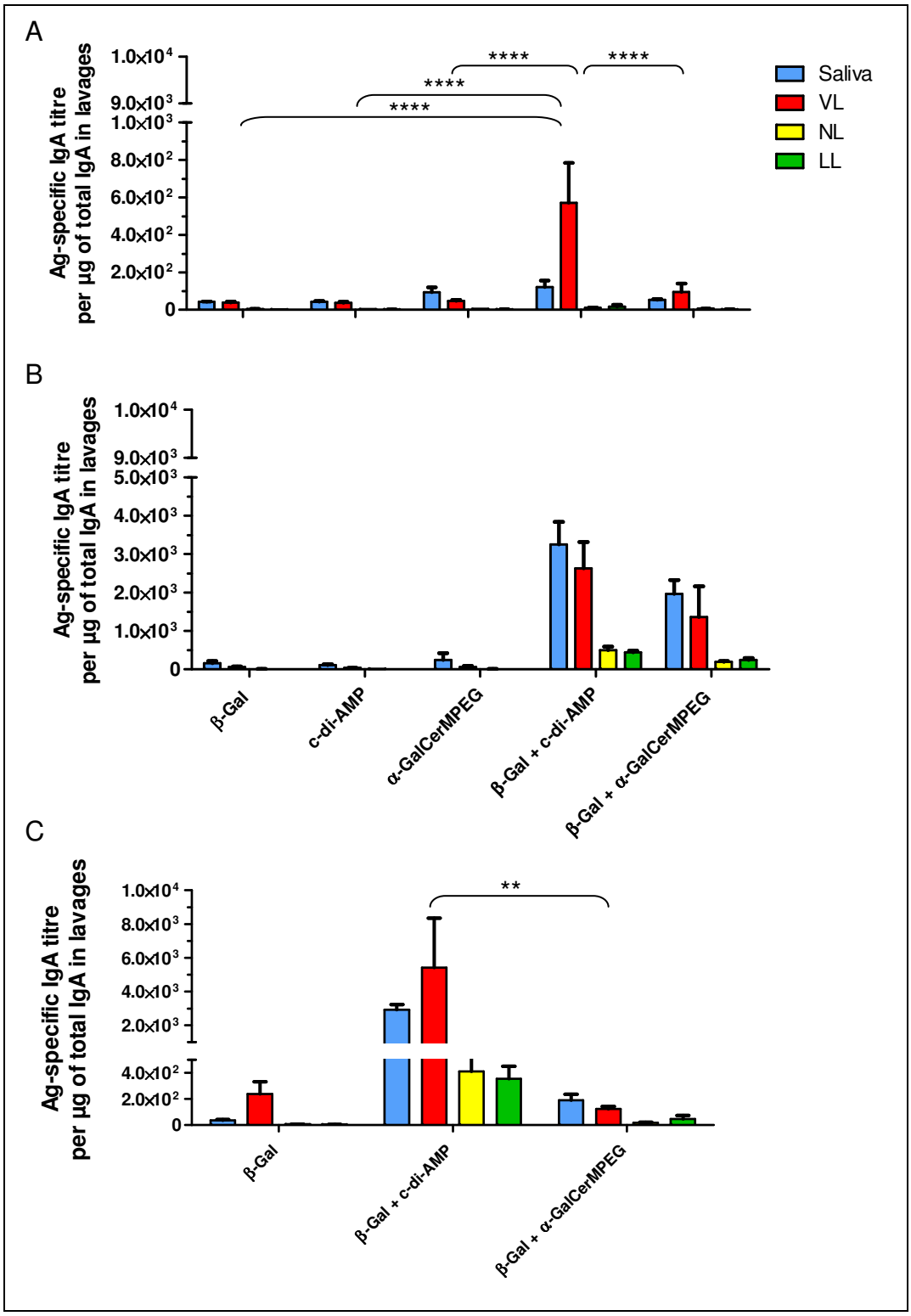

Figure 47: Humoral immune responses in mucosal lavages after i.vag and s.l. immunisation, alone and in combination.
Humoral mucosal immune responses of BALB/c mice immunised three times with β-Gal + c-di-AMP via the (A) i.vag., (B) s.l or (C) combination of the routes. SEM is indicated by vertical lines. The statistic significance of the observed differences was evaluated by Two-way ANOVA, $p<0.0001$ (****), $p<0.01$ (**).

4.3.4.3. Systemic and local cellular immune responses stimulated following combined immunisation via i.vag. and s.l. route

The cellular immune responses stimulated following i.vag., s.l. and combined vaccination strategies were compared analysing the proliferative capacities of cells derived from spleen and the corresponding draining LNs. The only vaccine formulation stimulating strong proliferative immune responses at systemic level was β-Gal + c-di-AMP, independently of the application route (Fig. 48). In any case, the stimulated proliferation rates were significantly stronger as compared to those observed using PBS and adjuvant alone, respectively, or α-GalCerMPEG in combination with β-Gal ($p<0.0001$) (Fig. 48). The strongest proliferation rates were stimulated after s.l. immunisation (Fig. 48B). Thus, combination of both immunisation routes did not further increase the cellular responses at systemic level (Fig. 48B and C).

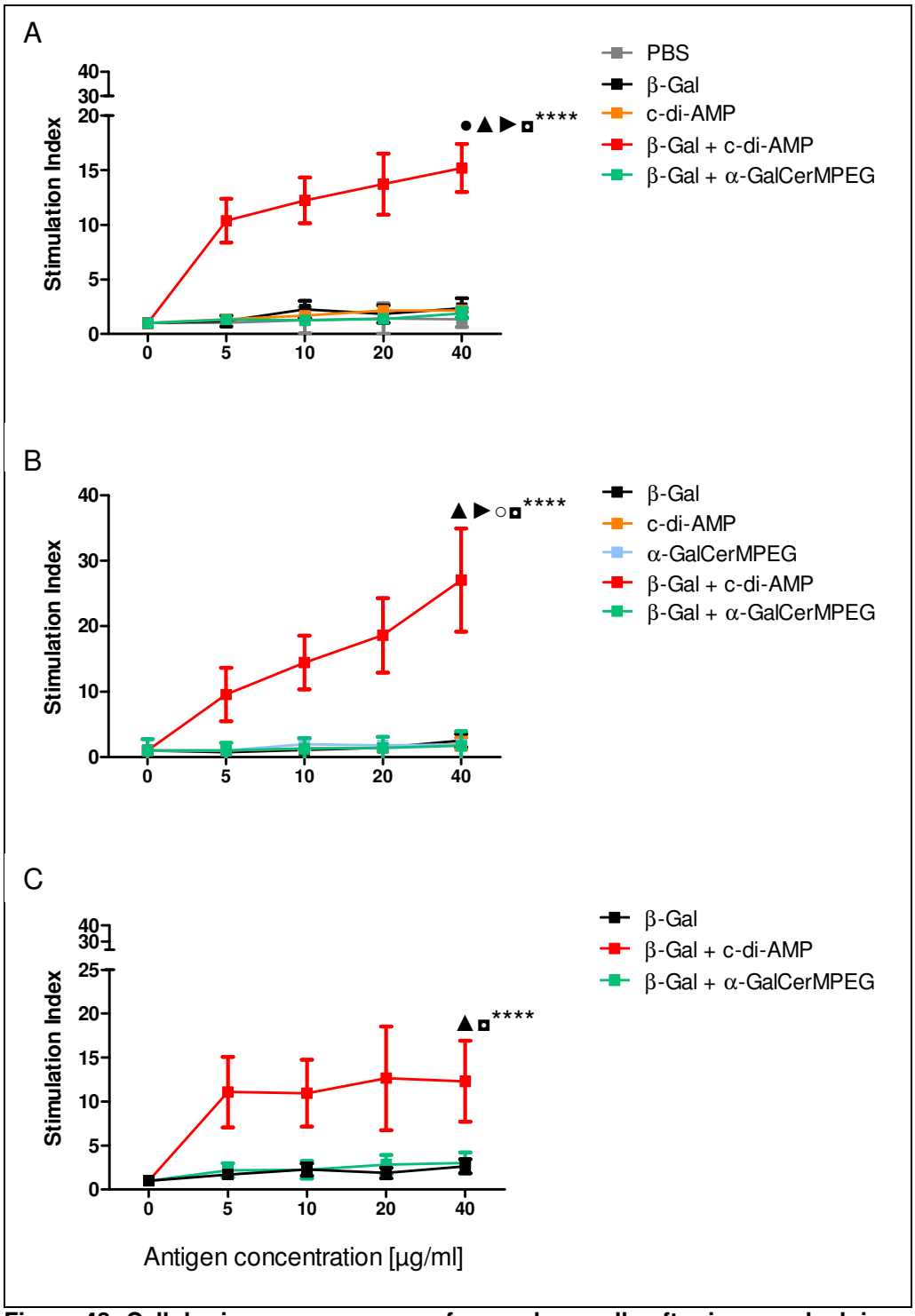

Figure 48: Cellular immune responses from spleen cells after i.vag and s.l. immunisation and a combination of these routes.
The groups received β-Gal and c-di-AMP via the (A) i.vag., (B) s.l. and (C) a combination of both routes. SD is indicated by vertical lines. The statistic analysis was performed using Two-way ANOVA, $p<0.0001$ (****).The differences were statistically significant in comparison with the control groups receiving PBS (●), β-Gal (▲), c-di-AMP (►), and α-GalCerMPEG (○), and the group receiving β-Gal + α-GalCerMPEG (◘).

Evaluation of the cellular responses stimulated at local level showed that only immunisation with β-Gal + c-di-AMP resulted in strong cellular responses (Fig.

49 + 50). Nevertheless, the observed differences in the proliferative capacities of cells derived from iLN were only significant following i.vag. immunisation ($p<0.01$) (Fig. 49A). When investigating cells derived from cLN, immunisation via s.l. route stimulated the strongest proliferation rates which were significantly higher as compared to all other vaccination groups ($p<0.0001$) (Fig. 50B). Neither i.vag. immunisation stimulated strong local responses in cLN, nor did s.l. immunisation in iLN (Fig. 49B + 50A). In any case, the combined vaccination strategy was demonstrated to be unefficient to further increase the proliferative capacities of LN derived cells (Fig. 49C + 50C).

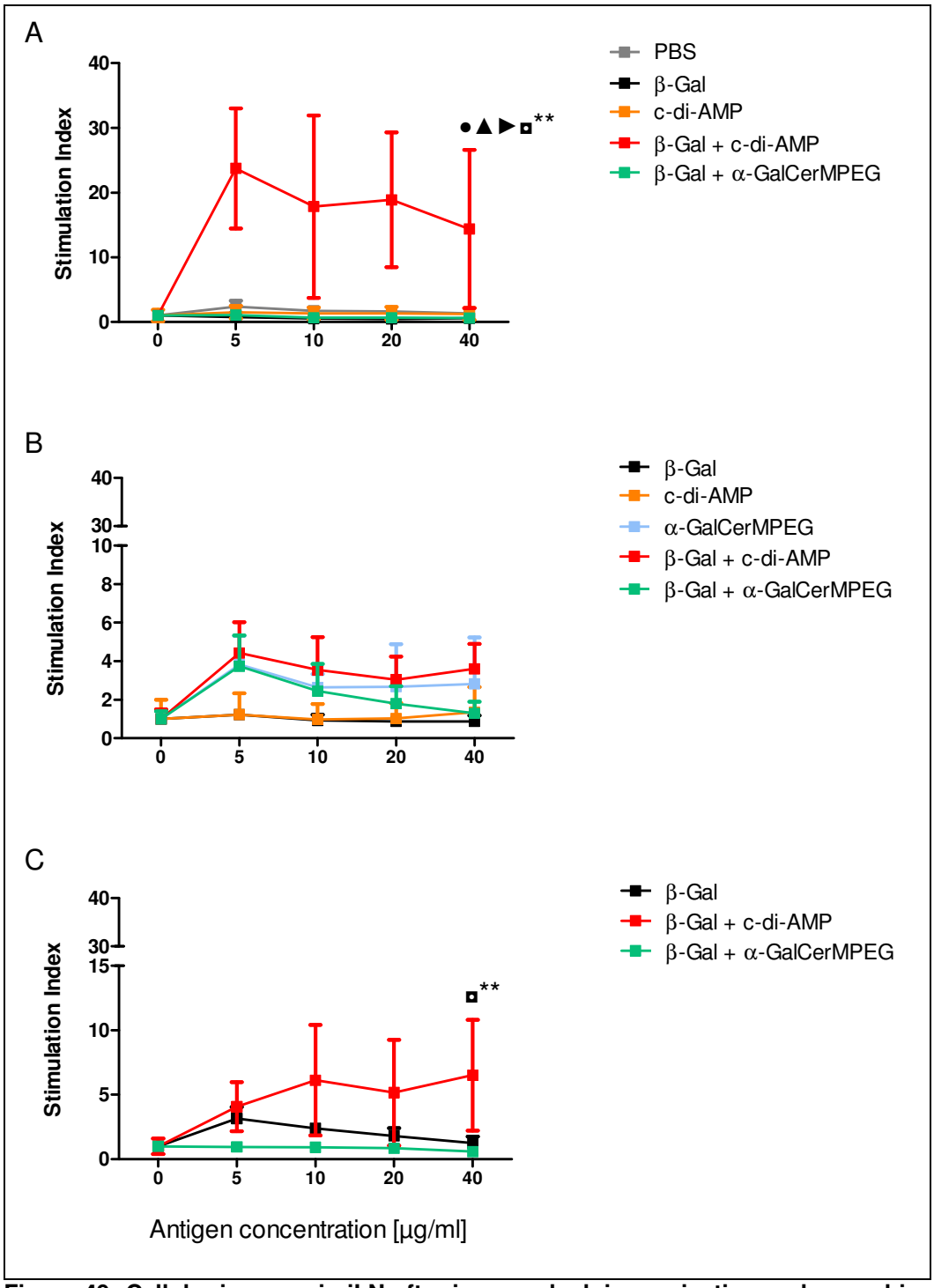

Figure 49: Cellular immune in iLN after i.vag and s.l. immunisation and a combination of these routes.

The immunisation groups received β-Gal + c-di-AMP via the (A) i.vag., (B) s.l. and (C) a combination of both routes. SD is indicated by vertical lines. The statistic analysis was performed using Two-way ANOVA, $p<0.01$ (**).The differences were statistically significant in comparison with the control groups receiving PBS (●), β-Gal (▲), c-di-AMP (►), and α-GalCerMPEG (○), and the groups receiving β-Gal + α-GalCerMPEG (◘).

