Infections of Common Marmosets with Calpox Virus:

A Model for Smallpox Virus Infections

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…to whom I love

Abstract

The intentional re-introduction of Variola virus (VARV), the agents of smallpox, into the human population remains of concern today. Moreover, zoonotic infections with Cowpox (CPXV) and Monkeypox virus (MPXV) cause severe diseases in humans. Smallpox vaccines presently available can have severe adverse effects that are no longer acceptable. The efficacy and safety of new vaccines and antivirals have to be demonstrated by different animal models. The existing primate models, using VARV and MPXV, need very high viral doses that have to be applied intravenously to induce a lethal infection in macaque monkeys.

To overcome these drawbacks, the main objective of this study was to develop a primate model in which a smallpox-like disease could be induced by a CPXV virus designated calpox virus which was isolated from a lethal orthopox virus (OPV) outbreak in New World monkeys (marmosets).

The new non-human primate model has three major advantages: 1. Working with calpox virus is less challenging and can be done under bio-safety-level two. 2. Mimicking the natural route of VARV infection, intranasally infected marmosets *(Callithrix jacchus)* reproducibly developed clinical symptoms of an OPV infection and died within two to three days after onset of the first symptoms. High viral loads of calpox virus were detected in blood, saliva and all analyzed organs. 3. Intranasal titration of the virus resulted in a 50 % monkey infectious dose (MID_{50}) of $8.3x10^2$ pfu, a lethal infectious dose 10,000 lower than those used in any other primate model. Moreover, we showed the aptitude of the primate model for the testing of new vaccines since nine to ten weeks after immunization with Vaccinia virus Lister-Elstree marmosets were completely protected against intranasal challenge with 10 MID_{50} of calpox virus.

As the calpox virus/marmoset model overcomes major limitations of current primate models it is suitable to evaluate new vaccines, new vaccination strategies and antiviral therapies.

Keywords: non-human primate model, intranasal infection, vaccination, *Callithrix jacchus*, smallpox

Abstract in German (Zusammenfassung)

Die vorsätzliche Freisetzung von Variola Virus (VARV) und schwere Erkrankungen des Menschen durch zoonotische Affen- (MPXV) und Kuh- (CPXV) pocken Viren stellen nach wie vor eine Bedrohung für die Bevölkerung dar. Klassische Pockenimpfstoffe bergen die Gefahr einer schweren Erkrankung. Deshalb ist die Entwicklung neuer Impfstoffe und Therapeutika von entscheidender Bedeutung. Deren Wirksamkeit und Sicherheit muss zunächst in verschiedenen Tiermodellen bewiesen werden. Existierende Makakken-Primatenmodelle leiden unter sehr artifiziellen Bedingungen der letalen Krankheitsinduktion durch VARV oder MPXV.

Aus diesem Grund wurde das Calpox Virus/Krallenaffen-modell etabliert, welches auf einem CPXV aus natürlich infizierten Neuweltaffen (Marmosets) basiert.

Das neue Modell hat drei wesentliche Vorteile: Die Arbeit mit Calpox Virus kann unter Sicherheitsstufe 2 durchgeführt werden und ist folglich einfacher in der Handhabung. 2. Die intranasale (i.n.) Infektion von Marmosets (Krallenaffen; *Callithrix jacchus*) spiegelt den natürlichen Infektionsweg von VARV wieder. Infizierte Affen entwickelten Pocken ähnliche Symptome und verstarben innerhalb von 2-3 Tagen nach Auftreten erster Symptome. Hohe Viruslasten wurden im Blut, Speichel und allen untersuchten Organen nachgewiesen. 3. Die i.n. Titration des Calpox Virus ergab eine 50 % Affen-Infektions-Dosis (MID_{50}) von $8.3x10^2$ pfu. Diese ist um den Faktor 10000 niedriger als in anderen Pocken-Primatenmodellen. Neun bis zehn Wochen nach einer Immunisierung mit dem Lister-Elstree Impfstoff waren alle Krallenaffen gegen eine letale Dosis des Calpox Virus (10 $MID_{50)}$ geschützt. Damit konnte der Nutzen des Calpox Virus/Krallenaffen-modells für die Erforschung neuer Impfstoffe gezeigt werden.

Das Calpox Virus/Krallenaffen-modell überwindet wesentliche Nachteile bestehender Primatenmodelle und ist somit ein geeignetes Model für die Evaluierung von neuen Impfstoffen, Impfstrategien und antiviralen Therapien.

Schlagwörter: Primatenmodell, intranasale Infektion, Immunisierung, Krallenaffen, humane Pocken

List of Abbreviations

Viruses

CLMV	Camelpox virus
CPXV	Cowpox virus
ECTV	Ectromelia virus
GBLV	Taterapox virus
MPXV	Monkeypox virus
VACV	Vaccinia virus
VACV MVA	Vaccinia virus, strain Modified Vaccinia virus Ankara
VACV LE	Vaccinia virus, strain Lister-Elstree
VACV LE-BN	Vaccinia virus, strain Lister-Elstree, produced by Bavarian Nordic
VARV	Variola Virus

Others

A.D.	Anno Domini
AP	alkaline phosphatase
bp	base pairs
BSL	bio-safety-level
CDC	Centers for Disease Control and Prevention
CEV	cell-associated enveloped virus
CJ	*Callithrix jacchus*
CMC	carboxymethyl-cellulose
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dUTP	deoxyuridine triphosphate
ELISA	enzyme-linked immuno sorbent assay
EEV	extracellular enveloped virus
EM	electron microscopy
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
HE	haematoxylin and eosin
IEV	intracellular enveloped viruses

IFA	immunofluorescence assay
IFN	interferon
IgM	immunoglobulin M
IgG	immunoglobulin G
IgY	immunoglobulin Y
IL	interleukin
IMV	intracellular mature virus
i.c.	intracranial
i.ce.	intracerebral
i.d.	intradermal
i.n.	intranasal
i.p.	intraperitoneal
i.t.	intratracheal
ITR	inverted terminal repeats
i.v.	intravenous
IV	immature virion
Kb	kilo base pairs
mRNA	messenger ribonucleic acid
NHP	non-human primate
OPV	orthopox virus
ORF	open reading frame
oro.	oropharyngeal
p.ch.	post challenge
PCR	polymerase chain reaction
pfu	plaque forming unit
p.i.	post infection
p.im.	post immunization
PRNT	plaque reduction neutralization assay
RKI	Robert Koch-Institut
RNA	ribonucleic acid
ROX	6-carboxy-X-rhodamine
RPMI	Roswell Park Memorial Institute
s.c.	subcutaneous
T_m	melting temperature
TNF	tumor necrosis factor
U	unit

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1 Introduction

This chapter reviews current literature on orthopox virus (OPV) infections, their clinical and biological features and the development of new therapeutics highlighting the need and the possible significance of the development of a new non-human primate (NHP) model for Variola virus (VARV) infections.

Following an historical overview of smallpox and its eradication, a description of the virus, its transmission, general clinical features and pathogenesis of the disease will provide the necessary OPV-related background knowledge. The growing concern about zoonotic OPV infections and the implications of the lack of licensed vaccines and antiviral agents are outlined. A short overview of current animal models used in OPV research is pointing out their limitations regarding their application in evaluating new vaccines and therapeutic agents. Since common marmosets as well as the calpox virus are the basis of the primate model developed here, the final sections of the introduction will give a concise overview of the history of calpox virus and the biology of the common marmoset.

1.1 Historical overview – past and present of orthopox virus infections

1.1.1 Smallpox and its eradication

Smallpox, caused by infection with VARV, has once been a human disease prevalent throughout the world. One variety, VARV major, resulted in case-fatality rates between 5 % and 40 % whereas VARV minor, the second variety of smallpox, produces less severe illness and was associated with case-fatality rates between 1 % and 2 %. Without an animal reservoir, the VARV spread continuously and exclusively between humans. Early methods of protection against naturally acquired smallpox infections were practiced in India and Asia as early as A.D. 1000 [1]. It became common practice to inoculate scabs or pustular material from smallpox patients by nasal insufflation or by scarification into the skin. In 1796, Edward Jenner first demonstrated that material from a human pustular lesion caused by cowpox virus (CPXV) provided a protection against smallpox if inoculated into the skin of another person [2]. He called the material vaccine, from the Latin word vacca (cow) and the process vaccination. Later, vaccination was performed by transmission of the vaccine between individuals also accidentally transmitting other diseases. Therefore, the development to grow the virus on calf skin offered an adequate but safer supply of vaccine material [3]. In the late 1940's, a commercial process for large-scale production of stable freeze-dried vaccine was

established [4]. In 1950, the Pan American Sanitary Organization decided to implement an eradication program and, by 1967, succeeded in eliminating smallpox in almost all countries of the Americas except for Brazil, Argentina, Colombia and Ecuador. In 1959 the World Health organization (WHO) initiated a global eradication program. By October 1977, the last naturally occurring case of smallpox was reported, and the World Health Organization certified the eradication of smallpox on May 8, 1980 [5].

Many different strains of Vaccinia virus (VACV) have been used for vaccination in the last two centuries, but little is know about their origins or history of serial passage. Jenner assumed to have used CPXV for vaccination, but VACV strains that have been used later on are phylogenetically clearly different from CPXV. Genome analyses of the different VACV strains show that they are similar to each other but distinct from other OPVs including CPXV or VARV. Thus the origin of the present VACV strains remains unclear. For the eradication of smallpox by the WHO program different VACV strains were used: the Lister-Elstree strain (Lister Institute, Elstree, England; propagated by the National Public Health Institute, Netherlands), the New York City Board of Health strain (NYCBH) (propagated by Wyeth Laboratories, USA) [3], the Temple of Heaven strain (used in Asia) and the EM-63 strain (used in Russia). Application was performed by scarification, jet injectors or by multi-puncture vaccination with a bifurcated needle (figure 4). Serious side effects of vaccination were eczema vaccinatum, progressive vaccinia, generalized vaccinia, postvaccinal encephalopathy and encephalitis.

1.1.2 The potential threat of bioterrorism and zoonotic events

Soon after smallpox was officially eradicated in 1980, general vaccinations with VACV were discontinued worldwide to avoid the severe side effects. In the USA routine population-wide smallpox vaccinations were already abolished in 1971 [6] and discontinued for health-care workers in 1976 [7]. In Europe routine smallpox vaccination ended between 1976 and 1984 (Germany: in the BRD in 1976 and in the GDR in 1980) [8]. The production and licensing of the “old” vaccines was discontinued in the late 80’s, but new vaccines like the ACAM2000 (licensed in August 2007 by the FDA, USA) [9] or VACV LE-BD (Lister-Elstree strain produced by Bavarian Nordic, Martinsried, Germany) [10] are available today. However, their use is mainly intended to protect military personnel and that of selected laboratories working with OPVs.

Although naturally occurring smallpox was declared eradicated it was recommended to destroy VARV in the laboratories worldwide and to transfer the virus stocks to one of two WHO reference laboratories: the CDC in Atlanta, USA, and the Vector Institute in Novosibirsk, Russia. Experiments with whole VARV or parts of it are since that time controlled by an expert committee of the WHO. Nevertheless, the re-introduction of VARV into the human population by a bioterroristic attack is one of the greatest threats [11,12,13,14]. The use of VARV as a biological weapon in bioterrorist attacks is of concern as the virus is easily transmitted from person to person and because of its high mortality and the low or non-existent smallpox immunity in the majority of the human population [15]. Therefore, smallpox is included in the A category of the CDC's list of potential biological weapons (for details see [16]).

In addition to the possible intentional release of smallpox, over recent years an increasing zoonotic potential of OPVs was observed as both, CPXV [17] and Monkeypox virus (MPXV), were found to cross the species barrier [18,19,20]. These human zoonotic OPV infections are rare but at the same time encountered with increasing frequency outside their usual geographical range. Outbreaks of human MPXV have been reported from Central and Western Africa since the 1970s [21,22,23,24,25], but unexpectedly an MPXV outbreak took place in the U.S. in 2003, where most cases had acquired MPXV from infected prairie dogs (*Cynomys spp*.) [26]. Investigations identified imported African rodents as the original source of infection. In contrast, CPXV is limited to Europe and Asia with a wide host range including humans [27,28]. CPXV is endemic to wild rodents, with bank voles (*Myodes glareolus*) and wood mice (*Apodemus sylvaticus*) as the main reservoirs [29] and sporadic occurrences in rats (*Rattus norvegicus*) [30,31]. The domestic cat, in the meantime recognized as a common host of CPXV, is responsible for the majority of human CPXV infections [32,33]. In addition, CPXV has been isolated from a variety of zoo animals [34]. Increasing worldwide travel and importation of exotic animal species continue to increase the risk of OPV infections outside endemic areas. The majorities of these infections, particularly CPXV infections, have a mild course and are often self-limiting with low morbidity. However, immunosuppressed individuals are at risk because a variety of severe complications can develop, including widespread dissemination and death [33,35,36]. On the other hand, an MPXV infection is associated with a significant morbidity and childhood mortality in immunocompetent infected individuals [37].

1.2 Orthopox virus diseases

1.2.1 Virus transmission

An infection with VARV is established via the respiratory tract, by inoculation through the skin, or rarely via the conjunctiva [38] or the placenta, respectively. The respiratory route is the main route of transmission of VARV. Infected patients exhale virus-containing droplets from the oral, nasal or pharyngeal mucosa which are then inhaled by persons in close contact to the patients. A transmission is possible from the onset of symptoms and is frequent during the first week after the appearance of the exanthema when the titer in oropharyngeal secretions reaches up to 10^5 pfu/ml [39]. VARV is also present in high titers in scabs that had separated from the skin lesions [40], but epidemiological studies have shown that scabs play a negligible role in virus transmission. In very rare cases infection via the conjunctiva had been described [41]. In pregnant women VARV major infections are always more severe with abortion or stillbirth than in non-pregnant women or men [42]. The fetus is presumably infected by the growth of virus in the placenta which is infected during the second viremic phase [43]. There is no evidence that infection has ever occurred via the alimentary tract or that arthropods of any kind were involved.

In comparison to smallpox, human MPXV infections are not well understood, but it seems likely that infections usually occur through contact with infected wild animals including NHPs. The rate of transmission from person to person is getting higher (73 %) [44] than previously reported (30 %) [25]. Transmission chains beyond secondary infection have been rare [45,46] but may occur [21,47]. Therefore, it is unlikely that MPXV infections could persist in a human population without repeated re-introduction of the virus from the main reservoir [48].

CPXV infections are usually acquired through skin lesions resulting in a local self-limiting infection at the site of inoculation. Skin lesions develop most commonly on fingers and hands. Infection of the face is usually caused by virus-transfer from skin lesions on hands and fingers. A generalized infection with severe symptoms, resulting in hospitalization or even death, can occur in persons with atopic dermatitis or immunodeficient persons [35,49].

1.2.2 Clinical symptoms

The most common clinical form produced by both VARV major and minor is the ordinary type (figure 1A). According to the severity of the rash on the face and upper limbs, ordinary smallpox is subdivided into the confluent subtype (confluent rash on face and

forearms), the semi-confluent subtype (confluent rash on face and discrete rash elsewhere) and the discrete subtype (areas of normal skin between the pustules).

Three other clinical types of smallpox are recognized and classified as follows:

(i) modified type which covers the great majority of cases of VARV major, is usually nonfatal and the rash evolves quickly with more variation in lesion size than in discrete ordinary smallpox

(ii) flat type which is similar to the ordinary type but with an accelerated course in which confluent or semi-confluent pustules remain flat and soft and nonsuppurative

(iii) hemorrhagic type with widespread hemorrhages in skin and mucous membranes

For the hemorrhagic type (iii) two subtypes are distinguished: early type with purpuric rash, and the late type with hemorrhages into the base of pustules. The flat type (ii) and the hemorrhagic type (iii) of VARV major are usually fatal [42].

The average incubation period for smallpox is 12 days, ranging from 7 to 17 days. A two- to five-day period of high fever, malaise, prostration, headache and backache is followed by the development of a maculopapular rash. The rash appears first on the mucosa of the mouth and pharynx, the face and the forearms and spreads to the trunk and legs. Lesions appear occasionally on the palms and soles. The rash becomes vesicular and then pustular within one to two days. The pustules are characteristically round, tense and deeply embedded in the dermis. Crusts begin to form on day eight or nine and leave a pigment-free skin when they fall off. In lethal infections death occurs late in the first week or in the second week of illness and is commonly due to the effects of an overwhelming viremia. The severe and fatal hemorrhagic form (94-96 % mortality) [42] occurs with extensive bleeding into the skin and gastrointestinal tract, followed by death within a few days. Illness caused by VARV major is generally more severe, with a more extensive rash, a higher fever and a greater degree of prostration than illness caused by VARV minor.

For MPXV the average incubation period is 12 days, ranging from 7 to 21 days. The onset of fever occurs around day six after infection. The rash appears after approximately seven days after the onset of fever. The obvious clinical feature that differentiates human MPXV from smallpox is the pronounced lymphadenopathy seen in most cases of MPXV. Lymphadenopathy is sometimes found only in the neck or inguinal region, but more often a generalized lymphadenopathy is observed already at the onset of fever, but usually one to three days before the rash [50,51]. The eruption begins after a prodromal illness that lasts one to three days with fever, prostration and lymphadenopathy. Similar to smallpox, the lesions

develop more or less simultaneously (figure 1B) and evolve together at the same rate through the stages papules, vesicles and pustules [52]. Pustules usually measure about 0.5 cm in diameter, but sometimes may be up to 1 cm in diameter. Confluent lesions that are distributed over the body similar to flat-type or hemorrhagic-type smallpox have not been reported [37,53]. The generalized distribution of rash has been reported for most cases in African outbreaks [53], but peripheral distribution of the rash has also been found [37]. Rash usually lasts two to three weeks after onset. Especially in smallpox-vaccinated persons, MPXV infection has been observed to have a mild course. The case-fatality rate of MPXV infection among non-vaccinated patients ranged between 0 % in the outbreak in 2003 in the USA [54] and 1-5 % (1997 outbreak) [23,24] and 10-17 % (1970-1986 outbreaks) [25,48], depending on the origin of the virus strain and the route of infection [54,55,56].

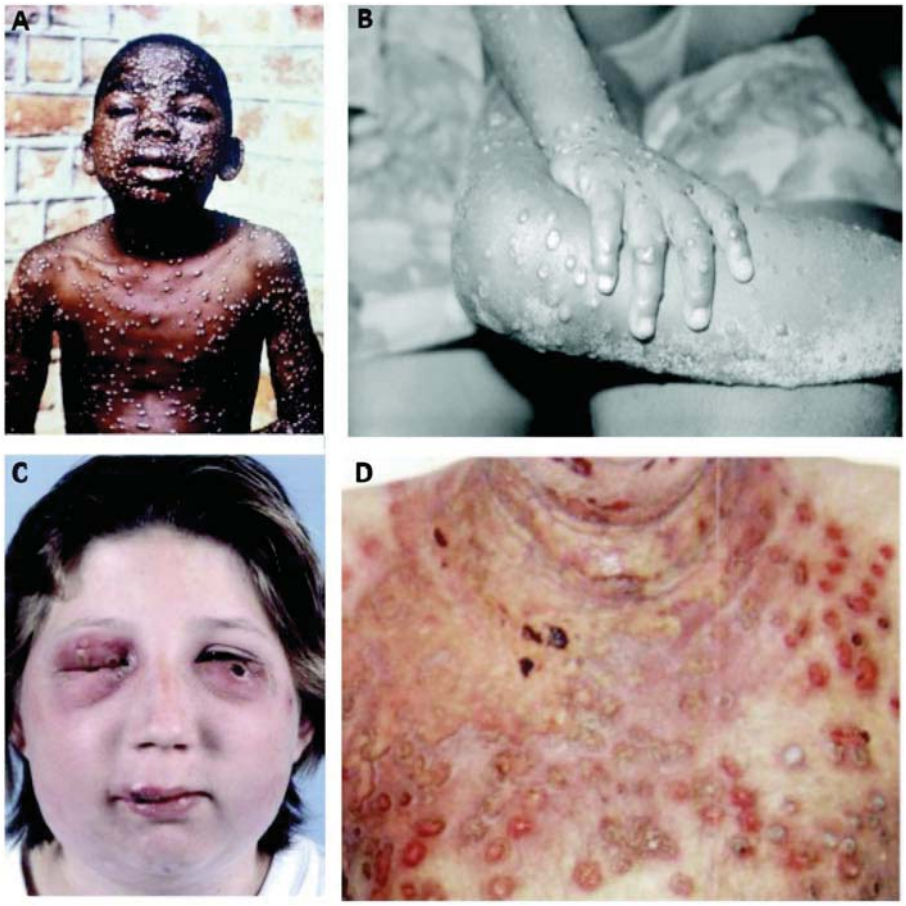

Figure 1: Typical OPV infections caused by **A)** Variola virus (VARV) major (ordinary-type smallpox) [57], **B)** Monkeypox virus (MPXV) [58], **C)** Cowpox virus (CPXV) (infection of the eyelid) and **D)** CPXV (infection of a patient with atopic dermatitis) [31]; D: [59]

The clinical manifestations of CPXV infections vary. The typical lesion begins as an inflamed macula, and over a period of seven to 12 days it passes through papular and vesicular stages. The vesicle becomes pale blue to purple and increasingly hemorrhagic and begins to ulcerate and becomes incrusted by the end of the second week [60,61]. The lesions can measure 1-3 cm in diameter and the degree of inflammation varies. The lesions are usually extremely painful and local lymphadenopathy occurs, which is often marked and prolonged. Most patients report systemic symptoms like fever, malaise, lethargy and occasionally vomiting and sore throat that can last for three to ten days. The time until complete recovery can take ten to 12 weeks or longer. In some cases ocular infections with the involvement of conjunctiva and cornea have been reported [36,62] (figure 1C). Besides localized CPXV infections, more serious generalized infections have been reported. Atopic eczema, dermatitis, allergic asthma or immunosuppression are generally associated with severe or lethal progression (figure 1D) [35,36,59].

1.2.3 Pathogenesis

After inhalation of VARV, the first targets of infection are the mucosal membranes of the mouth, the nasal cavity, the oropharynx or the alveoli of the lungs. Virus migration to regional lymph nodes via the lymph system and subsequent multiplication of the virus results in a first asymptomatic viremia between day five and six of infection [63]. The first viremia is followed by an extensive propagation of the virus in lymphoid organs like spleen, bone marrow and lymph nodes. With virus entry into the bloodstream, a secondary viremia commences around the day eight of infection, leading to a complete dissemination of the virus through the body including the blood vessels of the dermis of the oral and pharyngeal mucosa. The second viremia is accompanied by fever and extensive cytokine secretion ("cytokine storm"). Mostly because of the infected macrophages, VARV migrate from the small vessels in the dermis into the epidermis where edema, ballooning degeneration and splitting of the epidermis occur and lead to the classical 'pox' lesion. In the skin, the characteristic maculopapular lesions and later the vesicular and pustular lesions appear between days ten and 14 after infection. Histological analyses reveal numerous Guarnieri bodies and extensive necrosis in the epithelial cells of affected skin tissue. Lesions in the mouth and pharynx ulcerate quickly because of the absence of the stratum corneum and release large amounts of virus into the saliva even before a cutaneous rash becomes visible [39]. Virus titers in saliva

were found to be highest during the first week of illness [64], and in fatal cases virus is shed until death. In addition to saliva, in some cases virus has also been detected in urine.

Pathological evidence of viral replication was also found in the pharynx, larynx, tongue, the upper part of the trachea and esophagus (in descending frequency). Lesions in the bronchi and in intestines were rare, except for some cases of hemorrhagic-type smallpox [65]. Other organs were rarely involved in VARV infection, but reports exist of hyperplasia of bone marrow and spleen. Endothelial cells of the liver and testis were often found to be swollen and occasionally necrotic. Death probably resulted from the massive secretion of cytokines associated with circulating immune complexes and soluble VARV antigens [66] as well as overwhelming virus replication in the organs, all leading to multiple organ failure.

1.3 Orthopox viruses within the *Poxviridae* family

The OPV genus within the subfamily *Chordopoxviridae*, which belongs to the *Poxviridae* family, is very homogenous. The individual viruses are primarily named based on the host from which they were isolated. The most important indicators for characterization are the host range, pock morphology and ceiling temperature on the chorioallantoic membrane. Different virus species with a wide host range along with virus species restricted to a single host belong to the OPV genus. Table 1 lists the names, the host range and the geographical distribution of naturally occurring OPV species [67]. All nine species show extensive serological cross-reactivity. The most important pathogen among the OPVs is the VARV, the causative agent of smallpox, which only infects humans. Other OPVs with a narrow host tropism are the Camelpox virus (CLMV) and Ectromelia virus (ECTV), infecting camels or mice, respectively. Both viruses are apathogenic for humans. Although CLMV was described as “extremely closely related” (genetically) to VARV [68], it behaves differently in cell culture [69]. In contrast, VACV and CPXV have a broad host range and are able to infect humans, cattle, cats and rodents. Many strains of both VACV and CPXV with different biological properties were described. However, VACV and CPXV are phylogenetically distinct [70]. MPXV, first isolated from a cynomolgus monkey in 1958, is able to infect primates in general, but zoonotic events of human MPXV infections have also been observed. Today rodents are regarded to be the natural reservoir of MPXV [71]. Other known OPV species are Raccoonpox virus (RCNV) infecting the North American raccoons, the Taterapox virus (GBLV) found in the African gerbil and Volepox virus (VPXV) infecting the California piñon mouse and voles.

Table 1: Species of the genus OPV

Species	Strain* (prototype)	Host range	Animals naturally infected	Geographical distribution[a]
Variola virus (VARV) (eradicated)	Variola major virus Bangladesh-1975 Variola virus minor Garcia-1966 Variola major virus India-1967	Narrow	Man	Formerly world-wide
Camelpox virus (CMLV)	Camelpox virus CMS	Narrow	Camels	Africa and Asia
Ectromelia virus (ECTV)	Ectromelia virus Moscow	Narrow	Mice	Europe
Vaccinia virus (VACV)	Vaccinia virus Lister-Elstree Vaccinia virus Western Reserve Vaccinia virus Copenhagen Buffalopox virus Rabbitpox virus Utrecht	Broad	Man, cows, pigs, rabbits, buffalos etc.	Worldwide
Cowpox virus (CPXV)	Cowpox virus Brighton Red Cowpox virus GRI-90 Calpox virus #	Broad	Cows, man, rodents, rats, cats, large felines, elephants, etc.	Europe
Monkeypox virus (MPXV)	Monkeypox virus Zaire 96-1-16	Broad	Monkeys, great apes, squirrels, prairie dogs, rodents, man	Western and Central Africa
Raccoonpox virus (RCNV)	Raccoonpox virus	Broad ?	Raccoons	USA
Taterapox virus (GBLV)	Taterapox virus	Narrow	Gerbils	Western Africa
Volepox virus (VPXV)	Volepox virus	Narrow?	California pine voles	USA

Information according to the International Committee on Taxonomy of Viruses (ICTVdB-Index of Viruses); * = not all strains listed; # = not fully characterized, a = natural infections; ? = not really known

1.4 Virus structure

OPVs are enveloped viruses with a maximum of 350 nm in length and 260 nm in diameter. The morphology of the virion is generally brick-shaped and sometimes pleomorphic. Embedded into the surface membrane is a lipoprotein displaying irregular tubules, which consists of small globular subunits (10–40 nm) (figures 2B and D) [72]. The surface membrane encloses a biconcave core with the genomic DNA and proteins organized in a nucleoprotein complex (figure 2A). One or two lateral bodies appear to be present in the concave region between the core membrane and the surface membrane (figure 2D). During VACV infection four different types of virions are produced by each infected cell: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV). These virus particles have different morphology, containing different proteins and different numbers of membrane layers.

Additionally, acidophilic-type inclusion bodies (ATI), or A-type inclusion bodies can be found in the cytoplasm of CPXV or ECTV infected cells [73]. B-type inclusion bodies or Guarnieri bodies, are observed in infected epithelial cells which represent the site of replication, including the aggregation of virus particles during infection (figure 2C).

1.5 Virus genome

The OPV genome consists of one large single linear and complex dsDNA molecule of between 165 kb (VACV MVA 1721) and 220 kb (CPXV Germany 91-3) [74] encoding over 250 genes (VACV). All OPV genomes have inverted terminal repetitions (ITRs) at the two ends of the genome [75]. The ITRs are variable in length and consist of an A+T-rich, incompletely base-paired hairpin loop that connects the two DNA strands. In addition, ITRs have a highly conserved region of less than 100 bp that is required for the processing of replicating concatemeric forms of DNA [76,77,78]. The members of the OPV genus have about 100 open reading frames (ORFs) in common. The ORFs that are involved in essential functions like transcription, translation and replication are conserved and located in the center of the genome. They are found in sets and in a conserved order and do not usually overlap. The ORFs coding for genes involved in less essential functions are variable and located towards the outer regions of the genome. mRNAs of OPVs are not spliced.

