Long Term Immunological Memory to Vaccinia Virus

Halla Halldórsdóttir

Ritgerð til meistaragráðu
Háskóli Íslands
Læknadeild
Líf- og læknavísindi
Heilbrigðisvísindasvið

HÁSKÓLI ÍSLANDS

Langtíma T- og B-frumu ónæmisminni gegn kúabóluveiru

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Ritgerð til meistaragráðu í líf- og læknavísindum

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ÁGRIP

Kúabólubóluefnið gegn bólusótt hefur mikla sérstöðu. Notkun bóluefnisins varð til þess að bólusóttarveirunni var útrýmt í náttúrunni, en ekkert annað bóluefni hefur náð slíkum árangri enn. Sýnt hefur verið fram á að bóluefnið vekur bæði frumu- og vessabundin ónæmissvör sem geta varað lengur en hálfa öld. Í ljósi þeirra hryðjuverka sem framin hafa verið í seinni tíð hafa vaknað áhyggjur varðandi möguleikann á notkun bólusóttarveirunnar í hryðjuverkaárásum. Slíkar áhyggjur hafa meðal annars blásið lífi í rannsóknir á bólusóttarveirunni og ónæmissvarinu gegn henni. Í rannsókninni sem hér verður fjallað um var langtíma T- og B-frumu ónæmisminni gegn kúabólubóluefnininu rannsakað í einstaklingum sem voru bólusettir fyrir meira en þremur áratugum síðan. Í þessum hópum voru: 1) einstaklingar sem upplifðu aukaverkanir, 2) viðbragðslausir einstaklingar sem ekki fengu bólu og ör þrátt fyrir ítrekaðar bólusetningar og 3) einstaklingar sem brugðust eðlilega við bólusetningunni með bólu og síðar öri. Langvinn ónæmissvör hafa aldrei áður verið rannsökuð í einstaklingum með slík sjaldgæf og óvenjuleg svör gegn kúabólubólusetningu. Í rannsókninni var kúabólu veiran, *Vaccinia* veira, sem hafði verið óvirkjuð með háum hita (VV) notuð til þess að örva einkjarna frumur einangraðar úr blóði þátttakendanna. Lykil boðefni sem frumurnar seyttu eftir örvunina voru mæld með ELISA og Luminex aðferðum. Einnig voru B-frumur örvaðar og VV-sértækar IgG^+ mótefnaseytandi frumur taldar með ELISPOT aðferð.

Bæði VV sértæk B- og T-frumu ónæmissvör voru til staðar í öllum þremur hópunum. B-frumu minni var ekki jafn öflugt í aukaverkana hópnum og í hinum tveimur hópunum en T-frumu svörin, bæði Th1 og Th2 svör, voru hinsvegar öflugri í aukaverkana hópnum heldur en í hinum tveimur. Svo virðist sem einstaklingar sem upplifðu aukaverkanir hafi myndað sterkari Th1 og Th2 minnisfrumur á kostnað kraftminni B-minnisfruma. Athygli ber einnig að veita því að viðbragðslausi hópurinn sýndi ekki lakari B- og T-frumu minnisviðbrögð en eðlilegi hópurinn þrátt fyrir að bregðast ekki við bólusetningu með eðlilegum hætti.

Niðurstöður rannsóknarinnar hafa aukið við skilning okkar á langtíma T- og B- frumu minni gegn kúabóluveiru og hvernig upprunaleg viðbrögð við bólusetningu tengjast því. Þær hafa einnig veitt okkur tækifæri til þess að skoða niðurstöðurnar í samhengi við þær erfðafræðilegu upplýsingar sem eru til staðar fyrir einstaklingana sem sýna þessi sjaldgæfu og óvenjulegu viðbrögð við kúabólubólusetningu. Aðrar rannsóknir hafa þegar sýnt að breytileika í bæði frumu- og vessabundna ónæmissvarinu er að hluta til stjórnað af HLA genum. Með því að bera kennsl á breytileika í HLA genum og öðrum genum sem gætu aukið líkurnar á slæmum aukaverkunum gegn bóluefnum, og mögulega aukið líkurnar á langvinnum bólgusjúkdómum, væri mikilvægt skref tekið í átt að þróun öruggari bóluefna og jafnvel í átt að fyrirbyggjandi meðferðarúrræðum fyrir einstaklinga með aukna áhættu.

ABSTRACT

The smallpox vaccine is a vaccine of considerable importance. It made the eradication of smallpox possible and has been shown to elicit humoral and cellular immunity that can last for over half a century. Due to concerns about smallpox being used as a biological weapon, the immune response to the vaccine is of considerable interest. In this study, long term B- and T-cell responses were investigated in individuals with extreme responses to the smallpox vaccine when they were immunised over three decades ago. They were divided into three groups: 1) Those that experienced adverse reactions to the vaccine, 2) those that had "no take" or no response and 3) those that had a "take" or a normal response. This is the first study to analyse long term B- and T-cell responses to *Vaccinia* virus in subjects grouped according to these rare extreme type reactions. Heat inactivated *Vaccinia* virus (VV) was used to stimulate blood cells isolated from the subjects and key cytokines secreted were measured by ELISA and Luminex. Additionally, B-cells were stimulated and VV specific IgG^+ antibody secreting cells (AbSCs) were enumerated by ELISPOT assays.

Both VV specific B- and T-cell responses were efficiently elicited in all three groups. B-cell memory responses were found to be weaker in the adverse reaction group than in normal responders and nonresponders. The subjects in the adverse reaction group had increased T cell responses, both Th1 and Th2 responses, when compared with normal responders and nonresponders. It therefore seems as if individuals that suffered adverse reactions to the smallpox vaccine may have been induced to produce stronger Th1 and Th2 memory cells at the cost of a less robust memory B-cell response. It is noteworthy that the nonresponders had measurable B- and T-cell responses despite showing "no take" at the time of vaccination.

The novel results of this study have increased our understanding of long term T- and B-cell memory to VV and how it relates to the primary reaction to vaccination with the *Vaccinia* virus. They have also provided the unique opportunity to overlay the immunological results for the two groups of extreme vaccine responders onto the extensive genotype data available. Other researchers have already demonstrated that variations in both antibody and cellular immune responses are genetically controlled by HLA genes. The identification of HLA and other genes which could increase the risk of adverse reactions to vaccines, and possibly increase the risk of developing chronic inflammatory diseases, would be an important step towards the design of a safer vaccine formulation and perhaps towards preventative treatment for high risk individuals.

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ABBREVIATIONS

Ab	Antibody
AbSC	Antibody secreting cell
APC	Antigen presenting cell
AP	Alkaline phosphatise
AIDS	Acquired immune deficiency syndrome
BCR	B-cell receptor
Blimp-1	B-lymphocyte-induced maturation protein 1
BSA	Bovine Serum Albumin
CKBP-II	Chemokine-binding protein type II
CrmB	Cytokine response modifier B
CRP	Complement regulatory protein
DC	Dendritic cell
DIs	Dairen I Strain
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
FCS	Foetal calf serum
FDC	Follicular dendritic cell
GPCR	G protein-coupled receptor
HA	Hemagglutinin
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP-10	IFN-γ induced protein 10
LC16m8	Lister clones 16m8
MAC	Membrane attack complex
MHC	Major histocompatibility complex
MIG	Monokine induced by IFN-γ
MIP	Macrophage Inflammatory Protein
MITF	Microphthalmia-associated transcription factor
MVA	Modified Vaccinia Ankara
NK	Natural killer (cells)
ORF	Open reading frame
PAX5	Paired box protein 5
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
Pfu	Plaque forming units
PRC	Patient recruitment centre

PWM	Pokeweed mitogen
Rpm	Rotations per minute
RT	Room temperature
SAC	*Staphylococcus aureus*
SECRET	Smallpox virus-Encoded Chemokine Receptor
SNP	Single-nucleotide polymorphism
SPICE	Smallpox inhibitor of complement enzymes
SCF	Stem cell factor
T_{CM}	Central memory T-cell
TCR	T-cell receptor
T_{EM}	Effector memory T-cell
Th	T helper (lymphocyte)
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TIMP-2	Tissue inhibitor of metalloproteinase 2
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine peroxidase substrate
TNF	Tumour necrosis factor
Treg	T regulatory (cell)
vCCI	Viral chemokine inhibitor
VCP	Vaccinia virus complement-control protein
VV	Heat Inactivated *Vaccinia* Virus
WHO	(The) World Health Organisation
XBP-1	X-box binding protein 1

1 INTRODUCTION

1.1 The history of the fight against smallpox

On May 8th in the year 1980, the World Health Organisation triumphantly declared that one of mankind's oldest enemies had been defeated (1). Smallpox had been eradicated. It was an enormous achievement, and incontrovertible proof of the power of vaccination. However, the journey toward this thrilling conclusion was a long and arduous one.

1.1.1 The beginning

Poxviruses, the largest and most complex viruses that infect humans, are presently distributed worldwide among mammals, reptiles, insects and birds. It is therefore considered likely that poxviruses descend from infectious agents that plagued early forms of life (2). One of those descendants was the causative agent of smallpox; a very host-specific poxvirus called *Variola virus*. Since it only infected humans, its origins are somewhat obscure. It is known, however, that camelpox virus and taterapox virus (isolated from a West African rodent) are more closely related to *Variola* virus than other poxviruses, and it is believed that they share a common ancestor, presumed to be a rodent virus (3-5).

Analysis of archive data and comprehensive phylogenetic comparison of the many *Variola* virus isolates has allowed for the conclusion that the most probable time of separation of *Variola* virus from its ancestor is 3,400 ± 800 years before the present day (4).

1.1.2 Variolation

The first active measures taken to protect against smallpox were based on the common knowledge that survivors of the disease became immune to it. Physicians infected healthy individuals with smallpox organisms from patients with mild symptoms, hoping that resulting infection would be less severe than a naturally occurring one, and that immunity would be formed. These measures were called *variolation,* a term related to the word *Variola* (smallpox) which was used for the first time in AD 570, by Bishop Marius of Avenches. *Variola* either came from the Latin word *varius*, which means "stained", or from *varus*, which means "mark on the skin". Alternatively, the term *inoculation* was used, a word derived from the Latin *inoculare*, meaning "to graft", and the two terms were often used interchangeably (6, 7).

Variolation was practised in many places, in slightly different ways. In China, scabs of smallpox pustules were powdered and blown into the nostrils of healthy individuals through a tube. The Chinese also took pills made from the fleas of cows to protect against smallpox – a hundred years before Edward Jenner's cowpox vaccine. This practise is the first recorded example of oral vaccination. In India, the most common form of variolation consisted of applying scabs or pus from a patient to the intact or scarified skin of a healthy individual (6).

It was in 1717, when Edward Wortley Montague was appointed Ambassador to the Sublime Porte, that variolation found its most ardent English supporter. It was the Ambassador's wife, Lady Mary

Wortley Montague, who had a passionate interest in the technique. Smallpox had recently robbed her of both her 20-year-old brother and of her beautiful looks. When she learned of it, she became determined to spread variolation to England. Thus, on her return to London in April 1721, she had the Embassy surgeon, Charles Maitland, inoculate her 4-year-old daughter in the presence of the court physicians. This became the first professional variolation performed in England. News of the technique quickly reached the ears of the Royal family, and Maitland was granted royal license to test variolation on six prisoners at Newgate. The prisoners were promised full pardon in exchange for their cooperation in the "Royal Experiment". They all survived and they were all pardoned. Maitland moved on to treating charity children in London and even successfully treated two daughters of the Princess of Wales on the 17th of April in 1722. Following this latest success, variolation gained wide-ranging approval and acceptance, both in Britain and in America (6, 7).

Although two or three percent of variolated individuals died of smallpox or complications from the procedure, case-fatality rates were 10 times lower than those of the naturally occurring smallpox. James Jurin, innovatively applying statistics to a medical and social problem in the year 1722, observed that the smallpox-associated case-fatality rate was 1:14 in noninoculated children and 1:91 in inoculated children. Therefore, variolation was a justly popular practise until Jenner popularised the cowpox vaccination method (6).

1.1.3 Vaccination

Being a milkmaid must have been a somewhat sought after position when smallpox was at large. It was commonly known that milkmaids became immune to smallpox after developing cowpox. Additionally, cowpox did not pockmark their faces, allowing them both immunity against smallpox and a chance to keep their looks (6).

It was Dr. Fewster of Thornbury, Gloucestershire, who in 1765 wrote to the Medical Society in London, reporting that variolation failed to provoke a reaction in individuals who had previously been infected with cowpox. About a decade later, in 1774, a farmer by the name of Benjamin Jesty is known to have vaccinated his wife and sons using material taken from cowpox infected udders. However, because Jesty's wife had reacted badly to the cowpox vaccine the technique did not catch on. Later, in 1791, a schoolmaster by the name of Peter Plett tried his hand at cowpox vaccinations. He vaccinated three children in Hasselburg, Holstein, protecting them from a terrible smallpox epidemic which swept through Holstein three years later. He, like Jesty, was deterred from testing the technique again because one of the children became inflamed at the vaccination site (6).