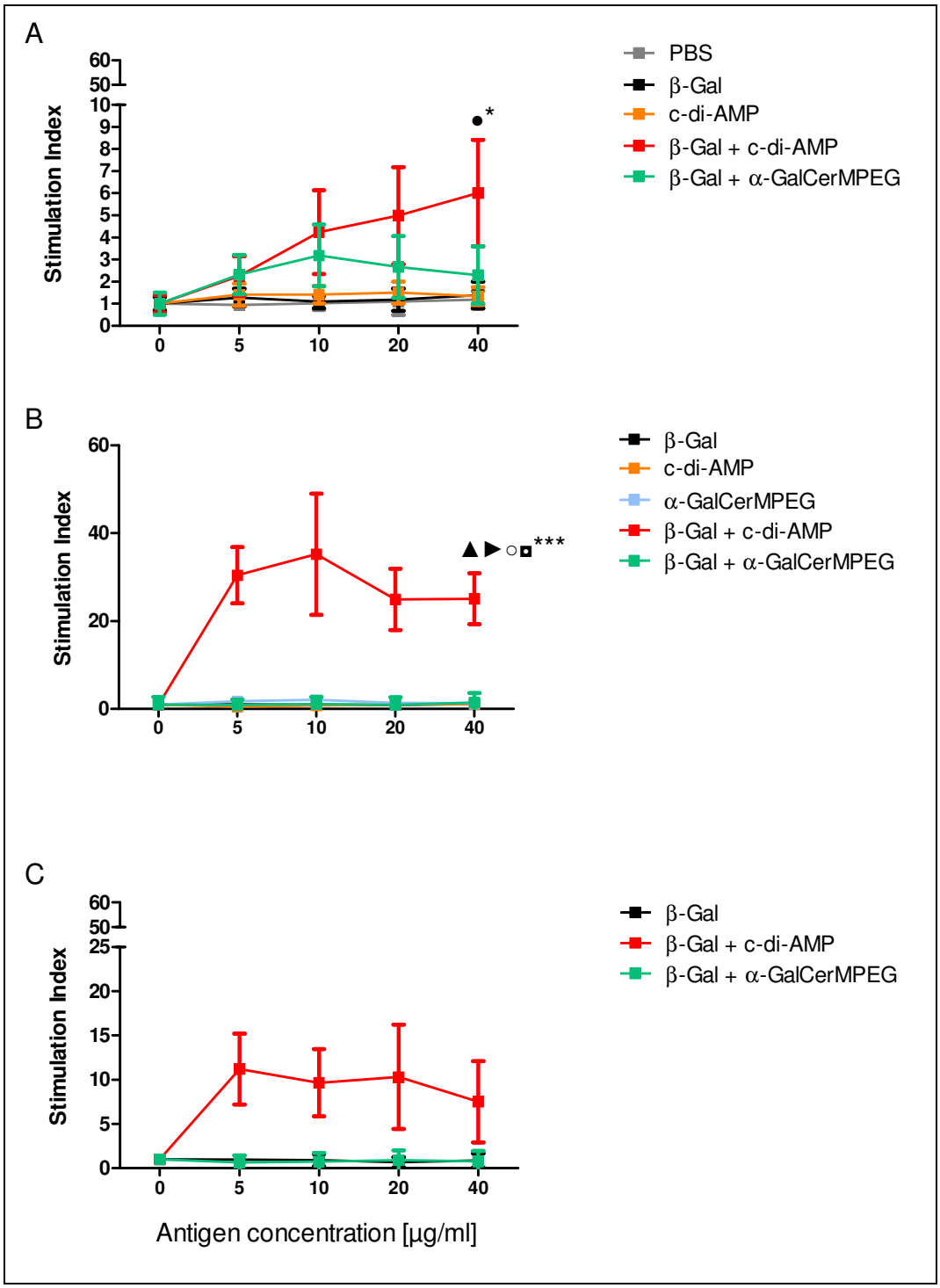

Figure 50: Cellular immune responses in cLN after i.vag and s.l. immunisation and a combination of these routes.
The groups were immunised with β-Gal and c-di-AMP and α-GalCerMPEG, respectively, via the (A) i.vag., (B) s.l. and (C) after combination of i.vag. and s.l. immunisation route. SD is indicated by vertical lines. Statistical test is Two-way ANOVA, $p<0.005$ (*), $p<0.0001$ (****).The differences were statistically significant in comparison with the PBS group (●), β-Gal (▲), c-di-AMP (►), α-GalCerMPEG (○), and the groups receiving β-Gal + α-GalCerMPEG (◘).

Interestingly, no significant differences in the values of cytokine producing cells have been observed when comparing the combined vaccination strategy with

the individual immunisation protocols (Fig. 51). Again, only vaccination with β-Gal + c-di-AMP stimulated strong values of cytokine producing cells (Fig. 51). In addition, IL-17 secreting splenocytes were stimulated the most, followed by those secreting IL-4 (Fig. 51). In contrast, only low levels of IFN-γ secreting cells were observed (Fig. 51). As shown before, only low levels of IFN-γ and IL-4 secreting splenocytes have been stimulated using α-GalCerMPEG as adjuvant (Fig. 51).

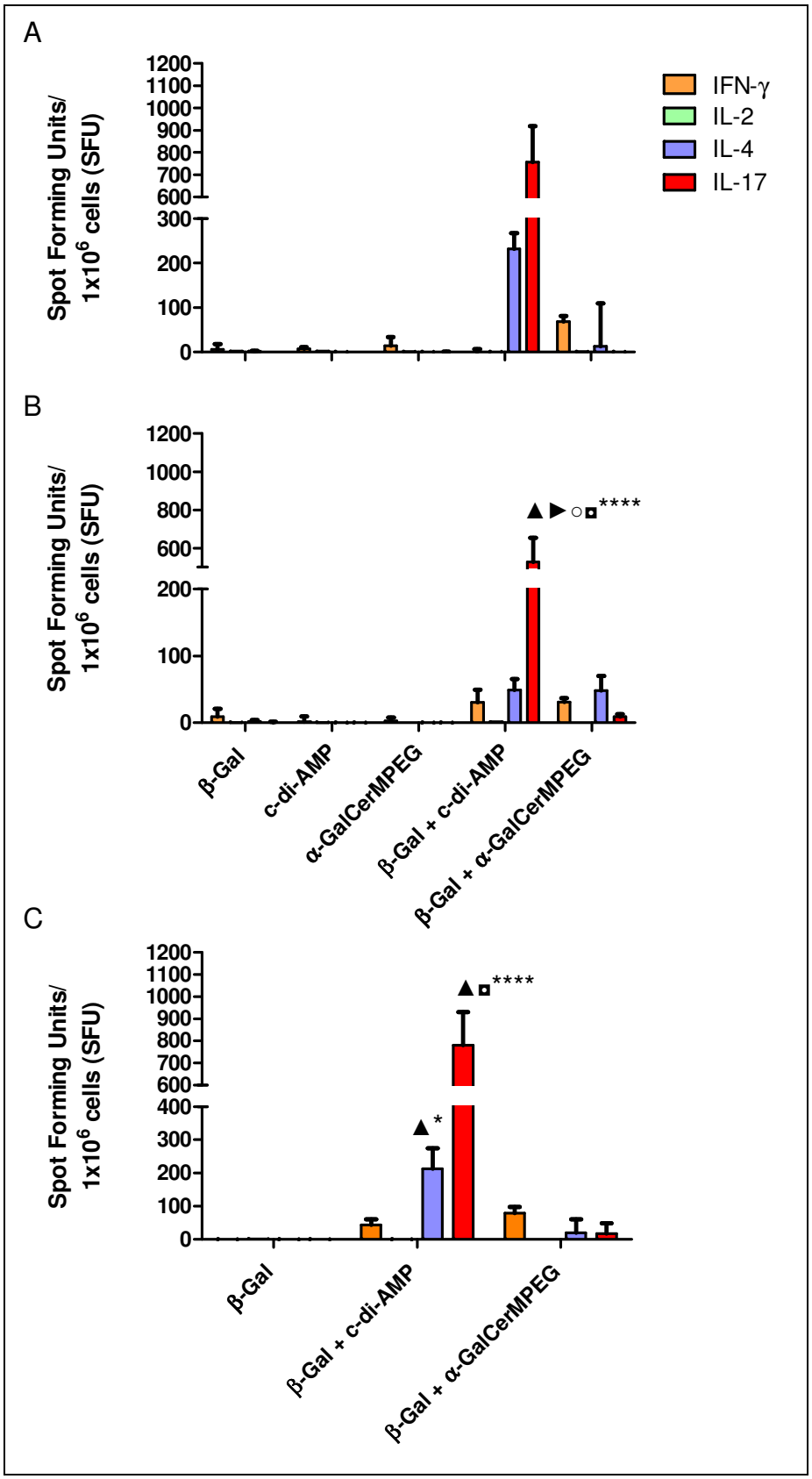

Figure 51: Cytokine profile of spleen cells stimulated following i.vag and s.l. immunisation and a combination of these routes.
The groups of mice were vaccinated via (A) i.vag., (B) s.l. and (C) a combination of both routes. Cells were incubated for 24 or 48 h in the presence of a peptide encompassing the

immunodominant Ld-restricted epitope of β-Gal (TPHPARIGL), which is specific for MHC class I presentation (for IFN-γ), or the β-Gal protein (for IL-2, IL-4 and IL-17). SEM is indicated by vertical lines. The statistic analysis was performed using Two-way ANOVA, $p<0.0001$ (****). The differences were statistically significant in comparison with the control groups receiving PBS (●), β-Gal (▲), c-di-AMP (►) and α-GalCerMPEG (○), and the test group receiving β-Gal + α-GalCerMPEG (◘).

In conclusion, combination of the i.vag. and s.l. immunisation routes in a single vaccination protocol did not further increase the efficiency of the single route vaccination strategies.

In order to characterise more in detail the source of IL-17 in mice immunised with β-Gal + c-di-AMP via i.vag. or s.l. route, spleen cells of vaccinated animals were restimulated with 40 µg/ml of ag and subsequently characterised by specific T-cell marker (CD4, CD8, γδ-TCR) based on iterative chip cytometry (ICBC). In brief, splenocytes of immunised mice were pooled and transferred to the chip. After fixation, cells were stained by fluorochrome-conjugated marker-specific antibody, and the type and number of cells were evaluated. About 1% of spleen cells were IL-17$^+$ cells (Fig. 52). While no clusters of IL-17 producing cells could be detected in splenocytes isolated from mice receiving PBS, 40% of the IL-17 producing cells isolated from spleens of mice immunised with β-Gal + c-di-AMP via the i.vag. route were CD4$^+$, followed by 2.7% of CD8$^+$ and 0.9% of γδ T-cells (Fig. 52A + B). However, more than half of the analysed cells were not specified, but it is likely that most of them were CD4$^-$ NKT cells (Fig. 52B) [208]. In contrast, when analysing spleen cells derived from mice immunised with β-Gal + c-di-AMP via the s.l. route, 15% of the IL-17 producing splenocytes were characterised by the expression of γδ-TCR and the number of CD4$^+$ cells was increased by 10% (Fig. 52B+C). The amount of non specified IL-17 producing cells was reduced by 20%.

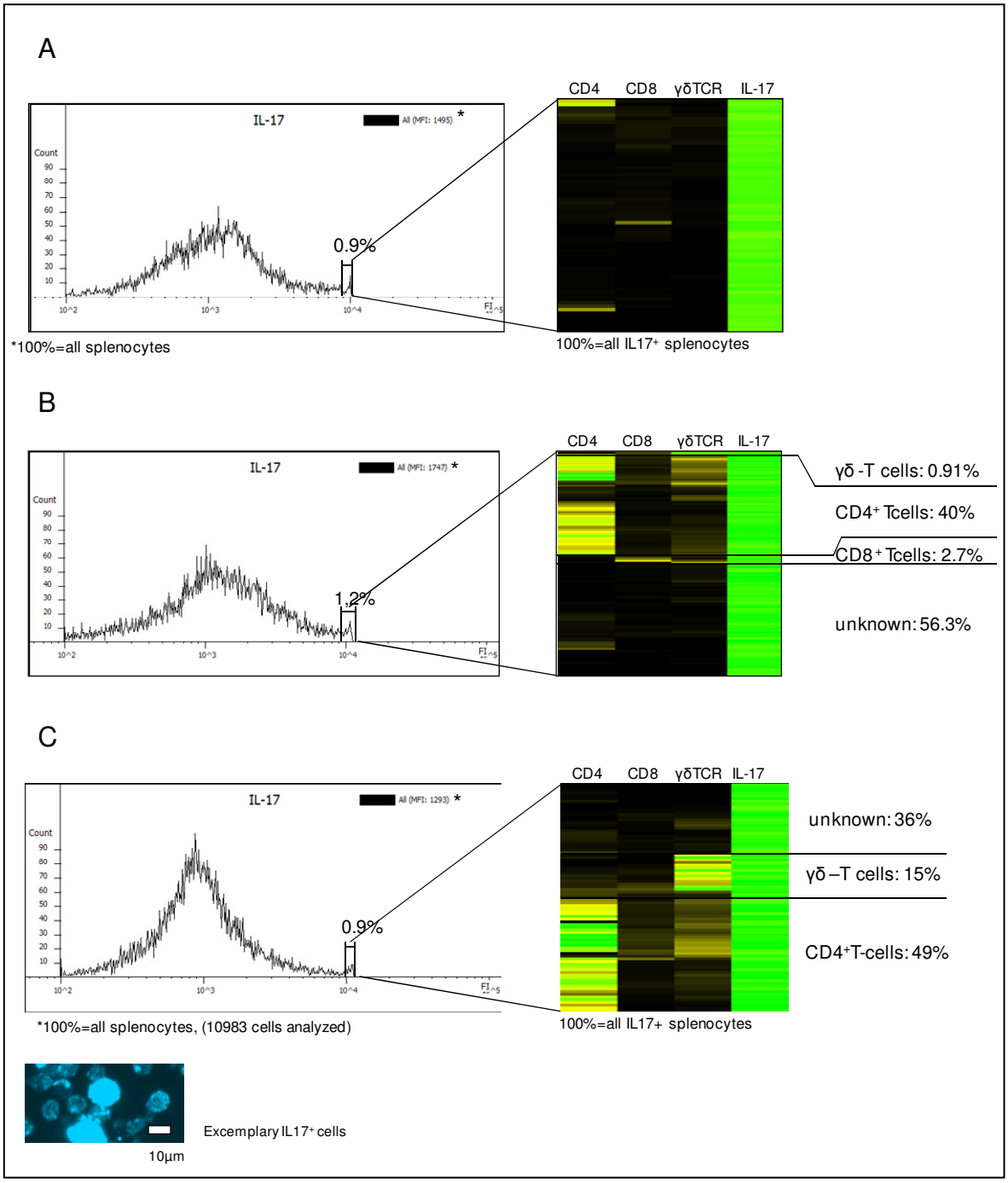

Figure 52: Analysis of the IL-17 producing cells stimulated following immunisation with β-Gal + c-di-AMP.
Cluster of IL-17 producing cells isolated from spleen of mice immunised by i.vag. route with (A) PBS, β-Gal + c-di-AMP by (B) i.vag. and (C) s.l. route. Results are given as percentage of IL-17 producing splenocytes.

Taken together, the main populations producing IL-17 following immunisation with β-Gal + c-di-AMP seemed to be $CD4^+$ and perhaps NKT cells. However, also γδ T-cells were found to produce IL-17, whereby higher numbers of these cells were found in spleen of mice immunised via s.l. route compared to those

obtained following i.vag. vaccination. If this result is rooted in a different stimulation capacity of c-di-AMP depending on the route of application or in a different functionality of the γδ T-cells activated at different mucosal territories needs to be further investigated [209], [210].

4.4. Summary of the humoral and cellular responses obtained following different immunisation strategies

The obtained results demonstrated that s.l. immunisation is an efficient alternative to i.n. immunisation (Table 1). Vaccination by both routes stimulated similarly strong humoral and cellular immune responses. In addition, administration of vaccine formulations by the s.l. route promoted as strong mucosal immune responses as administration by i.n. route, showing the same compartmentalisation profile. Thus, s.l. vaccination stimulated strong production of ag-specific IgA not only at local level but also at distant mucosal areas, such as the genital tract. Furthermore, the titres obtained at distant territories were even higher, as compared to those obtained following i.vag. immunisation. However, although immunisation via s.l. and i.n. route stimulated the strongest humoral immune responses using the adjuvant c-di-AMP, immunisation via the i.vag. and i.n. routes promoted the strongest cellular immune responses. An approach to stimulate optimal humoral and cellular immune responses based on the combination of these two vaccination strategies was not proven efficicacious.