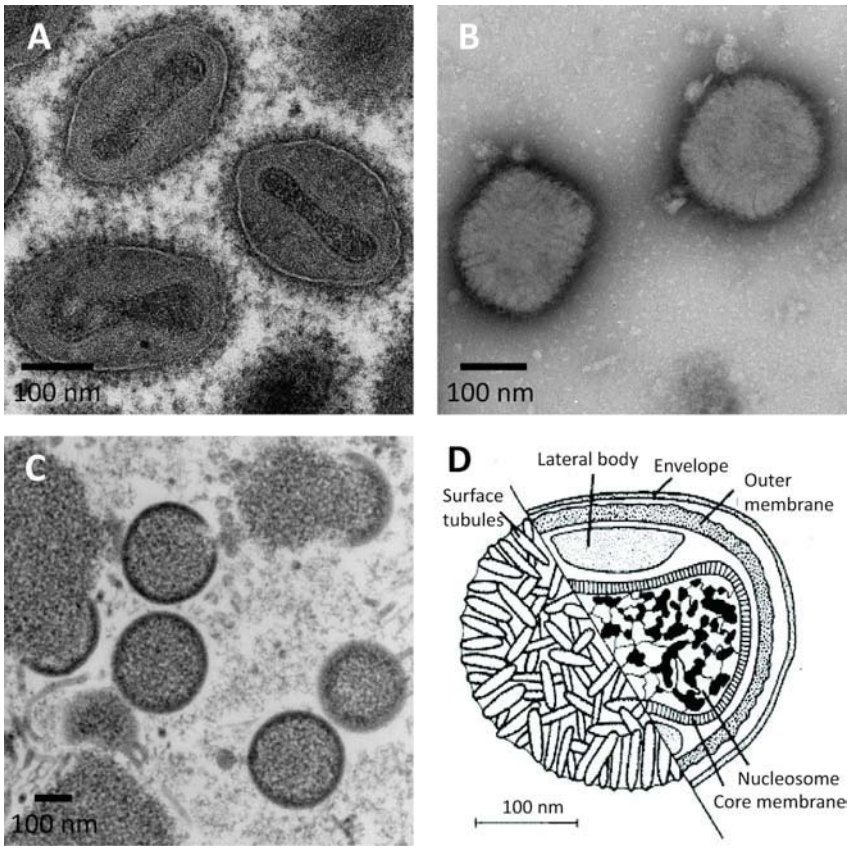

Figure 2: Virion morphology of OPVs by electron microscopy (A-C) and drawing (D); **A)** VARV with biconcave core formation, ultrathin sectioning; **B)** VACV with irregularly arranged surface tubules, negative staining; **C)** area of a viral factory with different stages of immature VACV during assembly, ultrathin sectioning (all kindly provided by H.R. Gelderblom, RKI, Berlin); **D)** drawing of an EEV particle [79]

1.6 Virus life cycle

OPVs code for their own mRNA and DNA synthetic machinery and bring their own viral enzymes that are essential for early virus transcription. They are thus able to replicate in the cytoplasm of infected cells.

1.6.1 Virus entry and uncoating

IMVs are generally thought to enter cells by fusion with the plasma membrane or vesicles formed by surface invaginations [80,81,82,83,84,85,86]. In contrast, the knowledge on EEV entry is limited. Evidence that IMV and EEV have different binding sites includes the efficiency with which the two virus types bind to different cell lines [87]. Cell attachment of EEVs involves different proteins or attachment occurs via heparin sulfate [83,88,89]. Some data suggest that EEVs enter cells by endocytosis followed by low pH disruption of the EEV

outer membrane and fusion of the released IMV with endosomal membranes [90,91]. Another mediator for the binding of the virus is chondroitin sulfate which binds to the virus envelope protein D8L [92]. Some OPVs can infect a variety of cells, leading to the assumption that they use either many cellular receptors or one that is ubiquitous. So far, no specific cellular receptor has been identified for any of the OPVs.

1.6.2 Virus gene expression

Viral gene transcription is divided into three phases: early, intermediate and late transcription. The transcription occurs in the cytoplasm of the host cell. OPVs providing an autarkic mechanism for the initial synthesis of their own mRNAs: a complete early transcription system is packaged within the core of an infectious poxvirus particle, and viral mRNAs do not need to undergo splicing processes. The early mRNAs encode enzymes and factors necessary for host interactions, viral DNA synthesis and transcription of the intermediate class of genes [93]. The early transcription takes place already inside the mature virion. OPV mRNAs are capped, methylated and polyadenylated like most eukaryotic mRNAs [94].

Following early transcription, the intermediate genes are expressed. They encode transcriptional transactivators for late gene expression.

The transcription of late genes follows that of intermediate genes. During this time high levels of late proteins accumulate in the infected cell including structural viral proteins and enzymes that are packaged in the progeny virions.

1.6.3 DNA replication

DNA replication takes place in the cytoplasm in discrete foci of replication, termed virus factory areas or Guarnieri bodies (figure 2C). For VACV, DNA replication results in the generation of about 10,000 genome copies per cell, half of which are ultimately packaged into virions [95]. Because of the absence of a defined replication origin, a self-priming replication model is proposed. DNA replication begins with the introduction of a nick close to one or both of the hairpin termini, providing a free 3’-end for priming. If the replication is initiated at only one end, the polymerase continues to the other end to generate a concatemer or even very large branched concatemers. If initiation occurs on both ends, replication takes place without concatemer formation [96]. The onset of late transcription is essential for the resolution of concatemers and the generation of unit-length genomes [97].

1.6.4 Virion assembly, maturation and release

The virus assembly and morphogenesis occur together with the transcription of late genes and genome replication in viral factories [98]. It is assumed that vesicles bud from cellular organelles and then coalesce to form the viral membrane [99]. In subsequent stages of development the crescent membranes are transformed into spherical immature virions (IV) (figure 2C) with a dense nucleoprotein mass already containing the viral genome. Maturation to infectious particles (intracellular mature virus, IMV) occurs by condensation of the virus core and by proteolytic processing of the major structural proteins [100] which enter the immature envelopes just before they are completely sealed [101]. IMVs remain within the cell or are released after cell lysis. The movement of IMVs out of the assembly area into the cell periphery is microtubule dependent [102]. IMVs acquire additional membranes, derived from the trans-Golgi or early endosomal network [103,104] to form the intracellular enveloped viruses (IEVs) that are then transported to the cell surface and exported by exocytosis [105], losing the outermost Golgi-derived membrane (CEV and EEV). Only some of the externalized virions are found in the medium of infected cultures as free EEVs that are important for long-range dissemination of the virus [106], whereas CEVs remain attached at the cell surface and mediate efficient cell-to-cell spread.

1.7 Immune response

1.7.1 Humoral immune response

Both humoral and cellular immunity play an important role in the response to an OPV. During infection, neutralizing antibodies directed against enveloped and non-enveloped virions are generated. They can be detected around day six of illness (about 18 days after infection) in non-hemorrhagic smallpox with increasing titers over the course of the disease [66,107]. In hemorrhagic-type smallpox patients a delayed, reduced or even failing antibody response was found [63].

Vaccination with VACV induces an antibody response that is developing much faster than after natural VARV infection. Antibodies can be detected as early as ten days after primary vaccination and within four to seven days after re-vaccination [108]. The prompt immunologic reaction to the re-vaccination also results in an increase in titers of neutralizing antibodies.

Neutralizing antibodies against OPV are directed against different forms of viral particles (IMV as well as EEV virions) [109,110,111,112] and gene products [113,114].

Studies have shown that antibodies against EEV particles cause the aggregation of EEV particles on the cell surface [115] and immunological protection is provided by preventing the release rather than by neutralization [116] of the EEV particles.

Animal studies suggested that neutralizing antibodies are essential and sufficient for an immunological protection [117]. Depending on the antigenic relationship, antibodies against specific OPVs were found to have a wide cross-reactivity among the whole virus genus.

1.7.2 Cell-mediated immunity and virus immune evasion

Invading OPVs alter the host cell in several ways to make it an ideal environment for virus survival and replication. Interactions of the virus with their host occur by (i) the inhibition of host macromolecular synthesis, which is poorly understood, and (ii) the defense against host antiviral mechanisms.

A whole array of viral defense proteins is expressed by OPVs to modulate and combat the host's antiviral response. These proteins are capable of preventing apoptosis, capturing chemokines and counteracting the complement system; they interfere with the interferon response, intercept interleukins and induce the synthesis of steroids [118,119,120,121,122]. Interestingly, these immunomodulatory proteins do not seem to be generally shared across all OPVs, on the contrary, each virus species or even strain encodes its own unique combination of proteins. Deletion of some genes results in an attenuated or altered disease [123].

Nevertheless, intensive early inflammatory response by the host is thought to inadvertently damage the host to such a degree that it causes illness and even death after infection [124].

1.8 Animal models – why is there a need for a new primate model?

On the background of (i) the threat by bioterrorism, (ii) zoonotic OPV infections and (iii) a decreasing immunity among the population, more research into therapeutic agents for the prevention and treatment of OPV infections is required [125]. Vaccination during the very first days after exposure to VARV might still have a protective effect. But still there remains a period of seven to ten days before antibodies can be detected. In primate and mouse models a protection against an OPV infection is not observed before the development of antibodies [126,127]. Antiviral therapies can fill this gap and complement vaccination by reducing viral titers regardless of immune status. To date, only cidofovir, a nucleoside analog which has been used for the treatment of Cytomegalovirus infections, is approved for the treatment of

OPV infections [128]. The orally deliverable compound ST-246, currently in the FDA approval procedure, is a specific inhibitor of EEV formation and resulted in 100 % protection against a lethal infection of different OPVs [129,130,131,132] in different animal models. At present ST-246 has the status of an orphan drug and was recently used very efficiently in the treatment of a 2-year-old child with a severe eczema vaccinatum [133]. Numerous other compounds showed anti-OPV activity in cell culture or in animal models [134,135,136,137,138,139,140,141,142]. New approaches for antiviral therapy like passive immunotherapy with anti-VACV immune serum [143], siRNAs against potential OPV targets [144] or the use of DNA aptamers with antiviral activity [145] are under development. New and safer vaccines like DNA vaccines are also under development [146]. New-generation vaccines using attenuated VACV with specific and defined deletions grown in tissue culture have shown promising immunogenicity in initial studies [147,148,149,150,151,152].

However, efficacy and safety of new vaccines and antiviral agents have to be shown in clinical trails. In case human trials are considered to be unethical, the Food and Drug Administration (FDA) demands studies in at least two different animal models for approval of the drug. So far, there are six different animal models for OPV infections.

The ECTV/mouse model (different mice strains, e.g. BALB/c or DBA/2) is the most common animal model for studying the pathogenesis of OPVs and their interactions with the host's immune system. ECTV can be applied via intradermal (i.d.) or intranasal route (i.n.). In both cases a low infectious dose of one to ten pfu is sufficient to induce a systemic disease and death [153,154]. The VACV/mouse model as well as the CPXV/mouse model (using BALB/c mice) are used to measure the efficacy of vaccines and antiviral components. Routes of infection are i.d., i.n., intravenous (i.v.) and intracranial (i.c.) [155,156,157]. Lethal infection requires high virus doses between 10^4-10^6 pfu. Development of skin lesions as well as systemic disease depends on the inoculation route and the dose [158]. Another model is the infection of rabbits with rabbitpox virus. As little as 15 pfu applied by i.d. inoculation (into the footpad) or i.n. inoculation are sufficient to induce a systemic and lethal infection with the development of rash [159].

The closest animal model for the study of human disease as well as for the testing of vaccines and antiviral compounds are NHP which are relatively closely related to humans [158]. So far, there are two well-established NHP models: MPXV infection of cynomolgus macaques (*Macaca fascicularis*) [160] and rhesus macaques (*Macaca mulatta*) as well as VARV infection of cynomolgus macaques [161]. Both models have major limitations: a very high infectious dose has to be administered, using mostly i.v. inoculation to induce a severe

disease. For the VARV/cynomolgus macaques model at least 10^8 pfu by i.v. application are required to cause smallpox symptoms [161] and a mortality of at least 33 %. For the MPXV/rhesus macaques model different inoculation routes like i.v., i.n., subcutaneous (s.c.) and intratracheally (i.t.) application are described. But these routes of infection also require high viral doses of 10^7-10^6 pfu to induce severe symptoms. Infections with lower doses than 10^6 pfu result in a milder course of disease, and most animals survive [126,160,162,163,164]. Additionally, working with MPXV and VARV is technically challenging, as it requires BSL 3 or BLS 4 laboratories, respectively. Additionally, macaque monkeys are relatively big and expensive compared to other animal models.

Therefore, the development of a new NHP model in which infections with OPVs other than VARV and MPXV induce a disease comparable to human smallpox is highly recommended. In addition, it would be very helpful if the infection occurred with a low virus dose via a more natural application route to mimic naturally acquired smallpox infection.

1.9 Lethal orthopox virus outbreak in New-World monkeys and calpox virus isolation

In 2002 an lethal atypical epizootic poxvirus infection was observed in a colony of 80 New-World monkeys in Lower Saxony, Germany [165]. A group of *Callithrix jacchus, C. penicillata, C. geoffroyi, C. Saimiri sciureus, Callimico goeldii* and various tamarin species were affected. Macroscopic examination and necropsies revealed typical OPV symptoms with erosive-ulcerative lesions of the oral mucous membranes and typical hemorrhagic skin lesions distributed randomly over the body. Other observations were mild-to-moderate edema and severe lymphadenopathy of the mandibular and axillary regions.

Electron microscopy revealed virus particles with OPV-like morphology within intracytoplasmatic inclusions. Using two OPV-specific quantitative real-time PCR assays targeting the OPV genes *crmB* (tumor necrosis factor receptor II homologue) and A13L (IMV membrane-associated protein), approximately 10^7 OPV genomes per 10^5 cells were detected in different tissues of all animals, concomitantly excluding VARV. A conventional PCR, targeting the HA gene (hemagglutinin glycoprotein, that forms part of the extracellular virus outer envelope), also led to positive results. The *crmB* and HA PCR products from liver and spleen were sequenced and identical sequences were found in all animals, indicating that they had been infected with the same virus.

Virus was isolated in Vero E6 cells from skin tissue of infected animals. Cell cultures

showed a cytopathogenic effect already one day after infection. Transfer of the supernatant to new cells again resulted in cytopathogenic effects, indicating a reproductive replication of virus in cell culture. The virus was grown for 3 passages on Hep2 cells to achieve a higher virus titer and stored in aliquots at -70 °C. The newly isolated OPV was named calpox virus according to its host *Callithrix jacchus*. Calpox virus was characterized by specific real-time PCRs [166], electron microscopy, partial genome sequencing and immunofluorescence assays using human anti-VACV immunoglobulin.

Moreover, complete nucleotide sequences of the *crmB* and HA genes were translated into amino acids and phylogenetic analyses were performed, as these genes are known to have important functions. The deduced protein sequences were aligned with published OPV sequences. A phylogenetic tree was constructed using the Protdist and Neighbor softwares from the Phylip program package [167] to elucidate the genetic relationship of the calpox virus to other species of the genus OPV (figure 3). The trees showed that VARV, VACV, MPXV, CLMV and ECTV sequences (ECTV only in the HA tree since this virus has a large deletion in the *crmB* region) separated into distinct individual clusters. But CPXV sequences generated at least three or four well-separated clusters, almost all supported by high bootstrap values [168] (figure 3). The calpox virus was found to be most closely to CPXV, although the phylogenetic grouping to CPXV strains varied depending on the sequence used for analysis (Ellerbrok et al, unpublished). Since most of the CPXV HA and *crmB* sequences represent individual genebank entries it was not evident sequence might originate from an individual CPXV isolate, therefore making it difficult to compare the CPXV clusters for the HA tree and the crmB tree. Thus, HA and *crmB* sequences from CPXV strains Bighton Red (BR) and GRI-90, the only two complete CPXV genome sequences available, were used for orientation. While calpox virus HA clustered with CPXV BR (figure 3A), calpox virus *crmB* was much closer to CPXV GRI-90 (figure 3B). Therefore, the ancestor of the calpox virus remains unclear until more sequence data is available (work in progress).

However, phylogenetic analyzes revealed that calpox virus is related to, but distinct from, known CPXV. Results also indicate that the “CPXV group” is extremely heterogeneous and thus the calpox virus could be considered as a new CPXV strain.

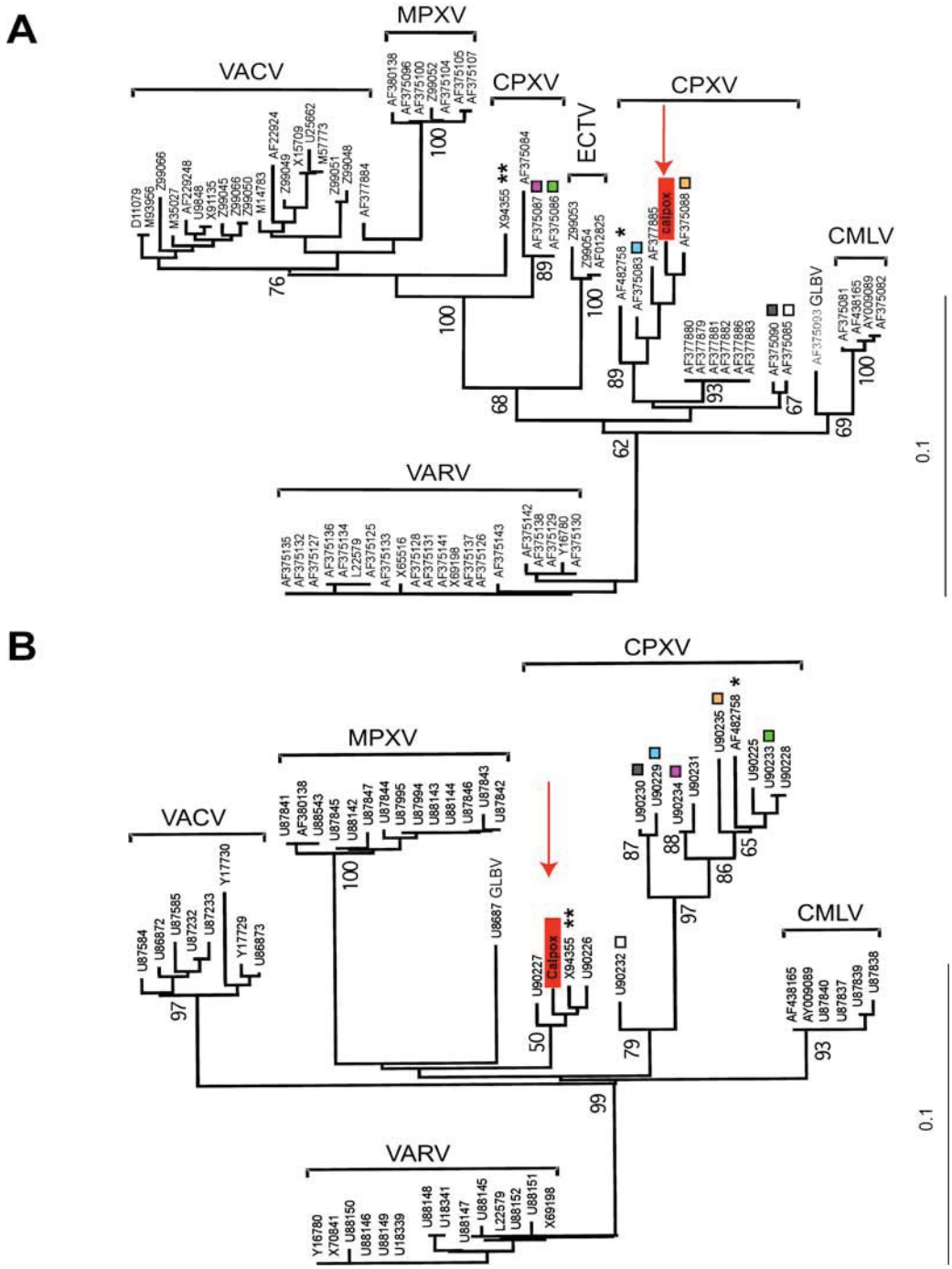

Figure 3: Unrooted phylogenetic tree of the calpox virus and other OPVs based on **A)** hemagglutinin (HA) amino acid sequences and **B)** tumor necrosis factor receptor II homolog (*crmB)* amino acid sequences. The deduced amino acid sequence for HA and for *crmB* were aligned with published sequences. Phylogenetic trees were constructed with the Neighbor Joining method. Bootstrap values (1000 bootstraps) for the major branching points are given in percent. Sequences are identified with their accession numbers and the different clusters are indicated. The calpox virus is boxed and indicated by arrow and CPXV isolates Brighton Red and GRI-90 are labeled with one and two stars, respectively. HA and *crmB* sequences most likely originating from an individual virus isolate are indicated with identical symbols in figures A and B (Ellerbrok et al., unpublished). GBLV: Taterapox virus; CLMV: Camelpox virus; CPXV: Cowpox virus; ECTV: Ectromelia virus; MPXV: Monkeypox virus; VACV: Vaccinia virus; VARV: Variola virus

1.10 The common marmoset – *Callithrix jacchus*

The common marmoset is the smallest NHP used in bio-medical research. It is a well-established animal model for cardiac arrhythmia [169], stroke [170], Parkinson's disease [171,172], multiple sclerosis [173,174], infectious diseases [175,176,177] as well as for immuno-toxicological studies [178]. These New-World monkeys are members of the family *Callitrichidae* genus *Callithrix*. The species classification is "jacchus" [179]. Other names were "true marmoset" or "white-tufted-ear marmoset". The trapping of marmosets from the wild is illegal, and animals used in research are bred in captivity. Common marmosets are small-bodied monkeys that are colored brown, grey, and yellow with white ear tufts and long, banded tails [180]. Males and females are about the same size (16 to 21 cm) and weight (300 to 350 g) [180]. Members of the genus *Callithrix* have some characteristics that are unique to this group and necessary for their diet and arboreal lifestyle. On all but the hallux (big toe), they have claw-like nails instead of the characteristic flat nails (of other primates, including humans) [181]. Common marmosets originate from Brazil. They are an adaptable species with high fecundity and a complex behavioral repertoire. In the wild they are found in small groups or in family organizations with well-defined territories [180]. Their social structure is very flexible; while depending on the group, different pairing systems are reported [182]. Furthermore, they have a tendency to give birth to non-identical twins which is unusual for primates [183]. Inbreeding is usually not a problem because subordinate sexually mature females within the family group do not ovulate [184]. The average lifespan of a wild common marmoset is 12 years [180]. They are exudativore-insectivores and feed on plant exudates. They utilize gum, sap, latex and resin much more than other species [183,185,186]. Other important food sources for common marmosets are insects, fruits, seeds, flowers, fungi, nectar, snails, lizards, tree frogs, bird eggs, nestlings and young mammals.

2 Aims of Study

The main objective of this study was to establish and evaluate an advantageous, cost-efficient and relevant NHP animal model for OPV infections in which a smallpox-like disease can be induced via a natural route of infection. From the natural outbreak in 2002 it was hypothesized that the calpox virus/common marmoset system could be used as a model for smallpox virus infections in humans. This animal model should induce a disease similar to smallpox and should overcome the limitations of the other primate models used in OPV research.

Therefore, in the first part of the study the focus was on the development of transmission routes that reflect natural routes of smallpox infection. Besides the intravenous infection route, intranasal infection was studied. In addition, pathogenesis and the development of clinical symptoms were characterized and compared to other OPV diseases, including smallpox. After establishment of a natural route of infection (intranasal), in titration experiments the lethal dose of calpox virus in marmosets was determined. Immunological parameters were investigated during the course of infection which should give insights into the immune response after lethal and non-lethal infections.

To evaluate the suitability of the calpox virus/marmoset model for the testing and validation of new vaccines and antiviral substances *in vivo*, marmosets were vaccinated with the classical VACV Lister-Elstree strain (VACV LE-BN) and challenged with a lethal dose of calpox virus. In addition, infected animals should be treated with ST-246, a recently developed antiviral agent with a high therapeutic efficacy against OPV infections *in vitro* and *in vivo*.

3 Materials and Methods

This chapter describes the experimental approach and presents technical equipment, material and methods used in this study. Animal experiments like virus infection and necropsy are explained as well as virological and molecular biological analyses. All work was done in collaboration of the Robert Koch-Institut, Berlin, Germany, the German Primate Center, Göttingen, Germany and the Paul-Ehrlich-Institut, Langen, Germany.

3.1 Technical equipment

All technical equipment not mentioned in the description of the methods is listed below.

Spectral photometer Coulter DU 640 B	Beckman, Krefeld, Germany
Nanodrop spectral photometer	peQ Lab Erlangen, Germany
Light optical microscope Axiophot	Zeiss, Oberkochen, Germany
Light optical microscope Axioskop	Zeiss, Oberkochen, Germany
Light optical microscope Axiovert 40cFL	Zeiss, Oberkochen, Germany
Micropipettes	Eppendorf, Hamburg, Germany
PIPETBOY acu	VWR, Darmstadt, Germany
Pipettes Gilson	Abimed Analysen Technik, Langenfeld, Germany
Cell culture incubator Hera cell 150	Heraeus, Hanau, Germany
Lamina flow cabinet HERA Safe	Heraeus, Hanau, Germany
Precision scales	Sartorius, Göttingen, Germany
Scales	Sartorius, Göttingen, Germany
Thermo mixer comfort	Eppendorf, Hamburg, Germany
Vortexer REAX 2000	Heidolph, Munich, Germany
-70°C Freezer	Sanyo, London, England
Lab centrifuge Heraeus Sepatech	Heraeus, Hanau, Germany
Varifuge 3.0R	Heraeus, Hanau, Germany
Table centrifuge 5415C	Eppendorf, Hamburg, Germany
Table centrifuge 5417R	Eppendorf, Hamburg, Germany

3.2 Consumables

Cell culture

Cryo tubes (0.8 ml, 1.2 ml)	Nunc^{TM}, Wiesbaden, Germany
Pipettes (for cell culture) (1-25 ml)	Nunc^{TM}, Wiesbaden, Germany

Cell culture plates (24-well; 48-well; 96-well)	TPP, Trasadingen, Switzerland
Cell culture flasks Nunclon	Nunc^{TM}, Wiesbaden, Germany
Cell scraper	TPP, Trasadingen, Switzerland
Falcon tubes (15 ml, 50 ml)	TPP, Trasadingen, Switzerland
Polymerase Chain Reaction (PCR)	
TaqMan PCR-plates (96 well)	Applied Biosystems, Weiterstadt, Germany
TaqMan-PCR Optical Tubes and Caps	Applied Biosystems, Weiterstadt, Germany
Optical Adhesive Covers	Applied Biosystems, Weiterstadt, Germany
Others	
Toothpicks	BTS Biotech, St-Leon-Rot, Germany
Parafilm	American National Can, USA
Tips for micropipettes	Eppendorf, Hamburg, Germany
Microcentrifuge tubes, safe lock (0.5-2.0 ml)	Sarstedt AG & Co., Nümbrecht, Germany
Microcentrifuge tubes, snap cap (0.2 ml)	Rapidozym, Luckenwalde, Germany
Microcentrifuge tubes, screw top (2 ml)	Sarstedt AG & Co., Nümbrecht, Germany

3.3 Viruses

Calpox virus	Calpox virus, isolated from skin tissue of an infected *Callithrix jacchus,* 2002, RKI, Berlin, Germany
CMLV CP-19	Camelpox virus, strain CP-19, kindly provided by Dr. Sandra Eßbauer, LMU Munich, Germany
CPXV 81-02	Cowpox virus, strain 81-02, kindly provided by Dr. Sandra Eßbauer, LMU Munich, Germany
CPXV BR	Cowpox virus, strain Brighton Red (only DNA), kindly provided by Dr. Hermann Meyer, Institute for Microbiology (German Federal Armed Forces), Munich, Germany
MPXV AP-1	Monkeypox virus, strain AP-1 (only DNA), kindly provided by Dr. Hermann Meyer, Institute for Microbiology (German Federal Armed Forces), Munich, Germany
VACV LE-BN	Vaccinia virus, strain Lister-Elstree Bavarian Nordic, LELS-2003-007; lot 120103, 2003; Bavarian Nordic GmbH, Martinsried, Germany
VACV VR15	Vaccinia virus, strain New York City, ATCC-VR 1536
VACV M1	Vaccinia virus, strain M1, kindly provided by Dr. Sandra Eßbauer, LMU Munich, Germany

VACV WR	Vaccinia virus, strain Western Reserve, ATCC-VR 1354
ECTV NU	Ectromelia virus, strain Nuremberg, kindly provided by Dr. Sandra Eßbauer, LMU Munich, Germany

Abbreviations are according to the 7th Report of the International Committee on Taxonomy of Viruses.

3.4 Non-human primates

3.4.1 Animal husbandry

All animal experiments were performed in close collaboration with Dr. C. Stahl-Hennig und Dr. K. Mätz-Rensing at the German Primate Center (DPZ, Göttingen, Germany). Marmosets (*callithrix jacchus)* of different age were bred and housed at the DPZ. Before infection, marmosets were moved to the animal L3 facility of the DPZ. Living conditions were 25°C, 60 % humidity and single cages (130 cm x 53 cm x 80 cm) for each animal, with visual and acoustical contact to each other. All animals were adequately fed, tended and maintained according to the German Animal Protection act. All animal experiments were approved by responsible authorities (approval number 33.42502/08-07.0514:19 and 33.42502-04019/07).

3.4.2 Virus application

The marmosets were either infected intravenously (i.v.), oropharyngeally (oro.) or intranasally (i.n.) with different infectious doses of calpox virus. For virus application marmosets were narcotized by injecting 0.1 ml Göttinger Mischung II (GMII)/200 g body weight into the hamstring muscle. Calpox virus was i.v. administered in a maximal volume of 500 µl into the *vena saphena*. Oropharyngeal virus application was performed by dropping 100 µl virus solution directly onto the tonsils. Intranasal infection was carried out as described elsewhere [187,188] by pipetting 50 µl virus solution into each nostril.

Before immunization with VACV LE-BN, the vaccination site, a small skin area on the shoulder, was shaved. Marmosets were immunized intradermal (i.d.) using the multi-puncture method with standard bifurcated needles (Precision Medical Products, Inc. Denver, USA), based on the immunization in humans: a bifurcated needle holding a drop (3 µl which corresponds to approximately $1x10^5$ pfu) of the vaccine (figure 4) was pressed three times into the skin at the vaccination site.