Where Jesty and Plett failed, Edward Jenner succeeded. As Francis Galton said, "In science credit goes to the man who convinces the world, not the man to whom the idea first occurs." (7)

Edward Jenner was born in the year 1749 in the UK western county of Gloucestershire. He became apprentice to Daniel Ludlow in Sodbury when he was 13 years old. During Jenner's stay there, he is reported to have heard a milkmaid say "I shall never have smallpox for I have had cowpox. I shall never have an ugly pockmarked face." Little did the milkmaid know what a profound effect her words would have on the course of human history (6).

When Jenner reached his 21st year, he moved to London and became John Hunter's apprentice at St. Georges Hospital. Hunter instructed Jenner well, teaching him the use of the scientific method. Hunter also encouraged Jenner's interest in natural science, suggesting that he write scientific studies. He offered Jenner a place as his assistant for his teaching and dissecting efforts at the Hospital, but Jenner decided to return to his hometown of Berkley and set up a general practice (8).

Jenner continued to be fascinated by the connection between cowpox and smallpox despite his many successes in other areas. He was convinced that cowpox had the power to protect against the human smallpox. When he came into contact with a milkmaid named Sarah Nelmes, who had recently developed cowpox he used the opportunity to experiment. On 14th of May, 1796, Jenner extracted fluid from a pustule on Sarah Nelmes' hand, and used it to inoculate a boy called James Phipps. Phipps was 8 years old at the time. Jenner proceeded to allow six weeks to pass before variolating Phipps, testing to see if there would be a reaction. Excitingly, the variolation produced no reaction. Some months later, Jenner repeated the procedure and confirmed that Phipps did not respond to it. Jenner's findings indicated that the cowpox vaccination had been a success (6).

His promising findings prompted Jenner to write an article, describing 13 cases of people who did not respond to variolation. All 13 individuals had previously had cowpox. The article also included Jenner's experiment with James Phipps. Once the article was ready, near the end of 1796, Jenner sent it to the Royal Society. The members of the Council of the Royal Society were unimpressed by the paper (6).

Determined to succeed in spreading the knowledge he had obtained, Jenner rewrote his manuscript and included additional case histories. Jenner arranged to have his enhanced manuscript published in London at his own expense, circumventing the Royal Society (8). It appeared, the year 1798, under the title "An inquiry into the causes and effects of the *variolae vaccinae*, a disease discovered in some the western counties of England, particularly Gloucestershire, and known by the name of the cow pox." (6, 9)

The initial reaction to the "Inquiry" was not entirely favourable. Jenner was faced with much criticism from sceptics and from those with a vested financial interest in the lucrative practice of variolation. However, with time and effort, Jenner's cowpox vaccine started to become the most popular way to defend against smallpox (6). Remarkably, it was not until seventeen years after Jenner's death, in the year 1840, that Parliament outlawed the practice of variolation, making cowpox vaccination the official UK policy (8).

1.1.4 The eradication

Jenner's contribution to the struggle with infectious illness can scarcely be overestimated. The World Health Organisation (WHO) had a World Health Assembly early in 1950 where the idea of the eradication of smallpox was first considered. However, it was not until 1967 that things really started to move forward. It was then that the Health Assembly formally signed up to the goal of global smallpox eradication (10).

On the 26th of October in 1977, Ali Maow Maalin, a resident of the village Merka in Somalia, became sick with the smallpox. He was the last man to contract the disease as a result of direct contact with another smallpox-afflicted human being. As already mentioned, the eradication of smallpox was announced by the WHO three years later, in 1980 (11).

Today the *Variola* virus, which causes smallpox, only exists in two laboratories. The strains are kept in the high-security facilities of the Centre for Disease Control and Prevention in Atlanta, the United States, and at the State Research Centre of Virology and Biotechnology (the Vektor Institute) in Novosibirsk, Russia. The final destruction of all smallpox virus strains has been postponed many times, due to a lack of consensus among the members of the WHO's executive board (12, 13).

Regrettably, the eradication of smallpox has led to a bitter irony. Because large scale vaccination programs against smallpox have been discontinued, and herd immunity is therefore not being actively maintained, the population of the world has been rendered susceptible to *Variola* virus and its kin again. Aside from relatively benign problems, such as an increase in cowpox infections in humans (14-16), there is the much more serious worry of *Variola* virus somehow being unleashed on the population once more. *Variola* has the potential to become a devastating biological weapon should it fall into the wrong hands (17-21).

Because of the growing terrorist threat, and the distressing potential that *Variola* virus has as a weapon, there has been a resurgence of interest in smallpox and smallpox vaccines. Researchers have been spurred into action. They have attempted to answer questions about the poxvirus family, to map out the response of the immune system to the virus or the vaccine (22-25), understand and improve the vaccine (26-29) and quantify the longevity of smallpox immunity (30-32). In this thesis, the poxviruses, the disease that smallpox causes, immunological memory, the interaction poxviruses have with the human immune system and the immune memory created by the smallpox vaccine will be explored. Additionally, the findings of the present study, comparing long term B- and T-cell memory responses in subjects with different reactions to the smallpox vaccine, will be detailed and analysed.

1.2 The poxviruses

The viruses of the *Poxviridae* family infect most vertebrate and invertebrate species. They cause an assortment of diseases, some of which are of veterinary and medical importance (33).

Variola virus is of the *Orthopoxvirus* genus. Orthopoxviruses cause skin lesions in mammals, and *Variola* virus is the most renowned member of the group. However, it is *Vaccinia* virus, the virus used in the smallpox vaccine, which is the type species of the genus. It is widely used as a model poxvirus in the laboratory (34).

1.2.1 Poxvirus characteristics

Poxviruses have large (130 – 280 kb) double stranded DNA genomes with cross-linked ends. The central regions of their genomes include genes for all the proteins needed for DNA synthesis and production of viral mRNAs. The regions of the poxviral genome that flank the central region have genes that encode for proteins that modify the host cell environment to promote viral replication and

spread. The terminal regions encode for immunomodulatory proteins and virulence factors (2, 23). Furthermore, orthopoxviruses have genome sequences that encode for the hemagglutinin (HA) protein, an infected-cell membrane antigen that distinguishes orthopoxviruses from other poxvirus genera (35).

The virions of the *Poxviridae* family are large and brick-shaped with three components. The components are designated: the outer membrane, the lateral bodies and the core. Additionally there is the inconstant component of an envelope (11). Virions released naturally from infected cells are enclosed with a lipoprotein envelope which, in the case of *Vaccinia* virus, contains *Vaccinia* HA protein and several other virus-specific polypeptides (11).

Virions released when an infected cell lyses lack an envelope. Without the envelope the virions are infectious, but it is believed that the envelope plays an important role during their spread inside the animal body, and thus in pathogenesis. It has been suggested that the low protection power of inactivated vaccines is partly due to the fact that they consist of inactivated non-enveloped virions, while live virus vaccines produce envelope proteins during the process of replication (11).

Poxvirus virions contain a great number of polypeptides, each with several epitopes. Three aspects of the composition of the polypeptides and their antigenic makeup are relevant: Firstly, some antigens show cross-reactivity across the whole subfamily *Chordopoxvirinae*; secondly, many antigens, among them those important in generating a protective immune response, show cross-reactivity within the genus *Orthopoxvirus;* thirdly, some antigens are species specific (11).

Poxviruses are only able to replicate with assistance from host cells. However, they can survive in aerosol form, particularly in cool, dry environments. They are vulnerable to heat, but they can be protected to a considerable extent if they are freeze-dried. Ultraviolet light and most kinds of commonly used hospital disinfectant will “kill” the virus (36).

1.2.2 *Variola* virus and smallpox disease

Variola virus exists in two forms and causes two distinct variations of smallpox disease. The difference is indicated by denoting the different forms: *Variola* major and *Variola* minor. *Variola* major infections have a much higher mortality rate on average than *Variola* minor infections. More specifically, it has been found that *Variola* major had a mortality rate of 5 – 40% while *Variola* minor had a mortality rate that ranged from 0,1% to 2% (11).

Variola virus shares most basic features with other orthopoxviruses. Its linear genome contains about 200 genes and the central region of the genome. Indeed, much of the interest in *Variola* virology stems from *Variola*’s ability to evade the host immune response. *Variola* is thought to encode for proteins that affect many different components of the immune system, as well as proteins that manipulate host cell signal transduction pathways (36).

The evidence also indicates that excretions from the mouth and nose of infected individuals were the most important sources of infectious virus, rather than their scab material (11). Infection by inhaled virus could have occurred through the mucous membranes of the mouth, the nasal cavity, the oro- or

nasopharynx, or via the alveoji of the lungs (11). Once an individual became infected, the clinical course of the illness began. It is thought that after first infection by inhaling the *Variola* virus, the virus replicated in the respiratory or oropharyngeal epithelium (11, 37). It was then likely taken up by macrophages, probably after primary viraemia. After that it likely entered the reticuloendothelial system where asymptomatic replication could continue. A second viraemia is thought to have occurred thereafter at the end of the incubation period, signalling the beginning of the next phase of the disease. Additionally, the incubation period is thought to have been an important time for the development of the subject's immune response.

The sudden onset of fever and malaise were the first symptoms of the prodrome phase (Figure 1), following directly after the incubation period (11). *Variola* major infected subjects would also commonly complain of painful headaches and backaches. The appearance of a macular rash signalled the end of the prodrome phase of the illness, and the beginning of the eruptive stage (11).

The eruptive stage was characterised by the appearance of lesions on mucous membranes and skin, mostly on distal limbs, face, soles and palms. This stage usually persisted for at least fourteen days (Figure 1). It was at the eruptive stage that the virus could most likely be found in the skin lesions, bone marrow, spleen, kidneys, and liver as well as in other organs.

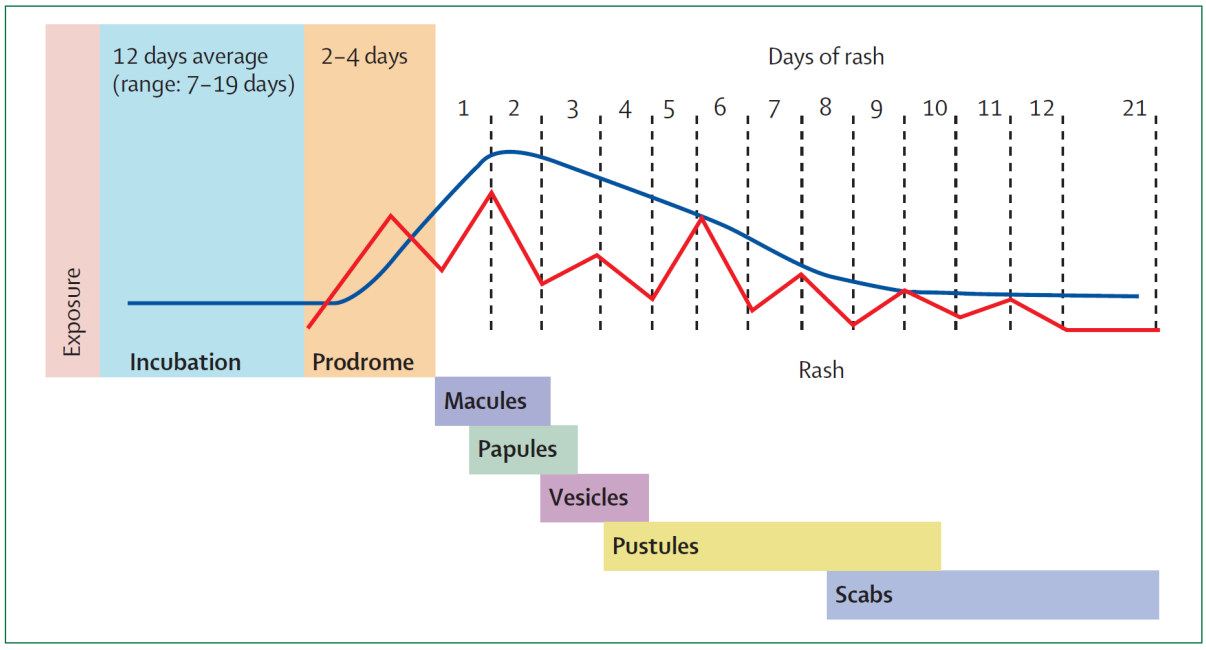

Figure 1. The course of smallpox infection. Fever, rash development, and viral shedding in ordinary-type smallpox. Red line signifies temperature; blue line signifies respiratory viral shedding (36). Reprinted from The Lancet, Vo. 367, Zack S. Moore, Jane F. Seward, J. Michael Lane, Smallpox, page 426, ©2006, with permission from Elsevier.

Smallpox infected individuals were at their most contagious stage around the onset of the rash (Figure 1). Smallpox sufferers would continue to be contagious through the entire course of their illness, and those who survived could remain contagious for up to 13 days after the onset of fever (11).

For those who did not survive smallpox, usually expiring between the tenth and sixteenth day of illness (11), the cause of death is not well understood, but often attributed to “toxemia” (2). Direct viral cytopathic effects, inflammatory mediators, circulating immune complexes and soluble *Variola* antigens are also thought to have played a role (38).