Table 1: Immune responses stimulated according to adjuvant and route of administration.

	Humoral immune response				Cellular immune response	
Route of immunisation	c-di-AMP		α-GalCerMPEG		c-di-AMP	α-GalCerMPEG
	mucosal	systemic	mucosal	systemic		
i.n.	+++	+++	o	+	+	+
i.vag.	++	+++	+	+/o	++	+/o
s.l.	+++	+++	+	+/o	+	o
i.vag. + s.l.	++	+++	+	+/o	+	o

No induction (o); low responses (+), intermediate responses (++), strong responses (+++).

4.5. Immunisation strategies to protect against mucosal challenge with HSV-1

4.5.1. Protection against β-Gal expressing HSV-1 after s.l. vaccination with β-Gal

The results obtained until now were based on subunit vaccine formulations using the model-ag β-Gal in combination with different adjuvants. However, it is essential to assess if the adjuvants administered by s.l. route would be able to stimulate a protective immune response. Thus, the potential of the used adjuvants co-administered with β-Gal by s.l. route to protect mice against a lethal viral challenge was investigated. To this end, a challenge model was implemented based on a HSV-1 strain expressing β-Gal, which operationally acts as viral ag [211], [212]. BALB/c mice (n=10) were immunised using either β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG administered via the s.l. route. After three immunisations on day 0, 14 and 28, animals were challenged on day 42 with $7.4x10^6$ PFU of LVLacZ via i.vag. route. The weight loss and vitality of infected mice indicated that a certain degree of protection can be achieved by using the α-GalCerMPEG-based formulation. However, despite the trend, the differences were not statistically significant and no virus could be determined in plaque-assay of VL after day 7 post challenge in all groups. This suggests that the level of viral expression of β-Gal is not sufficient to measure a clear protective effect under these experimental conditions.

4.5.2. Protection against HSV-1 challenge after s.l. vaccination with virus co-administered with adjuvants

To this end, BALB/c mice were immunised via the s.l. route with either PBS, or a live attenuated derivative (still able to replicate) of the LV strain expressing β-Gal (LVLacZ, 10^3 PFU/dose) co-administered with either c-di-AMP or α-GalCerMPEG. Two weeks after immunisation, mice were challenged with 10^6 PFU/dose of the LV wt strain.

Table 2: Vaccination protocol

Group*		Adjuvant (5 µg)	Live virus (10^3 PFU)	Immunisation route	Challenge Route (LV wt 10^6 PFU)
1	PBS	-	-	i.vag.	i.vag.
2	PBS	-	-	s.l.	s.l
3	PBS	-	-	i.n.	i.n.
4		c-di-AMP	LV LacZ	s.l.	s.l.
5		c-di-AMP	LV LacZ	s.l.	i.n.
6		c-di-AMP	LV LacZ	s.l.	i.vag.
7		α-Gal-CerMPEG	LV LacZ	s.l.	i.vag.

***** BALB/c mice (n=5)

All control animals treated with PBS died within 14 days after i.vag. challenge, whereas 60 and 80% survived following s.l. and i.n. challenge, respectively (Fig. 53). This suggests that the HSV-1 challenge model based on the LV strain is most efficient when infecting mice via i.vag. route. The analysis of the survival rates of mice immunised with the formulations based on c-di-AMP showed that only 20% survive after the i.vag. challenge, whereas 80 and 100% survive the i.n. and s.l. challenge, respectively (Fig. 53). This suggests that the c-di-AMP based formulations conferred marginal protection, if any at all. In contrast, 100% of animals immunised with the formulation adjuvanted with α-GalCerMPEG were able to survive the i.vag. challenge (Fig. 53).

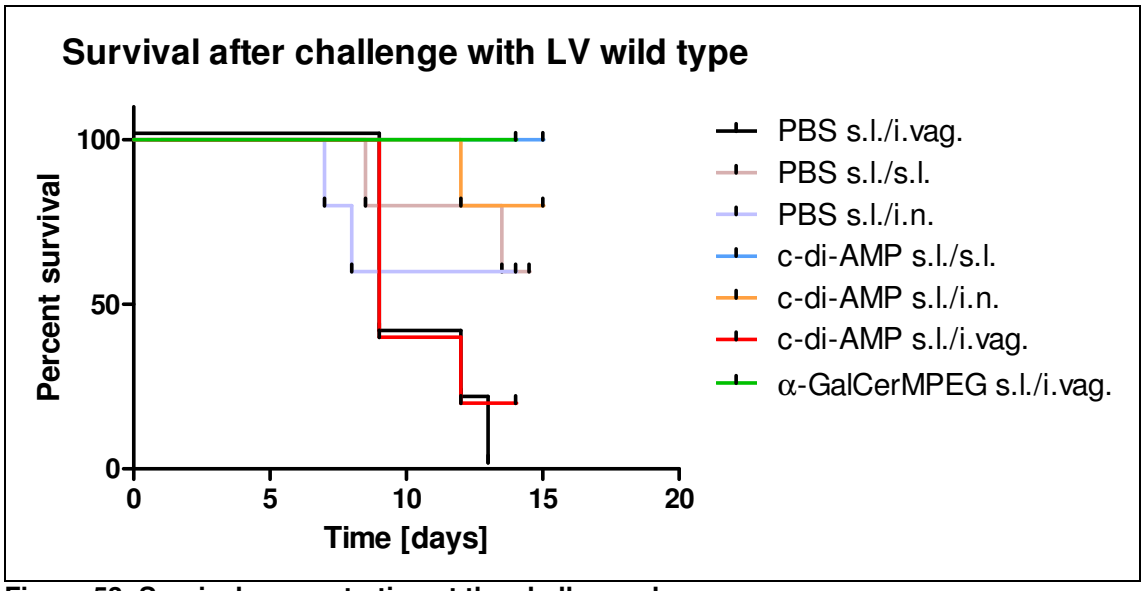

Figure 53: Survival curve starting at the challenge day.

Despite the stronger adjuvant properties of c-di-AMP, only the formulations containing α-GalCerMPEG were able to confer protection. In fact, vaccination of mice using c-di-AMP induced strong humoral and cellular immune responses as well as high numbers of IL-17 secreting splenocytes 21 days after the last immunisation. In contrast, α-GalCerMPEG did not result in late IL-17 secretion, stimulating only weak cellular responses. This suggests that differences in the quality rather than in the strength of the immune response might explain the differences in survival. More specifically, α-GalCerMPEG blocked Th17 responses, thus, to further investigate the role of IL-17 in host defence against HSV-1 studies were perfomed using IL-17 KO mice. To this end, the susceptibility of IL-17 A/F$^{-/-}$ mice (C57BL/6 background) to an i.vag. challenge with LV wt was evaluated and compared to that of C57BL/6 wt mice. According to preliminary studies, the optimal challenge dose was 6.9 x 10^8 PFU (LD60). As indicated in figure 54, in contrast to what expected, KO-mice were more susceptible to virus infection and died between day 9 and 14 after challenge, whereas 40% of the C57BL/6 survived more then 20 days. This is in line with recently published studies showing that IL-17 is needed for neutrophil recruitment to efficiently combat viral infections [213].Thus, while by adjuvant-mediated modulation Th17 polarisation was specifically blocked, in the IL-17 KO animals production of IL-17 by any cell subset is missing (*i.e.* also early IL-17

production by innate immune cells). This prevents a dissection of the specific contribution of Th17 cells to the overall protection conferred by vaccination.

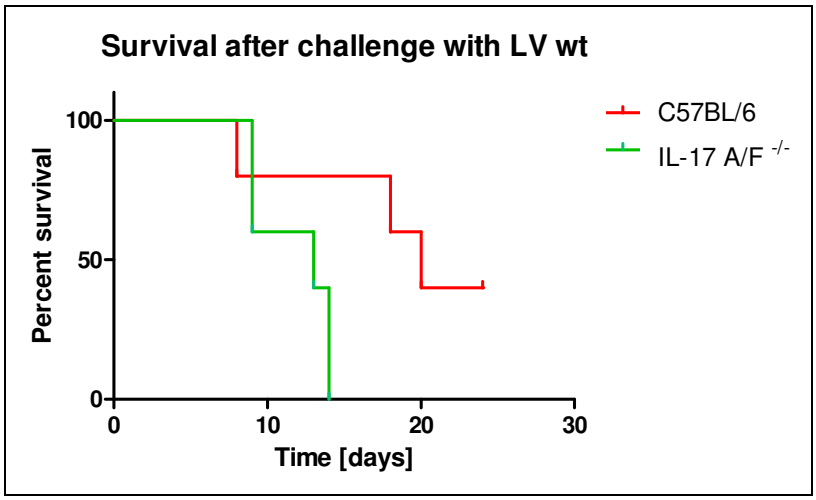

Figure 54: Susceptibility of IL-17 A/F $^{-/-}$ mice to HSV infection.
Wild type C57BL/6 and IL-17 A/F $^{-/-}$ mice were challenged with 6.9x10^{8} PFU of HSV-1 wt strain LV by i.vag. route. Results are expressed as percentage of survival.

5. Discussion

5.1. Mucosal immunisation

Millions of people die each year from infectious diseases [1]. Most of these cases occur in developing countries, since hygiene standards and medical care are less efficient respect to industrialised countries. Nevertheless, despite improved hygiene standards, therapeutic and vaccination approaches, infectious diseases still represent a huge burden, also in the developed world. The reasons for this lay in the fact that more and more pathogens develop resistance against anti-microbial agents and that new pathogens emerge. In general, vaccination constitutes the most efficient tool to prevent infectious diseases as it prepares the immune system to successfully combat pathogens before the disease develops, making cost intensive therapies redundant. In addition, vaccination also minimises the spread of infectious agents and could even eradicate a pathogen, such as in the case of small-pox in 1980 [1].

However, due to safety concerns, the acceptance of the public for vaccinations is only poor and until now only few vaccines are licensed for human use. Therefore, the development of novel vaccination strategies is urgently needed. In this context, vaccination via mucosal surfaces represents a promising approach, since most pathogens enter the human body via mucosal surfaces. Consequently, stimulation of protective immune responses in these territories would not only protect against disease, but also against infection. Furthermore, in contrast to parenteral vaccines that only stimulate systemic immune responses, mucosal immunisation is able to promote both mucosal and systemic immune responses. Due to the compartmentalisation of the mucosal immune system, local application of vaccines can also elicit protective immune responses at distant mucosal territories. Therefore, mucosal vaccination can in principle be superior to parenteral vaccine application.

However, in order to increase the safety profile of vaccine formulations, modern vaccines often consist only of parts of infectious agents rather than of whole inactivated or attenuated pathogens. Unfortunately, this increases safety at the

expense of an impaired immunogenicity. This can be explained by the fact that subunit vaccines are generally less immunogenic than whole cell vaccines, since many components with built-in adjuvant properties are missing. The immune responses to subunit vaccines are even poorer when they are administered by mucosal route. To overcome this obstacle, subunit vaccines should include adjuvants in the formulation, to strengthen and broaden the elicited responses (reviewed in [214]). Nevertheless, up to now only a handful of adjuvants have been licensed for human use. Furthermore, there is no mucosal adjuvant in the market [8]. Thus, the development of mucosal adjuvants represents a priority in vaccinology.

5.1.1. Establishment of successful immunisation protocols

Beside the stimulation of mucosal and systemic immune responses which are able to protect against infection, mucosal vaccination is hallmarked by a painless and easy administration logistic. For example, oral immunisation was successfully used for vaccination against polio, since both children and their parents could be easily motivated to comply. However, it turned out that depending on the route of administration, mucosal vaccination can stimulate immunity only locally or in very restricted distant mucosal territories. For example, oral and i.vag immunisation usually promote mucosal responses only in the gastrointestinal and genital tract, respectively. In contrast, vaccination by i.n. route elicits immune responses in both the respiratory and genital tracts [112]. For this reason, i.n. immunisation would be a promising strategy to vaccinate against both respiratory and STDs. Unfortunately, concerns about the safety of i.n. administered vaccines containing A-B moiety toxins or their derivatives as adjuvants. More specifically, neurological adverse effects (*i.e.* Bell´s palsy) were observed following administration of a virosome vaccine against flu adjuvanted with the heat labile toxin (HLT) of *E. coli* [127]. Similarly, side effects were reported in clinical trials performed using vaccine formulations containing a genetically inactivated derivative of HLT as adjuvant [128]. HLT can be redirected to the CNS via retrograde axonal transport, since neurons express receptors for the B subunit of the toxin. Nevertheless, it is not clear if the

observed side effects are directly linked to the transport of the adjuvant to the olfactory bulbs. It might also be, for example, that the inflammatory process triggered by the adjuvant resulted in a mechanic trauma of the nerve or the reactivation of a latent viral infection. Therefore, one aim of this work was to investigate the potential of the s.l. route as alternative strategy to i.n. vaccination.

To this end, comparative studies of i.n., i.vag. and s.l. immunisation were performed in a murine model, using as mucosal adjuvants the new promising compounds c-di-AMP and α-GalCerMPEG [207], [68]. The c-di-AMP stimulates ag-specific humoral and mixed Th1/Th2/Th17 responses. The adjuvant activity is at least in part mediated by its recognition by STING [68], [69]. STING is a transmembrane protein in the endoplasmatic reticulum that supports production of type I IFNs [215], [216], [217], [218]. In contrast, α-GalCer and its pegylated derivative α-GalCerMPEG are recognised by NKT cells in the context of the CD1d receptor expressed by APCs, promoting the stimulation of Th2 dominated humoral and cellular responses [78]. These two adjuvants were of further interest, since they exert a differential effect in the stimulation of IL-17 production and differentiation of Th17 cells following vaccination. The c-di-AMP promotes differentiation of Th17 cells with high levels of IL-17 in the late phase of the immune response. In contrast, α-GalCer blocks Th17 differentiation, but stimulates early IL-17 responses which peak after two hours and revert to baseline levels after 24 hours [80], [219], [77], [220], [68]. Production of IL-17 as well as the differentiation of Th17 cells were shown to play an important role in host mucosal defence by linking the innate and adaptive immune responses (reviewed in [178]). However, the role played by IL-17 and Th17 cells in HSV infection is still a matter of controversy. It has been shown that HSV infection stimulates IL-17 production in mice [221], and that IL-17 and Th17 cells contribute to the pathogenesis of HSV induced stromal keratitis [180]. A high number of IL-17 (*e.g.* produced by NKT-cells) might increase the mortality after HSV-infection [222]. On the other hand, immunisation of mice using α-GalCer as adjuvant co-administered with gD resulted in protective immunity against i.vag. challenge with HSV, which seemed to be triggered by strong IFN-γ responses

[205]. However, the potential effect of IL-17 early produced by iNKT cells activated by α-GalCer was not dissected [205]. Thus, second aspect of this study was to investigate induction of IL-17 and Th17 cells following different mucosal vaccination strategies, and exploit a HSV infection model to gain insights on the role of IL-17 in host defence to viral infections.