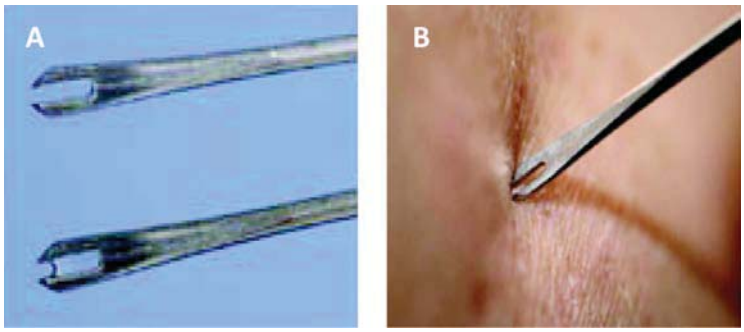

Figure 4: **A)** bifurcated needle with and without a drop of vaccine [189], **B)** scarification of the skin [190]

After immunization as well as infection all animals were inspected daily for signs of infection, and areas of the breast and abdomen were shaved for better inspection. At predetermined time points twice a week, marmosets were narcotized with 10 mg cetamine (Ketalar, Parke-Davis, USA) intramuscularly, and 1 x 0.8 ml EDTA blood and 1x 0.2 ml EDTA blood were collected. Simultaneously throat swabs were obtained.

Narcotization solution:

1 ml GMII: 10 mg xylazine (Rompun®, Bayer AG, Leverkusen, Germany), 50 mg atropine (Boehringer, Ingelheim, Germany), 50 mg cetamine (Ketalar®, Parke-Davis, USA)

Additional equipment:

Vacutainer® Plus and Vacutainer® system K2E 4.5 ml (Becton Dickinson, Heidelberg, Germany)

3.4.3 Necropsy and tissue handling

All animals were euthanized when showing clear clinical symptoms. During necropsy, a wide spectrum of tissue samples including skin, heart, liver, lung, kidney, spleen, lymph nodes, bladder, stomach, colon, small intestine and brain were immediately conserved in 2.5 % glutaraldehyde for electron microscopy, in 10 % formaldehyde for histological examination, in RNA-later (Ambion, Darmstadt, Germany) for RNA extraction and in liquid nitrogen for DNA extraction and virus titration.

Buffers and solutions:

10 % formaldehyde (1 l): 270 ml 37 % formaldehyde (Carl Roth, Karlsruhe, Germany), 730 ml phosphate-buffered saline (PBS, in-house production RKI)

1 x PBS buffer, (1 l, pH 7.2) without Mg^{2+} and Ca^{2+}: 8.0 g NaCl 0.2 g KCl, 1.44 g Na_2HPO_4 (all: Merck, Darmstadt, Germany), ad 1 l ddH_2O, sterile filtration

2.5 % glutaraldehyde (pH 7.2): 10 ml glutaraldehyde 25 % (Serva, Heidelberg, Germany), 90 ml 0.05 M HEPES-buffer (Sigma-Aldrich, Deisenhofen, Germany)

3.5 Mouse experiments

All mouse experiments were performed at the Paul-Ehrlich-Institut (PEI) (Langen, Germany) in close cooperation with Dr. Y. Süzer and Prof. G. Sutter. All mouse experiments had been approved by the German Animal Protection Agency. BALB/c mice (seven weeks old) were bred and housed at the PEI. Mice were kept three animals per cage with water and fed ad libituum. Virus application was carried out via the i.n. infection route. For virus application mice were narcotized with a mixture of 4 mg/kg cetamin (Ketalar®, Parke-Davis, USA) and 100 mg/kg xylazin (Rompun®, Bayer AG, Leverkusen, Germany). Afterwards they were laid on their back and virus was administered into each nostril in a maximal volume of 30 µl. Body weight and signs of illness were monitored daily until the end of experiment.

3.6 Cell culture

All cells were maintained according to the recommendations of ECACC (European Collection of Cell Cultures) or ATCC (American Type Culture Collection). Vero E6, Hep2 and B95-8 cells were split 1:3 or 1:5, depending on cell density, twice a week. ML2 cells were split 1:2 once a week or fortnightly. In the cultivation of adherent growing cells, cell culture medium was aspired and cells were washed once with PBS. After washing, cells were trypsinised with a 1:2 mixture of trypsin (PAA, Pasching, Germany) and EDTA (PAA). Afterwards new medium was added and one third of the cell suspension was transferred into a new cell culture flask. For B95-8 suspension cells one third of the cell suspension was transferred into a new cell culture flask and two thirds of new medium was added. All cells were incubated at 37°C and 5 % CO_2.

If a defined number of cells was needed, cells were counted using a "Neubauer cell counting chamber" (Blaub GmbH& Co. KG, Wertheim, Germany). The respective cell dilution was then seeded into cell culture plates or flasks.

Cells:

Vero E6	cell line from kidney tissue of an African green monkey (*Cercopithecus aethiops*); ECACC: 85020205
Hep2	human cervix carcinoma cell line, HeLa derivate, ECACC: 86030501
B95-8	peripheral blood lymphocytes from a marmoset (*Saguinus oedipus*); transformed with EBV; ATCC CRL 1612

ML2	cell line from lung tissue of a common marmoset (*Callithrix jacchus*); H. Ellerbrok and J. Reiche, unpublished
PBMC	peripheral blood mononuclear cells, preparation from fresh *Callithrix jacchus* blood

Cell culture medium:

Vero E6	D-MEM, 1 % L-glutamine, 10 % FCS
Hep2	D-MEM, 1 % L-glutamine, 5 % FCS
B95-8 and ML2	RPMI-1640, 1 % L-glutamine, 10 % FCS
PBMC	RPMI-1640, 10 % FCS

(D-MEM/ RPMI-1640: Gibco BRL®, Eggenstein, Germany; L-glutamine: Biochrom, Berlin, Germany; FCS: Gibco BRL®)

3.7 Virus propagation

3.7.1 Virus stock production

Hep2 cells were used for the propagation of all viruses. 80-90 % confluent Hep2 cells (in 175 cm^2 cell culture flasks) were infected with the respective virus in 10 ml medium and incubated for 1 h. After 1 h 25 ml culture medium/flask was added and cells were incubated for further three to five days until an overall cytopathic effect (CPE) could be observed. Cells and supernatant were shock-frozen over night at -70°C or -20°C. After thawing at room temperature cells were scraped off the cell culture flask and the cell suspension transferred into 50 ml Falcon tubes containing glass beads (for better cell destruction). After intensive vortexing, the suspension was centrifuged for 10 min at 200 g to pellet the cell debris. The virus-containing supernatant was frozen in aliquots (stock virus). The titer of the stock virus was determined and used for infection experiments. For the preparation of concentrate virus, the supernatant was overlaid onto a 30 % sucrose cushion (50 ml sucrose for 250 ml centrifuge tubes) and ultracentrifuged for 3 h at $3.4x10^3$ g (ultracentrifuge Sorvall WX Ultra 80, rotor R19, Thermo Fischer Scientific Inc, Waltham, MA, USA). The sucrose and supernatant were aspired and pelleted virus was re-suspended in a small volume of 0.9 % NaCl (infection of marmosets) or in PBS (infection of mice) or in cell culture medium (cell culture experiments).

Solutions:

0.9 % NaCl: 0.9 g NaCl (Merck, Darmstadt, Germany), 100 ml ddH_2O; 30 % sucrose: 300 g sucrose (Merck) ad 1 l PBS, autoclaved

3.7.2 Preparation of virus-infected cells on glass slides

Calpox virus and VACV LE-BN-infected cells were grown on glass slides (Teflon 12 wells/5 mm, Carl Roth, Karlsruhe, Germany) for immunofluorescence tests. $3x10^6$ Hep2 cells were infected with calpox virus (moi 1.5) or VACV LE-BN (moi 0.8-1.0) in 15 ml Falcon tubes for 1 h at 37°C and 5 % CO_2. Uninfected Hep2 cells ($1x10^6$) treated in parallel served as control. After 1 h, cells were washed twice with cell culture medium (centrifuged at 200 g for 5 min) and the cell pellet was resolved in 15 ml medium (infected cells) or in 3 ml medium (uninfected cells). Cells were seeded (30 µl/cavity) on glass slides and incubated over night at 37°C and 5 % CO_2. On the next day supernatant was aspired from each cavity and slides were dried and fixed in acetone (Carl Roth, Karlsruhe, Germany) for 30 min at room temperature. After fixation, slides were dried, labeled and stored at -20°C.

3.8 Plaque assay

Titers of infectious virus in plasma, saliva, tissue homogenates and virus stocks are determined using the plaque assay and expressed as plaque forming units (pfu). Briefly, 100 µl of tenfold serial dilutions of the samples were mixed with 100 µl Vero E6 cells ($1.5x10^6$ cells/ml) in wells of 48-well plates for 4 hours at 37°C and 5 % CO_2. Cells were then overlaid with 200 µl 1.6 % carboxymethylcellulose (CMC) and further incubated for 4 days. Finally the supernatant was aspired and cells were fixed in 1 ml 3.7 % formaldehyde for 30 min at room temperature. Cells were stained with naphthalene blue black for 10 min and surplus stain was removed by washing cells once with water. Plaques were counted and the virus titer was calculated based on plaque numbers as follows:

titer/ml = (n * a) / v

n = ∑ of counted plaques of the whole plate

a = dilution factor (smallest dilution at which plaque counting was possible)

v = ∑ of virus volume used in each dilution step

Solutions:

staining solution: 1 g naphthol blue black (Sigma-Aldrich, Deisenhofen, Germany), 13.6g sodium acetate (Merck, Darmstadt, Germany), 60 ml glacial acetic acid (Merck), ad 1 l ddH_2O

fixative: 100 ml 37 % formaldehyde (Carl Roth, Karlsruhe, Germany), 900 ml PBS

CMC overlay medium: 8 g carboxymethylcellulose (Carl Roth) in 500 ml D-MEM (with 1 % L-glutamine and 10 % FCS)

3.9 Plaque reduction neutralization test (PRNT)

The previously described PRNT [191] was modified. Briefly, $9x10^4$ Vero E6 cells/well were seeded into 48-well plates and incubated over night. To inactivate virus in plasma or serum as well as complement, samples were incubated for 30-60 min at 56°C. Twofold serially dilutions (50 µl) of the plasma samples were mixed with an equal volume of calpox virus or VACV LE-BN (virus titer varied between different stocks used for PRNT). After an incubation for 1 h at 37°C and 5 % CO_2, 100 µl serum-virus mixture/well was added to pre-seeded Vero E6 cells in 100 µl cell culture medium. Virus was allowed to adsorb for 1 h at 37°C and 5 % CO_2. Cells were overlaid with 200 µl of 1.6 % CMC medium and further incubated for 4 days at 37°C and 5 % CO_2. Medium was aspirated and cells were fixed in 1 ml 3.7 % formaldehyde for 30 min, stained with naphthalene blue black and washed once with water. The number of plaques/well was counted and the titer for 50 % plaque reduction was calculated in comparison to the virus titer obtained for OPV-negative marmosets serum which served as control. Human anti-VACV immunoglobulin was used as a positive control.

Human anti-VACV immunoglobulin:

Omrigam 5 % (G2H50CN HO4021, Israel, 2003), VIG (VACV immunoglobulin) (CBER/FDA Bethesda, MD 20892)

3.10 IFNγ ELISpot

The Enzyme-Linked ImmunoSpot (ELISpot) assay is a highly sensitive immunoassay, allowing the detection of secreted cytokine at the single cell level [192] with detection levels that can be as low as one cell in 100 000. The ELISpot has proven particularly useful when studying small populations of activated cells like those regularly found in specific immune responses.

3.10.1 Isolation of peripheral blood mononuclear cells (PBMC)

Blood, diluted 1:1 in 1xPBS, was added carefully onto a ficoll cushion (Merck, Darmstadt, Germany). After centrifugation at 400 g for 30 min at room temperature the PBMC containing phase was aspirated. The blood plasma in the layer above the PBMCs was collected and used for serological analyses. PBMCs were washed twice with 1xPBS and centrifuged at 400 g and 300 g for 10 min, respectively. RPMI-1640 medium was added and cell numbers were determined.

3.10.2 IFNγ ELISpot

For the detection of IFNγ-secreting cells the ready-to-use human-IFNγ ELISpot^{Pro} kit (Mabtech, Nacka Strand, Sweden) was used according to the manufacturer's protocol. PBMCs were added into anti-IFNγ monoclonal pre-coated wells (96-well format) and incubated at 37°C and 5 % CO_2 for 2 days. After removal of PBMCs by washing, anti-IFNγ-HRP (horseradish peroxidase)-conjugated antibodies were added. Finally, TMB (3.3', 5.5'-tetramethylbenzidine) substrate was added which formed a colored, insoluble precipitate when enzymatically converted by the HRP. A visible spot was formed at the site of IFNγ-producing cells. An automated ELISpot reader counted the spots and the frequency of positive cells were determined.

3.11 Extraction of nucleic acid

3.11.1 DNA extraction from virus stock

For viral DNA extraction from virus stocks the QIAamp Blood Kit (Qiagen, GmbH, Hilden, Germany) was used according to the manufacturer's standard protocol. 200 µl virus stock was used and DNA was eluted in 200 µl AE buffer.

3.11.2 DNA and RNA extraction from blood samples

In a first step whole blood samples were centrifuged for 10 min at 200 g. Plasma was aspirated, aliquoted and stored at -20°C. The rest of the blood sample was mixed well and 50 µl or 100 µl were used for DNA extraction, depending on the blood volume available. Remaining blood was used for RNA extraction.

Prior to DNA extraction, PBS was added to a final volume of 200 µl. For the preparation of total DNA from blood the QIAamp Blood Kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer's protocol. Elution was performed in AE buffer in a volume equal to the starting blood volume (50 µl or 100 µl) to avoid further dilution.

For the isolation of RNA from blood a preceding erythrocyte lysis step was performed with RCLB (Red cell lysis buffer). A minimum of 150 µl blood were added to 10 ml RCLB and incubated for 5 min at room temperature for efficient lysis. Samples were then centrifuged for 5 min at 220 g at 4°C. Supernatant was discarded and cells were washed once with PBS. Cell pellet was resolved in 350 µl RLT buffer. RNA extraction was performed with the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA was eluted in

50 µl RNase-free water. DNA digestion was performed with the Ambion TURBO DNase Kit (Ambion, Darmstadt, Germany) to remove residual DNA. 2 µl of DNase was incubated with 50 µl RNA for 10 min at 37°C. Then additional 1.5 µl DNase was added and samples were incubated for further 30 min at 37°C. All other preceding steps were performed according to the manufacturer's protocol.

Buffer:

RCLB (red cell lysis buffer): 8.29 g NH_4Cl (Merck, Darmstadt, Germany), 1.0 g $KHCO_3$ (Merck), 0.372 g EDTA (Sigma-Aldrich, Deisenhofen, Germany), ad 1 l ddH_2O, sterile filtration, autoclaved

Chemicals:

ethanol (100 %) (RNase-free) (Carl Roth, Karlsruhe, Germany), diethyl pyrocarbonate (DEPC) (Merck)

3.11.3 DNA and RNA extraction from tissue samples

For tissue samples a defined weight of tissue that was shock-frozen in liquid nitrogen was homogenized in 1 ml PBS using Precellys-ceramic beads (peQ Lab, Erlangen, Germany) and a rotor-stator homogenizer (FastPrep®FP120 machine, MP Biomedicals, Heidelberg, Germany). Homogenization was performed for 2x20 sec at 4 or 6 m/sec, depending on the type of tissue. Homogenates were kept at 4°C immediately after homogenization. Equal volumes of 100 µl and 200 µl of homogenates were stored at -70°C until use. For DNA extraction 100 µl homogenate and the QIAamp Blood Kit (Qiagen, GmbH, Hilden, Germany) were used according to the manufacturer's protocol. Elution was performed in 100 µl AE buffer. In addition, DNA extraction was also done with the DNeasy Tissue Kit (Qiagen) according the manufacturer's standard protocol to compare the DNA yield for both kits. For RNA preparation 100 µl tissue homogenate and the RNeasy Mini Kit (Qiagen) were used according to the manufacturer's protocol. RNA was eluted in 50 µl RNase-free water. For difficult tissue samples tissues conserved in RNA-later were homogenized in 700 µl RLT-buffer, and RNA was extracted using also the RNeasy Mini Kit according to the manufacturer's recommendations. Elution was done in 50 µl RNase-free water. In addition, a DNA digestion was always performed after RNA extraction to eliminate residual DNA using the Ambion TURBO DNase Kit (Ambion, Darmstadt, Germany). RNA was incubated with 2 µl DNase for 10 min at 37°C, then additional 1.5 µl DNase was added and samples were incubated for further 30 min at 37°C. All other preceding steps were performed according to the manufacturer's protocol.

Chemicals:

ethanol (100 %) (RNase-free) (Carl Roth, Karlsruhe, Germany), diethyl pyrocarbonate (DEPC) (Merck, Darmstadt, Germany)

Additional equipment:

forceps (tweezers) and microscopic scissors (both stainless steel) (both Carl Roth)

3.11.4 cDNA synthesis

cDNA was generated by reverse transcription (RT) in a total volume of 20 µl. 10 µl RNA, 500 ng calf thymus DNA (Sigma Aldrich, Hamburg, Germany) and 500 ng oligo $dT_{(18)}$ primer (Metabion, Martinsried, Germany) were incubated at 65°C for 5 min before 4 µl 5x buffer, 2 µl 0.1 M DTT, 0.4 µl 25 mM dNTP and 200 U Superscript II reverse transcriptase (all Invitrogen®, Karlsruhe, Germany) was added. Samples were then incubated for 52 min at 37°C and for 10 min at 70°C. cDNA samples were stored at -20°C.

Additional equipment:

thermal cycler MJ Research PTC-200 (Biozym, Oldendorf, Germany)

3.12 Polymerase chain reaction (PCR)

3.12.1 Primer design

To distinguish calpox virus from VACV-specific DNA and RNA, two real-time PCR assays were established. Because only parts of the calpox virus genome sequence were available, the clone 12/13 of calpox virus was used for primer and probe design. The genome sequence of CPXV BR (AF482758) was used as a reference sequence. All other real-time PCR assays had previously been established at the RKI. In addition, marmoset-specific cytokine real-time PCR assays were established. For primer and probe design the respective marmoset cytokine sequences (NCBI database) were used. The IL1b human-specific real-time PCR had been published previously [193]. Primer and probe selection was done in collaboration with Dr. A. Nitsche [194] using the BioEdit program, the website *www.idtdna.com/SciTools/SciTools.aspx* and the NCBI nucleotide blast search (blastn) [195]. In table 2 all oligo nucleotides are listed. Primer and TaqMan probes were synthesized by TIB Molbiol (Berlin, Germany) and TMGB-probes were produced by Applied Biosystems (Foster City, USA).

Table 2: Details of oligo nucleotides used in PCR reactions

Oligo name	Oligonucleotide sequence 5'→ 3'	S/A	Ta (°C)	Tm (°C)
Cal	**Amplicon length: 138 bp**		**62.0**	
Cal F	gTCTTTCTCgTTTACCAAgTgC	S		55.0°
Cal R	ACAgAgAAAACATTTAAggATgAATCTATA	A		55.1
Cal TMGB	F-ATAgCTCCgTTTATTTTgTTA NQF MGB	S		66.4
OPV	**Amplicon length: 140 bp**		**62.0**	
OPV F	gCCAATTgTCTTTCTCTTTTACTgA	S		56.2
OPV R	gAAAACATTTAAggATgAATCCATCT	A		55.4
OPV TMGB	F-CCTTCTATAgATCTgAgAAT NQF MGB	S		65.5
c-myc	**Amplicon length: 80 bp**		**60.0**	
c-myc F	gCCAgAggAggAACgAgCT	S		59.4
c-myc R	gggCCTTTTCATTgTTTTCCA	A		54.2
c-myc TM	F-TgCCCTgCgTgACCAgATCC-T	S		65.9
M13/ pCR2.1	**Amplicon length: 243 bp + insert**		**55.0**	
M13 F	gTAAAACgACggCCAg	S		50.7
M13 R	CAggAAACAgCTATgAC	A		47.0
CJ IL1β	**Amplicon length: 200 bp**		**62.0**	
HU IL1β F	TCTTCgAggCACAAggCAC	S		57.9
HU IL1β F	CAgAggTCCAggTCCTggAA	A		58.5
CJ IL1β TM	F-ACCTgAgCTCgCCAgTgAAATgATggCTT-T	S		65.1
CJ IL2	**Amplicon length: 219 bp**		**62.0**	
CJ IL2 F	TTTACTgCTggACTTACAgATgCTT	S		57.3
CJ IL2 R	gCTgATTATATCCCTggTgTCTCTTA	A		56.4
CJ IL2 TMGB	F-CTCCAgAggTTTgAgTTC NFQ MGB	S		69.0
CJ IL6	**Amplicon length: 65 bp**		**62.0**	
CJ IL6 F	CCTCAggAACCCAgCTATgAAC	S		58.4
Hu IL6 R	CCCAgggAgAAggCAACTg	A		58.2
CJ IL6 TM	F-CTCTCTCCACAAgCgCCTTCAgA-T	A		63.6
CJ IL10	**Amplicon length: 195 bp**		**62.0**	
CJ IL10 F	gCACCCACTTTCCAggCA	S		59.9
CJ IL10 R	ggCATCACCTCCTCCAggTAA	A		59.4
CJ IL10 TMGB	F-CTTTTgAAAgAAAgTCTTC NFQ MGB	S		67.0
CJ IL13	**Amplicon length: 116 bp**		**62.0**	
HU IL13 F	ggAgCTggTCAACATCACCC	S		58.2

HU IL13 R	CgTTgATCAgggATTCCAgg	A		55.4
CJ IL13 TM	Y- AACCAgGCCCCCCTCTGCAATg -NFQ	S		64.5
CJ IFNγ	**Amplicon length: 186 bp**		**62.0**	
CJ IFNγ F	TTTgggTTCTCTTggCTgTTAC	S		57.2
CJ IFNγ R	ATgTCTTCCTTgATggTCTCCA	A		56.0
CJ IFNγ TM	V-CTCTTTTggATgCTCTggT-NFQ	A		70.0
CJ TNFα	**Amplicon length: 213**		**62.0**	
CJ TNFα F	ACTTCTCTCTAATCAgCCCTCTgg	S		57.9
CJ TNFα R	gggAgTAgACgAggTACAgCC	A		57.1
CJ TNFα TM	F-CCTgTAgCCCATgTTgTAgCAAACCCTC-T	S		67.9

The base "G" is given as a small letter to avoid confusion with "C". **F** = FAM label, **T** = TAMRA label, **V** = VIC label, **Y** = YAK label, **TM** = hydrolysis probe, **TMGB**= hydrolysis probe coupled to an MGB moiety; **MGB** = Minor Groove Binder, **NFQ** = Non-fluorescent quencher, **Tm** = Melting temperature (calculated by nearest neighbor method), **Ta** = Annealing temperature, **A** = antisense orientation, **S** = sense orientation, **CJ** = *Callithrix jacchus*, **HU** = human; location of the oligonucleotide in reference to the respective NCBI GenBank entry: Cal: calpox virus clone 12/13; OPV: AF482758, c-myc: V00568, CJ IL1β: BT007213, IL2: DQ826674, CJ IL6: DQ658153, CJ IL10: DQ658154, IL13:NM002188, CJ IFNγ: X64659, CJ TNFα: DQ520835

3.12.2 Conventional PCR

Primer combinations were tested for their optimal annealing temperatures (best sensitivity and specificity) using a gradient cycler (Mastercycler ep gradient, Eppendorf, Hamburg, Germany) [196]. PCR was also used to generate plasmid standards. PCR components and cycling conditions are given in table 3.

The colony PCR allows a rapid screening for correct DNA vector constructs in bacterial colonies (*E. coli*). Bacterial colonies were picked with a sterile toothpick and added into the PCR master mix. For all colony PCR reactions the vector primer M13 F and the M13 R primer were used. All other components were the same as those used in conventional PCR (table 3).

Chemicals:

10xbuffer, $MgCl_2$, Platinum® *Taq* DNA-Polymerase (all Invitrogen™, Karlsruhe, Germany), dNTP (Amersham, Freiburg, Germany), water (DNase-free, Fluka) (Sigma-Aldrich, Deisenhofen, Germany)

Additional equipment:

GeneAmp PCR System 2400 and 9700 (Perkin Elmer, Norwalk, CT, USA)

Table 3: Components and cycling conditions for conventional PCR

	Approach 25 µl	Cycling conditions		
Water	14.3 µl			
10xbuffer	2.5 µl	95°C	5 min	
Mg_2Cl (50 mM)	2.0 µl	95°C	30 sec	
dNTP (25 mM)	2.0 µl	55-62°C#	30 sec	45 repeats
Primer S* (10 µM)	0.75 µl	72°C	30 sec	
Primer A† (10 µM)	0.75 µl	72°C	10 min	
Taq polymerase	0.2 µl			
DNA/cDNA	2.0 µl			

***S** = sense orientation, †**A** = antisense orientation, **#** annealing temperature depending on primer melting temperature

3.12.2.1 Agarose gel electrophoresis

To separate PCR products according to their size, agarose gel electrophoresis was used. Depending on the PCR fragment size, 1-2 % agarose-TAE solutions were boiled, and the liquid agarose gel was poured into the gel chamber. After the agarose solidified, 30 ml 1xTAE-EtBr buffer was added. Samples and a 100-bp size marker (Fermentas, St. Leon-Rot, Germany) were added to the preformed slots. By applying an electric current of 90-100 V for 30 to 60 min, amplified fragments moved through the gel matrix toward the anode because of their negative charge. Migration speed depended on the fragment length. Using ethidium bromide, which intercalates with double-stranded DNA, visualization of the PCR fragments was done under ultraviolet lights.

Buffers and solutions:

loading buffer (6x) for gel electrophoresis: 10 mM Tris-HCl pH 7.5 (Merck, Darmstadt, Germany), 2 mM EDTA (Sigma-Aldrich, Deisenhofen, Germany), 15 % (V/V) Glycerin (Merck), 0.1 % bromphenolblue (LKB, Bromma, Sweden)

TAE-buffer (50x) (pH 8): 242 g Tris Base (Merck), 57.1 g glacial acetic acid (Merck), 100 ml 0.5M EDTA (Sigma-Aldrich), ad 1 l ddH_2O

TAE-EtBr buffer (1x): 20 ml 50x TAE-buffer, 100 µl ethidium bromide (10 mg/ml) (Serva, Heidelberg, Germany), ad 1 l ddH_2O

peq GOLD Universal agarose (peQ Lab Erlangen, Germany)

Additional equipment:

gel electrophoresis power supply ST304 (Gibco BRL®, Eggenstein, Germany), gel electrophoresis chamber Horizon 58 (Gibco BRL®), microwave oven Privileg 8520 (Privileg, Fürth, Germany), video documentation system (Herolab, Wiesloch, Germany)

3.12.3 Real-time PCR

The real-time PCR is based on the polymerase chain reaction and can be used to simultaneously amplify and quantify a specific DNA sequence using fluorescence-labeled DNA oligonucleotide probes. After hybridization of these probes with complementary DNA strands a fluorescence signal is generated after each amplification cycle, accounting for the number of generated amplicons in real-time. Advantages of real-time PCR are the speed, the low risk of carry-over contaminations and the option of on-line analysis. The additional specificity obtained by the binding of a third oligonucleotide probe makes a real-time PCR assay much more reliable than a conventional PCR assay [197,198]. For quantification a plasmid standard with the respective target sequence and the housekeeping gene *c-myc* were measured in each run. All real-time PCR assays run on the ABI Prism™ 7500 or the ABI Prism™ 7900 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). The real-time PCR components and cycling conditions are listed in table 4.

Table 4: Components and cycling conditions for real-time PCR

	Approach 25 µl
Water	15.3 µl
10xbuffer	2.5 µl
Mg_2Cl (50 mM)	2.0 µl
dUTP (25 mM)	1.0 µl
Primer S† (10 µM)	0.75 µl
Primer A& (10 µM)	0.75 µl
Probe (10 µM)	0.25 µl
ROX*	0.25 µl
Taq polymerase	0.2 µl
DNA/ cDNA	2.0 µl

Cycling conditions		
95°C	10 min	
95°C	30 sec	45 repeats
60 or 62°C#	30 sec	

† **S** = sense orientation, & **A** = antisense orientation, * concentration depending on TaqMan instrument (for 7500 instrument 10 µM, for 7900 instrument 100 µM), # annealing temperature depending on primer melting temperature, varied for different real-time PCR assays, ROX: 6-carboxy-X-rhodamine

Chemicals:

10xbuffer, $MgCl_2$, Platinum® *Taq* DNA-Polymerase (all Invitrogen™, Karlsruhe, Germany), ROX (TIB Molbiol, Berlin, Germany), dUTP (Amersham, Freiburg, Germany), water (DNase-free, Fluka) (Sigma-Aldrich, Deisenhofen, Germany)

3.13 Cloning

3.13.1 Purification of PCR fragments after gel electrophoresis

If additional unwanted fragments were generated during PCR amplification, the desired PCR fragment was cut out of an agarose gel with a scalpel and purified with the JetQuick Gel Extraction Spin Kit (Genomed GmbH, Bad Oeynhausen, Germany) according to manufacturer's protocol. DNA elution was done in 25 µl DNase-free water.

3.13.2 TOPO TA cloning

To generate plasmid standards for real-time PCR, respective target sequences were cloned into a pCR2.1 vector (TOPO TA Cloning Kit, Invitrogen®, Karlsruhe, Germany). Preceding PCRs were performed with dNTPs and Platinum® *Taq* DNA-Polymerase. The Platinum® *Taq* DNA-Polymerase has a template-independent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector pCR2.1 features single overhanging 3´ deoxythymidine (T) residues that allow PCR inserts to ligate efficiently with the vector. Ligation and transformation reaction was performed according to manufacturers protocol using 2 µl PCR product and 50 µl or 100 µl of chemically competent TOP10 cells (Invitrogen™, Karlsruhe, Germany). Finally 20-100 µl were plated on LB-Amp++ plates and incubated over night at 37°C. As a transformation control 1 ng pEMBLE (Invitrogen™, Karlsruhe, Germany) was used.