1.2.3 *Vaccinia* virus

Edward Jenner’s original “variolae vaccinae” was cowpox virus. However, through some twist of fate, it was *Vaccinia* virus which became the most widely used for vaccination. The two viruses have similar clinical effects on humans, producing only local lesions and causing minimal systemic disturbance. Both provide cross-protection against *Variola* virus should one become infected. Therefore, both cowpox and *Vaccinia* virus were ideal vaccination agents. The reason why *Vaccinia* virus prevailed as the more widespread of the two may never be truly known (11).

There are many strains of *Vaccinia* virus, each with distinct biological properties. However, the strains share several key features, such as wide host ranges, distinctive genome maps and the ability to grow rapidly on a chorioallantoic membrane. Like all orthopoxvirus genomes, the *Vaccinia* virus genome is a single linear molecule of double-stranded DNA. The molecular mass of the DNA of different *Vaccinia* virus strains varies between 118 million and 125 million, and when *Vaccinia* virus DNA is denatured, it behaves rather unusually. The two sister strands of the DNA molecule do not separate, instead they form quite a large single-stranded circular molecule. This happens because the terminal fragments of the genome are able to cross-hybridise with each other, or indeed, with the terminal fragments of other orthopoxvirus genomes (11).

The *Vaccinia* virus’ wide host range might explain the confusion that shrouds its origins. When it was still in extensive use for human vaccination, accidental infections of domestic animals were not uncommon. Serial transmissions could have happened naturally between different species of animals in such cases. Whether such events contributed to the mysteries of the *Vaccinia* virus background is unknown. Some theories suggest that *Vaccinia* is a hybrid between cowpox and *Variola* virus, others that it may have been derived from cowpox virus, or some other orthopoxvirus, by serial passage under laboratory conditions of culture. Still another theory proposes that *Vaccinia* virus is the laboratory survivor of a virus that has become extinct in nature (11).

The clinical features of *Vaccinia* virus infection in immunocompetent individuals are localised to the site of vaccination. A cutaneous lesion, also known as a Jennerian pustule, will most often appear and progress through the classic stages of orthopoxvirus disease. First it will become a papule, then a vesicle; finally it will become a pustule before scabbing over and falling off. Usually the vaccinated individual will be left with a pockmark or a scar as a souvenir. Occasionally there will be adverse reactions to the vaccine (12).

Most clinical descriptions of *Vaccinia* virus are associated with vaccination. However, over the past decade zoonotic outbreaks of *Vaccinia* have been reported in Brazil. The infected individuals have usually had direct contact with infected cattle, but it is not known whether the *Vaccinia* virus strain in question is a wild type or a surviving vaccine strain. Those who contract the disease develop lesions

on the hands and forearms, as well as fever, malaise and lymphadenopathy. Milk from infected animals may also cause a drinker to develop oropharyngeal lesions (39).

1.3 Immunological Memory

“Fool me once, shame on you; fool me twice, shame on me.” Immunological memory provides a vital selective advantage to organisms equipped with it. In essentials, it prevents a pathogen from fooling an individual twice. Upon re-exposure to a particular pathogen, an individual with functioning immunological memory will not experience a full blown infection. Instead, the disease manifestation might be very mild or even nonexistent.

Long-lasting protective immunity is generated during the primary, adaptive immune response. During an adaptive response to a particular pathogen, a population of specialised memory cells is formed. These memory cells persevere, protecting the individual upon re-exposure to the same pathogen in a memory response. Memory responses, also known as secondary or tertiary immune responses, are very different from primary immune responses (40).

During primary immune responses B- and T-cell memory cells are formed. Upon re-exposure they are able to respond to the invading pathogen swiftly and precisely. B- and T-cell-derived memory functions on two levels. On the protective level, memory cells immediately defend peripheral tissues from familiar invaders. On the reactive level, they mount recall responses to recognised antigens in secondary lymphoid organs. B-cell memory for these different functions is carried out via distinct cell types. Protective memory is mediated by long-lived plasma cells that secrete Abs, and the reactive memory is mediated by memory B-cells that proliferate and differentiate into plasma cells. Memory T-cells are thought to divide their labour in a similar way (41). They have been suggested to mediate protective immunity via effector memory T-cells (T_{EM}) and reactive immunity via central memory T-cells (T_{CM}). In response to a familiar pathogen, T_{EM} cells migrate to inflamed peripheral tissues and display immediate effector function while T_{CM} cells home to T-cell areas of secondary lymphoid organs and differentiate into effector cells in response to antigenic stimulation (42).

1.3.1 Memory B-cells and plasma cells

Among the many types of differentiated B-cells that arise when the immune system is challenged there are plasma cells and memory B-cells. Plasma cells bestow immediate protection from pathogens by the secretion of specific antibodies (Abs), while memory B-cells confer a rapid and superior response to secondary challenge by the pathogen they recall (43).

Plasma cells and memory B-cells both start out as naïve B-cells. Becoming a plasma cell or a memory B-cell is an involved process for the naïve B-cell and is made up of several phases. First the naïve B-cell must be activated. During Phase I of the activation process a naïve B-cell receives the first of three signals. First, its B-cell antigen receptor (BCR) is appropriately triggered (44). This involves transmitting a signal directly to the cell’s interior when the BCR binds its antigen, and

delivering the antigen to the inside of the cell, where it is degraded and returned back to the B-cell's surface as peptides bound to major histocompatibility complex (MHC) class II molecules. Second, it receives T-cell help in the T-cell zone of the secondary lymphoid organ. Armed helper T-cells, having already encountered antigen presenting cells and undergone clonal expansion, are able to recognise the peptide:MHC class II complexes on the surface of the B-cell. This stimulates the T-cells to produce cytokines such as interleukin (IL)-2, IL-4, and IL-6 and cause the B-cell to proliferate and differentiate (Phase II). Finally, it either receives a signal delivered by Toll-like receptor (TLR) agonists or by cytokines which are produced by activated dendritic cells (DCs) (45, 46).

A successfully activated naïve B-cell migrates away from the T-cell zone and either proliferates and forms a primary focus where plasmablasts are formed, or it migrates to a primary lymphoid follicle where it ultimately enters Phase III and forms a germinal centre (Figure 2).

Germinal centres are specialised microenvironments where B-cells undergo very important modifications. They are composed mainly of proliferating B-cells, but antigen-specific T-cells, called follicular helper T-cells (Tfh cells), comprise about 5 – 20% of germinal centre lymphocytes. Their main purpose is to help the B-cells, and as such they are an indispensible part of the germinal centre (47, 48). Germinal centre Tfh cells are a distinct T helper cell lineage that can arise from naïve $CD4^+$ T cells (49). Tfh cell derived CD40L, IL-4 and IL-21 play important roles in germinal centre B-cell proliferation, survival and affinity maturation (50-52).

Affinity maturation is one of the important modifications that B-cells undergo in the germinal centre. The others are called somatic hypermutation and isotype switching. Somatic hypermutation generates diversity by introducing a flurry of point mutations into the V regions of the rearranged heavy- and light-chain immunoglobulin (Ig) genes, forming mutant BCRs on the surface of the B-cells. Some of these mutants have a higher affinity for the antigen, most do not. Due to the process called affinity maturation B-cells with ever higher affinity for their antigen are selected for survival, while those with lower affinity become apoptotic. Finally, isotype switching allows the same assembled V region to be expressed in IgG, IgA or IgE Abs. This allows the selected B-cells to express a range of different effector functions (47).

Following these modifications the selected B-cells will differentiate into memory B-cells or plasma cells (Figure 2). Due to the modifications, these plasma cells will secrete higher-affinity and isotype-switched Ab in the later stages of the primary immune response. A subset of these plasma cells will migrate to the bone marrow and live there for a long period. They are the source of long-lasting high affinity Abs (47).

Memory B-cells are the long-lived descendants of cells that were originally stimulated by antigen. It has been pointed out that memory B-cells are considerably heterogenic, including unswitched IgM and unmutated memory B-cells (53). However, classical studies have focused mainly on the IgG isotype of isotype switched B-cells as it has long been considered a good marker for identifying memory B-cells (54, 55). Part of the reason for this is that IgG is able to confer signals necessary for long-term survival through its intracytoplasmic tail (56).

Conventional memory B-cells divide slowly, if at all, and they express surface immunoglobulin. They do not secrete Abs like the plasma cells do. If they secrete Abs at all, they do so at a very low rate. Since memory B-cell precursors took part in the germinal centre reaction, they inherit the somatic mutations and the isotype switching which occurred at that point. They seem to be distinctly "programmed" on the basis of their antigen receptor affinity to enter the long-lived memory cell pool (47, 48).

Once memory B-cells have been generated by the primary immune response, re-exposure will cause them to expand very rapidly, with the help of memory T-cells, and produce a burst of plasma cells. This is sometimes called Phase IV (57). The amount of plasma cells in peripheral blood peaks on day seven after the boost and can exceed by 100 fold the baseline level. Notably, the increase is accounted for almost entirely by antigen-specific plasma cells (58, 59). The increase in plasma cells coincides with a burst of serum Abs which plateaus on day ten. This plateau indicates that most of the plasma cells produced are short-lived (43).

Specific signals within the germinal centre initiate the pathways that lead to the differentiation of B-cells into memory B-cells, short-lived plasma cells and long-lived plasma cells (48, 60). The interaction between CD40 and its ligand have been found to be very important to the generation of long-lived plasma cells and memory B-cells (61). It has been shown that memory B-cell development but not germinal centre formation is impaired by *in vivo* blockade of CD40-CD40 ligand interaction (62). CD40 has also been found to stimulate CD137 which in its turn stimulates B-cell proliferation, enhances B-cell survival, and induced the secretion of tumour necrosis factors (TNFs), such as TNF-α and TNF-β, when it engages with its ligand and the time of B-cell activation (63).

The selective expression of transcription factors B-lymphocyte-induced maturation protein 1 (Blimp-1) and X-box binding protein 1 (XBP-1) encourage differentiation into short-lived and long-lived plasma cells (64-67). When B-cells commit to the plasma cell differentiation pathway, one gene expression program is turned off and another is turned on. The paired box protein 5 (PAX5) which is crucial for the maintenance of naïve or memory B-cell identity (68) is repressed, and the genes required for differentiation into plasma cells and Ab secretion, e.g. XBP-1, are no longer inhibited (69). The microphthalmia-associated transcription factor (MITF) must also be absent in order for the path to plasma cell differentiation to be clear (70). The differentiation of B-cells into plasma cells is terminal. This permanence is partly due to the repression of c-Myc by Blimp-1, which leads to an impaired cell-cycle progression (71). In plasma cells, Blimp-1 along with XBP-1, IRF4, and other regulators are known to not only impair the cell-cycle progression, but to also decrease signalling from the B-cell receptor and communication with T-cells, inhibit isotype switching and somatic hypermutation, down-regulate CXCR5, and induce abundant immunoglobulin synthesis and secretion (48, 72, 73).

Memory B-cells and naïve B-cells can be told apart on the basis of their different surface markers. A widely used marker for memory B-cells is CD27, although a considerable fraction of memory B-cells lack CD27 expression (74). They would be easy to mistake for naïve B-cells if it were not for the constitutive expression of TLR and other markers of human memory B-cells (75), and the lack of ABCB1 transporter which is exclusive to naïve B-cells (74).

Plasmablasts and plasma cells can also be identified using surface markers. It has been shown that newly formed plasmablasts express CD62L, human leukocyte antigen-DR (HLA-DR), and Ki67, while plasma cells that have been displaced from the bone marrow lack these markers (76).

Short-lived plasmablasts secrete high affinity Igs for a short period only unless they are recruited from the peripheral blood into survival niches e.g. in the mucosa or the bone marrow. If they express the appropriate chemokine receptors and make it into a survival niche, they are provided with factors that help them endure and differentiate into long-lived plasma cells (77). One theory suggests that in order to prevent the "fading" of memory for pathogens encountered in the distant past, newly generated plasmablasts and older plasma cells are thought to compete amongst themselves for survival niches. This elegant mechanism focuses memory provided by plasmablasts on recently encountered pathogens, without losing the protection provided by the old plasma cells (78). Long-lived plasma cells will be discussed further in chapter 1.4.3. "Immunological Memory and *Vaccinia Virus*".