In order to allow a proper comparison of the different immunisation strategies, the first set of experiments focused on the setup of the vaccination protocols resulting in optimal immune responses with high ag-specific Ig titres and strong cellular responses. For i.vag. immunisations it has been shown that for a successful application, vaccines should be given during the dioestrus [223], [130], [224], [129], [225]. During this phase the epithelium is the thinnest and surface receptors (*e.g.* nectin-1, allowing viral entry) are up-regulated on the mucosa [95]. Moreover, neutrophils and mucus are also increased during this stage. These properties facilitate the uptake and processing of vaccine formulations, as well as infectious pathogens. Immune responses are poorer at the other stages of the cycle, due to a thickened epithelia and a different cell compositions [130], [131], [226], [227], [228]. The need for application of formulations during dioestrus is valid for both humans and mice [223], [91]. In humans, however, the natural occurring phases of the cycle are satisfactory to elicit antibody responses following immunisation in dioestrus stage and do not need to be modulated by external interventions [91]. Therefore, in an attempt to mimic the situation in humans, immunisation studies were performed using mice at natural occurring dioestrus stage. However, this approach was not sufficiently robust for further implementation and resulted in inefficient immune responses (data not shown). Different mice should be vaccinated at different time points, since their cycles were not synchronised. This is consistent with results reported by Marks *et al*. [223]. To artificially synchronised the oestrus cycle of female mice prior to i.vag. immunisation different methods were tested. The first approach is based on the so called Whitten-effect, where female mice are placed in cages in which male mice were hosted before [190], [191]. Here, hormones present in male urine are known to influence the female cycle. Unfortunately, no lasting and constant cycle synchronisation was obtained by

this method (tested in C57BL/6 mice). Thus, a dioestrus synchronisation method based on subcutaneous injection of DEPO was used [229], [193]. Despite the fact that this method is very commonly used, it is important to remember that treatment of mice with non-physiological amounts of hormones might change not only the susceptibility to certain pathogens like HSV, but also affect cell compositions, thereby modifying the resulting immune response. In this context, progesterone treatment has been shown to result in immune responses by transient generation of VALT consisting of $CD11c^+$ DCs and $CD3^+CD4^+$ T cells in the lamina propria, leading to protection against HSV-challenge [193], [89]. Additionally, it has been described that hormones directly influence the presence of cytokines and chemokines that correlate with immune response severity [230]. Another aspect to take into account for the outcome of the immunisation studies is that progesterone can prevent DCs activation by binding to surface receptors, which in turn inhibit STAT-1 activity [231]. STAT-1 is activated by IFN-γ and leads to T-bet activation, the transcription factor of the IFN-γ gene and the main factor in Th1-lineage commitment [232]. This in turn might explain, at least in part, the missing $CD8^+$ response observed in the present work following i.vag. immunisation of DEPO treated mice with β-Gal co-administered with c-di-AMP. In fact, immunisation via i.n. route using c-di-AMP resulted in the stimulation of IFN-γ producing $CD8^+$ cells [68]. In conclusion, Th1-development might not be supported through DEPO-treatment. This can also affect the susceptibility of vaccinated mice to the viral infections performed in this work [233], [193], [194]. Cherpes *et al.* showed that hormone treatment favours reactivation of latent HSV-1 resting in the trigeminal ganglion due to direct effect on neurons and inhibition of $CD8^+$ T-cell responses [234]. Moreover, it is known that hormonal changes correlate with the susceptibility to HIV infection, because the expression of viral receptors on mucosal cells changes according to the stage of the female cycle [235].

In parallel to the establishment of the optimal protocol for oestrus cycle synchronisation, the optimal ag-adjuvant formulation for a specific mouse strain was identified. To this end, immunisation via the i.n. route was used as reference method, since this approach was known to induce strong humoral and cellular responses at both local and systemic level. In a first step the adjuvant

stimulating the strongest responses was identified. The MALP-2 derivative BPPCysMPEG tested in C57BL/6 mice stimulated only weak ag-specific immune responses after i.n. or i.vag. vaccination, compared to c-di-AMP which triggered robust responses following i.n. administration, and even stronger humoral and cellular responses after i.vag. immunisation. Thus, c-di-AMP was chosen for further experiments, since it was the most promising adjuvant independently of the administration route. After the adjuvant was identified, different mouse strains were immunised via different routes using OVA and β-Gal as model-ags. The strongest humoral and cellular immune responses were obtained immunising BALB/c mice by either i.n. or i.vag. route with the model-ag β-Gal co-administered with c-di-AMP. In contrast, vaccination of mice with OVA and c-di-AMP stimulated comparatively weaker immune responses. The strain-dependent immune responses reported here are consistent with previous reports [236], [237].

It was described that BALB/c mice are more susceptible to HSV infection, as compared to the more resistant mouse strain C57BL/6. This could be explained by the development of a fast IFN type I response of the innate immune system in C57BL/6 mice, which in turn promotes protection against viral infections [198], [199]. Thus, further comparative immunisation studies were performed using β-Gal in combination with c-di-AMP and BALB/c mice.

One of the aims of this work was also to investigate the contribution of IL-17 and Th17 cells in antiviral host defence. Thus, α-GalCerMPEG was included as adjuvant in the further experiments. Interestingly, α-GalCerMPEG also stimulated stronger antibody responses in BALB/c mice than in C57BL/6 mice, although to a lesser extent as compared to c-di-AMP. While the use of c-di-AMP as adjuvant resulted in the stimulation of both IgG1 and IgG2 subclasses (*i.e.* a mixed Th1/Th2 response), α-GalCerMPEG promoted an IgG1 dominated (*i.e.* Th2 biased) immune response. However, when the obtained mucosal immune responses were analysed, α-GalCerMPEG stimulated weaker IgA production than c-di-AMP. The same was true when comparing the cellular responses stimulated by α-GalCerMPEG and c-di-AMP. The proliferative capacity of spleen cells derived from BALB/c mice receiving c-di-AMP was about two-fold higher than the one of cells derived from C57BL/6 mice. Similar results were observed

regarding the levels of IL-2, IL-4, IL-17 and IFN-γ producing splenocytes. Co-administration of α-GalCerMPEG resulted in minimal cell proliferation and cytokine production, independently of the tested mouse strain. As expected, no IL-17 production has been observed after restimulation of splenocytes derived from mice immunised with β-Gal co-administered with α-GalCerMPEG. In contrast, when c-di-AMP was used as adjuvant, high numbers of IL-17 producing cells were detected. These findings are in line with former observations made by our group [207], [68].

5.1.2. Benefits and drawbacks following i.vag. immunisation

The i.vag. immunisation is one possible strategy to achieve protective immune responses at the genital tract. This route, however, mostly promotes a local mucosal immune response, since cells stimulated at the genital mucosa are usually not homing to distant sites (see section 1.3.3). Nevertheless, this approach is still of interest to promote protective immune responses against STDs since, in contrast to systemic immunisation, i.vag. application of the ag triggers both CTL responses in the draining LN and local IgA production [238], [239].

When c-di-AMP and α-GalCerMPEG were used as adjuvants, c-di-AMP always promoted stronger humoral and cellular immune responses at both local and systemic level. However, comparison of i.vag. and i.n. administration showed that i.vag. immunisation induced only weak humoral responses at systemic and mucosal levels, as indicated by lower titres of serum IgG and sIgA. In contrast, cellular immunity was stronger following i.vag. immunisation, as compared to i.n. vaccination. Remarkably, c-di-AMP containing formulations elicited strong $CD4^+$ T-cell responses but only low numbers of IFN-γ producing $CD8^+$ T-cell spleen cells when administered by i.vag. route. Beside the impact of DEPO treatment, the reduced number of IFN-γ producing $CD8^+$ T-cells might be explained by the fact that in vagina c-di-AMP mainly stimulated Langerhans DCs, rather than $CD207^-$ DCs [240]. This favours a $CD8^+$ T-cell-biased immune response, which results in IL-17-secretion rather than promotion of IFN-γ producing T-cells.

Accordingly, it was reported that vaginal Langerhans DCs contribute to the differentiation of ag-specific $CD8^+$ T-cells producing IL-17A and IL-10, which seem to have regulatory functions critical during viral infections [241]. Despite the fact that their true role has not been fully dissected, the high numbers of IL-17 producing cells obtained following immunisation with c-di-AMP further support this affirmation, that the stimulated $CD8^+$ T-cells mainly produce IL-17 and low IFN-γ [242], [231], [232].

As mentioned before, hormones affect the function of APCs, including their ability to activate T-cells. Therefore, DEPO-treatment might also contribute to the outcome of the stimulated immune responses [243]. This aspect would be of particular importance with regard to immunisations against viral infections, such as HSV, where IFN-γ secreting $CD4^+$ cells seem to be the key effector cells needed for viral clearance [244], [245]. Nevertheless, it is still controversially discussed if $CD8^+$ T-cells are the main source for IFN-γ and to which extent help provided by $CD4^+$ T-cells is needed for successful stimulation of $CD8^+$ responses [246], [247].

Vaccination with formulations containing α-GalCerMPEG as adjuvant were poor inducers of cellular immunity as compared with those containing c-di-AMP, particularly in terms of stimulating IFN-γ producing cells. Only IL-4 secreting cells were detected three weeks after the last immunisation, and, as expected, ag-specific Th17 cells were not induced. This is in contrast to a previous report in which gD co-administered with α-GalCer through the i.vag. route stimulated strong IFN-γ responses both in spleen and genital lymph nodes [205]. The differences might be explained in part by different intrinsic immunogenic properties of the ag (*i.e.* gD versus β-Gal) [205]. The α-GalCerMPEG containing vaccine formulation also stimulated strong antibody responses. These results are in line with previous studies, showing that this adjuvant mainly promote Th2-biased responses [207]. However, α-GalCer binds to CD1d on DCs, thereby stimulating NKT-cells to produce IL-17 [80]. $NK1.1^-$ NKT-cells are responsible for the IL-17 secretion observed 2-3 hours after stimulation, which is not restricted to a single subset [77], [248]. These cells also secrete low amounts of IFN-γ and IL-4, which contribute to the recruitment of neutrophils to the site of infection [80]. This can be in turn critical for shaping the microenvironment in a

form which is conductive towards the elicitation of an efficient adaptive immune response. It follows, that it is also critical to enable an appropriate window between the vaccination and any challenge aimed at evaluating the efficacy of the stimulated response. In fact, the early effector functions from α-GalCerMPEG might also contribute to microbial clearance, since γδ T-cells were described to survive around four weeks or even switch to memory cells [249].

5.2. Immunisation via s.l. route is a promising alternative to i.n. and i.vag. immunisation

The exploitation of the s.l. immunisation route can lead to strong mucosal and systemic immune responses, with minimal risk for side effect. The s.l. mucosa encompasses a pluristratified epithelium associated to immune cells, which as inductive site enables the stimulation of local and distant responses, as indicated by sIgA production, and stimulation of serum antibodies and CTL responses following vaccination (reviewed in [83]). Vaccine formulations administered via s.l. route directly enter the blood stream without passing intestine or liver [143], [144], [142]. In general, only minimal adverse side effects have been observed in patients after s.l. application of drugs or immune therapies for allergies [250]. However, before evaluating the s.l. route as a vaccination strategy, experiments were performed to rule out that under our experimental conditions ag leaking to the gut might be responsible for any observed immune response. While s.l. immunisation stimulated strong humoral and cellular immune responses at local and systemic level comparable to those obtained after i.n. immunisation, oral vaccination resulted in only marginal antibody and cytokine production. In addition, s.l. immunisation stimulated similar levels of ag-specific IgA production in the different mucosal territories, as compared to administration via i.n. route. In contrast, oral immunisation stimulated significantly decreased IgA titres with respect to the other routes. Thus, the protocol for s.l. immunisation used in the presented work is valid in order to investigate the potential of the s.l. route as vaccination strategy.

The experiments performed in this thesis also revealed that the ag and adjuvant dosages for s.l. immunisation should be approximately doubled respect to those applied by i.n. or i.vag. route. Thus, in order to allow a proper comparison of the different immunisation strategies, studies were performed in which animals were immunised via i.vag. route with the same dosage needed for s.l. vaccination. The side-by-side comparison of the two ag and adjuvant showed no significant differences in the level of stimulated humoral and cellular immune responses. Thus, this confirms that the comparability of the different vaccination strategies is allowed, although the amount of ag and adjuvant distinguish for s.l. immunisation compared to i.vag. or i.n. immunisation.

In this study, the humoral immune responses obtained following s.l. immunisation were comparable in strength and pattern to those observed after i.n. vaccination and superior to those elicited following i.vag. immunisation. The same is true when the cellular responses stimulated after s.l. and i.n. responses are compared. Again, ag-specific splenocytes from immunised mice showed similar proliferative capacities, as well as analogue levels and patterns of secreted cytokines. Interestingly, i.vag. vaccination stimulated stronger cellular responses with respect to the other application routes, as indicated by higher proliferative capacities of spleen cells and higher numbers of cytokine secreting cells in immunised mice. To assess if immune responses could be optimally tailored by combining approaches, a vaccination schedule was implemented in which s.l. and i.vag. immunisations were combined. To this end, mice received the same ag and adjuvant dosages as administered by the single route protocol, but divided and administered at two different routes. Interestingly, although it seemed that the combined mucosal vaccination strategy resulted in slightly increased cellular responses as compared to the s.l. immunisation alone, the differences were not statistically significant. However, as recently shown, a prime boost protocol in which the vaccine formulation is given first by one route and then a boost is performed by another route might be a more efficient approach to fine-tune the elicited response [251].

After characterisation of the immune responses stimulated following s.l. immunisation, the capacity of this route to promote protective immunity was investigated. To this end, mice immunised via s.l. route with a sub-lethal dose of the HSV type I strain LV co-administered with either c-di-AMP or α-GalCerMPEG, were subsequently challenged with a lethal dose of the homologous strain via the i.vag. route. When mice were vaccinated using α-GalCerMPEG as adjuvant, 100% of the animals survived the i.vag. challenge three weeks after the last immunisation. It seems unlikely that this protection results from a long lasting NK and/or NKT response stimulated by α-GalCerMPEG considering the interval between the last boost and the challenge. Therefore, protection appears to be mediated by the adaptive immune responses stimulated by the vaccine. This is further support by the fact that Lin *et al*. demonstrated that the beneficial effect of α-GalCer also depends on the time point of administration. The α-GalCer was able to protect mice only when administered one day before a viral challenge [252].