Positive clones were selected using the blue-white screening. White colonies were picked with a sterile toothpick and were directly used for colony PCR, to verify the insert (see 3.12.2), and inoculated into 3 ml LB-Amp for over-night cultures to generate plasmid DNA.

Bacterial medium:

Luria-Bertani (LB)-Agar++: 10 g bacto-trypton, 5 g bacto-yeast extract (both Gibco BRL®, Eggenstein, Germany), 10 g NaCl, 15 g agar (both Merck, Darmstadt, Germany), ad 1 l ddH_2O, pH 7, autoclaved; ad: 500 µl 1 M IPTG (BTS-Biotech, St. Leon-Rot, Germany), 600 µl 40 ng/µl X-gal (BTS-Biotech), 600 µl 100 mg/ml ampicillin (Sigma-Aldrich, Deisenhofen, Germany)

LB-Amp medium: 10 g bacto-trypton, 5 g bacto-yeast extract (both Gibco BRL®), 10 g NaCl (Merck), ad 1 l ddH_2O, pH 7, autoclave; add ampicillin (100 mg/ml) (Sigma-Aldrich) to a final concentration of 0.1 mg/ml directly before use.

Additional equipment:

lab-shaker (Kühner, Switzerland), water bath (P-D Industriegesellschaft GmBH Dresden, Germany)

3.13.3 Plasmid preparation

The LB-Amp cultures were incubated over night on a shaker at 800 rpm and at 37°C. For the preparation of plasmid DNA the NucleoSpin® Plasmid Kit (Macherey Nagel, Düren, Germany) was used according to the manufacturer's instructions. DNA was eluted in 50 µl sterile water. Finally DNA concentrations were measured using a spectral photometer.

3.13.4 Calculation of plasmid copy numbers

The plasmid copy number was calculated with the following formula:

Plasmid size [bp] = vector size [bp] + insert size [bp]

1 bp dsDNA correspond to 660 g/mol

1 mol = 6,023 * 10^{23} mol-1 (Avogadro constant)

plasmid size [bp] * 660 g/mol = molarity [g/mol]

molarity [g/mol] / 6,023 * 10^{23} = weight [g/plasmid or ng/plasmid]

DNA concentration [ng/µl] / weight [ng/plasmid] = $\underline{\text{n plasmid [plasmid/µl]}}$

3.14 Sequencing

3.14.1 PCR product purification

Before PCR products were sequenced, a purification step was performed to eliminate interfering components like primers with the help of the QIAquick PCR-Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Elution was performed with 25 µl sterile water. Plasmid DNA was directly used for sequencing reaction without a purification step.

3.14.2 Sequencing

To analyze DNA sequences, the Sanger method which is also known as dideoxychain termination method was used [199]. For the sequence reaction the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) was used. A separate reaction was performed for each primer. The components and cycling conditions are listed in table 5. The separation of the fragments was done using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). All established sequences were analyzed with the SeqMan software (DNAstar Lasergene software 7.2.1, DNASTAR, Madison, Wisconsin, USA).

Table 5: Sequence reaction mix and cycling conditions

	Approach 10µl	Cycling conditions		
Primer	0.5 µl			
BigDye	1.0 µl	96°C	2 min	
10xBuffer	1.5 µl	96°C	10 sec	
ddH_2O	ad to 10 µl	50-60°C*	5 sec	45 repeats
DNA	s.b.	60°C	4 min	
DNA concentration:				
100-200 bp	1-3 ng			
200-500 bp	3-10 ng			
Plasmids	200-300 ng			

s.b. see below (depends on DNA concentration); ***** annealing temperature depends on the melting temperature of the used primer

3.15 Immunofluorescence test (IF)

To test plasma and serum samples for virus-specific IgM and IgG antibodies, infected Hep2 cells on glass slides (see 3.7.2) were used. Plasma and serum samples were pre-diluted 1:5 in IF-staining buffer. Samples were heat treated for 30-60 min at 56°C before use to inactivate virus as well as complement components. For IgM antibodies a preceding elimination of IgG antibodies was performed using an anti-human IgG antibody (Mastsorb absorb reagent, Mast Diagnostika, Reinfeld, Germany). For this, 30 µl IF-staining buffer, 20 µl plasma and 50 µl Mastsorb absorb were mixed. Samples were incubated for 30 min at room temperature and then centrifuged at 9600 g for 5 min. IgG-free supernatant was aspired and used for IgM analyses. Inactivated plasma samples were serially diluted twofold in IF-staining buffer. 20 µl of each dilution were added per cavity and incubated for 1 h at 37°C under humid conditions. After two washing steps for 5 min with 1xPBS 20 µl goat-anti-human IgG FITC-labeled (1:50) antibody or goat-anti-human IgM FITC-labeled (1:30) antibody (both Caltag, Burlingame, CA) was used as secondary antibody. Additionally, Evans Blue (0.1 g/100 ml) (1:1000) (Sigma-Aldrich, Deisenhofen, Germany) was added to counterstain the cytoplasm of the cells. After an incubation of 1 h in a humid atmosphere slides were washed 3 times with PBS for 5 min. If a nuclear staining was necessary, DAPI (1:10) (Serva, Heidelberg, Germany) was applied and slides were incubated for further 5 min at 37°C. Slides were washed twice with 1xPBS for 5 min and covered with mounting medium (Dako, Hamburg, Germany) and cover glass (24x60 mm, Carl Roth, Karlsruhe, Germany)

before they were analyzed under a fluorescence microscope (Axiovert 40cFL) or a confocal laser scan microscope (both Zeiss, Oberkochen, Germany).

Additional antibodies and buffers:

positive control: Omrigam 5 % (G2H50CN HO4021, Israel, 2003), VIG (anti-VACV immunoglobulin, CBER/FDA Bethesda, MD, USA)

IF-staining-buffer: 200 ml PBS, 2 % BSA (Sigma-Aldrich, Deisenhofen, Germany), 0.2 % NaN_3 (Carl Roth, Karlsruhe, Germany)

3.16 Histological and immunohistochemical staining

The histological and immunohistochemical staining (IHC) were done in collaboration with Dr. K. Mätz-Rensing at the German Primate Center (Göttingen, Germany). All histological and IHC pictures in this manuscript are printed here with her personal permission. Fixed tissues were embedded in paraffin using the Hypercenter XP machine (Thermo Shandon, Frankfurt/Main, Germany) according to the manufacturer's protocol. Afterwards tissues were processed into 3 µm sections using the microtome HM 400R (Microm, Walldorf, Germany). Paraffin sections were placed on glass slides and dried over night at 37°C.

3.16.1 Histological staining

For histological examination, tissues were stained with standard eosin-hematoxylin staining (HE) using the Varistain Gemini staining automat (Thermo Shandon, Frankfurt/Main, Germany). Tissue sections were deparaffinized and rehydrated as follows: xylol for 5 min/2 min/2 min, 100 % ethanol for 2 min, 96 % ethanol for 2 min, 80 % ethanol for 1 min, 70 % ethanol for 1 min and ddH_2O for 1 min. For nucleolus staining, sections were incubated for 3 min in haemalaun (Merck, Darmstadt, Germany) and then for 10 min in ddH_2O. For counter-staining, sections were incubated for 5 min in hydrous eosin (Thermo Shandon, Frankfurt/Main, Germany) followed by 5 sec in ddH_2O. Afterwards sections were dehydrated using an ascending alcohol series: 70 %, 80 %, 96 % and 100 % ethanol each for 1 min, 100 % ethanol for 2 min and xylol for 1 min and two times for 3 min. Finally tissue sections were covered with Eukitt (Kindler, Freiburg, Germany) and analyzed under a light optical microscope.

Chemicals:

ethanol (100 %) (RNase-free) (Carl Roth, Karlsruhe, Germany)

xylol (Sigma-Aldrich, Deisenhofen, Germany)

3.16.2 Immunohistochemical staining

Sections of every tissue sample were analyzed immunohistochemically for calpox virus antigens using human anti-VACV immunoglobulin (Omrigam 5 %, see 3.15) (primary antibody, 1:100 dilution) and human anti-IgG-AP labeled antibody (secondary antibody, dilution to manufacturers instructions) (Dako, Hamburg, Germany). Sections were deparaffinized and rehydrated like for histological staining. For antigen demasking, sections were incubated in 0.05 % trypsin (diluted in 1xPBS) for 20 min at 37°C and washed three times with Tris-buffer. Using the NexES-IHC staining module (VENTANA, Illkirch, France), antigen staining was performed according to the manufacturer's instructions. Finally sections were dehydrated, embedded and analyzed like for histological staining (see 3.16.1).

Buffer:

Tris-buffer 0.01 M (stock solution): 60.5 g Tris Base (Merck, Darmstadt, Germany) in 500 ml ddH_2O, pH 7.6, ad ddH_2O to 1000 ml, 90 g NaCl (store at 4°C or room temperature), *working solution:* 1:10 dilution from stock buffer just before use

3.17 Electron microscopy

Electron microscopy analyses were performed in collaboration with Dr. A. Kurth (RKI). Selected tissues were fixed in 2.5 % glutaraldehyde, at the time of necropsy. For pre-staining, tissues were incubated in 1 % osmium solution, tannin solution and uranyl acetate solution each for 1 h at room temperature. Dehydration and embedding procedures were performed using a Lynx tissue embedding automat (EMS, Hatfield, PA, USA). After dehydration, samples were embedded in epoxy (Serva, Heidelberg, Germany), and polymerization occurred at 60°C for 2 days and curing for additional 7 days. Then samples were sectioned into thin sections using the TM 60 trim instrument and the ultracut S ultramicrotom (both Reichert, Vienna, Austria). Afterwards tissues were stained with lead and analyzed using a Zeiss EM 10A transmission electron microscope (Zeiss, Oberkochen, Germany).

Buffers and solutions:

0.05 M HEPES-buffer: 5 ml 1 M HEPES-buffer (Sigma-Aldrich, Deisenhofen, Germany), 95 ml ddH_2O, pH 7.2

2.5 % glutaraldehyde: 10 ml glutaraldehyde 25 % (Serva, Heidelberg, Germany), 100 ml 0.05 M HEPES-buffer, pH 7.2

1 % osmium solution: 4 g osmiumtetroxid (Merck, Darmstadt, Germany), 400 ml ddH_2O

1 % tannin solution: 1 g tannin acid (Mallinckrodt, St. Louis, USA), 100 ml 0.05 M HEPES-buffer

2 % uranyl acetate: 0.4 g uranyl acetate (Merck), 20 ml ddH_2O, pH 4.4

4 Results

This chapter describes the development of the calpox virus/common marmoset model and begins with the development of a specific calpox virus and a generic OPV real-time PCR assay, both essential tools for monitoring the course of the infection in the animal experiments. The natural OPV outbreak in New-World monkeys implied that the calpox virus was highly pathogenic for common marmosets. To investigate the reproducibility of the clinical picture observed during the natural outbreak marmosets were infected i.v. with calpox virus. Establishing a natural route of infection in common marmosets further refined the model. After a successful i.n. virus application, titration experiments were performed to determine the lethal infectious dose (median monkey infectious dose at which 50 % of the infected animals die [MID_{50}]).

A biological characterization of the calpox virus was achieved by examination of the pathogenicity of calpox virus in mice. In addition, marmosets were infected with CPXV strain 81-02 to explore whether this closely related virus also induced a similar disease.
To investigate the suitability of the model for the evaluation of new vaccines, marmosets were immunized with VACV LE-BN and challenged with 10 MID_{50} of calpox virus.

4.1 Development of calpox virus- and OPV-specific real-time PCR assays

For the characterization and establishment of the marmoset model, a calpox virus-specific (Cal assay) and an OPV-generic (OPV assay) real-time PCR were designed and established for the discrimination between calpox virus and all other OPVs, particularly CPXV and VACV. At the time these investigations were started, the calpox virus genome had not yet been fully sequenced. Primers and probes for the real-time PCR assays were referring to calpox virus genes sequenced in our workgroup. The sequences of CPXV BR (AF482758) and VACV LE (LC16m8; AY678276) served as reference sequences. The Cal assay and the OPV assay were designed to bind in the coding region of the CPXV 82 protein of isolate CPXV BR and in the gene of the VACV LE DNA-binding phosphoprotein. The Cal assay specifically only detected calpox virus but no other known OPVs. In contrast, the OPV assay could detect all known OPVs except calpox virus and ECTV. The ability to differentiate between calpox virus and VACV was especially important for following the course of vaccination studies in which distinct discrimination between vaccine and challenge virus was needed. For the validation of the PCR assays calpox virus, four different VACV strains, two MPXV strains, two CPXV strains, one CLMV and one ECTV strain were tested (table 6). As

intended, the Cal assay was reactive only with calpox virus, and no cross-reactivity with other OPVs could be observed. The OPV assay, on the other hand, detected all OPVs except for calpox virus and ECTV (table 6). In addition, all virus DNA samples were investigated with the Rpo18 real-time PCR assay capable of detecting all OPVs (C_T values between 19.79 and 27.23).

Furthermore, variability and efficiency of the Cal and OPV assays were determined. Results of intra- and inter-variability for plasmid dilutions using cloned target sequences and DNA dilutions prepared from virus stocks for the Cal assay and the OPV assay are given in table 7. PCR efficiency for the Cal assay varied between 112.1 % and 93.7 % and for the OPV assay between 110.2 % and 90.6 % depending on the target used for PCR. The variability for both assays was low, although variability of C_T values seemed to depend on plasmid concentrations: with low plasmid copy numbers (10 or 10^2) a variability could be observed that was slightly higher than that with high plasmid copy numbers (10^5 and 10^6). Both assays featured a very low detection limit reaching down to ten copies.

Table 6: Reactivity of Cal assay, OPV assay and Rpo18 assay with different OPVs

	TaqMan assay (C_T)		
DNA*	**Rpo18**	**Cal**	**OPV**
calpox virus [a]	19.79	19.57	na
calpox virus [b]	23.20	23.03	na
VACV MVA	27.23	na	28.67
VACV LE-BN	26.18	na	28.54
VACV VR15	25.48	na	26.81
VACV M1	25.02	na	26.26
ECTV-NU	24.42	na	na
MPXV AP1	20.89	na	22.28
CLMV CP-19	26.92	na	27.99
CPXV 81-02	29.19	na	31.72
CPXV BR	24.68	na	29.01

***** DNA extracted from virus stocks with a medium virus load; **#** mean value was calculated from triplicates of one PCR run; **a** and **b**: different stocks of calpox virus resulting in different DNA concentrations; **na**: no amplification (C_T values > 40); **C_T**: threshold cycle; VACV MVA: Vaccina virus, strain Modified Virus Ankara; VACV LE-BN: Vaccina virus, strain Lister-Elstree-Bavarian Nordic, VACV VR15: Vaccinia virus, strain New York City; VACV M1: Vaccinia virus, strain M1; ECTV-NU: Ectromelia virus, strain Nuremberg; MPXV AP1: Monkeypox virus, strain AP1; CLMV CP-19: Camelpox virus, strain 19; CPXV 81-02: Cowpox virus, strain 81-02; CPXV BR: Cowpox virus, strain Brighton Red

Table 7: Variability and efficiency of the calpox virus-specific and OPV-specific real-time PCR assays

Assay	Virus	Target	Variability C_T		Efficiency [%]*
			intra-variability#	inter-variability+	
Cal	calpox virus	plasmid copies			
		10^6	17.68±0.46	17.70±0.02	
		10^5	20.60±0.03	20.76±0.22	
		10^4	23.75±0.07	24.00±0.36	112.1
		10^3	27.45±0.23	27.59±0.20	
		10^2	31.04±0.18	31.72±0.96	
		10^1	34.21±0.11	34.92±1.01	
		DNA			
		1:10	19.64±0.12	20.12±0.68	93.7
		1:100	23.02±0.11	23.62±0.86	
		1:1000	27.38±0.07	27.71±0.46	
OPV	VACV LE-BN	plasmid copies			
		10^6	19.27±0.08	19.30±0.08	
		10^5	22.41±0.09	22.45±0.19	
		10^4	25.55±0.15	24.76±0.91	110.2
		10^3	28.39±0.07	27.53±1.12	
		10^2	31.70±0.18	31.03±1.21	
		10^1	35.35±1.46	35.73±1.53	
		DNA			
		1:10	23.38±0.13	23.29±0.26	90.6
		1:100	26.98±0.47	27.31±0.93	
		1:1000	30.54±0.11	30.61±0.21	

***** mean value of efficiency was calculated from 3 independent standard curves for plasmids and DNA, respectively; C_T threshold cycle; **#** mean value was calculated from triplicates (using plasmids and viral DNA) of one PCR run; **+** mean value was calculated from 3 independent runs (using plasmids and viral DNA) on different days

Cross reactivity in both assays in the background of samples containing both calpox virus and VACV DNA in variable DNA concentrations was tested. A dilution matrix with each, a high load of calpox virus and a low load of VACV and vice versa, was tested with both assays. The results (table 8) indicated that the Cal assay as well as the OPV assay were specific for their respective virus targets and the C_T values not influenced by the presence of heterologous OPV DNA.

Table 8: Determination of genome copies (C_T values) for the Cal and OPV assay in mixtures of two OPV DNAs

		Cal assay (C_T)*				**OPV assay (C_T)***		
Calpox virus		1:10	$1:10^2$	$1:10^3$		1:10	$1:10^2$	$1:10^3$
VACV LE-BN	1:10	19.71	23.72	27.34		24.11	24.07	24.47
	$1:10^2$	19.88	23.85	27.25		28.61	30.09	31.04
	$1:10^3$	19.96	24.15	27.06		35.28	34.93	35.87
VACV M1	1:10	19.73	23.37	27.53		25.26	26.59	26.44
	$1:10^2$	19.43	23.52	26.98		35.55	33.75	35.01
	$1:10^3$	19.69	23.75	27.30		37.03	39.96	36.22

Determination of assay specificity in a mixture of different DNA concentrations of calpox virus, VACV LE-BN and VACV M1: **$1:10^1$** = DNA was diluted 1:10 in water; **$1:10^2$** = DNA was diluted 1:100 in water; **$1:10^3$** = DNA was diluted 1:1000 in water **C_T** = C_T value (threshold cycle); ***** mean C_T value was calculated from triplicate measurements of one PCR run; VACV LE-BN: Vaccinia virus, strain Lister-Elstree (Bavarian Nordic), VACV M1: Vaccinia virus, strain M1

4.2 Reproducibility of the natural disease by calpox virus isolated from cell culture

In order to determine if the isolated virus was capable of inducing a disease comparable to that in the natural outbreak observed in the common marmosets colony [165], five marmosets (group I) were administered i.v. either with $1.0x10^7$ pfu (animals I-a, I-b and I-c) or with $1.3x10^7$ pfu (animals I-d and I-e).

4.2.1 Viral load in blood

After i.v. infection a fast progression to severe disease could be observed. All animals inoculated i.v. died, between day four and day seven post infection (p.i.) (table 9). To determine calpox virus loads, the Cal assay (calpox virus-specific) was used. In general, viral genomic DNA which is equivalent to virus genome equivalents [GE] as well as viral mRNA as an indicator for actively replicating virus were determined. Calpox virus GE were standardized to 1 ml blood and calpox virus mRNA copy numbers were standardized to 10^6 c-myc mRNA copy numbers corresponding to approximately 10^6 blood cells per ml blood. Blood samples analyzed at the time of death showed high levels of calpox virus DNA, between $1.0x10^6$ and $3.3x10^9$ calpox GE/ml blood (table 9). High levels between $5.1x10^6$ and

$1.4x10^8$ copies of calpox mRNA per 10^6 copies c-myc mRNA were determined, indicating replication of calpox virus in blood cells. Plasma samples tested for OPV-specific IgM and IgG antibodies were negative for all animals, implicating a fast progression of the infection leading to death prior to the development of a humoral immune response.

Table 9: Viral loads in blood of marmosets infected with calpox virus by i.v. inoculation

group	**animal no.**	**dose [pfu]**	**days survived**	**calpox viral load in blood (in the final stage of disease)** #	
				[GE/ ml blood]	[calpox mRNA copies/10^6 c-myc mRNA copies]
I	a	$1.0x10^7$	5	$1.0x10^6$	$5.1x10^6$
	b		7	$8.0x10^8$	$4.9x10^7$
	c		7	$6.9x10^8$	$2.5x10^7$
	d	$1.3x10^7$	4	$4.2x10^8$*	$3.5x10^7$
	e		6	$3.3x10^9$	$1.4x10^8$

calculation of the copy numbers is based on the mean value of duplicate measurements and a respective plasmid standard for each real-time PCR assay; * viral load in serum instead of blood; **GE**: genome equivalents;

4.2.2 Clinical symptoms and pathological findings

The first clinical symptoms observed were breathing difficulties, languishment and anorexia preceding death by only one day. Macroscopic inspection showed that all five animals had symptoms comparable to other OPV infections. They had few papular small skin lesions with a diameter of 2-3 mm on the face (figure 5A), breast and the inner sides of the thighs which often coincided with focal hemorrhages. All marmosets developed lymphatic hyperplasia of the spleen and severe lymphadenopathy of submandibular and inguinal lymph nodes accompanied by moderate hemorrhages. Acute moderate hemorrhages occurred in the intestine of all animals (figure 5B), urinary bladder (animals I-b, I-d), liver (animal I-c), lung (animal I-e) and testis (animal I-d).

Histological examination showed a multifocal vesicular dermatitis with intradermal and subepithelial bleeding in skin and oral mucosa. The dermal alterations were characterized by epidermal acanthosis and acantholysis leading to vesiculation (figure 5C). Typical intracytoplasmic inclusion bodies were found in degenerated keratinocytes (figure 5C). The Guarnieri bodies were of different size and were distributed randomly within the altered epithelium. Lymph node inspection showed follicular hyperplasia with activation of the

follicle centre, severe necrotizing lymphadenitis and wide area bleeding mainly in the submandibular lymph nodes. In the liver diffuse degeneration of the hepatocytes, peracute bleeding (animal I-c) (figure 5D) and a beginning necrotizing hepatitis were found. Investigation of colon and small intestine indicated severe peracute focal hemorrhages and focal erosive enteritis (figure 5E). Severe peracute focal hemorrhages could also be found within the testis (animal I-a and I-d) (figure 5F). Minimal focal bleeding, alveolar edema and hyperemia were detectable in all lung specimens as well as in heart and kidney tissue.

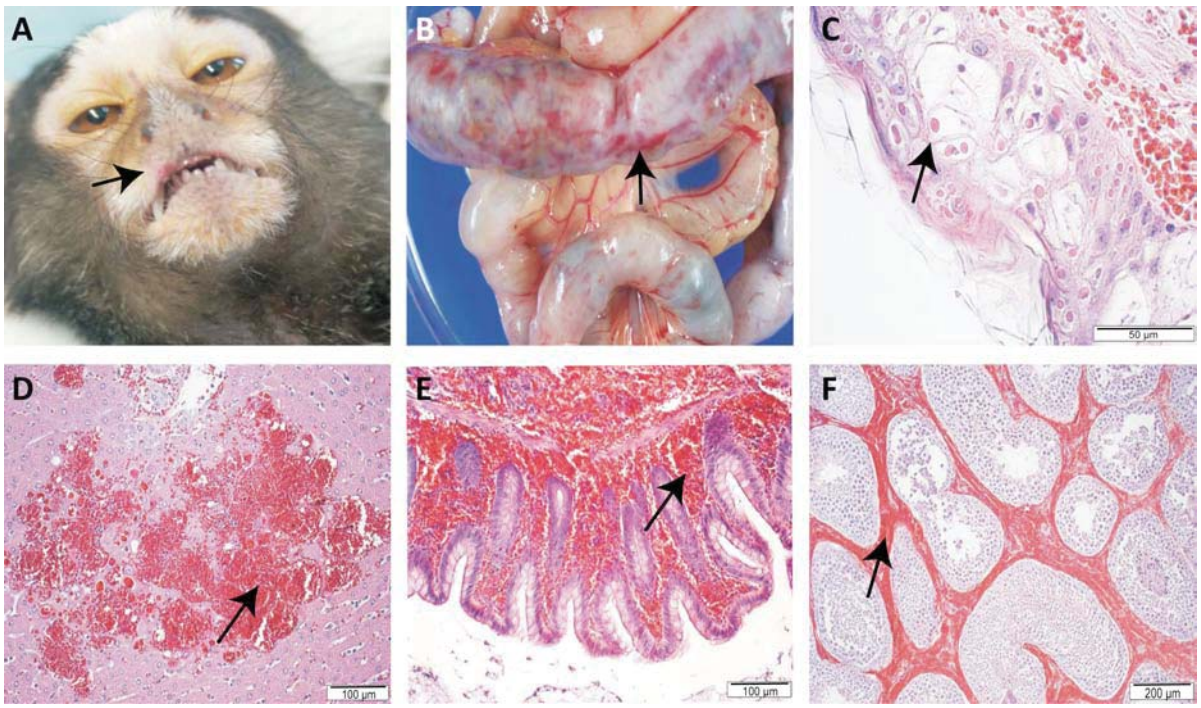

Figure 5: Macroscopic examination (A and B) and histological hematoxylin eosin (HE) staining of tissue sections (C-F) of i.v. infected marmosets; **A)** typical skin lesion in the area of the upper lip (indicated by arrow) (animal I-a); **B)** focal hemorrhages (arrow) in the intestine; **C)** skin area of a typical pox lesion, epidermal acantholysis leading to vesiculation, inclusion bodies (arrow) were distributed randomly within the altered epithelium; **D)** peracute bleeding in liver tissue (colored in red) surrounded by healthy liver tissue (colored in purple) (animal I-c); **E)** focal hyperemia and hemorrhages in the colon (colored in red) (animal I-d) and **F)** focal hyperemia and hemorrhages (colored in red) in the testis (animal I-a)

4.2.3 Viral load in tissues

Calpox virus DNA was detectable in all tested tissues (see figure 6A) of the five marmosets using quantitative real-time PCR. The number of calpox virus GE copies was standardized on 10^4 cellular c-myc DNA copies (c-myc GE) to compare the same number of cells. This allowed a comparison of the viral loads of different tissues within one animal and between different animals. Generally, copy numbers for calpox virus genomic DNA varied between different tissues but only slightly between the two applied infectious doses. The highest levels of calpox virus GE (median $>10^6$ calpox GE/10^4 c-myc GE in all i.v. infected marmosets) were found in skin, lung, liver, spleen, lymph node, heart and kidney, the lowest calpox GE levels, with medians as little as 10^4 calpox GE/10^4 c-myc GE, were found in stomach and colon. All other tested tissues had median viral loads between 10^4 and 10^6 calpox GE/10^4 c-myc GE (see figure 6A).

Actively replicating calpox virus was determined on the basis of viral mRNA, using RNA extraction followed by cDNA synthesis and quantitative real-time PCR (figure 6B). Viral mRNA copy numbers were standardized on the mRNA copy numbers of the cellular gene c-myc. Highest and lowest levels of viral mRNA (median levels of all i.v. infected marmosets) were found to correspond to the calpox DNA levels observed in the tissues: in skin, lung, liver, spleen, and lymph node $>10^5$ calpox mRNA copies/10^4 c-myc mRNA copies could be determined (figure 6B) and tissues with low viral mRNA expression $> 10^2$ calpox mRNA copies/10^4 c-myc copies were stomach, small intestine and colon. The detection of viral RNA in all tissues tested indicated active replication of calpox virus in these tissues.

Viral loads of calpox virus DNA (GE) and viral mRNA expression levels were higher for some tissues of animals I-d and I-e, which were infected with the higher dose and died one to two days earlier than the marmosets I-a, I-b and I-c. However, the detection of high copy numbers of viral DNA and mRNA in all tissues indicated a fulminate and generalized infection of all infected marmosets by the i.v. route.