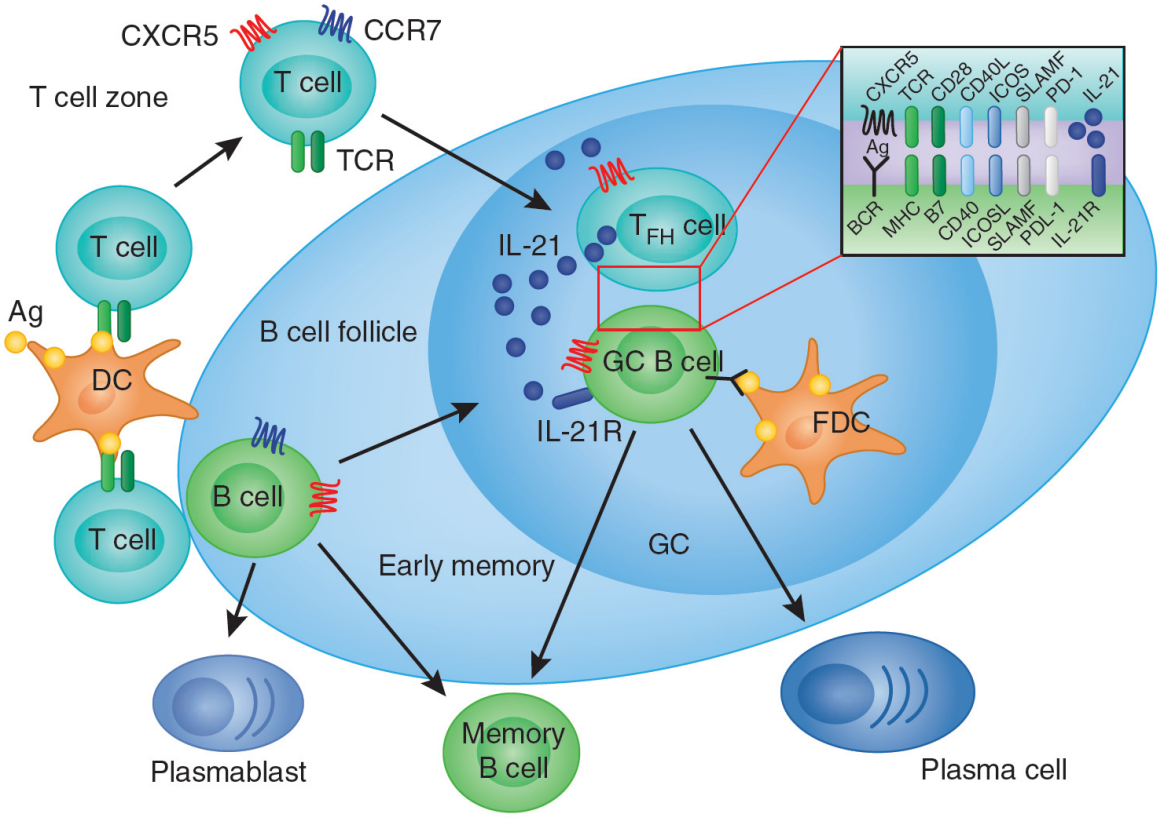

Figure 2. Multiple signals control the germinal centre output. Antigen-specific T cells, primed on DCs in the T cell zone migrate toward B cell follicles. They mature into Tfh cells after interacting with their cognate B cells. Follicular B cells encounter antigen, move to the border of the T cell zone to further differentiate into extrafollicular plasmablasts, give rise to early memory B cells or return to the follicle and undergo rapid proliferation to form a germinal centre. In the germinal centre, Tfh cells interact with germinal centre B cells through many molecular pairings, causing the T cell to secrete cytokines, particularly IL-4 and IL-21. The cytokines influence the formation of affinity-matured memory B cells and long-lived plasma cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology (48), © 2011.

1.3.2 Memory T-cells

Naïve T-cells, like naïve B-cells, are mature lymphocytes that have not yet encountered their specific antigens. They recirculate between blood and peripheral lymphoid tissue until they meet their specific antigen and are induced to proliferate and differentiate. Meanwhile, the T-cells receive survival signals from periodic encounters with self-peptide:MHC complexes and through their IL-7 receptors (79).

Not all naïve T-cells are the same. They can either be naïve $CD8^+$ T-cells or naïve $CD4^+$ T-cells. A naïve $CD8^+$ T-cell is predestined to become an armed cytotoxic effector T-cell once it has encountered its specific antigen, proliferated and differentiated. Naïve $CD4^+$ T-cells, however, can differentiate into regulatory T-cells (Treg) or into effector T helper type 1 (Th1), T helper type 2 (Th2), T helper type 17 cells (Th17) or Tfh cells (80). The various effector $CD4^+$ cells have different cytokine repertoires and also exhibit diversity in their homing behaviour. Some migrate to lymph node follicles where they help B-cells, while some migrate to inflamed tissues. Both $CD8^+$ and $CD4^+$ T-cells can differentiate into long-lived memory T-cells (81, 82).

Studies have shown that IL-12 and interferon-α (IFN-α) are key cytokines when it comes to regulating the aspects of T_{EM} and T_{CM} differentiation. IL-12 promotes the development of T_{EM} cells, while IFN-α drives the development of T_{CM} cells. In the presence of both IL-12 and IFN-α both T_{EM} and T_{CM} cells are simultaneously developed (83).

1.3.2.1 Memory $CD4^+$ T-cells

During the course of an infection, antigen-presenting cells (APCs) display complexes of foreign peptides and MHC class II to the outside environment. Naïve T-cell clones are able to bind to these complexes via their T-cell antigen receptors (TCRs) in secondary lymphoid organs. Costimulatory molecules derived from the APCs, such as CD80 and CD86 binding to CD28, along with signals through the TCR, trigger cell division and differentiation in the naïve T-cells. The naïve T-cells differentiate into different kinds of effector cells depending on which cytokines, produced by the innate immune system, are present in their environment (84).

If effector cell differentiation happens in the presence of IL-12 and IFN-γ, the expression of a transcription factor called T-bet is promoted, and this commits the cell to the Th1 program. Th1 cells are specialised to activate macrophages that are infected by pathogens, or have ingested pathogens that are able to incapacitate it. Thus, they are particularly effective when it comes to immune defence against intracellular bacteria, viruses and tumours. Once activated, the macrophage gains the ability to destroy its pathogen load. If effector cell differentiation happens in the presence of IL-4 however, the transcription factor GATA-3 is activated and the cell commits to the Th2 program. Th2 cells are specialised for promoting immune responses to parasites and they also promote allergic responses. They provide help in B-cell activation by secreting B-cell growth factors, inducing B-cell proliferation and isotype switching (40, 85).

Regulatory T-cells are induced when pathogens are absent in the naïve T-cell environment, and TGF-β is relatively abundant. This favours the expression of the transcription factor FoxP3 which commits the cell to its regulatory fate. Treg cells go on to secrete immune suppressive cytokines, such

as IL-10 and TGFβ1 at sites of inflammation, which inhibit local DC maturation and migration to draining lymph nodes. They are also known to migrate to lymph nodes and suppress effector cell differentiation (86).

Defining Tfh cells beyond their role in providing help to B-cells so that they may form long-lived antibody responses is quite challenging. It is difficult because T- and B-cell interactions take place at many phases of thymus-dependent antibody responses and T-cells tend to evolve in both phenotype and function during this response. It is also made complicated by the heterogeny of $CD4^+$ T-cells in B-cell follicles, and the confusion about whether T-cells that help B-cells at extrafollicular site belong to the Tfh lineage. Furthermore, the point at which a Th cell becomes a Tfh cell is still somewhat ambiguous (87). A common definition of a Tfh cell states that they are cells that help clear viral infections, bacterial infections, and the products or toxins of such infections via their ability to help B-cells produce potent antibodies. Additionally they express the transcription factor Bcl-6 and secrete IL-21. The definition of Tfh cells is often includes CXCR5 expression and follicular homing ability, but such is not always the case (87).

Finally, naïve Th cells differentiate into Th17 cell in the presence of IL-1β and IL-23. TGF-β is not required. Th17 cells are able to produce IFN-γ in the presence of IL-12, becoming a cross between Th17 and Th1 cells, or even morphing into a Th1 phenotype completely (88). However, some research indicates that IL-23 promotes the maintenance of rather than the commitment to the Th17 lineage (89). Human Th17 cells appear to express IL-12Rβ2 and CD161 in addition to IL-23R and the transcription factor T-bet in addition to RORγT (90, 91). They reportedly produce IL-17A, IL-17F, IL-22, IFN-γ and possibly IL-26 (90, 92).

Th17 secrete IL-17 and recruit neutrophils in order to mediate protection against extracellular pathogens (40). They are also said to help promote acute inflammation. Chronically inflamed human tissues, such as those in subjects with multiple sclerosis, rheumatoid arthritis, Chron's disease or psoriasis are also infiltrated by differentiated Th17 cells which produce inflammatory cytokines (91). It seems however, that Th17 derived Th17/Th1 cells and Th1 cells, rather than true Th17 cells, are the cells which play a pathogenic role in inflamed tissues of chronic inflammatory disorders (88).

The cytokines and transcription factors that have now been discussed are very important factors in the differentiation of naïve T-cells. However, the molecular pathways and epigenetic mechanisms behind the regulation of $CD4^+$ T-cell differentiation are quite a bit more complex. The different $CD4^+$ T-cell phenotypes depend on the integration of both extracellular and intracellular cues to produce signatures of master transcription factors, cytokines, chemokines, receptors and microRNAs, which together enforce lineage-specific programs. Epitope density, antigen duration, the costimulatory molecules expressed and the cytokines secreted by DCs collectively determine the signal strength that influences whether a naïve T-cell becomes a Th1, Th2, Th17, Tfh or a Treg cell (93).

Once naïve T-cells have been induced to differentiate, the number of effector cells most commonly peaks about a week into the response to infection. During the so called contraction phase which follows after and lasts for about 1 to 2 weeks, about 90% of the effector cells die. The remaining cells are a population of long-lived memory cells. They are capable of sporadic self-renewal and long-term survival in the absence of the inducing MHC class II:peptide complexes. Memory $CD4^+$ T-cells are

heterogeneous and exist as protective T_{EM} cells and reactive T_{CM} cells. T_{EM} cells express homing receptors that facilitate migration to peripheral sites of inflammation and secrete an assortment of cytokines, such as IFN-γ, IL-4 and IL-5, within hours of TCR stimulation. T_{CM} cells secrete IL-2 and proliferate extensively directly following TCR stimulation, they are thought to circulate through the T-cell areas of lymph nodes and mucosal lymphoid organs and undergo secondary responses there. As it has been shown in mice that IL-2 producing memory $CD4^+$ T-cells are present mainly in the lymph nodes it is considered a likely theory (84, 94).

A recent paper proposed a model for the generation of T_{EM} and T_{CM} cells that includes three pathways (Figure 3). The first pathway of the model suggests that after naïve T-cells have been stimulated to proliferate and form effector cells, some of the effector cells, perhaps those that do not interact with B-cells, commit to particular program (e.g. Th1 or Th2) and then either perish or develop into T_{EM} cells (Figure 3; Pathway I). The second pathway speculates that Tregs may be induced, but that they fail to develop into T_{EM}-cells (Figure 3; Pathway II). The third pathway proposes that some early effector T-cells interact with B-cells and receive signals through ICOS. Then they become Tfh cells that survive while the antigen and the antigen-specific germinal centre reaction persists, after which they may become T_{CM} cells (Figure 3; Pathway III). Alternatively, some early effector cells may become T_{CM} cell precursors while others become Tfh cells. In that case, all of the Tfh cells die once the germinal centre reaction ends, and the T_{CM} cell precursors go on to become full-fledged T_{CM} cells (Figure 3; Pathway III). In any case, it is clear that committed Th1, Th2 and perhaps Th17 cells survive the contraction phase to form T_{EM} cells while B-cells are an important driving force in the T_{EM} - T_{CM} “decision” (84).

Studies have shown that both Tfh cells and 20-25% of T_{CM} cells express CXCR5, a receptor which together with its ligand, CXCL13, is important for B-cell follicle formation in secondary lymphoid organs. It has been suggested that the CXCR5-expressing T_{CM} cells may represent a subset of memory-type Tfh cells, programmed for homing to follicles and provide B-cell help when stimulated by antigen. The results of experiments implied that a preferential recruitment of circulating $CXCR5^+$ T_{CM}-cells to B-cell follicles is required for the promotion of a quick and efficient protective secondary humoral immune response (95). These results indicate that both alternatives for pathway III (Figure 3) could be in play. Some Tfh survive the germinal reaction and go on to serve as a $CXCR5^+$ subset of T_{CM} cells while T_{CM} cell precursors develop into the $CXCR5^-$ T_{CM} cell subset.