The α-GalCer and its derivatives are known to activate iNKT, when presented in the context of CD1d receptor on DCs, which subsequently secrete Th1 (IFN-γ) and Th2 (IL-4) cytokines. While the ability of α-GalCer to induce Th2 cytokines seems to correlate with the amelioration of autoimmune diseases, the production of Th1 cytokines is shown to correlate with antitumor, antibacterial, and antiviral effects [253], [254], [252]. In this context, Adotevi *et al*. showed that co-administration of α-GalCer resulted in an improved ag delivery to DCs, which was reflected by a stronger and longer lasting $CD8^+$ T-cell response [255]. In addition, Gonzalez-Aseguinolaza and co-workers showed that vaccination using α-GalCer as adjuvant triggered protective immunity against malaria in mice. This protection seemed to be mediated by enhanced ag-specific $CD8^+$ T-cell responses and, to a lesser degree, by Th1-type cells, Vα14 NKT (iNKT) cells, and IFN-γ [253]. Moreover, in a corneal HSV infection model, IFN-γ was shown to be very important for protection. IFN-γ down regulates IL-17 by suppressing IL-23 synthesis, thereby hindering Th17 cells development [180]. Surprisingly, co-administration of c-di-AMP was not sufficient in order to protect mice against the homologous challenge, since 80% of the infected mice died. This is in contrast to what was observed by Ebensen *et al*. (unpublished data), since mice

vaccinated by i.n. route with inactivated flu virus co-administered with c-di-AMP were protected against a lethal homologous challenge via i.n. route.The adjuvant c-di-AMP stimulated IL-17 production three weeks after the last immunisation. The main source for IL-17 seems to be Th17 cells (up to 50%). These cells express the CD4, which is not expressed by most γδ T-cells. However, as shown here, also γδ T-cells were stimulated by c-di-AMP to secrete IL-17 (up to 15%). Like NKT cells, γδ T-cells are thought to bridge between innate and adaptive immune responses, whereby the adaptive responses were preceded by innate immune cells [256], [257]. Innate cell subpopulations, such as γδ T-cells, NK cells, and lymphoid tissue-inducer cells are also sources for other Th17 cytokines, like IL-22 [258]. The γδ T-cells can be classified according to their functional programming and show different tissue tropisms [160], [257]. Furthermore, γδ T-cells are commonly indirectly activated by PAMPs which, similar to NK cells and iNKT cells, lead to early induction of IFN-γ production [257]. However, PAMPs can also indirectly trigger IL-17 production by mouse Vγ6Vδ1$^+$ T-cells which can be mainly found in tongue, lung, uterus and vaginal epithelia [257]. Previous studies using various infectious disease models indicate that γδ T-cells can carry out different functions in response to infections. Their response is staged, occurring before or after the αβ T-cell response. This might be in part due to the specific distribution of γδ T-cells in different lymphoid tissues (reviewed in [210]). At mucosal sites, γδ T-cells contribute to the initial stages of the response to infection, thereby bridging the innate and adaptive immune system, and promoting αβ T-cell responses [210]. In addition, γδ T-cell involvement could also trigger chemokine mediated recruitment of other lymphoid populations to the site of infection. Finally, γδ T-cells seem to act as anti-inflammatory cells at the end of the effector response, contributing to tissue repair and cellular regeneration [210]. Thus, the controversial results obtained using c-di-AMP as adjuvant in vaccine formulations against flu and HSV might be also explained by the stimulation of different γδ T-cells subsets and adaptive immune responses at different mucosal territories.

In this context, infection of C57BL/6 mice by i.n. route with the influenza A virus stimulated the activation and expansion of two distinct populations of γδ T-cells

in lungs over time [210]. About 10 days post-infection, the number of Vγ4$^+$ T-cells, which are mainly found in lung, liver and spleen and epithelia, and primarily produce pro-inflammatory cytokines, increased, whereas about 13 days post-infection an increase of Vγ1$^+$ T-cells, which are normally localised in lymphoid tissues and spleen, and primarily produce immunoregulatory/anti-inflammatory cytokines, was observed [210]. Thus, the differences in the expressed cytokine profiles might be essential in order to protect mice against infection. Accordingly, s.l. immunisation of mice with HSV co-administered with c-di-AMP might stimulate mainly Vγ6$^+$ T-cells, which are programmed in the thymus to produce IL-17 and were believed to play a role in tissue remodelling and innate clearance of bacteria [257]. Furthermore, consistent with data presented here, Vγ6$^+$ T-cells are not found in spleens of mice, which could in turn explain the small amounts of IL-17 producing γδ T-cells in spleen of mice immunised with β-Gal co-administered with c-di-AMP by the i.vag. route. Vγ6$^+$ T-cells stimulation results in early production of IL-17 upon TCR signalling, whereas Vγ4$^+$ T-cells produce IL-17 and Th17 polarising cytokines, thereby resulting in different downstream processes [257]. However, also α-GalCer stimulates γδ T-cells to produce IFN-γ and IL-17, although it is not clear which specific subsets are responsible [259]. Nontheless, further studies need to be performed to address open questions. For example, by performing an in depth analysis of the cytokine profiles and cell subpopulations (numbers, phenotypic markers, functional properties) stimulated after immunisation of mice by different mucosal routes using c-di-AMP and α-GalCerMPEG as adjuvants. Also the specific impact of absence of IL-17 needs to be elucidated. According to our studies in IL-17-KO mice, deficiency in IL-17 production seems to have a negative effect in protection against HSV-1 infection. Similar effects were observed in the influenza model, where Th17 cells appear to be necessary to protect mice against a lethal challenge [260], and in certain bacterial infections [261], [262].

5.3. Conclusions

Mucosal vaccination exhibits many advantages as compared with the most often applied parenteral vaccination. The use of the mucosal route is cost efficient and results in (i) induction of humoral and cellular immune responses at both local and systemic level, (ii) stimulation of protective immunity at local and distant mucosal territories (*e.g.* vaginal tract), (iii) an easy administration logistics, and (iv) modest discomfort, stress or risk of cross-contamination [91], [115], [263].

However, there are also some disadvantages connected with mucosal vaccination. For example, although i.vag. application is a potent route to elicit local protective responses against STDs, but only female could be vaccinated, the optimal phase of the menstrual cycle needs to be considered and cultural factors might affect the degree of acceptance, making it unsuitable for mass vaccinations [91], [134]. Furthermore, in case of i.n. vaccination, the occurrence of adverse events following immunisation (*e.g.* Bell's palsy) raised concerns about the safety of certain candidate adjuvants.

In contrast, s.l. administration overcomes potential concerns of i.n. immunisation, retaining all the advantages of this appraoch [148], [119]. In fact, s.l. immunisation is able to elicit protection against lethal i.n. viral challenge, as well as promote protective antibody and CTL responses at the genital tract [148], [90]. Therefore, the s.l. administration is valid alternative to both i.n. and i.vag. vaccination, circumventing most of the safety and logistic concerns associated these routes [140].

However, independently of the administration route, there is a general consensus that IL-17-producing cells (*i.e.* Th17 cell, γδT-cells, NKT cells and LTI cells) play a substantial role in host defense against pathogens at mucosal sites. Thus, cytokines produced by these cells are important for the induction of innate and adaptive host responses. Nevertheless, IL-17 was also shown to contribute to inflammatory responses at mucosal sites, resulting in pathological manifestations linked to Th17 responses. Thus, it is becoming apparent that there is a fine balance at mucosal sites that defines immunity or inflammation (reviewed in [264]). In the present work, the mucosal adjuvants c-di-AMP and α-

GalCerMPEG showed opposed effects in terms of IL-17 production and Th17 development. While c-di-AMP seems to stimulate an early γδ T-cell response followed by a late Th17 response, α-GalCerMPEG stimulates NKT cells resulting in a Th1 dominated ag-specific response. Although α-GalCerMPEG is also able to stimulate γδ T-cells to secrete IFN-γ and IL-17, this adjuvant does not stimulate immune responses resulting in stimulation of Th17 cells [259]. In the HSV based experimental mouse model used in this work, this NKT activation and the subsequent Th1 responses were essential in order to protect mice against a lethal challenge. In contrast, the responses stimulated by c-di-AMP seem to favour a Th17/Th1 phenotype with production of both IL-17 and IFNγ, which is not able to protect against HSV challenge. Interestingly, Th17/Th1 cells are believed to be the main cause of pathogenic Th17 responses, rather than Th17 cells [265]. This might be an explanation why the mice are dying after the challenge.

6. References

1. Available from: http://www.who.int/research/en/.
2. *QUESTIONS AND ANSWERS; The Herpevac Trial for Women* 2010; Available from: http://www.niaid.nih.gov/news/QA/Pages/HerpevacQA.aspx.
3. Plotkin, S.A., Orenstein, W.A. & Offit, P.A., *Vaccines 5th edn*. 2008, Philadelphia: Saunders/Elsevier.
4. Pulendran, B. and R. Ahmed, *Immunological mechanisms of vaccination.* Nat Immunol, 2011. **12**(6): p. 509-17.
5. Janeway, C.A., Travers, P., Walport M., Shlomchik, M., *Immunbiologie*. 5 Edition ed. 2002: Spektrum Akademischer Verlag.
6. Bowen, J.C., et al., *Cholera toxin acts as a potent adjuvant for the induction of cytotoxic T-lymphocyte responses with non-replicating antigens.* Immunology, 1994. **81**(3): p. 338-42.
7. Simmons, C.P., et al., *Mucosal delivery of a respiratory syncytial virus CTL peptide with enterotoxin-based adjuvants elicits protective, immunopathogenic, and immunoregulatory antiviral CD8+ T cell responses.* J Immunol, 2001. **166**(2): p. 1106-13.
8. Mbow, M.L., et al., *New adjuvants for human vaccines.* Curr Opin Immunol, 2010. **22**(3): p. 411-6.
9. Glenny AT, P.C., Waddington H, Wallace U, *The antigenic value of toxiod precipitated by potassium alum.* J Pathol Bacteriol, 1926(29): p. 38-39.
10. Jordan, M.B., et al., *Promotion of B cell immune responses via an alum-induced myeloid cell population.* Science, 2004. **304**(5678): p. 1808-10.
11. Gavin, A.L., et al., *Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling.* Science, 2006. **314**(5807): p. 1936-8.
12. Kool, M., et al., *Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells.* J Exp Med, 2008. **205**(4): p. 869-82.
13. Flach, T.L., et al., *Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity.* Nat Med, 2011. **17**(4): p. 479-87.
14. Eisenbarth, S.C., et al., *Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants.* Nature, 2008. **453**(7198): p. 1122-6.
15. Sutterwala, F.S., et al., *Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1.* Immunity, 2006. **24**(3): p. 317-27.
16. Reed, S.G., et al., *New horizons in adjuvants for vaccine development.* Trends Immunol, 2009. **30**(1): p. 23-32.
17. Crepeau, E.B., *Achieving intersubjective understanding: examples from an occupational therapy treatment session.* Am J Occup Ther, 1991. **45**(11): p. 1016-25.
18. O'Hagan, D.T., *MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection.* Expert Rev Vaccines, 2007. **6**(5): p. 699-710.
19. Galli, G., et al., *Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts long-term persistence of protective antibody levels.* Proc Natl Acad Sci U S A, 2009. **106**(10): p. 3877-82.
20. Mosca, F., et al., *Molecular and cellular signatures of human vaccine adjuvants.* Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10501-6.

21. Seubert, A., et al., *The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells.* J Immunol, 2008. **180**(8): p. 5402-12.
22. Morel, S., et al., *Adjuvant System AS03 containing alpha-tocopherol modulates innate immune response and leads to improved adaptive immunity.* Vaccine, 2011. **29**(13): p. 2461-73.
23. Nicholls, E.F., L. Madera, and R.E. Hancock, *Immunomodulators as adjuvants for vaccines and antimicrobial therapy.* Ann N Y Acad Sci, 2010. **1213**: p. 46-61.
24. Didierlaurent, A.M., et al., *AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity.* J Immunol, 2009. **183**(10): p. 6186-97.
25. Harper, D.M., et al., *Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial.* Lancet, 2004. **364**(9447): p. 1757-65.
26. Harper, D.M., et al., *Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial.* Lancet, 2006. **367**(9518): p. 1247-55.
27. Kundi, M., *New hepatitis B vaccine formulated with an improved adjuvant system.* Expert Rev Vaccines, 2007. **6**(2): p. 133-40.
28. Hirschfeld, M., et al., *Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2.* J Immunol, 2000. **165**(2): p. 618-22.
29. Tapping, R.I., et al., *Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides.* J Immunol, 2000. **165**(10): p. 5780-7.
30. Evans, J.T., et al., *Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529.* Expert Rev Vaccines, 2003. **2**(2): p. 219-29.
31. Tiberio, L., et al., *Host factors impacting the innate response in humans to the candidate adjuvants RC529 and monophosphoryl lipid A.* Vaccine, 2004. **22**(11-12): p. 1515-23.
32. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses.* Nat Immunol, 2004. **5**(10): p. 987-95.
33. Pasare, C. and R. Medzhitov, *Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells.* Science, 2003. **299**(5609): p. 1033-6.
34. Dunham, E.W., et al., *Effects of essential fatty acid deficiency on prostaglandin synthesis and fatty acid composition in rat renal medulla.* Lipids, 1978. **13**(12): p. 892-7.
35. Alving, C.R., et al., *Effectiveness of liposomes as potential carriers of vaccines: applications to cholera toxin and human malaria sporozoite antigen.* Vaccine, 1986. **4**(3): p. 166-72.
36. Owais, M. and C.M. Gupta, *Liposome-mediated cytosolic delivery of macromolecules and its possible use in vaccine development.* Eur J Biochem, 2000. **267**(13): p. 3946-56.
37. Wilschut, J., *Influenza vaccines: the virosome concept.* Immunol Lett, 2009. **122**(2): p. 118-21.
38. Madhun, A.S., et al., *Intramuscular Matrix-M-adjuvanted virosomal H5N1 vaccine induces high frequencies of multifunctional Th1 CD4+ cells and strong antibody responses in mice.* Vaccine, 2009. **27**(52): p. 7367-76.
39. Alvin, C., *Immune reactions of lipids and lipid model membranes*. The antigens, ed. S. M. Vol. Vol. 4. 1977, New York: Academic Press.
40. Tsurudome, M., et al., *Lipid interactions of the hemagglutinin HA2 NH2-terminal segment during influenza virus-induced membrane fusion.* J Biol Chem, 1992. **267**(28): p. 20225-32.

41. Mischler, R. and I.C. Metcalfe, *Inflexal V a trivalent virosome subunit influenza vaccine: production.* Vaccine, 2002. **20 Suppl 5**: p. B17-23.
42. Gluck, R., et al., *Safety and immunogenicity of intranasally administered inactivated trivalent virosome-formulated influenza vaccine containing Escherichia coli heat-labile toxin as a mucosal adjuvant.* J Infect Dis, 2000. **181**(3): p. 1129-32.
43. Durrer, P., et al., *H+-induced membrane insertion of influenza virus hemagglutinin involves the HA2 amino-terminal fusion peptide but not the coiled coil region.* J Biol Chem, 1996. **271**(23): p. 13417-21.
44. Tyrrell, D.A., et al., *New aspects of liposomes.* Biochim Biophys Acta, 1976. **457**(3-4): p. 259-302.
45. Heath, T.D., D.C. Edwards, and B.E. Ryman, *The adjuvant properties of liposomes.* Biochem Soc Trans, 1976. **4**(1): p. 129-33.
46. Kimura, H.J., et al., *Application of innate immune molecules for a new class of drugs: infection, inflammation and beyond.* Endocr Metab Immune Disord Drug Targets, 2011. **11**(1): p. 68-75.
47. Kawai, T. and S. Akira, *TLR signaling.* Semin Immunol, 2007. **19**(1): p. 24-32.
48. McGreal, E.P., J.L. Miller, and S. Gordon, *Ligand recognition by antigen-presenting cell C-type lectin receptors.* Curr Opin Immunol, 2005. **17**(1): p. 18-24.
49. Carneiro, L.A., L.H. Travassos, and S.E. Girardin, *Nod-like receptors in innate immunity and inflammatory diseases.* Ann Med, 2007. **39**(8): p. 581-93.
50. Onomoto, K., M. Yoneyama, and T. Fujita, *Regulation of antiviral innate immune responses by RIG-I family of RNA helicases.* Curr Top Microbiol Immunol, 2007. **316**: p. 193-205.
51. Takeuchi, O. and S. Akira, *Recognition of viruses by innate immunity.* Immunol Rev, 2007. **220**: p. 214-24.
52. Palsson-McDermott, E.M. and L.A. O'Neill, *Building an immune system from nine domains.* Biochem Soc Trans, 2007. **35**(Pt 6): p. 1437-44.
53. Pashine, A., N.M. Valiante, and J.B. Ulmer, *Targeting the innate immune response with improved vaccine adjuvants.* Nat Med, 2005. **11**(4 Suppl): p. S63-8.
54. Pulendran, B., H. Tang, and S. Manicassamy, *Programming dendritic cells to induce T(H)2 and tolerogenic responses.* Nat Immunol, 2010. **11**(8): p. 647-55.
55. LeibundGut-Landmann, S., et al., *Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17.* Nat Immunol, 2007. **8**(6): p. 630-8.
56. Prajeeth, C.K., et al., *The synthetic TLR2 agonist BPPcysMPEG leads to efficient cross-priming against co-administered and linked antigens.* Eur J Immunol, 2010. **40**(5): p. 1272-83.
57. Takeuchi, O., et al., *Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway.* J Immunol, 2000. **164**(2): p. 554-7.
58. Borsutzky, S., et al., *The mucosal adjuvant macrophage-activating lipopeptide-2 directly stimulates B lymphocytes via the TLR2 without the need of accessory cells.* J Immunol, 2005. **174**(10): p. 6308-13.
59. Link, C., et al., *The Toll-like receptor ligand MALP-2 stimulates dendritic cell maturation and modulates proteasome composition and activity.* Eur J Immunol, 2004. **34**(3): p. 899-907.
60. Rharbaoui, F., et al., *The Mycoplasma-derived lipopeptide MALP-2 is a potent mucosal adjuvant.* Eur J Immunol, 2002. **32**(10): p. 2857-65.