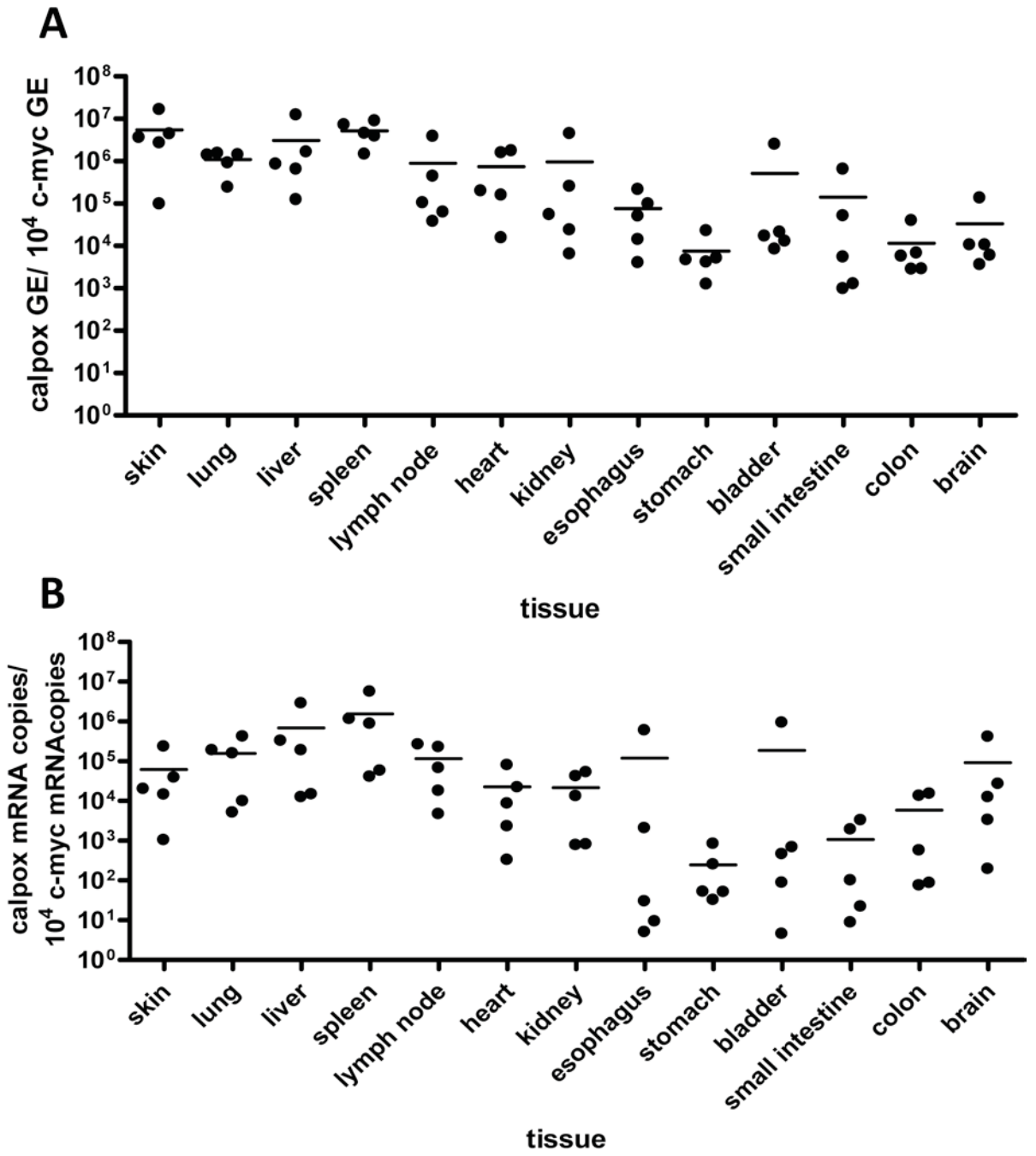

Figure 6: Determination of calpox virus in different tissues after i.v. infection by real-time PCR; **A)** calpox virus GE/10^4 c-myc GE ; **B)** calpox virus mRNA copies/10^4 c-myc mRNA copies; GE: genome equivalents (corresponding to DNA copy numbers); calculation of the copy numbers for calpox virus as well as c-myc is based on the mean value of duplicate measurements and a respective plasmid standard for each real-time PCR assay; lines indicate the median value for all five i.v. infected marmosets.

4.3 Establishment of a natural inoculation route

4.3.1 Oropharyngeal inoculation

Since the pathology of the naturally occurring infection of marmosets with the calpox virus was reproducible by i.v. inoculation, the subsequent step was to compare the course of infection of the i.v. application of the virus to a more natural route of infection. Therefore, three marmosets (group II a-c) were infected with $1.0x10^7$ pfu by oropharyngeal (oro.) inoculation. Only one marmoset (II-c) showed symptoms of an infection and had to be euthanized on day 13 because of extreme breathing difficulties. At this time point calpox virus GE and mRNA were detectable in the blood of this animal with titers of $3.6x10^6$ calpox GE/ml blood and $7.2x10^5$ calpox mRNA copies/10^6 c-myc mRNA copies (table 10). At no time neither OPV-specific IgM nor IgG antibodies were found.

Aside from breathing difficulties, no other clinical symptoms were obvious. Macroscopic examination showed focal petechial bleeding and alveolar edema in the lung together with splenomegaly and follicular hyperplasia of the lymph nodes. The main pathological finding was an edema localized in the larynx area with vesicular lesions, leading to the destruction of the larynx area which caused the breathing difficulties observed. All other organs were without findings. Histological analyses of the larynx and pharynx showed necrotizing laryngopharyngitis with multiple Guarnieri bodies in altered mucosal tissue and hyperplasia of the epidermis with focal degeneration and acantholysis. Additionally, massive bacterial secondary infections were found which had led to a severe inflammation in these tissues. In all organs of animal II-c calpox virus could be detected. Highest DNA copy numbers with up to 10^5 calpox GE/10^4 c-myc copies were found in skin and spleen. Lowest DNA copy numbers down to 10^2 calpox GE/10^4 c-myc copies were determined for bladder, colon and brain. Actively replicating calpox virus was detectable in all organs except for esophagus, bladder and small intestine (data not shown).

Pathological findings suggested that the marmoset had either a preceding injury in the pharyngeal area at the time of infection or was injured during inoculation by pipetting the virus on both tonsils. Both other marmosets (II-a and II-b) were found to be healthy with no signs of infection. Neither calpox virus in blood nor virus-specific antibodies were detectable over a period of 42 days after inoculation.

The oro. virus application was not able to induce reproducibly a disease comparable to the course observed after i.v. infection. Therefore, in the next step, an i.n. inoculation of calpox virus, mimicking the natural route of smallpox infection, was investigated.

4.3.2 Intranasal infection

Virus application was performed similar to i.n. infection of mice (G.L. Smith, personal communication). Marmosets were narcotized and after laying them on their back, calpox virus was administered directly into both nostrils (50 µl per nostril). Two marmosets were inoculated with $2.3x10^6$ pfu (group III) and two marmosets with $3.5x10^5$ pfu (group IV) (table 10). The i.n. infection induced a lethal disease in all animals. Three animals died nine and the fourth ten days after inoculation.

Table 10: Marmosets inoculated with calpox virus using the oropharyngeal or intranasal route of inoculation

group	**animal no.**	**dosis [pfu] (route*)**	**days survived**	**viral load in blood** [calpox GE/ml blood]/[calpox mRNA copies/10^6 c-myc mRNA copies] #	
				day 7 p.i.	at death
II	a	$1.0x10^7$ (oro.)	42	nd/nd	nd/nd
	b		42	nd/nd	nd/nd
	c		13	nd/nd	$3.6x10^6/7.2x10^5$
III	a	$2.3x10^6$ (i.n.)	10	nd/$5.0x10^3$	$4.0x10^6/3.8x10^7$
	b		9	$4.0x10^5/4.5x10^7$	$1.4x10^8/5.8x10^7$
IV	a	$3.5x10^5$ (i.n.)	9	$1.7x10^5/1.4x10^7$	$9.7x10^7/1.2x10^8$
	b		9	$8.9x10^4/6.9x10^6$	$3.6x10^7/3.1x10^8$

***** route of infection: oro.: oropharyngeal, **i.n.**: intranasal; **nd**: not detectable, the detection limits of the Cal assay and the c-myc real-time PCR assay are ≥10 copies; **GE**: genome equivalents; **p.i**.: post infection; **#** calculation of the copy numbers is based on the mean value of duplicate measurements and on a respective plasmid standard for each real-time PCR assay

4.3.2.1 Viral load in blood

Blood samples were taken on day seven p.i. and at the time of death. At day seven p.i., calpox virus DNA was detected by real-time PCR in three out of four animals (III-b, IV-a, IV-b) with viral loads between $1.7x10^5$ and $8.9x10^4$ calpox GE/ml blood (table 10). Calpox virus mRNA were detected on day seven p.i. in all four marmosets, with loads between $5.0x10^3$ and $4.5x10^7$ calpox mRNA copies/10^6 c-myc mRNA copies. At this time point none of the animals showed apparent clinical symptoms. Animals III-b, IV-a and IV-b died on day nine p.i. with final viral loads between $3.6x10^7$ and $1.4x10^8$ calpox GE/ml blood and $5.8x10^7$

to $3.1x10^8$ calpox mRNA copies/10^6 c-myc mRNA copies. Animal (III-a) which was calpox virus DNA negative on day seven p.i. died on day ten, with $4.0x10^6$ calpox GE/ml blood and $3.8x10^7$ calpox mRNA copies/10^6 c-myc mRNA copies (table 10). All four marmosets reached similar final viral loads in blood independent of the infectious doses. Neither IgM nor IgG OPV-specific antibodies could be detected in plasma samples of these marmosets until death.

4.3.2.2 Clinical symptoms and pathological findings

First clinical signs of infection appeared one to two days before death and were identical to i.v. infected animals (breathing difficulties, languishment and anorexia). Also macroscopic findings were similar to those of the i.v. infection, with symptoms in the upper respiratory tract and the lymphatic system accompanied by nasal discharge. All animals had pathological findings like edema in the mediastinum of the chest cavity and the larynx area (figure 7A), moderate purulent mediastinitis, splenomegaly, alveolar edema in the lung, edema in tonsils, lymphatic hyperplasia with focal bleeding and peracute necrosis (lymph node axillaris, inguinalis and submandibularis). Small sporadic pox-like lesions distributed on face, abdomen (figure 7B) and thighs were found in the skin except for animal III-a. These pathological findings indicated a systemic infection. Histological staining of the tissues revealed typical skin alterations characterized by focal vesicular dermatitis, syncytia formation and Guarnieri bodies. Minimal hemorrhage but severe hyperemia could be found in lung and liver. All other tissues were without specific pathological findings.

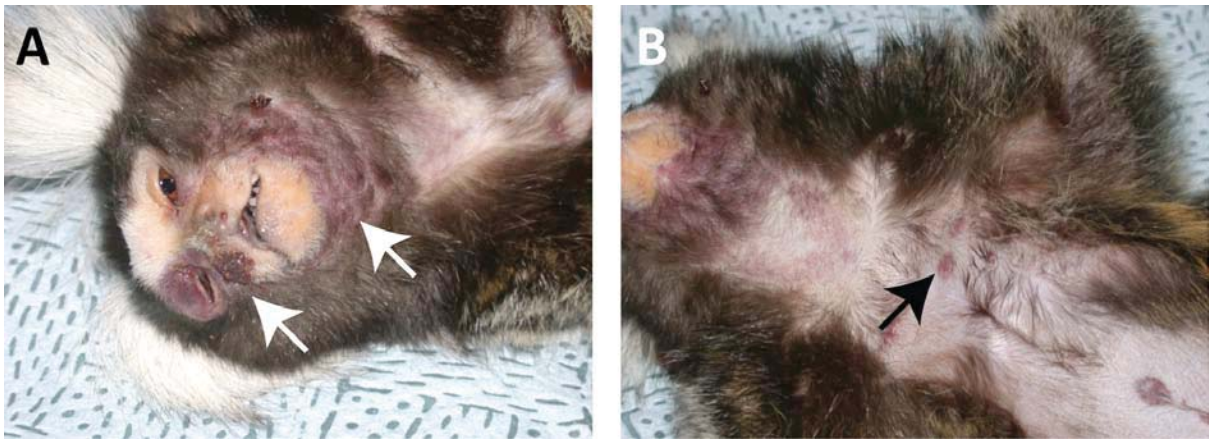

Figure 7: Typical macroscopic findings after i.n. infection of calpox virus; (shown for marmoset III-b); **A)** focal vesicular dermatitis in the face (focal papulation at the right ala of the nose) and hemorrhagic edema in the larynx area (both indicated by arrow) **B)** typical skin lesions in the breast area (indicated by arrow)

4.3.2.3 Viral load in tissues

Viral load in tissue samples of all animals showed similar virus distribution and final viral loads compared to the i.v. infection experiments (chapter 4.2.), with highest loads of calpox DNA in skin, lung, spleen, lymph node, esophagus and tonsils (above 10^5 calpox GE/10^4 c-myc GE) in all i.n. infected animals. Lower viral loads were found in heart, kidney, stomach, bladder, small intestine, colon and brain (down to 10^2 calpox virus GE/10^4 c-myc GE). Calpox virus mRNA was detected in all tissues tested, indicating active replication of calpox virus in these tissues. The highest virus expression was found in spleen and lymph nodes (above 10^5 calpox mRNA copies/10^4 c-myc mRNA copies). Lowest expression levels were determined in stomach, small intestine, colon and brain with as little as 10^2 calpox mRNA copies/10^4 c-myc mRNA copies. With both infectious doses used for i.n. infection, similar distributions of viral DNA and mRNA were found.

Using the i.n. route of infection a disease, with OPV symptoms and a lethal outcome, could be established in marmosets. Final viral loads of calpox virus DNA and mRNA in blood as well as in tissues indicated a systemic viremia and generalized infection. The successful induction of disease via a route of infection that resembles the natural way of transmission of VARV constituted a keystone in the development of this animal model as a model for smallpox disease.

4.4 Determination of calpox virus lethal dose

Having proved that calpox virus induced a lethal infection in marmosets with characteristic symptoms after both i.v. and i.n. inoculation, further experiments were performed using the i.n. inoculation route. To determine the MID_{50} (the amount of infectious virus that leads to the death of 50% of the infected animals), four additional groups (V-VIII) of each two marmosets were inoculated with decreasing doses of calpox virus (table 11).

4.4.1 Survival depending on the viral dose and calculation of the MID_{50}

Group V was infected with $3.5x10^4$ pfu. Continuously increasing calpox virus load was detected in blood from day seven p.i. on. Both animals died on day 14 (figure 8) with high viral loads $\geq 10^9$ calpox GE/ml blood and 10^8 calpox mRNA copies/10^6 c-myc mRNA copies (details table 11). In the two marmosets of group VI, inoculated with a ten times lower virus

dose ($3.5x10^3$ pfu), calpox virus genomic DNA was first detectable in the blood of animal VI-a on day seven. On day ten calpox virus DNA was found in the blood of both animals (VI-a and VI-b). Both marmosets died on day 14 with final viral loads $\geq 10^7$ calpox GE/ml blood (VII-a) (table 11). The last two groups (VII and VIII) were infected with $5x10^2$ pfu (group VII) and $1x10^2$ pfu (group VII). Only one (VII-a) out of four marmosets became infected and calpox virus could be detected in blood on day seven. The virus load increased continuously until day 14 when the marmoset died. The final calpox virus DNA load in this animal was $1.3x10^8$ calpox GE/ml blood (table 11). The other three animals did not develop any symptoms and survived 42 days, when they reached the pre-determined end of the study time frame. At no time point virus-specific IgM or IgG antibodies could be detected in these three animals. The surviving marmosets (VII-b, VIII-a, VIII-b) were re-inoculated with $5x10^2$ pfu of calpox virus. Animal VIII-a was successfully infected, and calpox virus genomic DNA was found in blood from day seven on. It died on day 14 with a final viral load of $3.6x10^7$ calpox GE/ml blood (table 11), showing the whole spectrum of OPV clinical symptoms. Animals VII-b and VIII-b showed no clinical signs of infection after 28 days. Neither calpox virus DNA/RNA nor virus-specific antibodies were detectable at any time point. These animals (VII-b and VIII-b) were inoculated with calpox virus for a third time, with a dose of $3.5x10^3$ pfu. One (VII-b) out of these two marmosets showed calpox virus DNA on day seven which then further increased to high final virus DNA copy numbers of $2.1x10^9$ calpox GE/ml blood on day 12 (death of animal VII-b). Animal VIII-b survived without developing any clinical symptoms and no calpox virus was detectable in blood or tissue. Furthermore, no virus-specific IgM and/or IgG antibodies were detectable during the observation period. The lack of OPV-specific findings in macroscopic and histological examinations confirmed that this animal was not infected.

Using the VACMAN 3.1 program [200], the MID_{50} of calpox virus was calculated. This program is generally used for statistical analysis of primate trials working with small animal numbers as well as virus dilutions other than 1:10 for virus titration. Standard statistical analysis programs do not take into account the effect of all the experimental errors incurred here, like dilution, virus stability or conspicuous variation between animals. The titration of the calpox virus used for infection resulted in a smallest infecting dose (SID) of $5x10^2$ pfu (2/5 died) corresponding to a virus stock dilution of 1:700. Hence, $8.3x10^2$ pfu are equivalent to one MID_{50} which is much lower compared to the MID_{50} used in other primate models.

Table 11: Calpox viral load in blood and survival after i.n. infection with different doses of calpox virus

group	# no.	dose [pfu]	first symptoms [day]	days survived	viral load in blood [calpox GE/ml blood]/ [calpox mRNA copies/10^6 c-myc mRNA copies][§]		
					day 7	day 10	day 14
V	a	$3.5x10^4$	14	14	$9.5x10^3/3.2x10^4$	$3.3x10^6/2.9x10^8$	$1.3x10^9/3.5x10^7$
	b		11	14	$1.5x10^4/2.2x10^5$	$1.3x10^7/3.2x10^8$	$2.2x10^9/2.1x10^8$
VI	a	$3.5x10^3$	11	14	$1.1x10^4/5.6x10^4$	$4.4x10^5/2.7x10^4$	$2.8x10^{7/}1.9x10^8$
	b		14	14	nd/nd	$1.3x10^4 7.9x10^5$	$3.5x10^7/1.1x10^8$
VII	a	$5.0x10^2$	14	14	$2.7x10^2/7.3x10^2$	$3.1x10^2/2.6x10^5$	$1.3x10^8/7.3x10^7$
	b		none	42[a]	nd/nd	nd/nd	nd/nd [a]
VIII	a	$1.0x10^2$	none	42[a]	nd/nd	nd/nd	nd/nd [a]
	b		none	42[a]	nd/nd	nd/nd	nd/nd [a]
1^{st} re-inoculation							
*VII	b	$5.0x10^2$	none	28[b]	nd/nd	nd/nd	nd/nd [b]
*VIII	a		14	14	$1.9x10^5/1.7x10^5$	$1.5x10^5/2.8x10^6$	$3.6x10^7/1.1x10^7$
*VIII	b		none	28[b]	nd/nd	nd/nd	nd/nd
2^{nd} re-inoculation							
					day 7	day 12	day 14
**VII	b	$3.5x10^3$	10	12	$4.4x10^4/4.2x10^6$	$2.1x10^9/2.8x10^6$	†
**VIII	b		none	28[b]	nd/nd	nd/nd	nd/nd[b]

 # no.: animal number**, a** virus negative until day 42 p.i. (pre-determined end of experiment) **b** virus negative until day 28 p.i. (pre-determined end of experiment); ***** first re-inoculation, ******second re-inoculation; **§** calculation of the copy numbers for calpox virus as well as c-myc is based on the mean value of duplicate measurements and a respective plasmid standard for each real-time PCR assay; †: animal already dead; **nd**: not detectable; the detection limit for the Cal and c-myc assay were ≥ 10copies per run; **GE**: genome equivalents

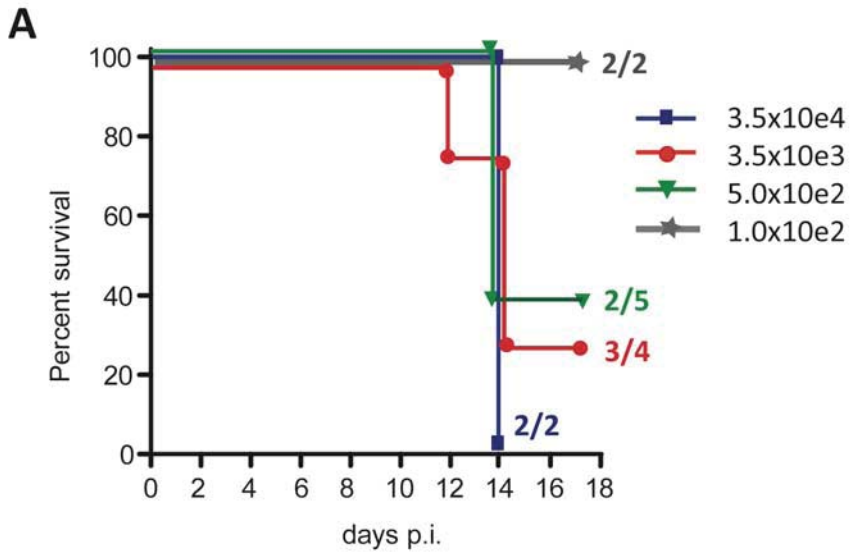

Figure 8: Kaplan Meyer plot of survival of marmosets infected intranasally with four different doses of calpox virus; percent survival post infection (p.i.)

4.4.2 The loss of body weight – a clinical prognostic marker for calpox virus infection?

The loss of body weight during an OPV infection in mice is a sensitive marker for the progression of the disease [201]. To test whether this may also be attributable to the calpox virus/marmoset model, body weight was determined twice a week at the same time when blood samples were taken. Daily examination of the body weight was not performed in an attempt to avoid additional stress with a potential effect on the body weight. Maximum weight loss was monitored in animals VI-b with 8.3 % and VII-b with 6.4 %. The body weight of all other animals varied in the range of ± 6.4 % from their initial body weight, independent of the infectious dose. These minor changes in body weight may be due on the short time frame between the onset of clinical symptoms and the noticeable loss of appetite just two days before death. Also, there was no placebo-infected control group and therefore these measurements could not be compared to body weight changes in the controls. The small changes in weight and the very short time frame in which the loss of appetite occurred render 'weight loss' unlikely to be a valuable clinical marker for the progression of calpox virus infection in common marmosets.

4.4.3 Clinical symptoms and pathological findings in animals inoculated with different doses of calpox virus

All marmosets that were infected died one to two days after the onset of clinical symptoms, a characteristic observed for all infections. After the onset of symptoms no

recovery from the disease could be observed independent of the infectious dose. All infected animals developed symptoms like sneezing, breathing difficulties, skin lesions, hemorrhagic edema in the larynx area, facial edema and severe lymphadenopathy.

Pathological findings were similar to the previous i.v. and i.n. infection experiments. Hemorrhagic hyperplasia, focal bleeding sites, inflammation processes and necrotizing lymphadenitis were typical for lymph nodes. Guarnieri bodies were found in skin, larynx, oral mucosa and in mucosa of the genitals. The larynx also showed focal bleeding and edema in muscle fiber tissue. In the lung, sub-acute interstitial pneumonia with formation of lymphatic inflammatory cell infiltrate was found. All animals showed the most substantial changes in tissues of the pharynx which seemed to be the primary area of virus manifestation. Macroscopic and histological examinations provide proof of a systemic infection in all infected marmosets.

Immunohistochemical (IHC) staining and electron microscopy (EM) of different tissues were performed to investigate the presence and localization of calpox virus in tissues. Both methods confirmed calpox virus infected cells in different tissue types. Calpox virus antigen was detected in spleen, lung, liver, salivary gland and lymph nodes using IHC staining (figure 9). EM examinations showed immature and mature virus particles, for example in spleen and liver tissue (figure 10).

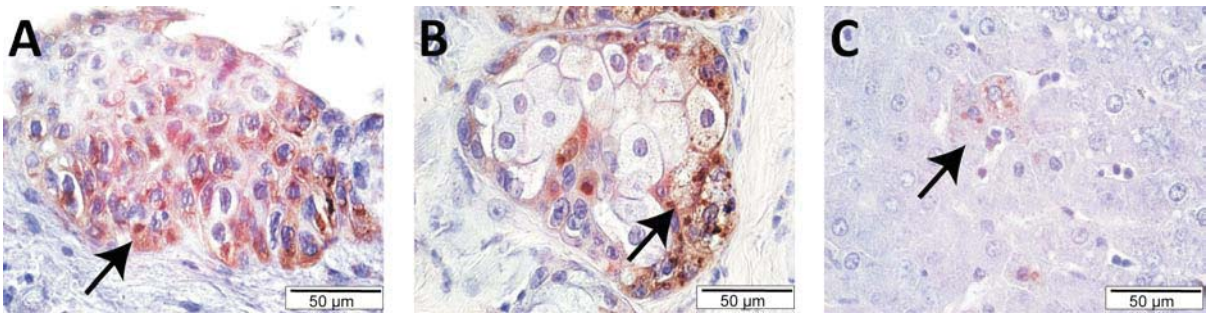

Figure 9: Immunohistochemical (IHC) staining of tissues after i.n. infection using human anti-VACV immunoglobulin (Omrigam 5 %) and anti-human IgG-AP-labeled antibody; **A)** focal vesicular dermatitis and staining of calpox virus antigen (colored in brown) (marmoset VI-b, infected with $3.5x10^3$ pfu); **B)** staining of calpox virus antigen (colored in brown) in sebaceous gland (marmoset VII-a, infected with $5x10^2$ pfu), **C)** staining of calpox virus antigen (colored in brown) in liver cells surrounded by uninfected cells (colored in blue) (marmoset VIII-a, infected with $5x10^2$ pfu); calpox virus infected cells are colored in brown and are further indicated by arrows

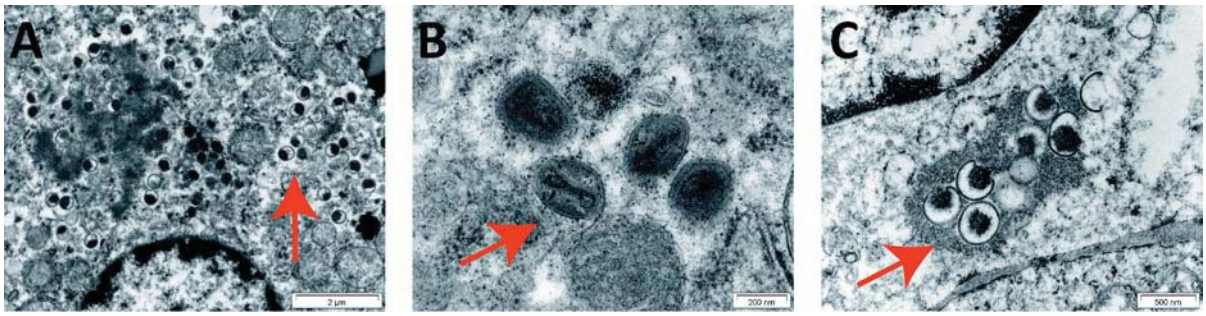

Figure 10: Electron microscopic micrographs of mature and immature calpox virus particles in different tissues of animal V-b; **A)** immature virus particles in liver tissue; **B)** mature virus particles in lung tissue and **C)** immature virus particles in spleen tissue; arrows indicate virus particles; all electron micrographs were taken with the help of Dr. A. Kurth (RKI, Berlin)

However, all animals that had been inoculated with calpox virus and survived showed no signs of infection. They were clinically healthy, and neither calpox virus DNA nor mRNA nor seroconversion was detectable.

4.4.4 Viral load in tissues of animals inoculated with different doses of calpox virus

Using real-time PCR, high levels of calpox virus DNA were detectable in all tissues of all infected marmosets. For all groups (except for group VIII in which all animals were inoculated but not infected) viral loads above 10^6 calpox GE/10^4 c-myc copies were found in skin, spleen and lymph nodes of all animals at the time of death. The lowest GE number was measured in heart, stomach, bladder, small intestine, colon and brain in all groups (under 10^3 copies) (data not shown). Detection of viral mRNA indicated actively replicating calpox virus in all tissues with the same distribution as calpox virus DNA. In summary, independent of the infectious dose, viral DNA and viral gene expression were highest in lymphatic tissues and tissues of nasopharynx and respiratory tract in all infected marmosets, whereas lowest copy numbers were found in tissues of the digestive tract.

Infectious viral particles were determined using the plaque assay. For this purpose the same tissue homogenates were used as for real-time PCR. Infectious calpox virus could be detected in all tissues analyzed. However, the infectious virus titers were about 3 to 5 logs lower than the number of GE measured by real-time PCR (figure 12A, animal VI-a infected with 3.5×10^3 and VII-a 5×10^2 pfu exemplarily for the respective group). The proportion of infectious calpox virus to viral genomic DNA (GE) within a tissue was comparable independent of the virus dose used for infection.

4.4.5 Infectious virus in saliva in animals inoculated with different doses of calpox virus

To investigate whether virus transmission could occur via droplets from aerosolized saliva, throat swabs were collected simultaneously to blood samples and analyzed for infectious calpox virus using the plaque assay. The appearance of calpox virus in salvia was approximately three days delayed compared to virus appearance in blood. Infectious calpox virus could be detected ten days p.i. in saliva of all animals except for marmoset V-a where calpox virus was detectable already on day seven p.i.. Calpox virus titers further increased reaching its highest titers at the time of death (figure 12B). The amount of infectious virus per throat swab was difficult to normalize because the volume of saliva collected could not be quantified (saliva in throat swab was resolved in 300 µl D-MEM medium). In addition, collected saliva was not cell-free and in consequence cell-bound virus might have been co-detected. These results underline a potential virus transmission from animal to animal via virus-containing droplets as described for naturally occurring smallpox infections.

4.4.6 Antibodies in plasma of animals infected with different doses of calpox virus

For immunological analysis plasma samples were routinely screened for virus-specific IgM and IgG antibodies, but only three out of eight marmosets (VI-a, VI-b and VII-a) showed seroconversion with maximal titers of IgM antibodies of 1:80 (figure 11) only detectable at the time of death. For none of the animals IgG antibodies were detected. Supposedly, all infected animals died before the development of a specific IgG antibody response.