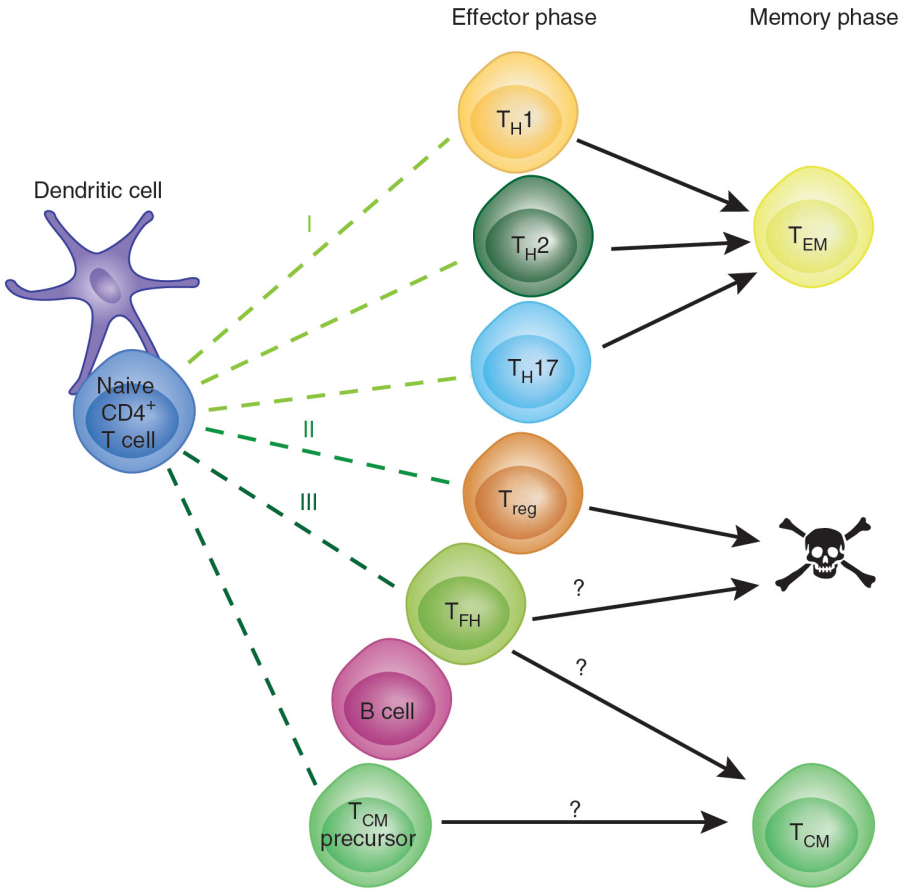

Figure 3. Simultaneous generation of T_{EM} and T_{CM} cells. Three pathways by which T_{EM} and T_{CM} cells are produced from a naïve $CD4^+$ T-cell. Pathway I: Effector cells successfully enter the memory phase as T_{EM} cells. Pathway II: Effector cells lack the potential to enter the memory phase and die. Pathway III: Cells present during the effector phase (follicular helper T-cells or precursor T_{CM} cells) become central memory cells after interacting with B-cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology (84), © 2011.

Memory $CD4^+$ T-cells are often characterised by an array of phenotypic and functional differences. It therefore seems likely that subsets such as the $CXCR5^+$ T_{CM} cell subset are more the rule than the exception. One theory proposes that the immune system evolved to express diversity in memory T-cell populations in order to provide increasingly flexible recall responses via heterogeneous effector cells that rapidly transition into correspondingly heterogeneous memory cells. When resting memory cells become reactivated by antigen they give rise to secondary effectors. Secondary effectors have superior functional attributes when compared with primary effectors, and are thought to play an important role in protective secondary immune responses (82). In addition to traditional helper roles for $CD8^+$ T-cell and B-cell responses, memory $CD4^+$ T-cells have been found to recruit innate inflammatory responses in the early stages of secondary immune responses, as well as enhance direct effector functions at later stages. They are thought to be involved in the recruitment of T- and B-cells, activation of DCs, licensing of DCs, inducing an antiviral state via cytokine production, and cytotoxic killing through cell to cell contact (96).

The question of how memory $CD4^+$ T-cells could be directed toward one phenotype or another is a fascinating one, and of some considerable importance. A key issue in the field is concerned with how to match memory T-cell responses to specific pathogens in order to get the most successful secondary response. One theory suggests that cellular and molecular regulators control the fate of the cell lineages and the functional capabilities of memory T-cells. However, another theory proposes that populations of memory T-cells are inherently unfixed and can be altered in function, and even in survival, at many stages during their long-term maintenance (97).

1.3.2.2 Memory $CD8^+$ T-cells

Cytotoxic $CD8^+$ T-cells recognise complexes of foreign peptides and MHC class I on cell surfaces and respond by producing cytokines, perforins and granzymes that kill the pathogen-infected cells. Thus, they aid in the control of infections. Once the pathogen has been cleared from the system, most of the pathogen-specific cytotoxic $CD8^+$ T-cells undergo apoptosis. However, 5 – 10% remain and mature into long-lived protective memory $CD8^+$ T-cells (81). This model, where memory $CD8^+$ T-cells are believed to be direct descendants of effector cells, is called the linear differentiation model. Another model, the branched differentiation model, suggests that memory T-cells are derived from a precursor that precedes effector T-cells and differentiates through a lineage parallel to the cytotoxic T-cells (98-101).

Studies have indicated that naïve $CD8^+$ T-cells are not preprogrammed to become either effector cells or memory cells, and that the cell fates are not determined until after T-cell activation. Indeed, a single precursor cell has been shown to have the capacity to give rise to both effector and memory $CD8^+$ T-cells (102, 103). The factors that determine the $CD8^+$ T-cell fates have not been conclusively recognised. Many signals have been proposed to serve a role in the process. The strength and duration of TCR stimulation, inflammatory cytokines, transcriptional regulations, metabolic switches and uneven segregation of lineage-determining factors have all been suggested as contributors. Additionally, the signal strength and duration received by any given T-cell could easily vary significantly due to differences in temporal and spatial exposure (81). Interestingly, studies have shown that without active signalling and maintenance, the functional phenotype of a memory $CD8^+$ T-cell is not sustained indefinitely, and can indeed begin to respond to stimulation like a naïve T-cell (104).

Regardless, memory $CD8^+$ T-cells can persevere for many years, even without any additional stimulation from antigen, and they have been shown to provide life-long protection (30). As such, they have a number of qualities in common with stem cells, such as the already mentioned longevity, but also telomerase expression, and the ability to self-renew (105, 106). Most importantly, they have the capacity to rapidly proliferate and differentiate into secondary effector $CD8^+$ T-cells once restimulated by antigen (79, 107).

The survival and self-renewal of memory $CD8^+$ T-cells is thought to be mediated through interactions with IL-7 and IL-15 (79, 107, 108). However, it is IL-2 which has been found to be of profound importance when it comes to promoting the differentiation of not only primary effector $CD8^+$

T-cells, but also of memory $CD8^+$ T-cells capable of differentiating into effector T-cells. Without high-avidity IL-2 receptors, memory $CD8^+$ T-cells fail to repopulate the effector pool upon secondary challenge (109).

Memory $CD8^+$ T-cells are heterogeneous like memory $CD4^+$ T-cells, and also exist as protective T_{EM} cells and reactive T_{CM} cells. Interestingly, recent studies have shown that in the absence of natural killer (NK) cells, differentiation of $CD8^+$ T-cells is strongly biased toward a T_{CM} phenotype. While T_{CM} cells that were generated in the presence or the absence of NK cells had similar functional features and recall capabilities, NK cell deletion caused a significantly higher number of antigen-specific $CD8^+$ T-cells and enhanced memory responses (110).

1.3.2.3 Key T-cell cytokines, chemokines and inflammatory mediators

The main Th1 cytokines are IL-2 and IFN-γ. IL-2 is able to affect a large number of target cells in the immune system. The primary biological activity of IL-2 where $CD4^+$ T-cells are concerned is the induction of expansion and differentiation of antigen specific clones. IL-2 is required for the differentiation of $CD4^+$ T-cells to Th1 and Th2 subsets, and it induces expansion via proliferative and anti-apoptotic mechanisms. It also increases the production of other cytokines. As for $CD8^+$ T-cells, IL-2 plays a very similar role. It induces expansion of antigen-specific clones and increases cytokine secretion. However, it also induces the proliferation of memory $CD8^+$ T-cells (111).

IFN-γ is secreted by cells of both the innate immune system (e.g. NK cells) and the adaptive immune system (e.g. $CD8^+$ T-cells and Th1 $CD4^+$ T-cells). An important role of IFN-γ is to control intracellular infection, especially viral infection, and tumour development. In addition to anti-viral activity IFN-γ has broad biological functions including: enhancing antigen presentation, influencing the cell cycle, influencing antibody isotype switching by B-cells, and immune regulating functions such as inhibiting Th2 $CD4^+$ T-cell development (112).

The main Th2 effector cytokines are IL-4, IL-5, IL-9 and IL-13. They are produced by numerous sources including antigen-stimulated $CD4^+$ T-cell subsets such as Th2 cells or antigen-stimulated basophils. The expression of these cytokines leads to IgE class switching, goblet cell hyperplasia, recruitment of eosinophils, basophils and mast cells, and enhanced proliferation and differentiation of $CD4^+$ T-cells (113).

IL-4 causes antigen-stimulated T-cells to develop into Th2 cells capable of producing IL-4, IL-5, IL-10 and IL-13 and suppresses IFN-γ producing $CD4^+$ T cells. IL-4 also determines the specificity of Ig class switching, causing B cells to switch to the expression of IgE and IgG4 (114). IL-13 was originally thought to be functionally redundant with IL-4, but studies indicate that although IL-13 mainly promotes IgE class switching like IL-4 it also has several distinguishing effector functions, e.g. the modulation of tumour cell growth (115). IL-5 has mainly pro-eosinophilic effects and is associated with antigen-induced eosinophilic inflammation in the lung (116)

IL-9 is a $CD4^+$ T-cell derived cytokine with pleiotropic activities on many different cell types. It acts on T-cells, B-cells, mast cells, eosiniophils, neutrophils and epithelial cells (117). Experiments with transgenic mice that over-express IL-9 indicate that it has a role in the development of airway

eosinophilia, mast cell hyperplasia, mucus production and airway hyperresponsiveness (118). A recent study has also shown that IL-9 mediated CCL11 may play a crucial role in airway inflammatory responses (119).

IL-15 is a growth factor and an antigen-independent activator for $CD8^+$ memory T-cells. IL-15 induces similar responses in memory $CD8^+$ T-cells as anti-CD3, promoting the synthesis of effector molecules such as IFN-γ, TNF-α, perforin and granzyme B, and increasing cytotoxicity and cellular proliferation (120). IL-15 and IL-2 share structural similarities and are both able to induce cell proliferation (121).

IL-1β is a pyrogen and a mediator of the acute-phase response. That is, it causes fever, induces hepatic acute-phase proteins, activates lymphocytes (such as T-cells), and upregulates prostanoid synthesis. It is mainly produced by macrophages and epithelial cells. Additionally, IL-1β is a potent anorectic cytokine which causes mice to lose weight when subjected to a local inflammatory process. Human studies have also shown a correlation between elevated levels of IL-1β and decreased food intake (122).

TNF-α is produced by macrophages, NK cells and T-cells, it has both beneficial effects for the host in inflammation and in protective immune responses against numerous pathogens. However, it can exert host-damaging effects in sepsis, tumour cachexia and in autoimmune diseases (123, 124)

C-X-C motif chemokine 10 (CXCL10) also known as IFN-γ induced protein 10 (IP-10) binds to the CXCR3 receptor which is important for chemotaxis, apoptosis, cell growth inhibition and angiostasis. Inflammation is strongly associated with the secretion of IP-10 from leukocytes, neutrophils, eosinophils, monocytes, keratinocytes, epithelial cells, endothelial cells, and stromal cells. They secrete IP-10 in response to IFN-γ. Predominantly T- and B-cells, NK cells, DCs, and macrophages express the CXCR3 receptor and are thus capable of responding to IP-10 secretion. Abnormal levels of IP-10 in body fluids have been observed in individuals with viral, bacterial, parasitic and fungal infections. This indicates a role for IP-10 in the pathogenesis of these diseases, including autoimmune diseases (125).

The chemokines CCL3 and CCL4, also known as Macrophage Inflammatory Proteins (MIP)-1α and -1β, are chemokines crucial for immune responses towards infection and inflammation. They were originally identified as secretory products of endotoxin-stimulated mouse macrophages, but since then they have been discovered to be secreted by numerous cell types, including neutrophils, fibroblasts and epithelial cells. They serve as chemoattractants for monocytes, macrophages and T-cells, and MIP-1α is a potent regulator of Th1 cells. In general, CCL chemokines have a well established effect on leukocyte activation and trafficking. However, they also play a role in tissue repair and are involved in skeletal muscle regeneration (126, 127).

1.4 Poxviruses and immunity

Large DNA viruses, such as the members of the family *Poxviridae*, defend against hostile immune systems by producing a variety of gene products that destroy key components of the inflammatory

response. Poxviruses are known to target many of the primary mediators of innate immunity, such as IFNs, TNFs, ILs, complement and chemokines. They are also known to hijack or manipulate intracellular signalling pathways, such as those that promote cell apoptosis. Although some of the genes employed by the poxviruses to produce their immune evasive products have demonstrated no clear resemblance to any known host genes, most of the genes involved seem to have originally been "borrowed" from the obliging hosts (128, 129).

The immunological targets and strategies used by poxviruses to disrupt the host immune response are astonishingly diverse and sophisticated. Knowledge concerning the different immune evasive tactics of the poxviruses is essential, as it furthers the understanding of how vaccines are able to protect against illness, and how they might be improved. It has also been suggested that as the immune evasive gene products are anti-inflammatory by nature, they might hold some promise as possible therapeutic agents for acute or chronic inflammatory conditions (128, 130, 131).