61. Michelsen, K.S., et al., *Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E.* Proc Natl Acad Sci U S A, 2004. **101**(29): p. 10679-84.
62. Israeli, E., et al., *Adjuvants and autoimmunity.* Lupus, 2009. **18**(13): p. 1217-25.
63. Leadbetter, E.A., et al., *Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors.* Nature, 2002. **416**(6881): p. 603-7.
64. Libanova, R., et al., *The member of the cyclic di-nucleotide family bis-(3', 5')-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant.* Vaccine, 2010. **28**(10): p. 2249-58.
65. McWhirter, S.M., et al., *A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP.* J Exp Med, 2009. **206**(9): p. 1899-911.
66. Hoebe, K., et al., *Identification of Lps2 as a key transducer of MyD88-independent TIR signalling.* Nature, 2003. **424**(6950): p. 743-8.
67. Yamamoto, M., et al., *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.* Science, 2003. **301**(5633): p. 640-3.
68. Ebensen, T., et al., *Bis-(3',5')-cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant.* Vaccine, 2011. **29**(32): p. 5210-20.
69. Burdette, D.L., et al., *STING is a direct innate immune sensor of cyclic di-GMP.* Nature, 2011. **478**(7370): p. 515-8.
70. Karaolis, D.K., et al., *Bacterial c-di-GMP is an immunostimulatory molecule.* J Immunol, 2007. **178**(4): p. 2171-81.
71. Sauer, J.D., et al., *The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides.* Infect Immun, 2011. **79**(2): p. 688-94.
72. Woodward, J.J., A.T. Iavarone, and D.A. Portnoy, *c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response.* Science, 2010. **328**(5986): p. 1703-5.
73. Witte, G., et al., *Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates.* Mol Cell, 2008. **30**(2): p. 167-78.
74. Oppenheimer-Shaanan, Y., et al., *c-di-AMP reports DNA integrity during sporulation in Bacillus subtilis.* EMBO Rep, 2011. **12**(6): p. 594-601.
75. Kawano, T., et al., *CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides.* Science, 1997. **278**(5343): p. 1626-9.
76. Funakoshi, c.l., *α-Galactosylceramide*, c.l. Funakoshi, Editor. 2009: Tokio, Japan.
77. Rachitskaya, A.V., et al., *Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion.* J Immunol, 2008. **180**(8): p. 5167-71.
78. Taniguchi, M., K. Seino, and T. Nakayama, *The NKT cell system: bridging innate and acquired immunity.* Nat Immunol, 2003. **4**(12): p. 1164-5.
79. Michel, M.L., et al., *Critical role of ROR-gammat in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation.* Proc Natl Acad Sci U S A, 2008. **105**(50): p. 19845-50.
80. Michel, M.L., et al., *Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia.* J Exp Med, 2007. **204**(5): p. 995-1001.
81. Czerkinsky, C. and J. Holmgren, *Mucosal Delivery Routes for Optimal Immunization: Targeting Immunity to the Right Tissues.* Curr Top Microbiol Immunol, 2010.
82. Brandtzaeg, P. and R. Pabst, *Let's go mucosal: communication on slippery ground.* Trends Immunol, 2004. **25**(11): p. 570-7.

83. Czerkinsky, C., et al., *Sublingual vaccination.* Hum Vaccin, 2011. **7**(1): p. 110-4.
84. Harandi, A.M., D. Medaglini, and R.J. Shattock, *Vaccine adjuvants: a priority for vaccine research.* Vaccine, 2010. **28**(12): p. 2363-6.
85. Edelman, R., *The development and use of vaccine adjuvants.* Mol Biotechnol, 2002. **21**(2): p. 129-48.
86. Surman, S.L., et al., *Clearance of HIV type 1 envelope recombinant sendai virus depends on CD4+ T cells and interferon-gamma but not B cells, CD8+ T cells, or perforin.* AIDS Res Hum Retroviruses, 2010. **26**(7): p. 783-93.
87. Kermode, M., *Unsafe injections in low-income country health settings: need for injection safety promotion to prevent the spread of blood-borne viruses.* Health Promot Int, 2004. **19**(1): p. 95-103.
88. Iwasaki, A., *Antiviral immune responses in the genital tract: clues for vaccines.* Nat Rev Immunol, 2010. **10**(10): p. 699-711.
89. Gillgrass, A.E., et al., *Protection against genital herpes infection in mice immunized under different hormonal conditions correlates with induction of vagina-associated lymphoid tissue.* J Virol, 2005. **79**(5): p. 3117-26.
90. Hervouet, C., et al., *Sublingual immunization with an HIV subunit vaccine induces antibodies and cytotoxic T cells in the mouse female genital tract.* Vaccine, 2010. **28**(34): p. 5582-90.
91. Johansson, E.L., et al., *Nasal and vaginal vaccinations have differential effects on antibody responses in vaginal and cervical secretions in humans.* Infect Immun, 2001. **69**(12): p. 7481-6.
92. Celum, C., et al., *Genital herpes and human immunodeficiency virus: double trouble.* Bull World Health Organ, 2004. **82**(6): p. 447-53.
93. Schneweis, K.E., *[Serological studies on the type differentiation of Herpesvirus hominis].* Z Immun exp ther, 1962. **124**: p. 24-48.
94. Zhou, Z.H., et al., *Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions.* J Virol, 1999. **73**(4): p. 3210-8.
95. Linehan, M.M., et al., *In vivo role of nectin-1 in entry of herpes simplex virus type 1 (HSV-1) and HSV-2 through the vaginal mucosa.* J Virol, 2004. **78**(5): p. 2530-6.
96. Susanne Modrow, D.F., Uwe Truyen, *Molekulare Virologie*. 2. Auflage ed. 2003, Heidelberg, Berlin: Spektrum Akademischer Verlag GmbH Heidelberg, Berlin, Gustav Fischer.
97. McGeoch, D.J., et al., *The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1.* J Gen Virol, 1988. **69 (Pt 7)**: p. 1531-74.
98. Dolan, A., et al., *The genome sequence of herpes simplex virus type 2.* J Virol, 1998. **72**(3): p. 2010-21.
99. R.J., W., *Herpes simplex viruses*, in *Fields Virology*, H.P. Knipe DM, Editor. 2001, Lippincott Williams & Wilkins: Philadelphia. p. 2461-2509.
100. Roizman B, P.P., *Fields virology, The family herpesviridae:A brief introduction*, in *Fields Virology*, H.P. Knipe DM, Editor. 2001, Lippincott Williams & Wilkins: Philadelphia. p. 2381–2397.
101. Chentoufi, A.A., et al., *The herpes simplex virus 1 latency-associated transcript promotes functional exhaustion of virus-specific CD8+ T cells in latently infected trigeminal ganglia: a novel immune evasion mechanism.* J Virol, 2011. **85**(17): p. 9127-38.
102. Cotter, C.R., et al., *The virion host shutoff protein of herpes simplex virus 1 blocks the replication-independent activation of NF-kappaB in dendritic cells in the absence of type I interferon signaling.* J Virol, 2011. **85**(23): p. 12662-72.
103. Melchjorsen, J., S. Matikainen, and S.R. Paludan, *Activation and evasion of innate antiviral immunity by herpes simplex virus.* Viruses, 2009. **1**(3): p. 737-59.

104. Roest, R.W., et al., *Prevalence and association between herpes simplex virus types 1 and 2-specific antibodies in attendees at a sexually transmitted disease clinic.* Int J Epidemiol, 2001. **30**(3): p. 580-8.
105. Nahmias, A.J., F.K. Lee, and S. Beckman-Nahmias, *Sero-epidemiological and -sociological patterns of herpes simplex virus infection in the world.* Scand J Infect Dis Suppl, 1990. **69**: p. 19-36.
106. Looker, K.J., Garnett G P, Schmid G P, *Bulletin of the WHO*, in *Past Issues*. 2008. p. 737-816.
107. Manservigi, R., et al., *Experimental keratitis in rabbits by human HSV-1 variants: prevention and treatment.* J Med Virol, 1990. **32**(3): p. 148-54.
108. Todar, K. 2009; Available from: http://www.textbookofbacteriology.net/themicrobialworld/AnimalViruses.html.
109. Novak, N., et al., *The immune privilege of the oral mucosa.* Trends Mol Med, 2008. **14**(5): p. 191-8.
110. Friedman, A. and H.L. Weiner, *Induction of anergy or active suppression following oral tolerance is determined by antigen dosage.* Proc Natl Acad Sci U S A, 1994. **91**(14): p. 6688-92.
111. Schuett, B., *Grundwissen Immunologie*. 2006: Spektrum Akademischer Verlag, Elsevier.
112. Holmgren, J. and C. Czerkinsky, *Mucosal immunity and vaccines.* Nat Med, 2005. **11**(4 Suppl): p. S45-53.
113. Quiding, M., et al., *Intestinal immune responses in humans. Oral cholera vaccination induces strong intestinal antibody responses and interferon-gamma production and evokes local immunological memory.* J Clin Invest, 1991. **88**(1): p. 143-8.
114. Eriksson, K., et al., *Specific-antibody-secreting cells in the rectums and genital tracts of nonhuman primates following vaccination.* Infect Immun, 1998. **66**(12): p. 5889-96.
115. Bergquist, C., et al., *Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina.* Infect Immun, 1997. **65**(7): p. 2676-84.
116. Nardelli-Haefliger, D., et al., *Specific antibody levels at the cervix during the menstrual cycle of women vaccinated with human papillomavirus 16 virus-like particles.* J Natl Cancer Inst, 2003. **95**(15): p. 1128-37.
117. Johansson, E.L., et al., *Comparison of different routes of vaccination for eliciting antibody responses in the human stomach.* Vaccine, 2004. **22**(8): p. 984-90.
118. Kozlowski, P.A., et al., *Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women.* Infect Immun, 1997. **65**(4): p. 1387-94.
119. Cuburu, N., et al., *Sublingual immunization induces broad-based systemic and mucosal immune responses in mice.* Vaccine, 2007. **25**(51): p. 8598-610.
120. Kuper, C.F., et al., *The role of nasopharyngeal lymphoid tissue.* Immunol Today, 1992. **13**(6): p. 219-24.
121. Davis, S.S., *Nasal vaccines.* Adv Drug Deliv Rev, 2001. **51**(1-3): p. 21-42.
122. Wu, H.Y., H.H. Nguyen, and M.W. Russell, *Nasal lymphoid tissue (NALT) as a mucosal immune inductive site.* Scand J Immunol, 1997. **46**(5): p. 506-13.
123. Pabst, R., *[Lymphatic tissue of the nose (NALT) and larynx (LALT) in species comparison: human, rat, mouse].* Pneumologie, 2010. **64**(7): p. 445-6.
124. Wu, H.Y. and M.W. Russell, *Nasal lymphoid tissue, intranasal immunization, and compartmentalization of the common mucosal immune system.* Immunol Res, 1997. **16**(2): p. 187-201.
125. Partidos, C.D., *Intranasal vaccines: forthcoming challenges.* Pharm Sci Technolo Today, 2000. **3**(8): p. 273-281.

126. van Ginkel, F.W., et al., *Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues.* J Immunol, 2000. **165**(9): p. 4778-82.
127. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland.* N Engl J Med, 2004. **350**(9): p. 896-903.
128. Lewis, D.J., et al., *Transient facial nerve paralysis (Bell's palsy) following intranasal delivery of a genetically detoxified mutant of Escherichia coli heat labile toxin.* PLoS One, 2009. **4**(9): p. e6999.
129. Parr, M.B., L. Kepple, and E.L. Parr, *Antigen recognition in the female reproductive tract. II. Endocytosis of horseradish peroxidase by Langerhans cells in murine vaginal epithelium.* Biol Reprod, 1991. **45**(2): p. 261-5.
130. Parr, M.B. and E.L. Parr, *Antigen recognition in the female reproductive tract: I. Uptake of intraluminal protein tracers in the mouse vagina.* J Reprod Immunol, 1990. **17**(2): p. 101-14.
131. Champlin, A.K., D.L. Dorr, and A.H. Gates, *Determining the stage of the estrous cycle in the mouse by the appearance of the vagina.* Biol Reprod, 1973. **8**(4): p. 491-4.
132. Edwards, H.E., et al., *Steroid hormones affect limbic afterdischarge thresholds and kindling rates in adult female rats.* Brain Res, 1999. **838**(1-2): p. 136-50.
133. Strauss, F., *Handbuch der Zoologie, Der weibliche Sexualzyklus*. Vol. Band 8. 1986, Berlin: Walter der Gruyter & Co.
134. Kozlowski, P.A., et al., *Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle.* J Immunol, 2002. **169**(1): p. 566-74.
135. Novak, N., T. Bieber, and J.P. Allam, *Immunological mechanisms of sublingual allergen-specific immunotherapy.* Allergy, 2011. **66**(6): p. 733-9.
136. Bohle, B., et al., *Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation.* J Allergy Clin Immunol, 2007. **120**(3): p. 707-13.
137. Mosges, R., E. El Hassan, and D. Passali, *Sublingual specific immunotherapy.* Discov Med, 2010. **10**(53): p. 348-54.
138. Kweon, M.N., *Sublingual mucosa: A new vaccination route for systemic and mucosal immunity.* Cytokine, 2011. **54**(1): p. 1-5.
139. Agostinis, F., et al., *Safety of sublingual immunotherapy with a monomeric allergoid in very young children.* Allergy, 2005. **60**(1): p. 133.
140. Cuburu, N., et al., *Sublingual immunization with nonreplicating antigens induces antibody-forming cells and cytotoxic T cells in the female genital tract mucosa and protects against genital papillomavirus infection.* J Immunol, 2009. **183**(12): p. 7851-9.
141. Song, J.H., et al., *CCR7-CCL19/CCL21-regulated dendritic cells are responsible for effectiveness of sublingual vaccination.* J Immunol, 2009. **182**(11): p. 6851-60.
142. Kildsgaard, J., et al., *Sublingual immunotherapy in sensitized mice.* Ann Allergy Asthma Immunol, 2007. **98**(4): p. 366-72.
143. Brimnes, J., et al., *Sublingual immunotherapy reduces allergic symptoms in a mouse model of rhinitis.* Clin Exp Allergy, 2007. **37**(4): p. 488-97.
144. Noonan, P.K. and L.Z. Benet, *Incomplete and delayed bioavailability of sublingual nitroglycerin.* Am J Cardiol, 1985. **55**(1): p. 184-7.
145. Cleary, J.F., *Pharmacokinetic and pharmacodynamic issues in the treatment of breakthrough pain.* Semin Oncol, 1997. **24**(5 Suppl 16): p. S16-13-9.
146. Johansson, E.L., et al., *Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin B subunit or conjugates.* Infect Immun, 1998. **66**(2): p. 514-20.