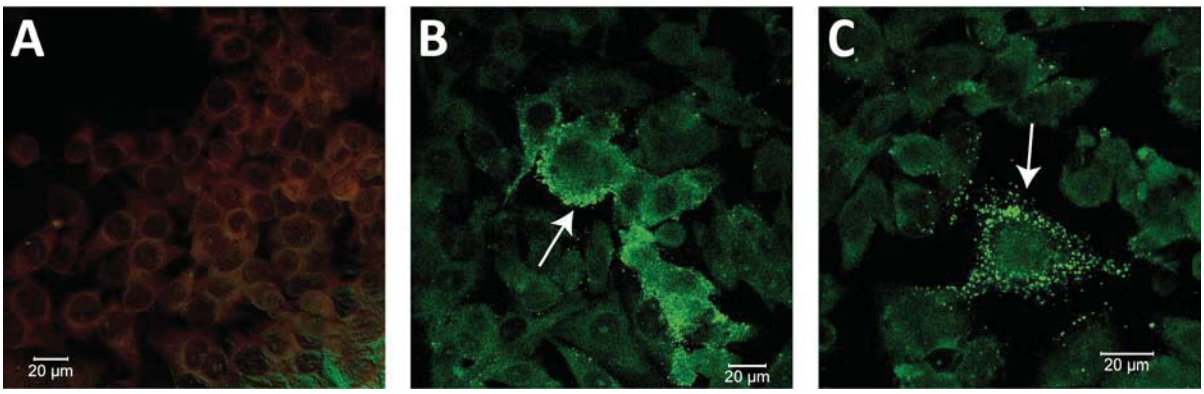

Figure 11: Determination of virus-specific IgM antibodies in the serum of infected marmosets at the time of death (day 14 p.i.) for different infectious doses. Calpox virus infected Hep2 cells were incubated with serum (1:10 diluted) and visualized with anti-human IgM-FITC labeled antibody 1:63 magnification; **A)** negative serum, **B)** animal VI-a, **C)** animal VII-a; virus antigens in the cytoplasm of infected cells are indicated by arrows

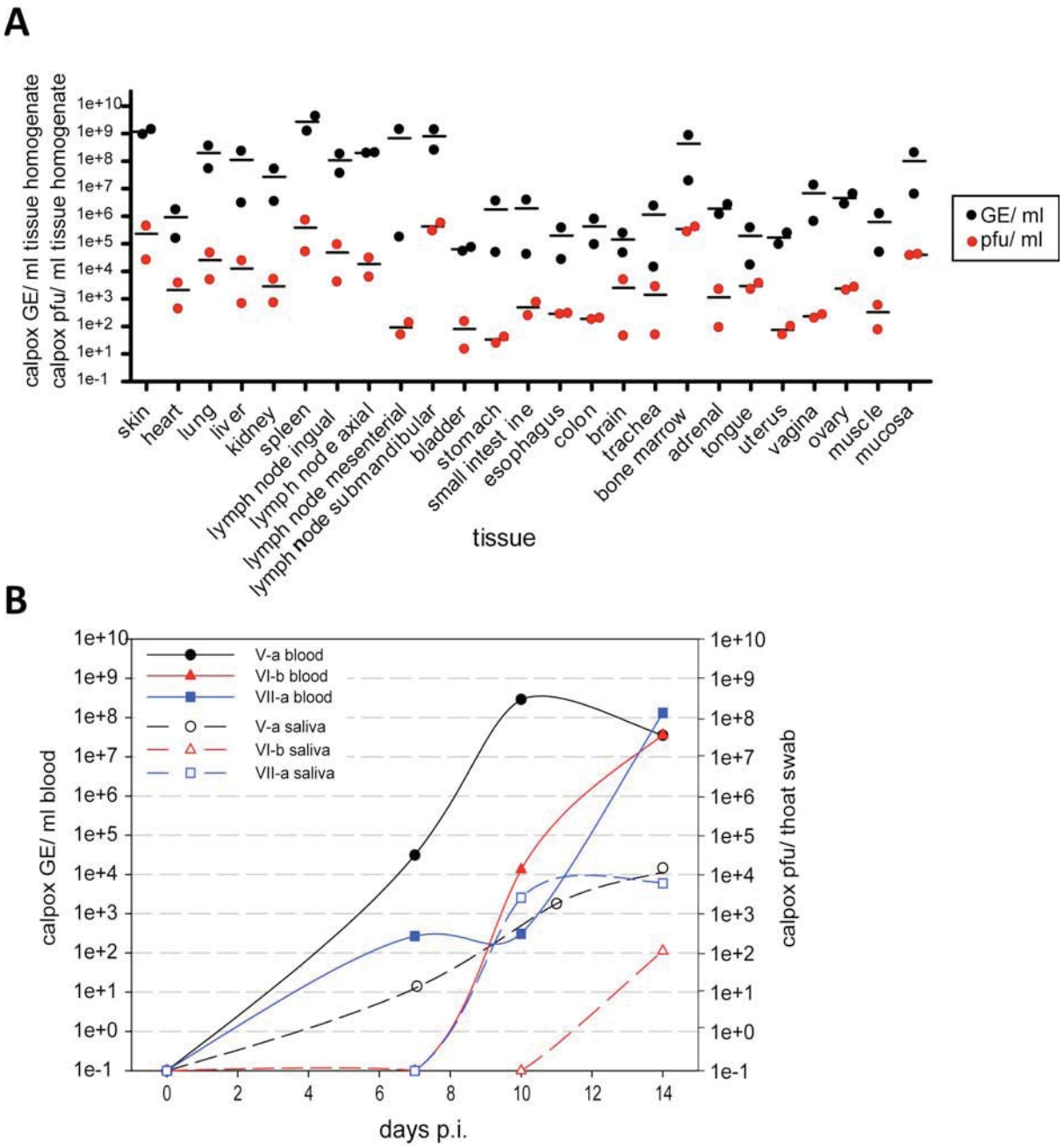

Figure 12: Detection of calpox virus in tissues and saliva; **A)** comparison of infectious calpox virus titer and calpox virus GE per ml tissue homogenate for two animals infected with $3.5x10^3$ (VI-a) and $5.0x10^2$ pfu (VII-a) (line indicates the median value for animal VI-a and VII-a); for real-time PCR: calculation of the copy numbers for calpox virus is based on the mean value of duplicate measurements and a respective plasmid standard; **B)** infectious calpox virus in saliva (normalization approximated by using throat swab as the denominator) compared to viral genome equivalents (GE) in blood (during course of infection) for three different infectious doses (group V: $3.5x10^4$, VI: $3.5x10^3$ and VII: $5.0x10^2$ pfu)

4.5 Infection of mice with calpox virus via intranasal inoculation

To further characterize the calpox virus in respect to its host range and pathogenicity in other animals, BALB/c mice as a common animal model for OPV infections were inoculated i.n. with $1x10^7$, $1x10^6$ and $1x10^5$ pfu (in 2x30 µl) of calpox virus (six mice each) to clarify its virulence in mice. A control group was inoculated with a lethal dose of $5x10^5$ pfu of VACV strain Western Reserve (VACV WR) and a further group was inoculated with 2x30 µl of PBS and served as negative control. Changes in body weight and typical signs of illness (SOI) like extensive ruffled fur, arched back, respiratory distress and reduction in motility as clinical markers for the disease progression were recorded daily during a period of 42 days.

Mice infected with $1x10^7$ pfu of calpox virus showed a significant reduction in body weight over the course of the infection (figure 13A). The first weight loss was measurable on day seven p.i., which further increased until day 13 with a peak of loss of 20 % of the initial body weight. After 14 days p.i. all mice gained weight, rebounding to initial weights on day 27. The group that was infected with $1x10^6$ pfu of calpox virus showed a marginal reduction of body weight of ≤ 3 % between day nine and 16 p.i.. The third group, infected with $1x10^5$ pfu of calpox virus as well as the PBS control group showed no significant loss of body weight during the observation time. Statistical analysis (table 12) using the Wilcox rank-sum test indicated a statistically significant reduction of the body weight after the infection with $1x10^7$ and $1x10^6$ pfu of calpox virus (p-value 0.0022) compared to the PBS control group. Mice infected with VACV WR showed rapid decrease of body weight from day three p.i. onwards. A peak of loss of 30 % of initial weight was reached already between days seven (two mice out of six) and eight (four mice out of six) when animals were sacrificed due to their bad health conditions.

In addition, the observed SOIs correlated with the loss of body weight (figure 13B). For mice infected with calpox virus only the $1x10^7$ infected group showed moderate signs at day seven p.i., which then deteriorated (ruffled fur and arched back) until day 13. All SOIs disappeared until day 18, nine days before the initial body weight was restored. The groups infected with $1x10^5$ and $1x10^6$ pfu of calpox virus as well as the PBS control group showed no signs of infection at any time. In contrast, the VACV WR-infected mice developed severe SOIs including pneumonia, shaking, huddled appearance, extensive ruffling and reduced mobility between day five and eight (euthanization).

Compared to VACV WR, calpox virus infection was less pathogenic in mice, since all inoculated mice survived even when inoculated with a high viral infectious dose of 10^7 pfu.

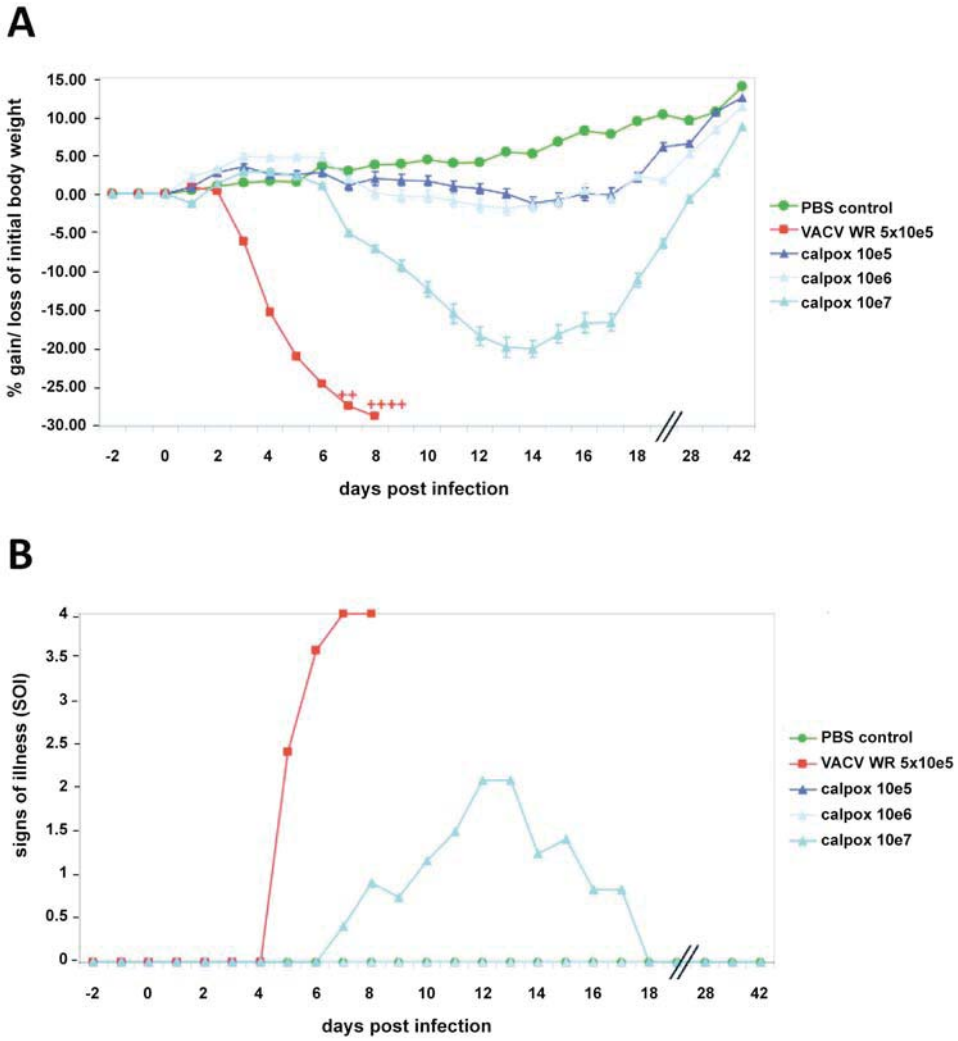

Figure 13: Intranasal infection of mice with calpox virus; **A)** changes in body weight (+ scarification of mice) and **B)** monitored signs of illness (SOI) during infection (0= healthy, 1= ruffled fur, 2= 1+ arched back, 3= 2+ respiratory distress, reduced motility, 4= 3+ diminished mobility); VACV WR: Vaccinia virus, strain Western Reserve

Table 12: Statistical analysis of body weight loss in calpox virus-infected mice using the Wilcoxon rank-sum test

group	**n**	**mean**[1]	**SD**[2]	**95 % -CI**[3]		**min**	**max**	**p-value**[4]
PBS control	6	76.0	5.91	69.8	82.2	68.6	85.6	/
VACV WR control $5x10^5$	6	-679.9	18.12	-698.9	-660.8	-704.6	-654.2	0.0022
Calpox virus 10^5	6	58.2	20.02	37.2	79.2	31.2	82.8	0.0931
Calpox virus10^6	6	34.4	24.18	9.0	59.8	3.2	64.6	0.0022
Calpox virus10^7	6	-26.2	20.63	-47.9	-4.6	-51.5	8.0	0.0022

[1] – mean AUC (area under the curve), [2] – standard deviation, [3] – 95% confidence limits, [4] – comparison with PBS control group

4.6 Infection of common marmosets with CPXV strain 81-02 via intranasal inoculation

To investigate whether other species of the OPV genus, besides calpox virus, have the ability to induce a severe disease in marmosets CPXV was used because calpox virus is phylogenetically closely related to other CPXV strains. Therefore, two marmosets (group XII) were infected with $5x10^5$ pfu of CPXV strain 81-02 by i.n. inoculation, in order to compare the pathogenicity and pathology to calpox virus. In the calpox virus/marmoset model this virus dose induced a lethal infection within nine days.

After inoculation CPXV DNA was detected in blood using the generic OPV real-time PCR not earlier than day 11 with viral loads of $1.6x10^4$ (XII-a) and $4.4x10^3$ (XII-b) CPXV GE/ml blood which further increased until day 18 p.i.. CPXV expression levels were similar in both marmosets and could be detected from day seven p.i. onwards. Infectious CPXV was also detectable in saliva from day seven (marmoset XII-a) and day 11 (marmoset XII-b) onwards, which further increased to final virus titers of $2.8x10^6$ (XII-a) and $1.1x10^3$ pfu/throat swab (XII-b) on day 18. As mentioned before, viral loads in saliva and blood were not directly comparable, but the progression over time was similar. Eighteen days after infection the experiment had to be terminated due to technical problems regarding animal husbandry. At the time of euthanization tissue samples of three organs (lung, liver and spleen) were taken. For both marmosets CPXV DNA as well as mRNA could be detected in these tissues. Standardization of CPXV copy numbers on 10^4 c-myc copies resulted in copy numbers between $1x10^5$ and $8x10^6$ CPXV GE/10^4 c-myc GE for animal XII-a and $1x10^2$ and $6.5x10^2$ CPXV GE/10^4 c-myc GE for animal XII-b for all three organs. Viral expression could also be detected in all three tissues of both animals with similar distribution in comparison to virus GE.

CPXV strain 81-02, applied i.n., displayed in comparison to infection with comparable doses of calpox virus a reduced viral load in marmosets together with a delayed detection of virus in the blood. In the blood of CPXV infected animals the viral load was approximately two to three log steps lower compared the to viral loads in calpox virus infected animals (comparing same time points after infection). Additionally, CPXV infected animals were still alive at day 18 p.i. and might even have survived the CPXV infection if the experiment did not have to be terminated through euthanization due to technical reasons. Marmosets infected with a comparable dose of calpox virus died on day nine p.i.. These preliminary data indicate that a classical CPXV (CPXV 81-02) is less pathogenic in marmosets than the calpox virus.

4.7 Immunization with Vaccinia virus Lister-Elstree (VACV LE-BN) and challenge with 10 MID_{50} of calpox virus

VACV LE-BN is one of the classical vaccines which was used in the smallpox eradication campaigns. To evaluate how suitable the newly established primate model is for the testing of vaccines, marmosets were immunized with $1x10^5$ pfu (in 3 µl) of VACV LE-BN, using the multi-puncture method with standard bifurcated needles identical to the technique used for immunization of humans. The titer of the VACV LE-BN vaccine was determined using the plaque assay and resulted in the expected final titer of $3.3x10^7$ pfu/ml. The first group (IX) was challenged with calpox virus, four weeks after immunization, and the second group (X) ten weeks after immunization. All animals were challenged i.n. with 10 MID_{50} of calpox virus ($8.3x10^3$ pfu). Non-immunized control marmosets (XI) were infected accordingly. Each group consisted of two marmosets except for the challenge control group with four marmosets. To validate the reliability of the results using such small animal numbers per group, the immunization experiment was repeated using same conditions with the exception of group X, which for technical reasons was challenged already after nine weeks instead of ten weeks, and only two control animals were infected instead of four.

4.7.1 Signs of successful immunization

After immunization the typical “vaccine take” with the development of a skin lesion at the site of immunization was detected in all immunized marmosets (figure 14). The skin lesion developed at day four to seven post immunization (p.im.) and reached a maximal size of about 1 cm in diameter two weeks p.im.. During the healing processes the size of the skin lesion began to decrease. After about four weeks the skin lesion had fully disappeared. No other clinical symptoms were observed after inoculation of VACV LE-BN.

After immunization VACV LE-BN DNA and mRNA were detected in the blood of all immunized animals except for animal IX-d, using the OPV real-time PCR assay (figure 16A). VACV LE-BN DNA was found as early as day seven p.im. in five out of eight animals and VACV LE-BN mRNA in six out of eight animals. Peak levels of DNA and mRNA copies in the blood were seen at day 14 p.im. (around 10^4 OPV GE/ml blood). VACV LE-BN was finally cleared from the blood 21 days p.im. and no circulating VACV LE-BN was detectable in the blood of all immunized marmosets at the time of challenge with a lethal infectious dose of calpox virus (10 MID_{50}).

To further characterize the course of immunization, saliva was taken (throat swabs) and analyzed for infectious VACV LE-BN particles (table 13). Only in four out of eight marmosets infectious VACV LE-BN was detectable as late as day 14 p.im. with low titers between $1.3x10^1$ and $5.5x10^3$ pfu/throat swab. In all other marmosets no infectious virus could be detected at any time.

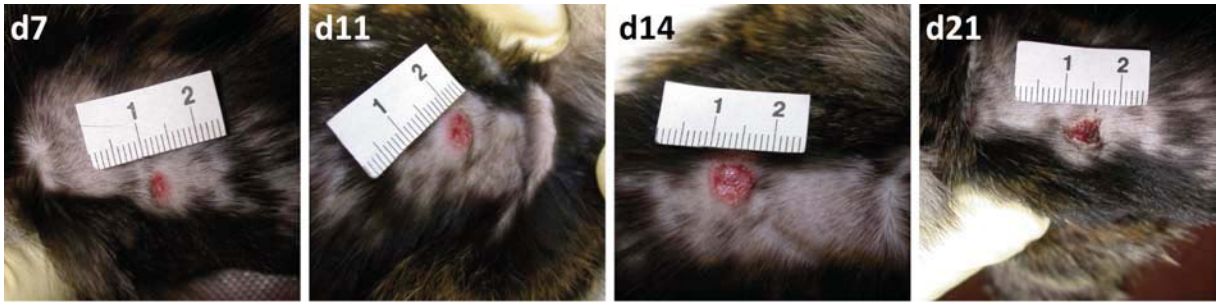

Figure 14: Progression of the skin lesion at the site of immunization; days after immunization are indicated with 'd' in the upper left corner of each picture, scale is stated in cm

4.7.2 Animal survival after challenge

None of the marmosets (four out of four animals) that were challenged with 10 MID_{50} of calpox virus nine or ten weeks p.im. (group X) developed clinical symptoms and were fully protected against the lethal infection (figure 15). In contrast, immunized animals challenged already four weeks p.im. (group IX) showed only a very limited protection. One out of four marmosets survived, but three marmosets died within 21 to 38 days post challenge (p.ch.). Although having a fatal outcome, the onset of symptoms as well as the time of death occurred was significant delayed compared to the non-immunized marmoset controls which died between day 12 and 15 p.ch.. These findings indicate that a period of four weeks is critical for the development of an effective protection against a lethal calpox virus infection after immunization.

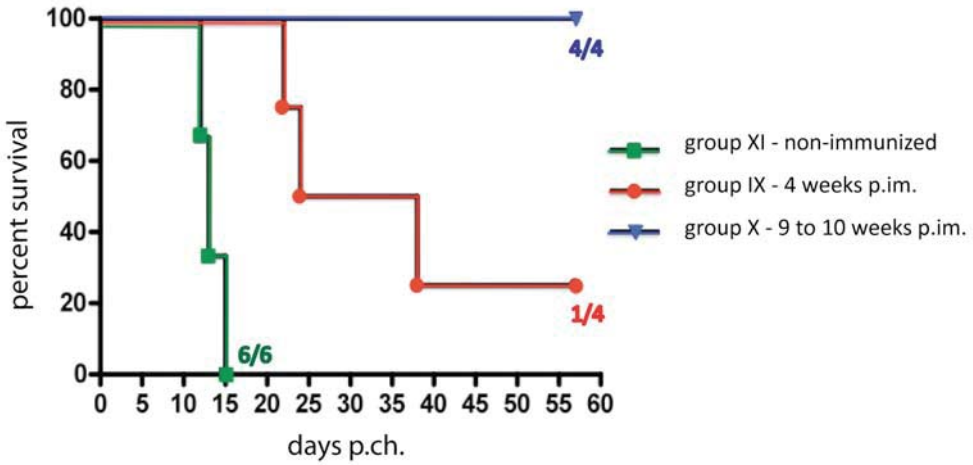

Figure 15: Kaplan Meyer plot of the survival of immunized and non-immunized marmosets after a lethal challenge with calpox virus; p.ch.: post challenge

4.7.3 Clinical symptoms and pathological findings after challenge

Clinical symptoms found in non-immunized animals (group XI) were similar to the above described disease progression for i.v. and i.n. infection. The onset of clinical symptoms of the three animals that died in group IX (challenged four weeks p.im.) was clearly delayed, and although the clinical picture was generally not as severe as in the control animals, death followed within 21 to 38 days. Macroscopic examination revealed that immunized animals developed only few skin lesions whereas non-immunized marmosets developed multiple skin lesions distributed over the body (face, breast, tail and genital area) and hemorrhages in skin areas of neck, breast, abdomen, inner thighs and mandible as well as edema with focal and petechial bleeding in the larynx. Moreover, these marmosets developed splenomegaly. Lymph nodes in regions corresponding to the skin lesions were marked by severe lymphatic hyperplasia. Surviving marmosets showed no clinical symptoms at any time during the infection.

4.7.4 Calpox viral load in the blood of challenged animals

The delayed onset of the disease as well as the severity of the disease in marmosets challenged four weeks p.im. and the complete protection of all marmosets challenged nine to ten weeks p.im. were reflected by the development of the calpox viral load in the blood of these animals.

Compared to non-immunized control animals (group XI), in the blood of group IX (challenged four weeks p.im.) calpox virus became detectable four to ten days delayed and in animals of group X (challenged nine to ten weeks p.im.) three to ten days delayed (figure 16B). In addition, at the time of death final copy numbers of calpox virus DNA and mRNA were reduced by a factor of two to four log scales in immunized animals compared to the final viral loads of non-immunized animals (XI) (figure 16B).

However, in group IX (challenged four weeks p.im.) calpox virus DNA was not detectable before day 14 p.ch. (IX-a and IX-b) or day 17 p.ch. (IX-c and IX-d) with copy numbers between $5.6x10^2$ and $9.4x10^4$ calpox GE/ml blood. From then onwards, viral load further increased until death. In the blood of animals IX-a, IX-b and IX-c final copy numbers between $3.1x10^4$ and $6.4x10^8$ calpox GE/ml blood were determined. Calpox virus copy numbers in the blood of the only surviving marmoset of group IX (IX-d) remained on the level of approximately $1x10^4$ GE/ml blood until the virus was finally cleared from blood after day 28 p.ch..

In marmosets challenged nine to ten weeks p.im. (group X) calpox virus was detectable in the blood from day ten p.ch. (X-c), day 14 p.ch. (X-a) and day 17 p.ch. (X-d) onwards, with viral loads between $8.2x10^2$ and $1.5x10^4$ calpox GE/ml blood. For animal X-c the viral load further increased until day 17 p.ch. Calpox virus was fully cleared from the blood of all three animals (X-a, X-c and X-d) after day 17 p.ch.. Only in the blood of animal X-b no calpox virus could be detected at any time during infection. All four marmosets remained healthy and survived the challenge.

Calpox virus was detectable in all non-immunized control marmosets (group XI) as early as day seven p.ch. (in two out of six animals) and then rapidly increased towards death to final copy numbers up to $9x10^8$ calpox GE/ml blood (days 12 to 15 p.i.; figure 16B).

Copy numbers of calpox virus mRNA also support active virus replication and showed a similar distribution to viral DNA in all animals (data not shown). In summary, all six non-immunized marmosets rapidly died with high viral loads in their blood whereas marmosets challenged four weeks p.im. (group X) died later, with lower final virus loads.

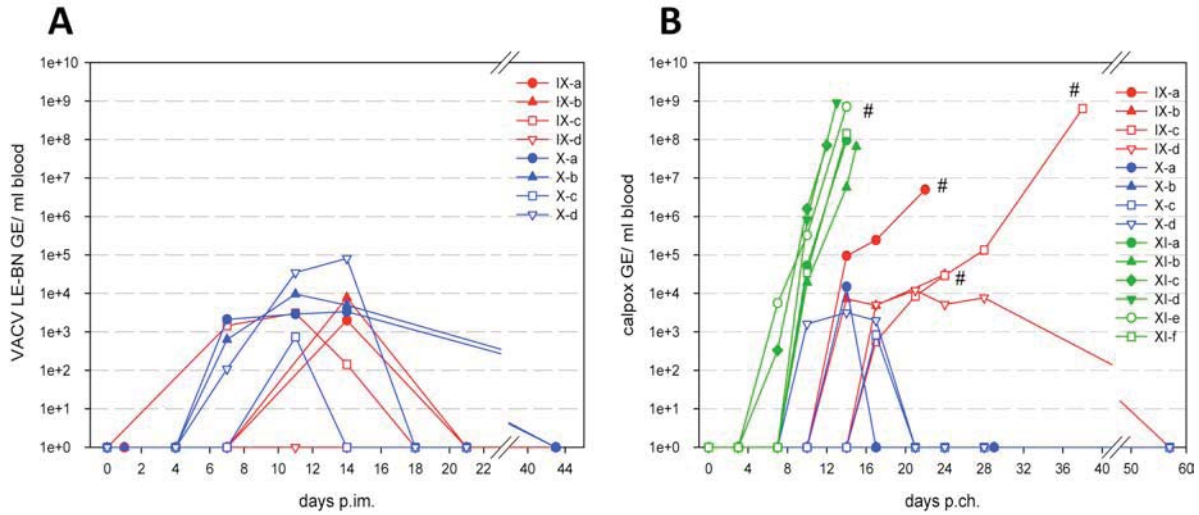

Figure 16: Viral load in blood determined by real-time PCR; **A)** VACV LE-BN viral load in blood after immunization and **B)** calpox virus viral load in blood after challenge; group IX: challenged four weeks after immunization; group X: challenged nine to ten weeks after immunization, group XI: non-immunized animals; filled symbols: animals of the first experiment; open symbols: animals of the second experiment; # time of death, p.im.: post immunization; p.ch.: post challenge

4.7.5 Changes in body weight after challenge

Although the monitored changes in body weight during titration experiments (see 4.4.2) were minimal, body weight was monitored during the immunization experiments, because the clinical course of the calpox virus-induced disease in immunized animals has never been described before. After immunization no significant changes in body weight could be observed (data not shown), but after challenge a loss in body weight could be seen in both immunized groups. Thus, the body weight at the time point of challenge was set as 100 % and changes were monitored during challenge.

Two animals of group IX lost less than 5 % of their initial weight (IX-c and IX-d). But animals IX-a and IX-b showed a significant loss of the body weight beginning around day 15 p.ch. and reaching the maximum at the time of death with approximately 15 % loss of body weight (figure 17A). Marmosets of group X showed a similar course (figure 17B). X-b showed a maximal weight loss of 6.2 % at day 17 p.ch. and X-d a maximal weight loss of 12.5 % at day 24 p.ch.. Both other animals, X-a and X-c, even gained weight. Body weight of non-immunized control animals remained stable except for animal XI-a which lost 8 % of initial body weight (figure 17B).

In summary, the maximum weight loss of 15 % for immunized animals was higher than for non-immunized animals. But still high variation in body weight within one group could be

observed. It seems that the change in body weight is not a good clinical marker for the course of the disease but it could be adducted.

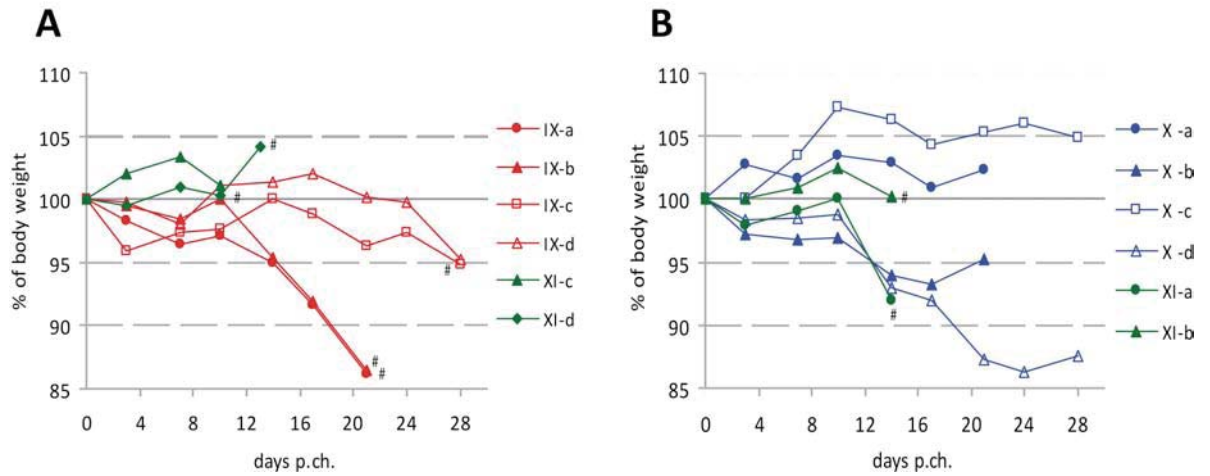

Figure 17: Changes in body weight after challenge with calpox virus in **A)** marmosets challenged four weeks after immunization and **B)** marmosets challenged nine to ten weeks after immunization; XI-a – XI-d: non-immunized marmosets, p.ch.: post challenge; # last measurement before death

4.7.6 Detection of infectious calpox virus in saliva after challenge

After challenge infectious calpox virus could be detected in the saliva of all non-immunized animals as early as day seven p.ch. (table 13) which increased until death to a maximal titer of approximately $4x10^4$ pfu/throat swab. In saliva of group IX infectious calpox virus was first detectable between day ten (IX-c), 17 (IX-b) and 21 (IX-a) p.ch. which then further increased until death to approximately $2x10^4$ pfu/throat swab (titer similar to control animals). For animal IX-d no calpox virus could be found after challenge. In group X in three out of four marmosets no infectious calpox virus could be detected. Only in saliva of animal X-c infectious calpox virus could be measured at day 17 p.ch.. The presence of calpox virus in saliva might serve as a predictive parameter for the outcome of the infection.