Long-lasting immune memory, induced by the administration of live *Vaccinia* virus vaccines against smallpox, or in the past by contracting and surviving the infection itself, is provided by an assortment of immune memory cells and serum Abs. The precise mechanisms behind protective immunity, the identification of major *Vaccinia*-specific T-cell and B-cell epitopes, and the quantification of how long "long-lasting" immunity really lasts, are all areas of particular interest in a world plagued by the threat of terrorist attacks. Just as the knowledge of poxviral immune evasive tactics aids in the quest to better understand and improve vaccines, the development of new, safer smallpox vaccines depends on a detailed understanding of the mechanisms behind protective, long-term immunity (24).

1.4.1 Human infection and immune evasion

The immune system is known to have responded to smallpox infections by producing neutralising Abs specific for both the extracellular virus and the mature virus proteins. These Abs appeared during the first week of the eruptive stage, but their production could be slowed if the infection was severe. HA-inhibition and complement-fixing Abs were generally detectable between days sixteen and eighteen of the infection. Neutralising Abs have been shown to be detectable in serum for at least 20 years following infection, and probably provide lifelong immunity along with memory B- and T-cells. The level of HA-inhibition and complement-fixing Abs has been found to start decreasing after one year (11, 31, 132). It seems then, that when the immune response to *Variola* does not fail, it is relatively robust. However, since *Variola* major was fatal to a grimly significant amount of people, it must follow that the immune evasive strategies it employed must have been highly sophisticated.

Variola virus could not cause skin lesions unless the host was human or one of the non-human primates. It is possible that other animals could not become infected with smallpox because the ability to prevent host cells from entering apoptosis was species-specific to humans (2). The fact that humans were the sole reservoir for *Variola* virus was a big part of why it was possible to eradicate it (11). The *Variola*'s species specificity is therefore one of its key features, and since it has been suggested that viral host specificity may be partially defined by effective immune evasion strategies,

they are an important subject to study (128). Genetic variation in humans may have an effect on these immune evasion proteins, which could partially explain individual differences in symptom severity.

Poxviruses have been shown to inhibit a variety of host immune response proteins. Among them are complement proteins (133, 134), chemokines (135), interleukins (136), serine proteases (137), interferons (138-140) and tumour necrosis factors (134, 141, 142). This knowledge, along with much of what is known about *Variola* virus and its immune evasion strategies, has, however, been inferred from studies of homologous genes in *Vaccinia*, cowpox, myxoma and other poxviruses. This is partly due to the difficulty of finding an appropriate animal model for *Variola* (143), and partly due to the scarcity of available *Variola* proteins after its eradication (144). Problematically, these inferences about *Variola* virulence are not easily supportable because other orthopoxviruses do not cause a similar disease in humans.

Some of the *Variola* proteins that have been convincingly characterised are the smallpox inhibitor of complement enzymes (SPICE) (133) and the *Variola* virus high-affinity secreted chemokine-binding protein type II (CKBP-II, CPB-II), also known as viral chemokine inhibitor (vCCI) (129). They have been shown to play a role in immunoregulation and both are encoded in the terminal regions of the *Variola* genome (23). Another important immune evasive protein is the viral soluble receptor called cytokine response modifier B (CrmB). CrmB is one of the so called “viroceptors”, or virally encoded proteins secreted from infected cells, which bind and inhibit biological activity of TNFs, different kinds of IFNs, IL-18, chemokines and other mediators of the host immune system. Previously CrmB was known only as a TNF-binding protein, but recently it has been shown that the C-terminal domain of the protein mediates chemokine-binding activity as well. The domain was therefore named Smallpox virus-Encoded Chemokine Receptor (SECRET) and has been found to be closely related to the cowpox virus vCCI protein (134).

SPICE allows the *Variola* virus to manipulate the host complement response by acting similarly to mammalian complement regulatory proteins (CRPs). Their function is to protect neighbouring cells from inadvertent injury during complement activation. Mammalian CRPs inactivate the complement proteins C3b and C4b, thus inhibiting complement activation, preventing membrane attack complexes (MACs) from being formed and averting the generation of proinflammatory complement fragments. As their name suggests, the MACs would under normal circumstances attack the membranes of virally infected cells while proinflammatory complement fragments like C3a and C5a would encourage local inflammation (23). The poxviral CRPs, such as SPICE and its homologue *Vaccinia* virus complement-control protein (VCP), parallel this function. They disable host C3b and C4b, which leads to an elegant circumvention of the host complement system (23).

SPICE has been shown to be more human complement specific than VCP (133). SPICE inhibits human and baboon complement better than dog or guinea pig complement. The opposite is true for VCP. Mammalian CRPs are known to function best against complement from phylogenetically related species, and can therefore be said to exhibit “homologous restriction” (145). It seems possible, since SPICE is more human specific than VCP, that viral CRPs exhibit “host complement restriction”. This host-specific characteristic of SPICE lends credence to the idea that viral CRPs are derived from their

host CRPs, which would of course confer a great selective immunoregulatory advantage to the virus (23).

The advantage that virally encoded CRPs provide can be visualised as a protective microenvironment, or shield, around a virally infected cell. Within the shield the infected cell cannot be attacked by the host complement system. An efficient viral CRP can be the key to allowing viral production and spread to continue unimpeded, and since SPICE is a more potent inhibitor of human complement than VCP, it may be associated with *Variola*'s species-specificity and virulence by "shielding" infected human cells and allowing for more viral progeny (23).

Variola CKBP-II and the SECRET domain of CrmB are both important mediators of chemokine binding. Chemokines are chemoattractant cytokines. They have been divided into groups based on the arrangement of conservative N-terminal cysteine residues. The groups are CCL, CXCL, CXL and CX3CL chemokines. All known cellular chemokine receptors are type III transmembrane proteins associated with G-proteins. Chemokines can be instrumental to the regulation of both innate and acquired immunity, playing important roles in orchestrating leukocyte migration to sites of injury and inflammation (134).

Homologues for CKBP-II have been found to be highly conserved among orthopoxviruses, with approximately 85% homology (23). CKBP-II proteins are absent in eukaryotes, however. This sets these proteins apart from other known orthopoxvirus virulence factors such as SPICE (129). Both *Variola* and *Vaccinia* CKBP-II proteins bind CC chemokines (β-chemokines) broadly, but not CXC chemokines (α-chemokines). The SECRET domain of CrmB, however, is known to bind the CCL25, CCL28, CXCL12β, CXCL13 and CXCL14 chemokines (134). By binding chemokines, the CKBP-II and CrmB proteins competitively inhibit the chemokines from binding to the host G protein-coupled receptors (GPCRs), interrupting and preventing host chemokines from playing their role in the host immune response (23). More of the host processes that are targeted by *Variola* virus are listed in Table 1.

Cowpox virus is most likely the orthopoxvirus with the widest host range. Additionally, as cowpox virus has the largest genome, and has been suggested to be the most ancient and the closest to the common ancestor virus (2, 146). Along with the highly conserved genes that are essential for virus replication, cowpox encodes for a large number of "nonessential" immunomodulatory proteins. In fact, it encodes for a much larger number of products that are involved in immune evasion than *Variola* virus. Collectively, these immune evasion proteins are able to target a wider range of anti-viral host responses. Cowpox encodes for proteins that are involved in complement evasion, suppression of cytokine and chemokine signalling, control of inflammatory cell influx via NF-κB inhibitors, inhibition of TNF-induced responses, blockade of IFN responses, inhibition of NK cell activation and T-cell evasion by downregulating MHC class I expression. Inhibition of NK cell activation is very important for successful virus dissemination, since NK cells are responsible for control of early virus spread and the efficient induction of the adaptive immune response (16, 147).

Table 1. The host processes targeted by *Variola* virus (2).

Site of action, virus-encoded protein	Host process targeted	Vaccinia[a]	Variola	Cowpox
Intracellular				
Serine proteinase inhibitor	Programmed cell death	crmA/SPI2	+	+
dsRNA BP	IFN response	E3L	+	+
eIF2α homologue	Protein synthesis	K3L	+	+
Extracellular				
Epidermal growth factor homologue	Keratinocyte proliferation	C11R	+	+
TNF-α BP	Inflammatory response	−	+	+
IFN-γ BP	Th1 response	B8R	+	+
IFN-α/β BP	Type I IFN response	B18R	+	+
IL-1β BP	Inflammatory response	B15R	−	+
IL-18 BP	Th1 response	B16R	+	+
Chemokine BP	Recruitment of neutrophils and macrophages	P35	+	+
Complement control protein	Lysis of virions and infected cells	C3L	+	+

NOTE. Virus species differ in the number of functional immunomodulatory proteins encoded in their genomes; for example, cowpox virus encodes 4 different TNF-α–binding proteins, whereas variola encodes 1 and vaccinia none. Similarly, variola differs from the others in lacking a functional IL-1β–binding protein (BP). crmA, cytokine response modifier A; dsRNA, double-stranded RNA; SPI, serine proteinase inhibitor; +, functional gene present; −, functional gene absent.

[a] Gene location(s) in vaccinia virus, the reference species.

Mike Bray and Mark Buller, Looking back at smallpox, Clinical Infectious Diseases, 15;38(6):885, by permission of Oxford University Press.

Other cowpox-encoded proteins which are thought to have immunomodulatory functions are semaphorin homologues and epidermal growth factor homologues, but they have not been characterised fully. These proteins can be grouped as intracellular and extracellular immunomodulators based on where they take effect. Extracellular immunomodulators can be grouped further based on their function, as virokines and viroceptors. Virokines are virally encoded proteins, often cytokine homologues that are secreted from the host cell. Similarly, viroceptors are virally encoded receptor homologues. Most of the “nonessential” genes in the cowpox virus genome are found in at least one other orthopoxvirus family member, suggesting that by studying cowpox virus-“specific” immunomodulators it will be possible to gain insights that are relevant for the entire viral family of pathogens (16).

Vaccinia virus, the type species of the orthopoxvirus family, has also been subjected to in depth analysis of its immune evasive techniques. As already mentioned, VCP is an important immunomodulatory protein which inhibits the host complement response. The importance of VCP becomes even clearer when it is taken into account that it has been shown that the neutralisation of enveloped virions is predominantly complement dependent and that targeting VCP with Abs reduces *Vaccinia* virus pathogenicity (148, 149). VCP is both secreted from and expressed on the surface of infected host cells. As VCP does not have a transmembrane domain, this surface-expressing quality was considered slightly puzzling. Further studies revealed that surface expression of VCP occurs through an interaction with the viral transmembrane protein A56 (130, 150).

MHC class II-mediated antigen presentation to $CD4^+$ T-cells is a part of the immune system's surveillance of bodily tissues for foreign and pathogenic material. Studies have shown that following

Vaccinia virus infection, the ability of professional and nonprofessional APCs to present antigen and peptides to $CD4^+$ T-cells was hindered. Biochemical and functional analysis revealed that *Vaccinia* virus infection directly interfered with ligand binding to MHC class II molecules. These observations indicate that disruption of MHC class II-mediated antigen presentation may be one of many methods that have evolved to allow *Vaccinia* virus to escape host immune surveillance (151).

Vaccinia virus is able to inhibit interferon activity via the immunomodulatory functions of the secreted viroceptor B18R and the cytoplasmic protein E3L. B18R neutralises IFN-α/β, while E3L blocks IFN-α/β effector function in infected cells. Studies on whether *Vaccinia* virus is also able to inhibit IFN-λ activity in host cells proved that it was able to do so, despite the fact that IFN-λ cytokines signal through a distinct receptor unrelated to the IFN-α/β signalling pathway (139).

The inhibition of IFNs, especially type I IFNs like IFN-α and IFN-β, prevents them from suppressing IL-17 expression and Th17 differentiation (152). It has been shown that IL-17 modulates the immune response to *Vaccinia* virus infections, as an increase in IL-17 expression correlates with a marked increase in virulence (153). An increase in IL-17 can have still further consequences. Individuals with atopic dermatitis are generally not vaccinated against smallpox as they have a tendency to develop eczema vaccinatum, a disseminated *Vaccinia* virus infection. Studies have shown that *Vaccinia* virus inoculation in sites of allergic skin inflammation causes a robust cutaneous IL-17 response (154). In a mouse model of eczema vaccinatum, eczematous mice exhibited lower NK cell activity than healthy mice. Critical failures in NK cell-mediated immunity seemed to allow for early spread of *Vaccinia* after cutaneous infection, causing eczema vaccinatum, and these early NK cell defects were, at least partly, due to the immunosuppressive effects of IL-17 (155).

1.4.2 Vaccines against smallpox

The history of smallpox and the first attempts to protect people against its devastating effects has already been detailed. However, the story of how crude inoculation techniques became sophisticated vaccination procedures is still incomplete. The *Vaccinia* virus smallpox vaccine was so successful that it is still widely considered the gold standard for vaccines. Despite this golden reputation and success, it is considered by many to be in some ways less than ideal. The serious side effects it has a propensity to cause is the main reason behind this concern. Ways to improve the vaccine are being actively explored, exciting hopes that in the case of a bioterrorist attack, safe and effective vaccines will be available for all those in need (27).