147. Parr, E.L., M.B. Parr, and M. Thapar, *A comparison of specific antibody responses in mouse vaginal fluid after immunization by several routes.* J Reprod Immunol, 1988. **14**(2): p. 165-76.
148. Song, J.H., et al., *Sublingual vaccination with influenza virus protects mice against lethal viral infection.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1644-9.
149. Dawson, R.M., *Characterization of the binding of cholera toxin to ganglioside GM1 immobilized onto microtitre plates.* J Appl Toxicol, 2005. **25**(1): p. 30-8.
150. O'Hanlon, G.M., T.R. Hirst, and H.J. Willison, *Ganglioside GM1 binding toxins and human neuropathy-associated IgM antibodies differentially promote neuritogenesis in a PC12 assay.* Neurosci Res, 2003. **47**(4): p. 383-90.
151. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* Nat Immunol, 2005. **6**(11): p. 1133-41.
152. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* Nat Immunol, 2005. **6**(11): p. 1123-32.
153. Pappu, R., et al., *The IL-17 family cytokines in immunity and disease.* J Clin Immunol, 2010. **30**(2): p. 185-95.
154. Korn, T., et al., *Th17 cells: effector T cells with inflammatory properties.* Semin Immunol, 2007. **19**(6): p. 362-71.
155. Khader, S.A., Kolls, J.K., *Th 17 cells: Role in inflammation and autoimmune diseases*. Progess in inflammation research. 2009, Basel, Switzerland: Birkhäuser Verlag.
156. Yao, Z., et al., *Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor.* Immunity, 1995. **3**(6): p. 811-21.
157. Kuestner, R.E., et al., *Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F.* J Immunol, 2007. **179**(8): p. 5462-73.
158. Toy, D., et al., *Cutting edge: interleukin 17 signals through a heteromeric receptor complex.* J Immunol, 2006. **177**(1): p. 36-9.
159. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.
160. Cua, D.J. and C.M. Tato, *Innate IL-17-producing cells: the sentinels of the immune system.* Nat Rev Immunol, 2010. **10**(7): p. 479-89.
161. Kolls, J.K. and S.A. Khader, *The role of Th17 cytokines in primary mucosal immunity.* Cytokine Growth Factor Rev, 2010. **21**(6): p. 443-8.
162. Kagami, S., et al., *IL-23 and IL-17A, but not IL-12 and IL-22, are required for optimal skin host defense against Candida albicans.* J Immunol, 2010. **185**(9): p. 5453-62.
163. Mitsdoerffer, M., et al., *Proinflammatory T helper type 17 cells are effective B-cell helpers.* Proc Natl Acad Sci U S A, 2010. **107**(32): p. 14292-7.
164. Fossiez, F., et al., *T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines.* J Exp Med, 1996. **183**(6): p. 2593-603.
165. Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.
166. Awasthi, A., G. Murugaiyan, and V.K. Kuchroo, *Interplay between effector Th17 and regulatory T cells.* J Clin Immunol, 2008. **28**(6): p. 660-70.
167. Yang, X.O., et al., *T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma.* Immunity, 2008. **28**(1): p. 29-39.
168. Zhou, L., et al., *TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function.* Nature, 2008. **453**(7192): p. 236-40.
169. Mangan, P.R., et al., *Transforming growth factor-beta induces development of the T(H)17 lineage.* Nature, 2006. **441**(7090): p. 231-4.

170. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells.* Immunity, 2006. **24**(2): p. 179-89.
171. Korn, T., et al., *IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells.* Nature, 2007. **448**(7152): p. 484-7.
172. Sakaguchi, S., et al., *Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in self-tolerance and autoimmune disease.* Curr Top Microbiol Immunol, 2006. **305**: p. 51-66.
173. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
174. Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J Exp Med, 2006. **203**(10): p. 2271-9.
175. Huang, W., et al., *Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice.* J Infect Dis, 2004. **190**(3): p. 624-31.
176. Conti, H.R., et al., *Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis.* J Exp Med, 2009. **206**(2): p. 299-311.
177. Ye, P., et al., *Interleukin-17 and lung host defense against Klebsiella pneumoniae infection.* Am J Respir Cell Mol Biol, 2001. **25**(3): p. 335-40.
178. Khader, S.A., S.L. Gaffen, and J.K. Kolls, *Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa.* Mucosal Immunol, 2009. **2**(5): p. 403-11.
179. Ye, P., et al., *Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense.* J Exp Med, 2001. **194**(4): p. 519-27.
180. Molesworth-Kenyon, S.J., et al., *IL-17 receptor signaling influences virus-induced corneal inflammation.* J Leukoc Biol, 2008. **83**(2): p. 401-8.
181. Hu, Y., et al., *The IL-17 pathway as a major therapeutic target in autoimmune diseases.* Ann N Y Acad Sci, 2011. **1217**: p. 60-76.
182. Chabaud, M., et al., *Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium.* Arthritis Rheum, 1999. **42**(5): p. 963-70.
183. Ziolkowska, M., et al., *High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism.* J Immunol, 2000. **164**(5): p. 2832-8.
184. Fujino, S., et al., *Increased expression of interleukin 17 in inflammatory bowel disease.* Gut, 2003. **52**(1): p. 65-70.
185. Yasumura, Y.K., Y. (1963). Vero cell line derived from the kidney of a normal, adult, African green monkey (Cercopithecus). Nippon Rinsho 21:1209., *Vero cell line derived from the kidney of a normal, adult, African green monkey (Cercopithecus).* Nippon Rinsho 1963. **21**: p. 1209.
186. Tognon, M., et al., *Analysis of HSV isolated from patients with unilateral and bilateral herpetic keratitis.* Int Ophthalmol, 1985. **8**(1): p. 13-8.
187. Barnden, M.J., et al., *Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements.* Immunol Cell Biol, 1998. **76**(1): p. 34-40.
188. Hennig, C., N. Adams, and G. Hansen, *A versatile platform for comprehensive chip-based explorative cytometry.* Cytometry A, 2009. **75**(4): p. 362-70.
189. Van den Broeck, W., A. Derore, and P. Simoens, *Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrl mice.* J Immunol Methods, 2006. **312**(1-2): p. 12-9.

190. Whitten, W.K., *Occurrence of anoestrus in mice caged in groups.* J Endocrinol, 1959. **18**(1): p. 102-7.
191. Champlin, A.K., *Suppression of oestrus in grouped mice: the effects of various densities and the possible nature of the stimulus.* J Reprod Fertil, 1971. **27**(2): p. 233-41.
192. Pruett, S.B., et al., *Quantitative analysis of the neuroendocrine-immune axis: linear modeling of the effects of exogenous corticosterone and restraint stress on lymphocyte subpopulations in the spleen and thymus in female B6C3F1 mice.* Brain Behav Immun, 2000. **14**(4): p. 270-87.
193. Kaushic, C., et al., *Progesterone increases susceptibility and decreases immune responses to genital herpes infection.* J Virol, 2003. **77**(8): p. 4558-65.
194. Miyaura, H. and M. Iwata, *Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids.* J Immunol, 2002. **168**(3): p. 1087-94.
195. Conradi, J., *Sublingual vs. Intranasal Immunization: an alternative approach for the administration of vaccines*, in *Helmholtz-Zentrum für Infektionsforschung, Inhoffenstr. 7, 38124 Braunschweig, Refers to data of Fig. 30-34 in this thesis*
2011, 3rd May, Stiftung Tierärztliche Hochschule Hannover, Bünteweg 2, 30559 Hannover;: Hannover.
196. Lohoff, M., et al., *Experimental murine leishmaniasis and the Th1/Th2 cell concept.* Tokai J Exp Clin Med, 1998. **23**(6): p. 347-50.
197. Brenner, G.J., N. Cohen, and J.A. Moynihan, *Similar immune response to nonlethal infection with herpes simplex virus-1 in sensitive (BALB/c) and resistant (C57BL/6) strains of mice.* Cell Immunol, 1994. **157**(2): p. 510-24.
198. Halford, W.P., J.W. Balliet, and B.M. Gebhardt, *Re-evaluating natural resistance to herpes simplex virus type 1.* J Virol, 2004. **78**(18): p. 10086-95.
199. Vollstedt, S., et al., *Interplay between alpha/beta and gamma interferons with B, T, and natural killer cells in the defense against herpes simplex virus type 1.* J Virol, 2004. **78**(8): p. 3846-50.
200. Gaffen, S.L., N. Hernandez-Santos, and A.C. Peterson, *IL-17 signaling in host defense against Candida albicans.* Immunol Res, 2011. **50**(2-3): p. 181-7.
201. Infante-Duarte, C., et al., *Microbial lipopeptides induce the production of IL-17 in Th cells.* J Immunol, 2000. **165**(11): p. 6107-15.
202. Schwarzenberger, P., et al., *IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines.* J Immunol, 1998. **161**(11): p. 6383-9.
203. Zygmunt, B.M., S.F. Weissmann, and C.A. Guzman, *NKT Cell Stimulation with alpha-Galactosylceramide Results in a Block of Th17 Differentiation after Intranasal Immunization in Mice.* PLoS One, 2012. **7**(1): p. e30382.
204. Jiang, J.Q., et al., *CD8+ T-cell-mediated cross-clade protection in the genital tract following intranasal immunization with inactivated human immunodeficiency virus antigen plus CpG oligodeoxynucleotides.* J Virol, 2005. **79**(1): p. 393-400.
205. Lindqvist, M., et al., *The mucosal adjuvant effect of alpha-galactosylceramide for induction of protective immunity to sexually transmitted viral infection.* J Immunol, 2009. **182**(10): p. 6435-43.
206. Price, G.A., M.W. Russell, and C.N. Cornelissen, *Intranasal administration of recombinant Neisseria gonorrhoeae transferrin binding proteins A and B conjugated to the cholera toxin B subunit induces systemic and vaginal antibodies in mice.* Infect Immun, 2005. **73**(7): p. 3945-53.
207. Ebensen, T., et al., *A pegylated derivative of alpha-galactosylceramide exhibits improved biological properties.* J Immunol, 2007. **179**(4): p. 2065-73.
208. Gapin, L., *The making of NKT cells.* Nat Immunol, 2008. **9**(9): p. 1009-11.
209. Shibata, K., et al., *Identification of CD25+ gamma delta T cells as fetal thymus-derived naturally occurring IL-17 producers.* J Immunol, 2008. **181**(9): p. 5940-7.

210. Carding, S.R. and P.J. Egan, *Gammadelta T cells: functional plasticity and heterogeneity.* Nat Rev Immunol, 2002. **2**(5): p. 336-45.
211. Marconi, P., et al., *HSV as a vector in vaccine development and gene therapy.* Adv Exp Med Biol, 2009. **655**: p. 118-44.
212. Evans, C., et al., *Progress in development of herpes simplex virus gene vectors for treatment of rheumatoid arthritis.* Adv Drug Deliv Rev, 1997. **27**(1): p. 41-57.
213. Hamada, H., et al., *Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge.* J Immunol, 2009. **182**(6): p. 3469-81.
214. Ebensen, T.F., B.; Schulze,K.; Guzmán,Carlos A, *Infection prevention: oil- and lipid-containing products in vaccinology*, in *Handbook of Hydrocarbon and Lipid Microbiology*, K.N. Timmis, Editor. 2009, Springer-Verlag: Berlin, Heidelberg. p. 3311-E-Page: 3331.
215. Ishikawa, H., Z. Ma, and G.N. Barber, *STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity.* Nature, 2009. **461**(7265): p. 788-92.
216. Ishikawa, H. and G.N. Barber, *STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling.* Nature, 2008. **455**(7213): p. 674-8.
217. Jin, L., et al., *MPYS, a novel membrane tetraspanner, is associated with major histocompatibility complex class II and mediates transduction of apoptotic signals.* Mol Cell Biol, 2008. **28**(16): p. 5014-26.
218. Zhong, B., et al., *The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation.* Immunity, 2008. **29**(4): p. 538-50.
219. Lee, K.A., et al., *A distinct subset of natural killer T cells produces IL-17, contributing to airway infiltration of neutrophils but not to airway hyperreactivity.* Cell Immunol, 2008. **251**(1): p. 50-5.
220. Wondimu, Z., et al., *Protective role of interleukin-17 in murine NKT cell-driven acute experimental hepatitis.* Am J Pathol, 2010. **177**(5): p. 2334-46.
221. Suryawanshi, A., et al., *Role of IL-17 and Th17 cells in herpes simplex virus-induced corneal immunopathology.* J Immunol, 2011. **187**(4): p. 1919-30.
222. Stout-Delgado, H.W., et al., *Aging promotes neutrophil-induced mortality by augmenting IL-17 production during viral infection.* Cell Host Microbe, 2009. **6**(5): p. 446-56.
223. Marks, E., et al., *CD4 T-cell immunity in the female genital tract is critically dependent on local mucosal immunization.* Eur J Immunol, 2011. **41**(9): p. 2642-53.
224. Seavey, M.M. and T.R. Mosmann, *Estradiol-induced vaginal mucus inhibits antigen penetration and CD8(+) T cell priming in response to intravaginal immunization.* Vaccine, 2009. **27**(17): p. 2342-9.
225. Parr, M.B. and E.L. Parr, *Langerhans cells and T lymphocyte subsets in the murine vagina and cervix.* Biol Reprod, 1991. **44**(3): p. 491-8.
226. Evans, G.S., et al., *Proliferative changes in the genital tissue of female mice during the oestrous cycle.* Cell Tissue Kinet, 1990. **23**(6): p. 619-35.
227. Gurevich, A.I., et al., *[Chemical-enzymatic synthesis of the amplifier of the T7 phage gene 10 and function of its structural elements].* Bioorg Khim, 1991. **17**(5): p. 647-52.
228. Domingo, P., et al., *Polyradiculopathy due to cytomegalovirus infection: report of a case in which an AIDS patient responded to foscarnet therapy.* Clin Infect Dis, 1994. **18**(6): p. 1019-21.
229. Parr, M.B., et al., *A mouse model for studies of mucosal immunity to vaginal infection by herpes simplex virus type 2.* Lab Invest, 1994. **70**(3): p. 369-80.
230. Gillgrass, A.E., et al., *Estradiol regulates susceptibility following primary exposure to genital herpes simplex virus type 2, while progesterone induces inflammation.* J Virol, 2005. **79**(5): p. 3107-16.