Table 13: Infectious virus in saliva (throat swabs) after immunization and challenge

A.)	**infectious VACV LE-BN (pfu/throat swab) after immunization**							
	immunized animals							
day p.im.	IX-a	IX-b	IX-c	IX-d	X-a	X-b	X-c	X-d
0	0	0	0	0	0	0	0	0
4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0
7	0	n.s.	0	0	$5.5x10^3$	0	0	n.s.
11	n.s.	n.s.	0	0	n.s.	n.s.	0	0
14	$1.3x10^1$	$1.3x10^1$	0	0	$3.4x10^2$	0	0	$1.3x10^1$
18	n.s.	n.s.	0	0	n.s.	n.s.	0	$1.3x10^2$
21	0	0	0	0	0	0	0	0

B.)	**infectious calpox virus (pfu/throat swab) after challenge**													
	immunized animals								**non-immunized animals**					
day p.ch.	IX-a	IX-b	IX-c	IX-d	X-a	X-b	X-c	X-d	XI-a	XI-b	XI-c	XI-d	XI-e	XI-f
3	0	0	0	1	0	0	0	0	0	0	0	$2.5x10^2$	0	0
7	0	0	0	1	0	0	0	0	n.s.	0	$4x10^2$	$5x10^2$	$2.6x10^2$	$3.5x10^2$
10	0	0	$2.5x10^1$	0	0	0	0	0	$6.3x10^2$*	$4.6x10^1$	$3x10^2$ *	$2.4x10^1$	$6.1x10^2$	$7.8x10^1$
14	n.s.	n.s.	$5.3x10^2$	0	0	0	0	0		$1.3x10^3$*		$2.8x10^3$*	$4x10^4$*	$4.3x10^4$*
17	n.s.	$2.3x10^1$	n.s.	0	0	0	$1.5x10^1$	0						
21	$1.3x10^1$*	$1.3x10^1$*	n.s.	n.s.	0	0	0	0						
28			n.s	n.s.	0	0	0	0						
38			$2x10^4$*	n.s.	n.s.	n.s.	n.s.	n.s.						

Infectious virus determined by plaque assay; **0** = no infectious virus detectable, ***** last collection before death of animal; **n.s.**: no sample available; **pfu**: plaque forming unit; **p.im.**: post immunization; **p.ch.**: post challenge

4.7.7 Calpox viral load in tissues of not protected marmosets

After death, tissue samples were analyzed for calpox virus using real-time PCR and plaque assays using the same tissue homogenate. Genome copy numbers (GE) and infectious virus titers (pfu) were standardized to one ml of tissue homogenate.

Immunized but not protected marmosets showed the highest DNA copy numbers of up to 10^8 calpox GE/ml tissue homogenate in skin, lung, lymph node inguinalis, trachea and bone marrow. Copy numbers as low as 10^4 calpox GE/ml tissue homogenate were found in kidney, bladder, small intestine, colon, brain, uterus and vagina. Compared to virus genome copy numbers, the infectious virus titer (pfu/ml tissue homogenate) was much lower in all organs (range 10^1 to 10^5 pfu/ml tissue homogenate) as already seen in previous i.n. infections. Surprisingly, no infectious calpox virus could be detected in stomach, bladder, small intestine, colon, brain, uterus and vagina.

Distribution of genome equivalents and infectious virus in non-immunized animals (group XI) was similar to previous i.n. infections in which calpox virus could be detected in all tissues with up to 10^9 calpox GE/ml tissue homogenate and infectious virus with up to 10^6 pfu/ml tissue homogenate.

4.7.8 Humoral immune response to VACV LE-BN and calpox virus

The seroconversion in immunized animals was followed with the help of immunofluorescence assays (figure 18). OPV-specific IgM antibodies could be detected between day 11 and day 21 p.im. with titer peak levels of 1:100 in all marmosets. The development of IgG antibodies is shown in figure 19A/B. OPV-specific IgGs became detectable in all immunized animals within two weeks p.im., Maximal IgG titers at day 21 p.im. varied between 1:100 to 1:1000.

At the time of challenge the antibody titers of all immunized animals were comparable (between 1:100 and 1:200) independent of the time p.im.. Three weeks after the challenge the IgG antibody titers of all immunized marmosets were boostered three- to fivefold (figure 19). The highest IgG titers were determined for animals IX-c, IX-d, X-c and X-d (second immunization experiment) with titers between 1:2000 and 1:8000 (at the endpoint: death or end of experiment) compared to endpoint IgG titers of 1:500 for animals of the first immunization experiment. All non-immunized control marmosets showed only OPV-specific IgM titers (between 1:10 and 1:80) but no IgG antibodies at the time of death.

Neutralizing antibodies were detectable from day 21 p.im. onwards. At the time of challenge neutralizing antibody titers varied between 1:10 and 1:80 in all immunized animals (figure 20). After challenge the neutralizing antibody titer in group IX was found to increase slightly to 1:80 in the surviving animal IX-d only. The final neutralizing antibody titer in animals of group X, which all survived the challenge, was 1:80 in animals X-a and X-b (first experiment) and 1:160 in animals X-c and X-d (second experiment). The very limited plasma volumes did not allow multiple repetitions of the PRNT assays.

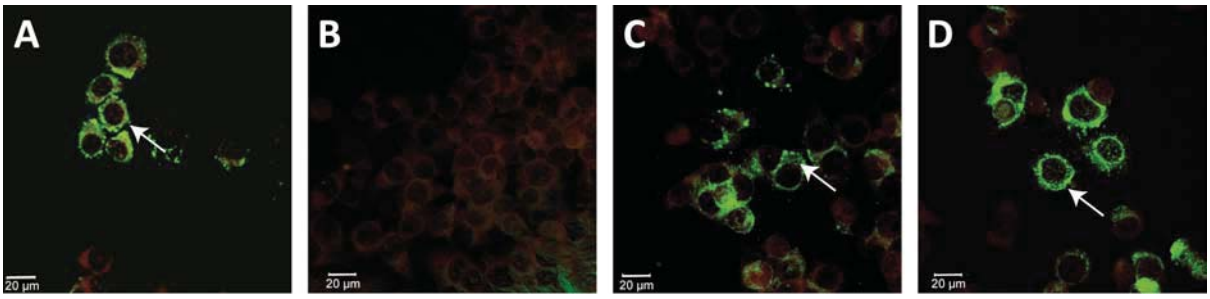

Figure 18: Detection of IgG antibodies after immunization and challenge using immunofluorescence assay; magnification 1:63; **A)** IgG-positive control (human anti-VACV immunoglobulin, **B)** marmoset OPV negative control serum, **C)** IgG after immunization (VACV LE-BN-infected Hep2 cells) and **D)** IgG after challenge (calpox virus-infected Hep2 cells); virus antigens in the cytoplasm of infected cell are indicated by arrows

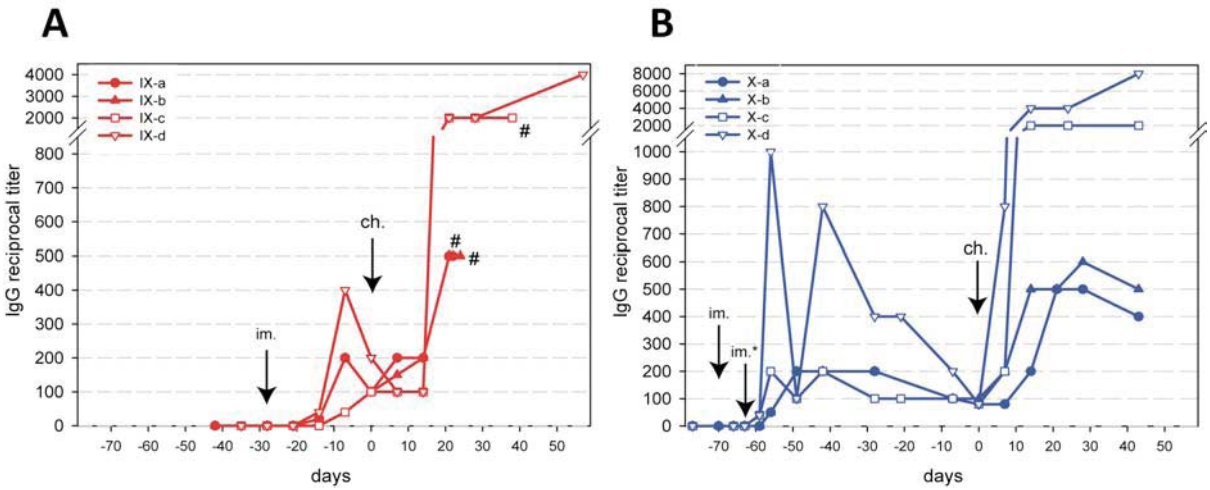

Figure 19: IgG development in marmosets after immunization with VACV LE-BN and challenge with calpox virus determined by immunofluorescence from plasma on calpox virus-infected cells (see figure 18); 0 is set as the day of challenge (ch.); **A)** group IX, challenged four weeks p.im. (immunization on day -28 indicated by arrow); and **B)** group X, challenged nine to ten weeks p.im. (immunization on day -70 [X-a and X-b] and day -63 (*) [X-c and X-d] indicated by arrow); time point of immunization (im.) and challenge (ch.) are indicated by arrows; # time of death

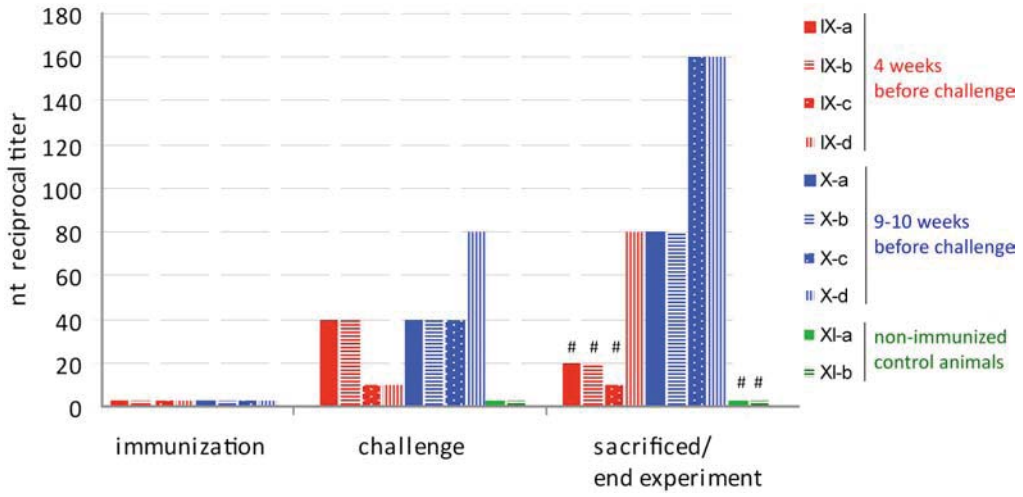

Figure 20: Detection of neutralizing antibodies after immunization with VACV LE-BN and challenge with calpox virus: at time of immunization, at the time of challenge and at the time point of euthanization; # death of animals; nt: neutralizing antibody titer (was determined using PRNT)

4.7.9 T cell-mediated immune response – IFNγ secretion after immunization with VACV LE-BN and challenge with calpox virus

The ELISpot assay was used for the analysis of IFNγ-secreting PBMCs after immunization and challenge. The number of IFNγ-secreting cells is expressed as SFU per 10^6 PBMCs (SFU: spot forming unit where one spot corresponds to one secreting cell). Figure 21A shows the results for group (IX) challenged four weeks p.im. and figure 21B group (X) challenged nine to ten weeks p.im..

Generally, the total number of secreting cells varied for each marmoset. In animals of both immunized groups IFNγ-secreting cells became first detectable around two weeks p.im. except for marmoset IX-a and IX-c were IFNγ-secreting cells could be detected not before three and four weeks p.im., respectively. Peak levels of IFNγ-secreting cells were detectable three weeks p.im. in most marmosets of group X with $\geq 10^3$ SFU/10^6 PBMC which then decreased until week eight p.im..

At the time of challenge IFNγ-secreting cells were still detectable in group IX (four weeks p.im.) with the exception of animal IX-d in which the number of INFγ-producing cells returned to pre-immunization base line levels. This animal survived the challenge with 10 MID_{50} of calpox virus. The three other animals were probably still in the IFNγ developmental phase. After challenge high variations in the number of IFNγ-producing cells were found in marmosets of group IX. However, the impact on the immunological protection against calpox

is difficult to estimate considering that three out of four animals died after the calpox challenge.

For animals of group X the number of IFNγ-producing cells had decreased to the initial level except for animals X-a and X-d ($6.7x10^2$ SFU/10^6 PBMC and $1.4x10^3$ SFU/10^6 PBMC, respectively) at the time of challenge. Three weeks p.ch. the number of IFNγ-producing cells had increased constantly to a new peak level (figure 21B) with $\geq 10^3$ SFU/10^6 PBMC. In addition, the number of IFNγ-secreting cells reached an overall higher level in group X compared to group IX in which the secretory cell counts remained below $1x10^3$ SFU/10^6 PBMC.

Non-immunized control animals showed a moderate increase with approximately 10^2 SFU/10^6 PBMC two weeks p.ch. (time of death) (data not shown).

However, these ELISpot data have to be treated as very preliminary. Due to the limited number of samples and the low blood volumes ELISpot data could not be verified by repeated experiments. Additionally, high background noise, likely to be due to sub-optimal cross-reactivity of the human anti-IFNγ antibody with the marmoset homologue, affected the analysis of the data.

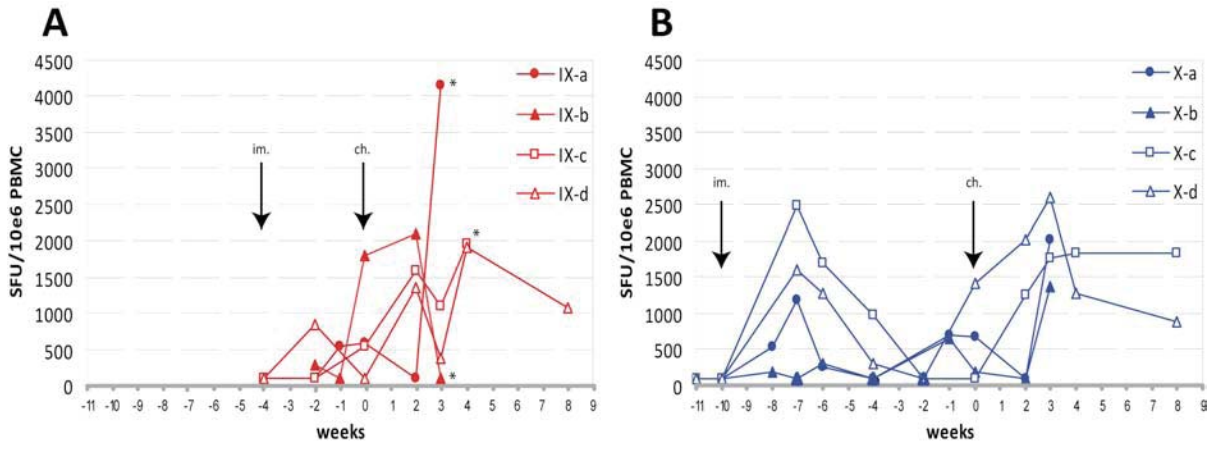

Figure 21: Analysis of IFNγ-secreting cells after immunization (im.) and challenge (ch.); **A)** marmosets challenged four weeks p.im. and **B)** marmosets challenged nine to ten weeks p.im.; the time points of immunization (im.) and challenge (ch.) are indicated by arrows; 0 is set as the day of challenge; * death of animals; SFU (spot forming units): one SFU corresponds to one IFNγ-secreting cell

In summary, both immunization experiments were performed independent from each other, but showed very similar results. All immunized marmosets developed antibodies (including neutralizing antibodies) before challenge. When marmosets were challenged nine to ten weeks p.im., a reliable immune response had developed, protecting all animals against a lethal calpox virus infection whereas only a limited protection could be observed in animals that were challenged already four weeks p.im.. Moreover, the five surviving marmosets of the first challenge were re-challenged with an increased dose of $3.5x10^5$ pfu (42 MID_{50}) of calpox virus. All animals survived the second challenge except animal IX-d. This animal died in narcosis during routine blood collection, without evidence of a calpox virus infection (macroscopic and histological examination and real-time PCR). In none of the surviving animals calpox virus DNA or mRNA could be detected after challenge. IgG and neutralizing antibody titers increased two to four times up to 1:4000 and 1:640. ELISpot data were similar to the first challenge.

These results revealed that this primate model is suitable to investigate new vaccines and vaccination strategies.

4.8 Development and evaluation of real-time PCR assays for marmoset cytokine expression

To improve the understanding of calpox virus pathogenesis, the secretion of different cytokines after infection was investigated using a Bio-Plex system (human 17-plex panel with 17 cytokines, Bio-Rad, Munich, Germany). Preliminary experiments were done *in vivo* using plasma of infected marmosets. Aditionally, cytokine secretion was investigated *in vitro* using calpox virus-infected marmoset (*Callithrix jacchus*) PBMCs (data not shown). As controls uninfected as well as infected PBMCs were stimulated with PMA, LPS and Ionomycin, respectively. Twenty-four and 48 hours after infection the supernatants were collected. Plasma samples as well as supernatants were analyzed for secreted cytokines (experiments were done in close cooperation with Prof. R. Lauster, German Rheumatology Research Centre, and the Technical University Berlin). These experiments were performed using antibodies specific for human cytokines, as no marmoset-specific antibodies were available. In parallel human PBMCs were infected, treated and analyzed similar to marmoset PBMCs. However, only in the supernatant of the human PBMCs secreted cytokines could be measured with this assay. Neither in the plasma nor in supernatant of infected or stimulated marmoset

PBMCs cytokines could be determined (data not shown), probably due to the lack of cross-reactivity between anti-human cytokine antibodies and marmoset-specific cytokines.

Therefore, marmoset cytokine-specific real-time PCR assays were established to further investigate the impact of calpox virus infection on the marmoset immune system. When this study was started, no genomic sequences of marmoset cytokines were available. Hence, previously published human-specific cytokine real-time PCR assays [193] were tested for their compatibility to marmoset cytokine sequences using calpox virus-infected as well as stimulated ML2 (established from marmoset lung tissue, H. Ellerbrok, unpublished) and B95-8 cells. Amplification products could only be generated for IL-1β and IL-6 and sequence analysis showed a similarity to human IL1β and IL6. As already seen in the lack of detection of secreted marmoset cytokines by human antibodies, the sequences of marmosets cytokine genes showed a certain degree of heterogeneity to homologue human cytokine sequences and were therefore not detected with primers specific for human cytokines.

During the course of this study sequences for *Callithrix jacchus* (CJ) IL-2, IL-10, IL-13, TNFα and IFNγ became available in the NCBI database allowing the design and evaluation of marmoset cytokine-specific real-time PCR assays. Amplified PCR fragments were sequenced and real-time PCR probe design was based on these sequences. PCR efficiencies between 91.6 % and 103.2 % were calculated for CJ-TNFα, CJ-IL10, CJ-IFNγ, CJ-IL-13 and CJ-IL1β (table 14). Lower efficiencies of 85.6 % and 86.3 % were determined for CJ-IL-2 and CJ-IL-6 real-time PCR assays, respectively. The correlation coefficient for all assays was 0.98 to 0.99. Intra- and inter-assay variability were higher for smaller copy numbers, but in any setting a minimum of ten copies of the respective target gene could be detected (table 14). Moreover, the combination of two real-time PCR assays was analyzed (duplex PCR). Combinations of plasmid standards for each assay were tested at different concentrations in one mix. Apparently, only the combination of CJ-IFNγ with CJ-TNFα and the combination of CJ-IL-6 with CJ-IL-13 worked satisfactorily without losing sensitivity or specificity. The variability was the same for both duplex real-time PCR assays compared to their corresponding single assays. Combinations of other assays resulted in a decreased sensitivity and in consequences a higher detection limit of up to 10^3 to 10^4 copies per assay.

In a first attempt only very preliminary data were obtained for immunized and non-immunized marmosets (data not shown). Small copy numbers for all cytokines could be determined, often very close to the detection limit (independent of the animal group) may resulting from the small used blood volume. However, to use the newly established real-time

PCR assays as a tool to characterize the immune response in marmosets further investigations have to be done. But the investigation of cytokine expression patterns is now possible using the newly developed marmoset-specific real-time PCR assays.

Table 14: Variability and efficiency of marmoset cytokine-specific real-time PCR assays

Assay name	**Plasmid copies**	**Variability (C_T)[a]**		**Efficiency (%)[a]***
		intra-assay[#]	**inter-assay[+]**	
CJ IL1β	10^6	17.18 ± 0.69	17.60 ± 0.11	103.2
	10^4	23.08 ± 0.12	24.50 ± 0.67	
	10^2	29.78 ± 0.19	30.98 ± 0.90	
CJ IL2	10^6	17.36 ± 0.11	18.42 ± 0.62	85.6
	10^4	24.45 ± 0.62	25.41 ± 0.92	
	10^2	32.45 ± 0.18	31.92 ± 0.36	
CJ IL6	10^6	19.81 ± 0.38	19.09 ± 0.12	86.3
	10^4	26.97 ± 2.12	25.86 ± 0.83	
	10^2	36.08 ± 0.98	33.32 ± 0.78	
CJ IL10	10^6	19.52 ± 0.92	17.83 ± 0.11	94.1
	10^4	26.66 ± 0.30	24.58 ± 0.22	
	10^2	33.57 ± 1.33	32.52 ± 0.51	
CJ IL13	10^6	18.45 ± 0.05	18.06 ± 0.31	98.2
	10^4	25.26 ± 0.33	24.85 ± 0.37	
	10^2	31.36 ± 0.67	31.29 ± 0.43	
CJ TNFα	10^6	19.22 ± 0.04	19.62 ± 0.52	91.6
	10^4	27.74 ± 0.66	26.88 ± 0.52	
	10^2	36.34 ± 1.06	33.80 ± 0.79	
CJ IFNγ	10^6	18.20 ± 0.36	18.06 ± 0.76	96.9
	10^4	25.10 ± 0.16	24.67 ± 0.85	
	10^2	32.13 ± 0.46	31.21 ± 1.03	

The detection limit for each assay was ≥ 10 copies; **a** determination of the intra- and inter-variability and efficiency was carried out with plasmid standards; ***** mean value of efficiency was calculated from three independent standard curves for plasmids; **#** mean value was calculated from triplicates of one PCR run; **+** mean value was calculated from three independent runs at different days; $\mathbf{C_T}$: threshold cycle

5 Discussion

Today the immunity against VARV is low or non-existent in the majority of the human population due to discontinuing of routine vaccination soon after the eradication of smallpox. Although VARV is generally unavailable, the intentional or unintentional re-introduction of VARV into the human population is of constant concern today [15]. In addition, an increase of zoonotic infections with other OPVs was observed over the last years especially with CPXV [17] and MPXV [20]. An increasing number of human MPXV infections has been reported from Africa and during the most recently reported MPXV outbreak from March until July 2008 in Bokungu in the Democratic Republic of the Congo (Congo-Kinshasa), 39 cases were described with three fatalities [202]. MPXV is especially important because a case fatality rate up to ten percent is reported for children and younger adults, which is probably a consequence of the decrease of protective immunity. An outbreak of human MPXV infections in the United States in 2003 occurred due to the inadvertent importation of MPXV infected rodents from West Africa [26,37]. MPXV was then transferred to prairie dogs. Several indigenous members of squirrel species are supposed to be the reservoir hosts, but the zoonotic features that predispose this virus to infect man and other primates are unknown [50]. If MPXV would be able to establish a reservoir status in a susceptible North American rodent species such as prairie dogs [203], the public health consequences would be considerable. Moreover, in Germany an increasing number of human CPXV infections has been reported in recent years (approximately: nine to ten suspected cases, and five to seven confirmed cases per year; personal communication Dr. A. Kurth and Dr. A. Nitsche, Consultant Laboratory for Poxviruses, Robert Koch-Institut, Berlin).

On this background an increased research effort into therapeutic agents for the prevention and treatment of OPV infections appear to be necessary [125]. Furthermore, many research groups worldwide attempt to understand OPV pathogenicity and virus pathology. The testing of the efficacy and safety of new vaccine candidates or antiviral substances has to undergo trials in different animal models, with an emphasis on primate models. Since the only two well-characterized NHP models working with VARV [161] and MPXV [126] have the drawbacks of using very high viral doses via the atypical route of infections to induce a severe disease in macaque monkeys, the main objective of this study was to overcome some of these drawbacks. Therefore, an alternative NHP model in which a smallpox-like disease could be induced by a CPXV under more physiological conditions would be extremely

helpful to study the course of OPV infections and to investigate the protective effect of vaccines candidates and potential antivirals.

5.1 Characterization of the calpox virus/ marmoset model in comparison to other non-human primate models for orthopox virus infections

As shown for NHP models using VARV or MPXV [117,161,204], only the i.v. inoculation, a non-physiological route of infection, which bypasses the mucosa leads to a lethal course of infection. In these primate models the i.v. inoculation route circumvents a primary replication of the virus in regional lymph nodes and the successive systemic spread via the lymphatic system (first viremia). Instead, the virus is immediately spread via the blood stream, inducing a disease manifestation with numerous lesions characteristic of a post-secondary viremia.

In a first attempt it could be shown, that the infection of marmosets by the i.v. inoculation route induced a disease pattern comparable to the diseases observed during the natural outbreak in 2002 [165]. The short incubation period of four to seven days and a zero survival rate after i.v. administration of a high virus dose showed that the calpox virus was lethal for the marmosets with very high final viral loads in blood (up to 10^9 calpox GE/ml blood) and in all analyzed organs (up to 10^6 calpox GE/10^4 c-myc copies) at the time of death. There was no sign of a preferential cell tropism of the virus, as high copy numbers of viral genomic DNA and mRNA were found throughout all tissues, indicating an effective replication in a wide spectrum of cells. The massive virus replication in all organs, resulting in high viral burdens in tissues, was probably associated with organ dysfunction and multisystem failure as shown for VARV infections in cynomolgus macaques [161]. The i.v. infection route overcomes the mucous membrane and evades the mucosal immune defense of the host. Due to the rapid and fulminate course of disease, the determination of parameters of the immune response is difficult and probably does not reflect a naturally acquired infection.

First targets for naturally acquired smallpox infection are the cells of the mucous membranes of the mouth, the nasal cavity, the oropharynx and/or the alveoli of the lungs. Although a high infection dose was used for the oro. application, two out of three animals survived, showing neither clinical symptoms nor detectable virus in blood. The animal showing severe symptoms was most probably injured during the inoculation implying that the subsequent viral infection was the result of a direct entry of the agent into the bloodstream and not due to a local infection of the mucous membranes of the oropharynx. As there were

no calpox virus-specific antibodies detectable in the serum of all three marmosets an immediate swallowing of the virus during the application of the virus suspension seems to be likely. Therefore, it was concluded that it appeared highly unlikely to establish a reproducible infection model with calpox virus via the oro. route.

Contrary, the i.n. infection as an inoculation route very similar to the major natural route of VARV infections proved to be a successful strategy of administering calpox virus to marmosets by dripping the virus solution directly into the nose of the animal. A close correlation was observed between the infectious dose and the course of infection. High virus doses of $2.3x10^6$ and $3.5x10^5$ pfu led to a rapid death within nine to ten days of infected marmosets, with final virus levels of up to 10^8 calpox GE/ml blood. A significant correlation between infectious dose and incubation period of the disease was observed when different calpox virus doses were investigated. No clinical signs or diagnostic markers of infection were observed when $1x10^2$ pfu were inoculated. Using different infectious doses of calpox virus the 50 % monkey infectious dose (MID_{50}) was calculated with $8.3x10^2$ pfu showing that calpox virus is highly pathogenic for marmosets. Independent of the virus dose, viral genomic DNA could be detected in the blood of marmosets developing symptoms from day seven p.i. onwards. It is noteworthy to mention that clinical symptoms developed only approximately two days before the death of the animals. Similar to the i.v. inoculation all organs were infected. The highest viral loads were determined in lymphoid organs like spleen, tonsils, bone marrow and lymph nodes, indicating a progressive and productive replication of the virus in these organs. Dissemination of calpox virus to other organs probably occurred through the blood stream (viremia). The generalized involvement of multiple organs is similar to lethal human MPXV and VARV infections where virus is found in various organs. Opposed to human VARV infections [63], an involvement of organs of the digestive tract was found in the calpox virus/marmoset model. In contrast to natural VARV and MPXV infections, where only part of the diseased patients had a lethal outcome, all marmosets developing clinical symptoms died. In animals exposed to calpox virus but without clinical symptoms neither viral genomic DNA nor virus-specific antibodies were found, pointing towards an unsuccessful infection. Infectious calpox virus was found in the saliva of all diseased marmosets, extending the i.n. calpox virus infection model even further: the i.n. inoculation caused a clinical outcome capable of spreading the virus via a natural route of transmission between marmosets – the same route of transmission that had been found in human smallpox infections.