1.4.2.1 The vaccinia vaccine

Smallpox vaccine is prepared from live *Vaccinia* virus and does not contain *Variola* virus, the causative agent of smallpox, or cowpox virus, Jenner's original smallpox vaccine. When *Vaccinia* virus is inoculated into the superficial layers of the skin, the virus grows and produces a protective immune reaction (156). Following vaccination in immunocompetent individuals a lesion will in most cases appear and progress through the stages of papule, vesicle, pustule and scab. The scar left behind

when the scab falls off serves as confirmation of the individual's immune status (39). This classic reaction is called "a take", or a "primary reaction" if it results from the first vaccination and a "major reaction" if it results from additional vaccinations. Some individuals do not experience the classic reaction; a circumstance termed "no take". The reasons behind this have been speculated to have something to do with the vaccination method; either the virus was implanted too deeply or without sufficient penetration of the external layer of the skin (156).

Childhood vaccination against smallpox was a routine in many countries until the early 1970s and until 1978 in Iceland. Children were vaccinated at 1 – 2 years of age, 6 – 7 years of age or at 12 years of age in Iceland with the Denmark strain of *Vaccinia* virus. If a pustule formed at the injection site on the seventh day following vaccination it was considered "a take" but if no take was observed, revaccination was recommended (11, 36, 157, 158). Lack of response or "take" to repeated vaccination attempts occurred in approximately 1.5% of individuals in Iceland (Jonsdottir I, unpublished data).

Regardless of whether vaccination produces "take" or "no take", it is generally a safe and effective way to prevent smallpox. For example, the proportion of "take" in an Israeli revaccination campaign, where 21000 individuals were revaccinated against smallpox, was 66.1%. This was similar to past vaccination programs when "take" also occurred in approximately two thirds of vaccinees. An Ab response occurred in 77.7% of all revaccinees: 94.4% of those with "take" and 56.6% of those with "no take" (159). However, some individuals experience adverse reactions in response to the vaccine. In the study mentioned above, as well as in another study, the most common side effects corresponded to symptoms of non-specific viral disease, such as weakness, fatigue, nausea, headache, joint pain, muscle pain, fever and chills (159, 160). Side effects such as these can happen to individuals with and without specific, preexisting susceptibilities, but vaccinees with preexisting susceptibilities run the danger of experiencing more rare and serious side effects than flu-like symptoms. These rare conditions range from being benign to life-threateningly serious (Table 2).

An example of a benign adverse effect is "erythema multiforme", where vaccinees develop erythematous papules, plaques or urticaria-like skin rashes after vaccination. Another example would be generalised vaccinia, a syndrome which is almost always benign and results from the viremic spread of the virus from the vaccination site. These types of reactions can look rather frightening but they are ultimately harmless. The more serious condition called eczema vaccinatum has already been mentioned. It can arise when individuals suffering from true atopic dermatitis receive the vaccine into diseased skin. This can have a fatal outcome. Other very serious, albeit rare, conditions that can follow vaccination are encephalitis or meningoencephalitis, myopericarditis, vaccinia keratitis – a condition where lesions of the cornea threaten eyesight – and perhaps the most serious of them all, progressive vaccinia. Progressive vaccinia occurs in individuals with immune defects, most commonly in those with T-cell deficiencies. In cases where T-cell defects are profound, the persistent and treatment-resistant vaccinia infection is nearly always fatal (161, 162).

Table 2. Adverse reactions that may occur after smallpox (vaccinia) vaccination (161).

Adverse event	Event subtype
Noninfectious rashes (erythema multiforme)	Macular
	Vesicular
	Pustular
	Urticarial
	Mixed
	Stevens-Johnson syndrome
Bacterial infection	Staphylococcal
	Streptococcal
	Enteric
	Anaerobic
	Mixed
Inadvertent inoculation	Autoinoculation
	Contact inoculation
	Vaccinia keratitis
	Eczema vaccinatum
Congenital vaccinia	Fetal/neonatal disease
Generalized vaccinia	Single occurrence
	Recurrent disease
Progressive vaccinia	In congenitally T cell–deficient individuals
	In antibody-deficient individuals (rare)
	In individuals with any disorder that suppresses T cell function (e.g., cancer and HIV infection/AIDS)
	Accompanying T cell–suppressive therapy (e.g., high-dose steroids and some forms of chemotherapy)
Postvaccinial encephalitis	Variable severity
Myopericarditis[a]	
From questionable case reports (temporal association only)	
Hemolytic anemia	
Arthritis	
Osteomyelitis[b]	
Cardiac lesions	
Thrombocytopenia	

[a] No known precursors at present.
[b] Vaccinia virus has been isolated from bone in some instances.

Factors that are known or thought to predispose to adverse events in the vaccinees include: pregnancy, breast feeding, extensive skin eruptions present at the time of vaccination, atopic dermatitis, presence or probability of T-cell immune defects or disease, immunosuppressive therapy and inflammatory or disruptive disease of the cornea or surrounding structures. Children less than one year of age are also thought to be predisposed to experience adverse events following vaccination. These predispositions are considered to be contraindications to smallpox vaccination. They only apply if the virus has not been introduced into the environment. In the event of a bioterrorist attack there are no contraindications to vaccination of exposed individuals (156).

In the 1960s, deaths resulting from smallpox vaccination were rare. They occurred approximately once in every million primary vaccinations, with fatalities resulting from progressive vaccinia, postvaccinial encephalitis and eczema vaccinatum. Deaths resulting from revaccinations were even rarer, and those fatalities occurred almost entirely from progressive vaccinia. However, today's population might be more susceptible to adverse events because the increased prevalence of immune deficiencies and atopic dermatitis (162). Thankfully, recent studies indicate that cases of eczema vaccinatum may be treatable. Monoclonal Abs capable of neutralising *Vaccinia* virus, anti-H3, which targets a mature virion antigen, and anti-B5, which targets an antigen of the enveloped virion, have been shown to successfully lessen the severity of both the disease kinetics and the erosive viral skin lesions caused by eczema vaccinatum in animal models (163, 164).

The development of such Ab treatments was made possible by the multitude of studies that have increased our understanding of how the immune system responds to the vaccinia vaccine. One such study showed that H3, a mature virion viral receptor involved in cell adhesion, is the most immunodominant *Vaccinia* virus neutralising Ab target. It is strongly conserved among orthopoxviruses, with *Vaccinia* and *Variola* virus sharing 97 – 98% amino acid identity, and may contribute to the observed cross-protection against smallpox provided by vaccinia immunisation. However, depletion or blockade of anti-H3 Abs revealed no significant reduction in neutralisation activity, indicating that though H3 is the immunodominant Ab target, the vaccinia vaccine succeeds in generating strong neutralising Ab responses by driving development of antibodies to multiple viral proteins targets, thus forming a "safety net" of redundant Ab responses that can vary from individual to individual. Therefore, it is not surprising that a recent study has showed that monoclonal Abs targeting another virion protein, the membrane protein A13, have been used to effectively treat orthopoxviral infections in mice (165). This redundancy and plasticity of the neutralising Ab response has been proposed to be a fundamental feature of the human immune response to the smallpox vaccine (24, 166).

The epitopes and antigens recognised by vaccinia specific immune responses, both cellular and humoral, are many and varied. Broadly, $CD4^+$ T-cell responses target late and structural antigens, while $CD8^+$ T-cells preferentially recognise early antigens, often distinct from those that are recognised by $CD4^+$ T-cells. The antigens recognised by helper T-cells are highly correlated with those that are recognised by Ab responses (167, 168).

Vaccinia-specific $CD8^+$ T-cells have been found to respond primarily to early virulence factor-related products. MHC class I peptides have been reported for the *Vaccinia* virus proteins B8R, D1R,

C10L, C19L, C7L, F12 and O1L. The immunodominant B8R epitope was recognised by approximately 10% of $CD8^+$ T-cells at the peak of the response to *Vaccinia* virus in a mouse study (169). Most $CD8^+$ T-cell responses are observed during primary vaccination, while long term memory responses are associated with $CD4^+$ T-cell compartments. Studies of MHC class II epitopes have been complicated by the finding that *Vaccinia* virus is able to disrupt MHC class II presentation of antigens by APCs *in vitro* (151). However, peptides from the I6L, D6R, A10L, D1R and A24R *Vaccinia* virus proteins have been found to bind to the MHC class II HLA-DR1 protein (170, 171). The *Vaccinia* virus envelope proteins A27 and B5 have also been found to include several epitopes recognised by both $CD4^+$ and $CD8^+$ T-cells (172). Furthermore, T-cell responses have been observed for peptides derived from 23 additional *Vaccinia* virus proteins, which represent a mixture of early, late and intermediate proteins, including proteins that are present in the virion as well as proteins that are only expressed in infected cells (173).

Clearly, both *Vaccinia*-specific $CD8^+$ and $CD4^+$ T-cell responses target a group of epitopes without a strong immunodominance hierarchy in humans. The evidence suggests that interactions between HLA alleles and other genetic or environmental factors result in many different patterns of *Vaccinia* virus epitopes recognised by human $CD8^+$ and $CD4^+$ T-cells. This indicates that the cellular immune response is in line with the “safety net” strategy of the humoral response mentioned earlier (24, 174).

Interestingly, studies focusing on the role that neutralising Abs play in primary *Vaccinia* virus infections have revealed an early neutralising T-cell dependent, but principally germinal centre reaction independent, IgM response. The anti-*Vaccinia* virus IgM Abs are instrumental to the early control of the infection, prior to the development of a vigorous IgG response (29).

1.4.2.2 Modified vaccinia vaccines

Vaccinia virus has been used more extensively for human immunisation than any other vaccine, and it is the only vaccine that has been employed to successfully eradicate a disease. However, since the eradication of smallpox, continued research on *Vaccinia* virus has produced a good number of modified vaccines with improved safety profiles. Some of these new, highly attenuated vaccinia virus vaccines have been considered for stockpiling against a possible re-introduction of smallpox through bioterrorism.

Vaccinia virus vaccines can be divided into four generations based on their origins.

First-generation vaccinia virus vaccines were based on the wild-type vaccinia virus. This was the generation used during the eradication campaign.

Second-generation vaccinia virus vaccines were newer forms of the wild-type vaccine.

Third-generation vaccinia virus vaccines were attenuated through sequential passage in alternative hosts.

Fourth-generation vaccinia virus vaccines are produced through “engeneering” of the *Vaccinia* virus genome.

The first generation vaccines were largely manufactured on the skin of live animals. Many different strains were used during the eradication drive. Once the eradication program had come to a successful end, the Wyeth Laboratories Inc.'s Dryvax® vaccine was the only commercially approved smallpox vaccine available for limited use in the United States. Dryvax® was manufactured from the lymph fluid of calves' skin infected with the *Vaccinia* strain that was used in the United States during the eradication drive. However, it was the Lister strain, developed at the Lister Institute in the United Kingdom, which was the most widely used in the world at the height of the eradication program. Additional vaccine strains included the Paris strain (France), the Copenhagen strain (Denmark), the Bern strain (Switzerland), the Ankara strain (Turkey), the Temple of Heaven and the Vaccinia Tian Tan (VTT) strains (China), and the Dairen strain (Japan) (175).

The use of live animals for the production of vaccine material has become an unacceptable process. This led to the production of second-generation vaccinia virus vaccines. These vaccines were produced using tissue culture systems or embryonated chicken eggs. ACAM2000™ is a second-generation smallpox vaccine licensed for use in the United States in 2007. It was derived from plaque purification of a Dryvax® isolate that was consequently manufactured in the Vero monkey cell line. Comparisons of the safety and immunogenicity of ACAM2000 and Dryvax®, along with ACAM1000™, in healthy vaccinia-naïve adults revealed that all three vaccines were able to produce "take", with 4-fold neutralising Ab rise and T-cell immune responses being similar between the groups, each of which included thirty individuals. However, both Dryvax® and ACAM2000™ are known to cause adverse events in the significant minority of the population with contraindications. For this reason, third-generation vaccinia virus vaccines were developed (175-177).

To create third-generation vaccines, the commonly used attenuation technique involves multiple passaging of the wild-type virus in tissue culture cells from alternative hosts. This technique has been shown to alter viral host range, virulence and genome composition. Through its use, the generation of non-replicating, highly attenuated *Vaccinia* strains that retain immunising properties against smallpox was made possible. There are three vaccine candidates that have been obtained in this way. Lister clones 16m8 (LC16m8), Modified *Vaccinia* Ankara (MVA) and Dairen I Strain (DIs) (175). MVA has been shown, through Ab profiling via proteome microarray, to have comparable immunogenicity with Dryvax® (178). Additionally, it has been shown to share epitopes with both *Variola* virus and one of the first generation *Vaccinia* virus strains, the Western Reserve strain (179). However, it has also been shown using mouse models that long-term protection from disease and $CD4^+$ T-cell levels were lower in mice vaccinated and boosted with MVA than the ones induced by the traditional vaccine (180).