231. Butts, C.L., et al., *Progesterone regulation of uterine dendritic cell function in rodents is dependent on the stage of estrous cycle.* Mucosal Immunol, 2010. **3**(5): p. 496-505.
232. Robinson, D.S. and A. O'Garra, *Further checkpoints in Th1 development.* Immunity, 2002. **16**(6): p. 755-8.
233. Baker, D.A. and S.A. Plotkin, *Enhancement of vaginal infection in mice by herpes simplex virus type II with progesterone.* Proc Soc Exp Biol Med, 1978. **158**(2): p. 131-4.
234. Cherpes, T.L., et al., *Medroxyprogesterone acetate inhibits CD8+ T cell viral-specific effector function and induces herpes simplex virus type 1 reactivation.* J Immunol, 2008. **181**(2): p. 969-75.
235. Yeaman, G.R., et al., *Human immunodeficiency virus receptor and coreceptor expression on human uterine epithelial cells: regulation of expression during the menstrual cycle and implications for human immunodeficiency virus infection.* Immunology, 2003. **109**(1): p. 137-46.
236. Packiam, M., et al., *Mouse strain-dependent differences in susceptibility to Neisseria gonorrhoeae infection and induction of innate immune responses.* Infect Immun, 2010. **78**(1): p. 433-40.
237. Darville, T., et al., *Mouse strain-dependent chemokine regulation of the genital tract T helper cell type 1 immune response.* Infect Immun, 2001. **69**(12): p. 7419-24.
238. Kato, H., et al., *Rectal and vaginal immunization with a macromolecular multicomponent peptide vaccine candidate for HIV-1 infection induces HIV-specific protective immune responses.* Vaccine, 2000. **18**(13): p. 1151-60.
239. Luci, C., et al., *Dendritic cell-mediated induction of mucosal cytotoxic responses following intravaginal immunization with the nontoxic B subunit of cholera toxin.* J Immunol, 2006. **176**(5): p. 2749-57.
240. Hervouet, C., et al., *Langerhans cells prime IL-17-producing T cells and dampen genital cytotoxic responses following mucosal immunization.* J Immunol, 2010. **184**(9): p. 4842-51.
241. Elrefaei, M., et al., *HIV-specific IL-10-positive CD8+ T cells suppress cytolysis and IL-2 production by CD8+ T cells.* J Immunol, 2007. **178**(5): p. 3265-71.
242. Intlekofer, A.M., et al., *Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin.* Science, 2008. **321**(5887): p. 408-11.
243. Gockel, C.M., et al., *Influence of the murine oestrous cycle on the induction of mucosal immunity.* Am J Reprod Immunol, 2003. **50**(5): p. 369-79.
244. Schon, E., et al., *Dendritic cell vaccination protects mice against lethality caused by genital herpes simplex virus type 2 infection.* J Reprod Immunol, 2001. **50**(2): p. 87-104.
245. Milligan, G.N. and D.I. Bernstein, *Interferon-gamma enhances resolution of herpes simplex virus type 2 infection of the murine genital tract.* Virology, 1997. **229**(1): p. 259-68.
246. Jing, L., et al., *Cross-presentation and genome-wide screening reveal candidate T cells antigens for a herpes simplex virus type 1 vaccine.* J Clin Invest, 2012. **122**(2): p. 654-73.
247. Rajasagi, N.K., et al., *CD4+ T cells are required for the priming of CD8+ T cells following infection with herpes simplex virus type 1.* J Virol, 2009. **83**(10): p. 5256-68.
248. Coquet, J.M., et al., *Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population.* Proc Natl Acad Sci U S A, 2008. **105**(32): p. 11287-92.
249. Tough, D.F. and J. Sprent, *Lifespan of gamma/delta T cells.* J Exp Med, 1998. **187**(3): p. 357-65.

250. Ciprandi, G. and G.L. Marseglia, *Safety of sublingual immunotherapy.* J Biol Regul Homeost Agents, 2011. **25**(1): p. 1-6.
251. Carmichael, J.R., et al., *Induction of protection against vaginal shedding and infertility by a recombinant Chlamydia vaccine.* Vaccine, 2011. **29**(32): p. 5276-83.
252. Lin, K.H., et al., *In vivo protection provided by a synthetic new alpha-galactosyl ceramide analog against bacterial and viral infections in murine models.* Antimicrob Agents Chemother, 2010. **54**(10): p. 4129-36.
253. Gonzalez-Aseguinolaza, G., et al., *Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines.* J Exp Med, 2002. **195**(5): p. 617-24.
254. Taniguchi, M., et al., *The regulatory role of Valpha14 NKT cells in innate and acquired immune response.* Annu Rev Immunol, 2003. **21**: p. 483-513.
255. Adotevi, O., et al., *B subunit of Shiga toxin-based vaccines synergize with alpha-galactosylceramide to break tolerance against self antigen and elicit antiviral immunity.* J Immunol, 2007. **179**(5): p. 3371-9.
256. Holtmeier, W. and D. Kabelitz, *gammadelta T cells link innate and adaptive immune responses.* Chem Immunol Allergy, 2005. **86**: p. 151-83.
257. Bonneville, M., R.L. O'Brien, and W.K. Born, *Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity.* Nat Rev Immunol, 2010. **10**(7): p. 467-78.
258. McAleer, J.P. and J.K. Kolls, *Mechanisms controlling Th17 cytokine expression and host defense.* J Leukoc Biol, 2011. **90**(2): p. 263-70.
259. Paget, C., et al., *Role of gammadelta T Cells in alpha-Galactosylceramide-Mediated Immunity.* J Immunol, 2012.
260. McKinstry, K.K., et al., *IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge.* J Immunol, 2009. **182**(12): p. 7353-63.
261. Lu, Y.J., et al., *Interleukin-17A mediates acquired immunity to pneumococcal colonization.* PLoS Pathog, 2008. **4**(9): p. e1000159.
262. Pasquevich, K.A., et al., *An oral vaccine based on U-Omp19 induces protection against B. abortus mucosal challenge by inducing an adaptive IL-17 immune response in mice.* PLoS One, 2011. **6**(1): p. e16203.
263. Del Campo, J., et al., *Intranasal immunization with a proteoliposome-derived cochleate containing recombinant gD protein confers protective immunity against genital herpes in mice.* Vaccine, 2010. **28**(5): p. 1193-200.
264. Guglani, L. and S.A. Khader, *Th17 cytokines in mucosal immunity and inflammation.* Curr Opin HIV AIDS, 2010. **5**(2): p. 120-7.
265. Annunziato, F. and S. Romagnani, *Mouse T helper 17 phenotype: not so different than in man after all.* Cytokine, 2011. **56**(1): p. 112-5.

7. Appendix

Figure index

Figure 1: Stimulation of TLRs leads to induction of specific Th-phenotype development [54]..... 24
Figure 2: Anatomy of the genital mucosa [88]..... 28
Figure 3: Structure of the HSV-1 virus. [108]..... 32
Figure 4: Hormonal changes in the female oestrus cycle in rats [132]..... 37
Figure 5: Histological section of vagina, cross section..... 38
Figure 6: Morphology of sublingual mucosa [138]..... 40
Figure 7: Mice were under narcosis and placed in this position, sitting head in ante flexion as long as narcosis is lasting..... 72
Figure 8: Immunisation strategy under DEPO treatment..... 73
Figure 9: Lymph nodes of a mouse. Lymph nodes 1-3 are here named sublingual lymph nodes and lymph nodes no. 21 are the iliac lymph nodes extracted in this study [189]..... 76
Figure 10: Stages of the murine oestrus cycle..... 89
Figure 11: *In vivo* proliferation of spleen and lymph node cells following i.vag. immunisation..... 90
Figure 12: Proliferation of $CD4^+$ spleen cells after adoptive transfer..... 91
Figure 13: Identification of the optimal formulation for further studies..... 94
Figure 14: Humoral immune response elicited in C57BL/6 mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route..... 95
Figure 15: Pattern of IgG subclass titres induced in mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route..... 97
Figure 16: Evaluation of cellular immune responses in C57BL/6 mice immunised with OVA or β-Gal + c-di-AMP by mucosal routes..... 98
Figure 17: Secretion profile of cytokines of spleen cells after ag-restimulation. 100
Figure 18: Induction of efficient systemic humoral immune responses in sera of BALB/c mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route..... 102
Figure 19: Distribution of IgG subclass titres in sera of BALB/c mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route..... 103
Figure 20: Local humoral IgA immune response of immunised BALB/c mice in VL..... 105

Figure 21: Proliferation of restimulated spleen cells of mice immunised with OVA + c-di-AMP or β-Gal + c-di-AMP by i.n. or i.vag. route. 106

Figure 22: Cytokine secretion profile of splenocytes derived from BALB/c mice immunised with OVA/c-di-AMP or β-Gal/c-di-AMP by the i.n. or i.vag. route. 108

Figure 23: Induction of efficient systemic humoral immune responses in sera of BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG. 111

Figure 24: Distribution of IgG subclass titres in sera of BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG. 112

Figure 25: Induction of local humoral immune response in VL of BALB/c mice. 113

Figure 26: Induction of mucosal immune responses in VL samples of BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG by i.vag. route. 114

Figure 27: Cellular proliferation of *ex vivo* ag-restimulated spleen cells..... 115

Figure 28: Cytokine profile of ag-restimulated spleen cells derived from BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG. 117

Figure 29: Cytokine secretion of IL-17 produced by iLN cells following i.n. immunisation. 118

Figure 30: IgG titre in sera of BALB/c mice immunised by s.l., i.n. or oral route. 120

Figure 31: IgG subclass pattern in sera of BALB/c mice after immunisation via s.l. or i.n. or oral route. 121

Figure 32: Elicitation of humoral immune responses at distinct mucosal sites following s.l., oral and i.n. immunisation. 122

Figure 33: Cellular proliferation of *ex vivo* ag-restimulated spleen cells derived from BALB/c mice immunised by s.l., oral or i.n. route. 123

Figure 34: Effect of vaccination by different routes in the secretion of cytokines by *ex vivo* ag-restimulated spleen cells. 124

Figure 35: Humoral immune responses stimulated in vaccinated BALB/c mice using c-di-AMP as mucosal adjuvant. 125

Figure 36: Humoral immune responses after i.vag. immunisation with low and high dose formulations of the ag and adjuvant..... 126

Figure 37: Cellular immune responses after i.vag. immunisation with low and high dose formulations of the ag and adjuvant..... 127

Figure 38: Cytokine secreting cells stimulated after i.vag. immunisation with low and high dose formulations. 128

Figure 39: Ag-specific IgG titre in sera of BALB/c mice immunised by i.n., s.l. and i.vag. routes..... 130

Figure 40: Distribution of IgG subclass titres in sera derived from BALB/c mice immunised by i.vag., s.l. and i.n. route. 131

Figure 41: Ag-specific IgA titre in lavage samples derived from BALB/c mice immunised with β-Gal + c-di-AMP or β-Gal + α-GalCerMPEG by s.l., i.n. or i.vag. route. 133

Figure 42: Evaluation of the cellular responses stimulated in mice vaccinated by different mucosal routes. 135

Figure 43: Evaluation of the cellular responses of LN cells derived from BALB/c mice vaccinated by different routes using c-di-AMP as adjuvant. .. 136

Figure 44: Analysis of the cytokine producing cells from vaccinated mice. 138

Figure 45: Ag-specific IgG in sera of BALB/c stimulated following single and combined vaccination strategies. 140

Figure 46: Humoral immune responses in sera after i.vag and s.l. immunisation and a combination of these routes. 142

Figure 47: Humoral immune responses in mucosal lavages after i.vag and s.l. immunisation, alone and in combination. 144

Figure 48: Cellular immune responses from spleen cells after i.vag and s.l. immunisation and a combination of these routes. 146

Figure 49: Cellular immune in iLN after i.vag and s.l. immunisation and a combination of these routes. 148

Figure 50: Cellular immune responses in cLN after i.vag and s.l. immunisation and a combination of these routes. 149

Figure 51: Cytokine profile of spleen cells stimulated following i.vag and s.l. immunisation and a combination of these routes. 151

Figure 52: Analysis of the IL-17 producing cells stimulated following immunisation with β-Gal + c-di-AMP. 153

Figure 53: Survival curve starting at the challenge day. 157

Figure 54: Susceptibility of IL-17 A/F $^{-/-}$ mice to HSV infection. 158

Table index

Table 1: Immune responses stimulated according to adjuvant and route of administration..... 154
Table 2: Vaccination protocol..... 156

Acknowledgement

I would like to thank Professor Dr Carlos A. Guzmán for providing me with the possibility to prepare this thesis in his lab. Many thanks also for the support and input to prepare this thesis. I thank Dr Kai Schulze for supervising me during my thesis and the fruitful discussions.

Especially I would like to thank Professor Dr Roland Jacobs for the co-review of this thesis and also as a member of my thesis committee. Thank you for being always open for discussion and offering always help whenever I needed.

Many thanks to Professor Dr Markus Kalesse for chairing the examination committee and supporting me as a member of my thesis committee.

I would like to thank all the members of the Vac group for the good collaboration, especially for the helping hands during the samplings.

I thank the group of Professor Dr Gesine Hansen, Department of Paedriatic Pneumology, Allergology and Neonatology, Hannover Medical School, and Dr Christian Hennig for the cooperation in analysing samples with their chip cytometry method. Special thanks also to Dr Tine Happle, Jana Bergmann and Björn Zweigait for the practical support.

I thank PD Dr Immo Prinz, Institute of Immunology, Hannover Medical School, for providing us with breeding pairs of his C57BL/6J-IL-17a IL17^{ftm1Prnz} knock-out-mice.

Special thanks to Dr Peggy Marconi, Department of Experimental Medicine, University of Ferrara, for delivering the HSV-1 strains and the teaching of the methods for viral stock preparation.

Especially I would like to thank my family for all the support and advice during my thesis.
Special thanks to Sascha for all his support….

Many thanks also to my friends, especially to Annika and Julia for support, discussions and corrections.

Curriculum vitae

Personal data:

Date of birth:	April 22nd, 1984
Place of birth:	Hannover
Nationality:	german

Education:

September 2008- March 2012	PhD thesis at the Helmholtz-Zentrum für Infektionsforschung, Braunschweig, Department Vakzinology and Applied Microbiology, Supervisor: Prof. Dr Carlos A. Guzmán, MD, PhD; submitted at the Faculty of natural science, Gottfried Wilhelm Leibniz Universität Hannover

Topic of the PhD thesis: New immunisation strategies via mucosal routes: comparative analysis of sublingual, intravaginal and intranasal immunisation

October 2003-July 2008	Gottfried Wilhelm Leibniz Universität Hannover, Course of studies: Diplom-Biologie Major subject: Cell-and developmental biology Minor subjects: Microbiology Immunology
July 31st, 2008	Graduate: Diploma

Topic of the diploma thesis: Charakterisierung von $CD20^+$ T-Zellen im Menschen, prepared at the Medical School Hannover, Klinik für Immunologie und Rheumatologie

1990-2003	Albert-Einstein-School Laatzen, cooperative comprehensive school, Abitur

Hannover, 21st of August 2012

Kirsten Scholz

Declaration

Herewith, I confirm that I have written the present PhD thesis with the topic: “New immunisation strategies via mucosal routes: comparative analysis of sublingual, intravaginal and intranasal immunisation” by myself and independently and that all used resources, utilities, sources and contributing institutions were completely indicated.

The PhD thesis was not used before as master-or diploma thesis or any other examination paper.

Ich erkläre hiermit, dass ich meine Dissertation mit dem Titel: “New immunisation strategies via mucosal routes: comparative analysis of sublingual, intravaginal and intranasal immunisation” selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, 21st August 2012

Kirsten Scholz