As mentioned before, no seroconversion was observed in diseased animals dying within 12 days. Only in longer living animals low titers of virus-specific IgM antibodies were detected at the time of death. In human smallpox infections the development of neutralizing antibodies became detectable between day six and eight after the onset of symptoms [42]. Intranasal infection of calpox virus probably resulted in an overwhelming infection and death occurred before the humoral immune system could respond by developing IgG and neutralizing antibodies. A correlation between the presence of serum antibodies and protection has already been rdeported for human OPV infections [205,206,207].

In human smallpox and MPXV infections a two to five-day period of high fever, malaise, prostration, headache and backache is followed by the development of a maculopapular rash. In the calpox virus/marmoset model the time between appearance of first clinical signs and death of the marmosets was drastically reduced as first clinical symptoms became obvious just one to two days before death. First symptoms rapidly developed to more severe clinical findings in the upper respiratory tract and the lymphatic system as well as hemorrhagic edema. In contrast to MPXV and VARV patients with a more or less confluent rash [20,25,42,53,63], only sporadic and small skin lesions distributed on face, abdomen and thighs were found in marmosets after i.n. infection with calpox virus. Even a high calpox virus dose was not able to induce an infection featuring skin lesions that were distributed confluently over the body of marmosets, a characteristic previously demonstrated in macaques infected with MPXV and VARV [160,161,164,208]. The clinical picture of calpox virus further differed in the morphology of the skin lesions, which remained flat and small in size (2-5 mm in diameter), whereas VARV or MPXV lesions can reach a diameter of up to 10 mm. This may result from the overall smaller size of the animals. The severe lymphadenopathy found in calpox virus infection is comparable to the clinical picture of CPXV and MPXV infections in humans as well as in NHP models [25,54,160,209] but could not be seen in human smallpox.

One major drawback in previous NHP models is that the virus has to be administered in very high doses to induce a severe or fatal disease. At least 10^6 pfu of MPXV have to be injected s.c. to achieve a lethal infection in cynomolgus macaques [163]. Intravenous inoculation of at least $5x10^7$ pfu of MPXV is reported to lead to a fatal infection in cynomolgus macaques and rhesus macaques [117,150,160,209]. The three inoculation routes established for the MPXV/macaque model that come closest to the natural route of smallpox infection are the following: the i.t. [126,164], the aerosolized [162] and the i.n. administration of MPXV [210]. Still, very high doses of 10^7 pfu are needed to induce a severe or lethal

infection. This is similar to the VARV/macaque model where extremely high virus doses of at least 10^8 pfu i.v. are needed to induce a systemic infection of cynomolgus macaques [161,204], whereas no systemic or serious disease could be established by its aerosolized administration [211]. In contrast, only a low virus dose given i.n. is needed in the calpox virus/marmoset model to induce a symptomatic infection in marmosets. The MID_{50} was determined with $8.3x10^2$ pfu that is approximately 10.000 times lower than that needed in any other NPH model for OPV infections and might be comparable to naturally acquired VARV and MPXV infections. Therefore, the low MID_{50} needed for the induction of disease by the i.n. inoculation route implies that the calpox virus/marmoset model is a reliable model and might have several advantages compared to other NHP model systems.

5.2 Calpox virus infection in the mouse model and comparison of marmoset infection with another CPXV strain

Laboratory mice are a suitable subject to study the pathogenicity of a number of CPXV strains [135,188]. Lethal virus doses for CPXV infections in mice are generally high (above 10^6 pfu), but lower doses also resulted in a symptomatic but mild infection followed by full recovery. CPXV naturally infects rodents, which seem to be a natural reservoir for the virus [27,212,213], but generally do not develop severe symptoms although the virus has a negative impact on their survival [214]. How the virus is transmitted to other vertebrates and how the virus survives in the rodent population is not fully understood, but evidence was found of a sexual transmission of the virus in mouse populations [215]. In this study the i.n. inoculation of mice with the calpox virus, although phylogenetically closely related to other CPXV strains, resulted only in a very mild infection indicating a low pathogenicity in BALB/c mice (100 % survival) even if the infectious dose was as high as $1x10^7$ pfu. This is in contrast to CPXV BR [188,201], a common CPXV strain used for infection of mice where i.n. inoculation of 10^6 pfu induced a severe infection (death within eight to ten days). Future research might address the underlying mechanism for the differences in viral pathogenicity of calpox virus and other CPXV in mice.

To investigate whether CPXV infections always lead to a fatal outcome in marmosets, CPXV strain 81-02 was used for the infection of marmosets by the i.n. route with a high dose of $5x10^5$ pfu. In comparison to calpox virus, the detection of viral DNA in CPXV 81-02-infected marmosets was delayed by approximately four days and DNA levels were lower in general. Whereas animals infected with a comparable dose of calpox virus died on day nine,

CPXV 81-02-infected animals were still alive on day 18 (very mild symptoms) when the experiment unfortunately had to be stopped due to technical problems in the animal keeping facility. These results, leading to the assumption that CPXV 81-02 has a lower pathogenicity in marmosets.

5.3 Development of assay systems for the detection of immune parameters

Besides the determination of virus-specific antibodies using immunofluorescence assays and neutralizing antibodies using PRNT, further parameters of the cellular immune response were to be characterized after calpox virus infection. Especially the role of the T lymphocyte (T cell) response against a lethal infection was to be investigated. Synthesized by natural killer (NK) cells, CD4+ and CD8+ T cells and CD8+ cytotoxic suppressor cells [216], IFNγ represents an early host defense marker and is involved in a range of mechanisms in addition to specific antiviral activity [217,218,219]. The interaction of T cell receptor and antigen-MHC I complex triggers the proliferation and expansion of antigen-specific T cells and the secretion of IFNγ [220]. Some of the antigen-responsive T cells develop further into antigen-specific memory T cells which is maintained within the host and provides an immune surveillance system. After a secondary exposure to the virus antigens, these virus-specific memory T cells are activated and expand with an even greater magnitude than seen during the initial response [221]. In this way specific memory T cells contribute to protect the host against further exposure to the virus.

IFNγ-secreting cells were determined using a commercially available ELISpot kit developed for the detection of human IFNγ. In addition to the general difficulties of working with small volumes of blood samples, a major problem was the restricted cross-reactivity of the anti-human IFNγ antibody with marmoset IFNγ resulting in high background noise. Nevertheless, this antibody showed the best cross-reactivity of all antibodies tested. ELISpot results were obtained for immunized and challenged marmosets and are discussed in chapter 5.4 revealing first insights concerning the IFNγ-release in marmosets after calpox virus infection.

Since OPVs use a wide repertoire of immune modulating mechanisms to evade the innate immune response like the secretion of immune modulating proteins interacting with the complement system [222], inhibition of the interferon response [223], protection of the infected cell from inflammatory responses and prevention of natural killer (NK) cell

activation, calpox virus immune modulation during the marmoset's immune response were to be investigated by measuring secreted cytokines. Considering that marmosets have an approximate 88-97 % genetic similarity to humans [224], it was surprising that none of the tested anti-human cytokine antibodies was cross-reactive with marmoset cytokines or marmosets cell surface markers used in Bioplex analysis and flow cytometric analysis (data not shown), respectively. The poor cross-reactivity of antibodies directed against human antigens with marmoset antigens has previously been shown in several studies. Human monoclonal antibodies (mAB) were tested for their applicability for flow cytometric analysis of cells from common marmosets [225,226] showing an overall cross-reactivity of different mABs of less than 40 % [226]. In addition, the fluorochrome labeling as well as the antibody-producing clone had an effect on the reactivity and binding of the mABs. These published results were taken into account in the design of our experiments, but several of the published antibodies were no longer available or had been replaced by other, non-cross-reacting clones. However, only a limited number of antibodies from different manufacturers were tested and further tests may identify some cross-reactive mABs for the staining of cell surface markers and secreted cytokines.

Due to the lack of cross-reacting antibodies marmoset-specific real-time PCR assays for IL1β, IL2, IL6, IL10, IL13 TNFα and IFNγ were established to investigate expression patterns of these cytokine genes. As seen for other organisms [197,227], the real-time PCR assays proved to be a reliable tool for the detection and quantification of cytokine mRNA copy numbers. Linear regression analysis of plasmid standard curves of each cytokine real-time PCR assay demonstrated reliable PCR efficiencies between 85.6 % and 103.2 % with the smallest correlation coefficient (R) of 0.98. The detection limit of ten plasmid copies observed for all developed assays is a common detection limit for many other real-time PCR assays used in virology, microbiology and for expression analysis [197,227,228]. Since blood and tissues obtained from different individuals usually varies in cell number, RNA integrity or quantity or experimental treatment all copy numbers determined by real-time PCR were normalized [227] against the reference gene c-myc that served at the same time as a positive control for the PCR reaction. The c-myc gene is expressed constitutively and independently from experimental conditions, different tissue or cell types, developmental stages and sample treatment. It is not affected by the infection with different OPVs (Dr. A. Nitsche, personal communication).

In preliminary experiments it could be shown that cytokine copy numbers were close to the detection limit preventing a reliable data interpretation. Due to the scarcity of test blood

all PCR experiments were done only once which is a clear limitation although all samples were measured in duplicates in all assays. Compared to reported protein levels for MPXV and VARV infected macaques monkeys, which were elevated for the proteins IFNγ, TNFα, IL2 and IL10 determined by ELISA [150,161,163,164] and intracellular staining [209,229] the marginal changes in mRNA levels measured in this study were unexpected. To place future expression data in a reliable context, it would have been interesting to determine the final cytokine protein levels since the regulation of translation and post-translational events plays an important role in the secretion of proteins which is unfortunately at present hampered by the lack of antibodies against the respective marmoset cytokines.

Despite all limitations, results allow the conclusion that the real-time PCR assays established in this study have the potential to become a useful tool for the determination of marmoset-cytokine expression. For a better characterization of the marmoset cytokine expression patterns, it is necessary to obtain larger blood volumes in order to assure measurements clearly above the detection limit. Furthermore, it would be worthwhile to investigate mock-infected marmosets as a negative control to compare the results obtained from infected animals.

5.4 Vaccine testing in the calpox virus/marmoset model

To investigate how suitable the calpox virus/marmoset model is for the testing and evaluation of vaccines, marmosets were immunized with the well-characterized live vaccine VACV LE-BN and later challenged with 10 MID_{50} of calpox virus. The challenge was done at two different times after immunization as it was not known how long the development of a protective immune response would take and how incubation time would impact on the possible survival of the marmosets. No recommended period for a challenge with a pathogenic OPV after immunization with VACV LE has been described in the literature. There are only data published regarding the immunization of cynomolgus monkeys with VACV LE followed by a MPXV challenge, showing that five weeks were sufficient to build-up a protective immune response [163]. After considering these reports, one group of marmosets was challenged four weeks and the second group nine to ten weeks after immunization. Two independent experiments were performed under comparable conditions resulting in four immunized animals per group and six non-immunized animals served as controls. Both experiments were analyzed together.

As described for live OPV vaccines the development of a characteristic lesion at the site of immunization is regarded as a sign for a successful immunization ("vaccine take") [229]. In humans the reported intensity and duration of the vaccine take depends on the immunization status of the individual. Both, naive and non-naive vaccine recipients, developed a major reaction by day seven with an average lesion size of 12.5 mm^2. The lesion healed completely within 23 to 26 days [230]. The progression and the development of the local lesion as well as its maximal size reported for macaque primates are similar to the "vaccine take" in humans. Cynomolgus macaques developed a pustular lesion with a size of 27 $mm^2 \pm 11$ mm^2 after immunization with LC16m8, a highly attenuated VACV strain that was developed from the original Lister-Elstree strain [163]. The immunization of marmosets with VACV LE-BN also induced a local cutaneous lesion in all immunized marmosets. But the development of the lesion was delayed by seven days and slightly reduced in size (approximately 10 mm^2) compared to humans. This may have been the result of the five times lower number of perpendicular insertions with the bifurcated needle as it is recommended for humans [231].

As VACV LE-BN has the ability to replicate in mammalian cells, the detection of VACV DNA in blood of human vaccine recipients is described in few cases [232,233]. Using PCR, Savona et al. measured about 10^3 VACV GE/ml blood in four out of 77 recipients as early as days six p.im.. Cohen et al. reported similar results where four out of 202 blood specimens were positive at low levels for VACV DNA as early as days four p.im.. Other human immunization studies using live VACV like Dryvax (Wyeth, USA) also demonstrated that viremia is a rare event but could occur in immunocompromised persons with multiple cutaneous lesions [234]. But there were also reports that no VACV DNA could be detected at all [235] (Nitsche and Ellerbrok, personal communication). However, VACV LE-BN DNA as well as mRNA was repeatedly detected in seven out of eight immunized marmosets beginning seven days p.im.. Peak levels of approximately 10^4 VACV LE-BN GE/ml blood were reached on day 14 p.im.. The virus was cleared from the blood of all animals before day 18 p.im.. The rate of vaccine-induced viremia found here was much higher (87.5 %) compared to humans where virus DNA was detectable only in 2 % to 6.5 % of the vaccinees. The general appearance of VACV LE-BN DNA and its detection by real-time PCR in our experiments is a sign of a successful infection by the vaccine virus and might be useful in the monitoring of live vaccine candidates in marmosets.

Because of the limited availability and low volumes of blood samples, the reported failure to isolate infectious virus after immunization in humans [232,233] and the low viral

load in infected marmosets, no attempts were made to isolate infectious VACV LE-BN from immunized marmosets. Panning et al. had shown that actively replicating VACV produced large quantities of defective particles, and only one infectious virus corresponds to 100 GE determined by PCR [236] suggesting that 50 to 100 µl of blood generally available from live marmosets would contain one to five infectious particles.

It is noteworthy to mention, that infectious VACV LE-BN could be isolated from saliva from 50 % of immunized marmosets with up to 10^2 pfu/throat swab which is in contrast to human vaccination studies [232,233].

During the mass vaccinations of the eradication era as well as in clinical trials with new live VACV vaccines against smallpox, it had been reported that almost all recipients who showed a lesion at the site of immunization seroconverted and developed neutralizing antibodies against the smallpox vaccine [237]. After immunization, antibodies, including neutralizing antibodies, developed rapidly in humans as well as in various animal models. In NHPs as well as in humans IgG antibodies could be detected around days ten to 11. Strong IgG responses (including multiple IgG isotypes) were clearly present at day 14 p.im.. Neutralizing antibodies develop parallel to the overall IgG response [117,238]. However, in immunized marmosets the development of IgM and IgG detectable by IFA as well as neutralizing antibodies was delayed compared to humans and other primates [109] and peak levels were detected around day 21 p.im. in all immunized marmosets. At the time of the challenge IgG antibody titers of all immunized animals were in the range of 1:100 and 1:200, but neutralizing antibody titers were low: 1:10 for 2/8, 1:40 for 5/8 animals and 1:80 for 1/8 animal. After challenge, IgG antibody titers of all immunized marmosets increased at least twofold 14 to 21 days p.ch.. Similar observations of antibody boosting were reported for humans [239,240]. Although the antibody titers were comparable in both immunization groups (challenged four or nine to ten weeks p.im.) at the time of challenge, there was no correlation to the rate of survival. Little is known about the protective effect of antibodies against a lethal OPV infection in humans as well as in primates. But historical clinical data suggest a correlative link between the presence of virus-specific serum antibodies and protection [205,206,207]. Moreover, recent studies showed that the development of neutralizing antibodies is obligatory for the protection against an infection with a lethal dose of virus and the level of virus-specific antibodies usually correlates with the grade of protection [241,242].

The secretion of INFγ and effective virus clearance are reported to play an essential role in protection [243,244], too. Although it is well known that the IFNγ response represents an

early host defense involved in a range of mechanisms to specific antiviral activity [217,218,219], it remains poorly understood which level of cell-mediated immune response is needed for an effective protection against an OPV infection. As mentioned before, the immune response to smallpox was monitored exclusively by measuring neutralizing antibodies. As the treatment of T-cell-deficient patients with anti-VACV immunoglobulin was relatively ineffective [245,246] it was suggested that the T cell immune response play a more important and dominant role in the protection and recovery from an OPV infection [218,247,248]. Recent studies have demonstrated that VACV-specific T-lymphocytes secrete IFNγ after immunization and that these cells are likely to have a prolonged lifespan [221,249]. Kennedy at al. determined IFNγ responses after immunization of VACV-naive and non-naive individuals: the responses began one week p.im. and peak levels were reached two weeks p.im. [250].

To investigate the role of the T cell response to VACV LE-BN in protecting against a lethal calpox virus infection, IFNγ-producing cells were measured using the ELISpot. In contrast to humans, IFNγ secretion in immunized marmosets was delayed and levels of IFNγ-producing cells peaked as late as three weeks p.im. At the time of challenge, group IX challenged four weeks p.im. generally showed a low number of IFNγ-producing cells. Moreover, in all these animals IFNγ-producing cells were observed to increase continuously at the time of challenge, a sign that these animals still underwent antigen-stimulation and clonal cell expansion. However, in group X in which the animals were challenged nine to ten weeks p.im. the number of IFNγ-secreting cells had returned to a baseline level before challenge. Considering that all animals of group X survived, the return to baseline levels of IFNγ-producing cells before a subsequent lethal virus exposure might play a significant part in the immunologic protection. To evaluate the T cell-mediated IFNγ production in detail, further experiments with a higher number of animals may provide additional and more reliable results.

As mentioned before, effective virus clearance from the blood also played an important role for the survival of infected marmosets. After challenge, four out of four (100 %) marmosets challenged nine or ten weeks p.im. survived without any clinical symptoms although calpox virus was detectable between days ten and 18 in the blood of three animals. Compared to non-immunized marmosets, viral loads were lower by four logs, and the calpox virus was finally cleared from the blood, indicating effective calpox virus elimination. Similar observations were reported for studies in other NHPs where the viral load in the blood of immunized animals in contrast to non-immunized animals was significantly lower [150] or

below the detection limit [160] after challenge. In addition, non-immunized macaque monkeys surviving a lethal-dose MPXV challenge cleared the virus from the blood [163]. However, in group IX, challenged four weeks p.im., only one out of four (25 %) animals cleared the virus from the blood and survived the lethal calpox virus challenge. Since virus elimination is linked to an adequate immune response (antibody development and cytokine release) it was not surprising that the only survivor in this group (IX-d) showed a twofold higher final neutralizing antibody titer compared to the deceased marmosets of this group. This neutralizing antibody titer was similar to the titers of the marmosets of group X which all survived the challenge. The deceased marmosets of group IX showed a significant delay in the progression of disease, dying approximately ten days later with a lower viral load at the time of death compared to non-immunized control animals (group XI). The lower viral load was may a result of a partial immunity in these animals, which were able to suppress virus replication to some degree, but finally failing to clear the virus from the blood.
Results imply that the titer of neutralizing antibodies as well as effective virus control play an important role in contributing to an efficient protection against a lethal calpox virus challenge.

In summary, the results obtained in these experiments indicate that the calpox virus/marmoset model is suitable for the evaluation of both, cellular and humoral immune responses after immunization with VACV, paving the way for its use in *in vivo* evaluations of new vaccine candidates and new vaccination strategies.

5.5 Advantages and limitations of the calpox virus/common marmoset model

Epidemiologic investigations of the natural calpox virus outbreak in the colony of New World monkeys gave no evidence for a transmission to humans even if they had close contact to diseased animals (Boll et al., unpublished). Sequence similarities of the calpox virus to published CPXV sequences group calpox virus tentatively to the species CPXV in the genus OPV. Since CPXV is classified as a BSL-2 pathogen, the experimental work with calpox virus can be done under BSL-2 conditions, opening research on this OPV isolate to a larger number of laboratories.

The investigations presented in this thesis showed, that the calpox virus/marmoset model is suitable to study a broad spectrum of basic and applied scientific research topics. Calpox virus induce a disease quite similar to human smallpox after i.n. infection with low viral doses (MID_{50} of $8.3x10^2$ pfu), which reflect to a large extent of the natural infection

route of VARV. The MID_{50} is approximately 10,000 times lower than that in other NHP models using VARV or MPXV. Major differences are apparent considering the outcome of an infection. Whereas part of humans or experimental animals infected with VARV or MPXV developed symptoms but convalesced, it has to be re-emphasized that all diseased marmosets died. Animals surviving virus inoculation showed neither clinical nor immunological nor molecular markers of an infection. The readout of the success or failure of vaccines or therapeutic treatments is in comparison to other NHP models straightforward, and there is no need to develop a score for clinical symptoms [126,150,160,163,209].

Using VACV LE-BN, a protective immune response could be induced including the development of IgG antibodies, neutralizing antibodies and the stimulation of IFNγ-secreting cells. The immunization with VACV LE-BN proved to be effective even against a lethal calpox virus challenge if marmosets were challenged nine to ten weeks p.im. demonstrating the potential and suitability of the calpox virus/common marmoset model for the testing and validation of new vaccines and vaccination strategies.

However, one major drawback attributable to all experiments presented here is the small animal number per group. As calpox virus seemed to be highly pathogenic for marmosets only low biological variations were expected. Therefore and also to keep the number of infected animal to a minimum, often only two animals per group were used for experiments. Due to the small sample size no statistical analysis was attempted. Considering the small number of marmosets used in the experiments, it was possible to obtain similar results for same experimental approaches in independently performed infections (e.g. infection with $8.3x10^3$ pfu resulted in death after 12-15 days in four experiments with a total number of nine animals) which support the validity of the results and indicate reliability of this marmoset model even thought it is based only on these very small groups of animals. As shown for other NHP models, numbers of three [163], four [117,204] and six animals per group [150,161] is a common group size in OPV research using macaque monkeys.

Advantages of using marmosets as experimental animal include: their small size, reasonably sized housing space and conditions, easy handling, animals can be socially housed, they reproduce well in captivity, they are inexpensive to keep, they have an economic purchase price, and wild populations are not endangered [251]. Despite these advantages the small size of this species bears some disadvantages for research that involves surgery or requires frequent sampling of blood. Furthermore, studies of the immune response in marmosets are hampered up to now by the lack of immunological tools for the detection e.g. of cell surface markers as well as cytokines.

6 Future Prospects

To investigate the marmoset immune response after calpox virus infection a wider spectrum of anti-human cytokine antibodies has to be tested for their cross-reactivity. In addition, cytokine expression patterns have to be obtained to compare and place them in a reliable context with levels of secreted cytokines.

The course of infections in marmosets infected with different OPV species will help to clarify the host range of these viruses, the pathogenic properties of the viruses and to identify genes essentially for virulence. In cooperation with the German Primate Centre several projects are in progress or planned: calpox virus infection in other monkey species, e.g. rhesus macaques used as model for MPXV will show whether the calpox virus/marmoset is a unique combination for a NHP model for OPV infections or if other monkeys, including Old World monkeys, can also be lethally infected by calpox virus. Furthermore, the aerosol transmission route of calpox virus will be explored by housing calpox virus infected together with uninfected marmosets.

We have started to investigate whether the orally administrable component ST-246 can be used for the therapy of infected marmosets to explore the suitability of the calpox virus/ marmoset model for the testing of new antiviral and therapeutic agents [129,132]. Animals will be infected with 10 MID_{50} of calpox virus and therapy (daily drug application) will begin either (i) directly after virus infection, (ii) when virus is first detectable in the blood (around day seven p.i.) or (iii) when first clinical symptoms are becoming obvious (days 12 to 14 p.i.).

Passive immunization is of major concern as only a limited amount of human immunoglobulin is available for treatment of OPV-infected patients or for people with severe reactions after immunization with VACV. Analogous to the human anti-VACV immunoglobulin, chicken IgY antibodies produced against calpox virus as well as VACV will be tested as a passive immune therapy against a lethal calpox virus infection in marmosets. IgYs, derived from egg yolk of immunized chickens, provide a cheap, economical and abundant source of polyclonal antibodies and have already been extensively explored in the preventive medicine and therapy of other infectious diseases [252,253,254,255].

Furthermore, in cooperation with Prof. G. Sutter, Paul-Ehrlich-Institute, derivate VACV MVA strains will be investigated for their potential to induce a protective immune response in marmosets. This will probably enable to get a closer insight for the development of optimized smallpox or MPXV vaccines.

7 References

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Declaration of Authorship

I certify that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigations, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other University.

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Curriculum Vitae

Personal data

Name: Marit Kramski

Education and Research Experience

2005 – now **Robert Koch-Institut and Humboldt University, Berlin, Germany**

Orthopox virus research project "Infections of Common Marmosets with Calpox Virus: A Model for Smallpox Virus Infections " leading to doctoral degree (PhD equivalent)

official enrollment: 07.06.2006

Research project was based on the collaboration between German Primate Centre, Göttingen and the Paul-Ehrlich-Institut, Langen; next to the experimental work responsibilities included oral and written presentations and lectures; teaching of students at the Technical University Berlin in molecular diagnostics; organization and leading University practical courses for students; supervision of students for practical trainings, student research projects and diploma thesis.

1999-2004 **Humboldt University, Berlin, Germany**

Diploma in Biology (MSc. equivalent degree in Biology); specialized in Virology, Microbiology, Genetics

2004 **Institute of Virology, Charité Medical School, Robert Koch-Institut, Berlin, Germany**

Hantavirus research project "Molecular Diagnostics and Epidemiology of Hantavirus in Central Europe" leading to Diploma in Biology (Honours thesis)

2003 **Institute for Medical Virology, Zurich, Switzerland**

HIV research project "siRNA mediated downregulation of the Proteins JM4 and JWA, which interact with the CCR5-Receptor" leading to admittance to final examinations

2002 **Internship at the Shands Hospital, University of Florida, Gainsville, USA**

Hypertension research project "Gene expression in response to hypoxia and normoxia in rat cardiomyocytes" was accepted by the Humboldt University as an equivalent for a module in biochemical techniques

2000 **Max-Delbrück Center for Molecular Medicine (MDC), Berlin, Germany**

Research Training

1992-1999 **Gauss- Gymnasium, Frankfurt (Oder), Germany**

A-Levels; School with main focus on mathematics and natural science; Abitur: Biology, Chemistry, German and History

Awards

2008 PEI Travel Grant

The Paul-Ehrlich-Institut (Institution of the Federal Republic of Germany), organizer of the XVIIth International Poxvirus and Iridovirus Conference, Grainau, Germany, June 2008, granted a stipend covering conference fees (465 Euro).

2007 DAAD Travel Stipend

The Deutsche Akademische Austauschdienst (DAAD) granted a travel stipend for the Participation at the Khon Kaen University Public Health Winter School, Thailand, February 2007 (1300 Euro).

2004 Charité Forschungsförderung

The Institute of Virology and Charité Medical School of the Humboldt University granted a three months undergraduate stipend for completing research for publication (960 Euro).

List of Publications and Presentations

Journals

Kramski M., Mätz-Rensing K., Stahl-Hennig C., Nitsche A., Kaup FJ., Pauli G., Ellerbrok H.; Common Marmosets and Calpox Virus: A new Primate Model for Orthopox Virus Infections. *Manuscript in preparation*

Kramski M., Dorz A., Dabrowski W., Ellerbrok H.; Rapid Detection of anti-Vaccinia Virus Neutralizing Antibodies. *Manuscript in preparation*

Kramski M., Achazi K., Klempa B., Kruger DH.; Nephropathia epidemica with a 6-week incubation period after occupational exposure to Puumala hantavirus; J Clin Virol. 2009 Jan; 44(1): 99-101. Epub 2008 Dec 5; *article*

Kramski M., Meisel H., Klempa B., Kruger DH., Pauli G., Nitsche A.; Detection and typing of human pathogenic hantaviruses by real-time reverse transcription-PCR and pyrosequencing.; Clin Chem. 2007 Nov; 53(11): 1899-905. Epub 2007 Aug 23; *article*

Conference Contribution

Kramski M., Mätz-Rensing K., Stahl-Hennig C., Kaup FJ., Pauli G., Ellerbrok H.; New World Monkeys and Calpox Virus: A New Model for Orthopox Virus Infections. XVII International Poxvirus and Iridovirus Conference 2008, 7-12 June 2008 in Grainau, Germany, *oral presentation*

Kramski M., Mätz-Rensing K., Stahl-Hennig C., Kaup FJ., Pauli G., Ellerbrok H.; A New Primate Animal Model for Orthopox Virus Infections. Third European Congress of Virology, 1-5 September 2007 in Nuremberg, Germany, *oral presentation*

Meeting report from the Third European Congress of Virology, 1-5 September 2007 in Nuremberg, Germany; Eurosurveillance monthly release, 2007 Dec; 12(12); *meeting report*

Nitsche A., Tesch J., Buwitt U., **Kramski M.**, Pauli G.; Pyrosequencing: A valuable tool in virus diagnostics; Third European Congress of Virology, 1-5 September 2007 in Nuremberg, Germany; *poster presentation*

Kramski M., Mätz-Rensing K., Stahl-Hennig C., Kaup FJ., Pauli G., Ellerbrok H.; A Primate Animal Model for Orthopox Virus Infections.; International Meeting on Emerging Diseases and Surveillance, February 2007 in Vienna, Austria; *poster presentation*

Kramski M., Meisel H., Klempa B., Kruger DH., Pauli G., Nitsche A.; Detection and quantification of the human pathogenic viruses of the Genus hantaviruses by real-time PCR; Annual Meeting of the German Association for Virology, April 2006, Munich, Germany; *poster presentation*