The advance made in biotechnology allowed for targeted attenuation of viruses and thus, fourth-generation vaccinia virus vaccines. The change made to *Vaccinia* virus involved the deletion of immune-modulating, host-range and accessory nucleotide metabolism genes, as well as the deletion of essential genes. The goal is to attenuate the virus while maintaining or even increasing its immunogenicity. One of the best characterised genetically attenuated mutants of *Vaccinia* virus is NYVAC, which has a deletion of 18 open reading frames (ORFs). Deleted regions and genes include the host range region, hemorrhagic region, the A-type inclusion region, the HA gene and the region encoding for the large subunit of ribonucleotide reductase. It was derived from the Copenhagen

Vaccinia virus strain, is highly attenuated in human cell lines and still retains the ability to produce a strong immune response. When compared with the MVA and Lister strains however, it becomes clear that vaccination with NYVAC results in lower levels of IFN-γ producing $CD4^+$ T-cells and neutralising Abs.

Other non-essential immunemodulatory genes that have been deleted are the C12L and A53R genes. C12L encodes for an IL-18 binding protein and A53R encodes for a TNF receptor homologue. These deletions have been shown to reduce virulence in mouse models, and the deletion of C12L by itself was enough to attenuate the virus in a rabbit model (181).

However, the deletion of genes may not be the best way to increase immunogenicity. At best, the deletion of certain genes appears to merely maintain immunogenicity. Therefore the effect of the insertion of genes has also been investigated (175).

Vaccinia virus has also been attenuated through the insertion of genes encoding host immune response modulators, such as IL-2, IFN-γ and IL-15. Studies on IL-2 and IFN-γ expression in *Vaccinia* virus have found that both IL-2 expressing viruses and IFN-γ expressing viruses are non-pathogenic in athymic nude mice. Studies on smallpox vaccines with integrated IL-15 have been shown to increase superior immunogenicity, efficacy and safety in mice and to confer long term protection against a lethal monkeypox challenge in cynomolgus monkeys. This indicates that although deletions may not increase immunogenicity, some additions might do the trick (175, 182, 183).

The expression of immune-stimulating genes may enhance immunogenicity of highly attenuated, non-replicating *Vaccinia* virus strains. MVA has been shown to infect APCs, which, in turn, activate $CD4^+$ and $CD8^+$ T-cells that ultimately induce an antigen-specific, adaptive and cell-mediated immune response. If MVA expressed cytokines that enhance the activation of APCs it could boost the overall response to the vaccine. Cytokines that have been proven to be successful in such a context are GM-CSF, CCL20 (or MIP-3α), and fms-like tyrosine kinase (Flt)3 ligand (175, 184).

It is important to note that *Vaccinia* virus-based vaccines are not the only new vaccine candidates. Considerable progress has been made in the development of protein, peptide or nucleic acid based subunit vaccines. Most researchers have focused on the inclusion of viral proteins targeted by humoral immunity, although the identification of $CD4^+$ and $CD8^+$ T-cell epitopes has allowed for cellular responses to poxviruses to be taken into account. It has been found that the combination of membrane and virion proteins (A27L, A33R, B5R, H3L, L1R) or DNA are far more effective than single protein vaccines. However, the genetic diversity of poxviruses must be considered. While the vaccines are based on *Vaccinia* virus, the most important pathogenic poxviruses are *Variola* and monkeypox. Cross-protection is the goal, and using whole viruses provides a pool of *Vaccinia* antigens, many of which are conserved enough to provide it. With protein or DNA based subunit vaccines the selection of antigens is much more limited, which may have a negative effect on vaccine efficacy (19).

Basing vaccine components on *Variola* homologues, or indeed *Variola* antigens, could be the key to making up for the limited selection. Subunit vaccines which include *Variola* virus antigens have already proved promising. It has been shown that codon-optimised DNA vaccines expressing three *Variola* antigens (A30, B7 and F8) and their recombinant protein counterparts induced a high-titre,

cross-reactive, *Vaccinia* virus neutralising Ab response in mice (185). However promising such results may seem, it is important to recall that DNA vaccines have not been successful in many larger animals, humans included. This may or may not change if the novel strategies that have been suggested to improve DNA vaccine immunogenicity in humans are implemented (186).

Another area of investigation is the use of different delivery routes, including mucosal vaccines and plant-based formulations (19). However, at the end of the day it will probably take a combination of different strategies to attenuate the wild-type *Vaccinia* virus while enhancing specific immune pathways. The continued efforts to genetically modify poxviruses will only improve the understanding of these viruses. It will also allow the preservation of safety, while taking advantage of the immunogenic benefits reaped from replication competence (175).

1.4.3 Immunological Memory and *Vaccinia* Virus

The true duration of immunological memory can only be properly studied if two conditions are met. First, the pathogen under consideration must not persist in the host. Persistent viruses, such as the Epstein-Barr or herpes viruses, can re-activate sporadically boosting the host immune response. Second, the pathogen must not be endemic to the population in question. Intermittent re-infection could skew the calculated duration of immune memory. Taking these conditions into consideration, it is clear that the memory formed via the smallpox vaccination, using *Vaccinia* virus, is an ideal model for study. The virus is typically completely cleared from the site of infection within a month and it does not spread systematically or persist in healthy individuals. Additionally, as smallpox has been eradicated and routine smallpox vaccination of the general public has ceased, the possibility of re-exposure to *Vaccinia* is exceedingly slight. Old smallpox vaccination scars are therefore being regarded in a new light, as they represent an opportunity to measure antiviral immunity over the course of many decades in the absence of re-exposure to viruses that could have reactivated the immune response (187).

The ultimate goal of a vaccine is to bring about long-lived immunological protection against pathogens via the development of a pool of memory cells and Abs (188). The vaccinia vaccine has been shown to provide vaccinees with immunity that involves both T- and B-cell responses. Defects in either the B- or T-cell compartment increase susceptibility to *Vaccinia* viral infections, although children lacking humoral immunity (i.e. agammaglobulinemic or hypogammaglobulinemic) are less susceptible to severe viral infections than children lacking cellular immunity.

The negligible consequences of diseases which impede humoral immunity have sometimes been cited as evidence that T-cells are the most important component of protective immunity against viral infections. However, even though people with T-cell defects are subject to more serious *Vaccinia*-related complications than patients with B-cell defects, this evidence is misleading (189). In the absence of humoral immunity, the T-cell response is functioning mostly intact, potentially capable of compensating for Ab deficiencies, while in cases of T-cell dysfunction, both humoral and cellular arms of the immune system are impaired because defects in the T-cell compartment are severely detrimental to the Ab response. It is, however, clear that humoral processes serve a very important

role in protective immunity. It has been shown that smallpox vaccine-induced Abs are necessary and sufficient for protection against monkeypox virus (190). It has also been shown that human *Vaccinia* Abs increase patient survival and can help treat postvaccinal complications (189, 191, 192).

Before the eradication of smallpox, T-cell-mediated immunity was poorly understood, largely due to technical considerations. Hence, studies focused mainly on the humoral response. It was found that neutralising Abs appeared at day six of illness during non-hemorrhagic smallpox, while Ab responses were lower and occurred later in patients suffering from the hemorrhagic smallpox. The neutralising Abs were shown to persist for many years, while HA-inhibiting Abs were demonstrated to fall to very low levels within 5 years of infection. Complement fixation Abs were shown to last an even shorter time, or only for about a year after smallpox recovery (191, 193). The question remained, however, whether those vaccinated individuals would maintain long-lived immunological memory in the absence of circulating smallpox in the world.

Groups studying long term vaccinia-specific memory have shown that 90% of long term vaccinees retained specific IgG Abs, and between 50 and 80% retained neutralising Abs (30, 194). These results have been corroborated by a longitudinal study where vaccinia immunised participants were shown to maintain high vaccinia-specific IgG and neutralising Ab titres practically indefinitely (195). These Ab levels have been shown to remain surprisingly stable between 1 and 75 years after vaccination, and even all the way up to 88 years (30, 31, 195). As for memory B-cells, it was observed that vaccinia-specific B-cell numbers initially declined after immunisation, but reached a plateau approximately 10-fold lower than the peak and were stable for more than 50 years after vaccination. The persisting vaccinia-specific memory B-cells were found at a frequency of approximately 0.1% of total circulating IgG^+ B-cells, and were able to mount a robust secondary Ab response upon revaccination (31). Another study found that vaccinia-specific memory B-cells accounted for 0.07% of circulating IgG^+ B-cells, while they accounted for 0.24% of all IgG^+ B-cells in the spleen. This indicates that long-lived memory B-cells have a specific anatomic distribution (196).

The humoral response to *Vaccinia* virus has been shown to improve with multiple vaccinations. A lower fraction of individuals retained long term vaccinia-specific Abs following one vaccination than those who received multiple immunisations (197). Ab titres were also lower among individuals who were vaccinated once, compared with individuals who were vaccinated two times. However, additional vaccinations failed to improve the Ab titre further (30). This led the researchers to conclude that while booster vaccinations may improve a poor primary Ab response, they are ‘unlikely to induce prolonged synthesis of higher Ab numbers above a certain threshold’ (30). Interestingly, it has been shown that the ability to neutralise either intracellular mature virions or extracellular enveloped viruses was not changed by the number of vaccinations received (198). It is also interesting to note that neutralising Ab titres following primary smallpox vaccination vary by gender with females having significantly higher titres, while the comparison of variables such as race and ethnicity revealed no significant differences (199). The neutralising Ab titre has been shown to be correlated with anti-vaccinia Abs that tend to decline over the years (31), but no correlation was found between vaccinia-specific T-cells and Ab titres at early and late points after vaccination. This indicates that cellular and humoral immunity are independently regulated (30).

The question of the quality of the persisting T-cell response remains unanswered. While $CD4^+$ Th1 cells have been shown to be the main component of the long term cellular memory to vaccinia (32, 200, 201), it is known that $CD8^+$ effector and memory cells play a crucial role in viral infections (188).

Studies concerned with vaccinia-specific $CD8^+$ T_{EM} and T_{CM} cells have become increasingly prevalent as the technology to detect these cells has become accessible. It became possible to show that cytotoxic T-cell memory persists for 35 to 50 years after immunisation (32) and that long term proliferative memory responses persist in more than 70% of vaccinated individuals 25 years after the end of the vaccination era (200).

The prevalence of memory responses depends on rapid effector immune responses, which can be measured by the short term production of IFN-γ by T_{EM} cells, and the slower T_{CM} responses, which involve clonal expansion of vaccinia-specific T-cells and the secretion of IL-2 (200). A study by Kennedy et.al. revealed that only about 20% of vaccinees exhibit T_{EM} responses more than 30 years after vaccination, while 42% exhibit a T_{CM} response (202).

During the primary response many $CD8^+$ T-cells do not immediately start secreting IFN-γ in response to antigen. In contrast, memory $CD8^+$ T-cells start producing IFN-γ in response to the same antigen at much faster rate. When immunodominance is being established during the primary response there is a strong correlation between the abundance of each epitope-specific T-cell population and the speed at which it initiates IFN-γ secretion. It has been proposed that the $CD8^+$ T-cells that are quickest to produce IFN-γ are able to dominate the developing T-cell response (203).

It has been shown that vaccinia-specific $CD8^+$ T-cells induced by both MVA and Dryvax® are highly polyfunctional cells. Upon restimulation with antigen they are able to kill infected cells, secrete cytokines such as IFN-γ, IL-2 and TNF-α, and proliferate exponentially. They also exhibited an unusual phenotype, $CD45RO^-CD27^{int}$ (157, 204). The fact that about 50% of long term vaccinees lose vaccinia-specific $CD8^+$ T cell responses is therefore cause for some concern (30, 201). It seems that in humans, $CD8^+$ T-cell memory predominates at earlier stages following antigen exposure, becoming less stable than $CD4^+$ T-cell memory over time (Figure 4). This data conflicts with the conventional perception that $CD8^+$ T-cells dominate in the memory of live viruses. Differences in the contraction phases of vaccinia-specific $CD4^+$ and $CD8^+$ T-cells have been shown in humans, and these differences are thought to partly explain why higher frequencies of vaccinia-specific memory $CD4^+$ T-cells manifest in the long term memory, compared with memory $CD8^+$ cells (201).

A proposed reason for the preference of memory $CD4^+$ T-cells over memory $CD8^+$ T-cells is that the maintenance of $CD4^+$ T-cells is more important to the persistence of B-cell memory and Ab production in the absence of circulating antigens (191). However, as studies have shown that long term Ab production can occur in the absence of continuous $CD4^+$ T-cell help, and that there is no statistical correlation between $CD4^+$ T-cell memory and long term serum Ab levels, it seems likely that maintenance of B-cell memory is not dependent on $CD4^+$ T-cell help (30, 31, 205, 206). It seems evident that while T-cell help is critical during the initiation of humoral immunity, the ensuing response does not call for continual antigen-specific T- and B-cell interactions (24).

The role of $CD4^+$ T-cell help following the reactivation of memory B-cells during secondary antigen-specific immune responses appears to be more complex. It has been shown that while $CD4^+$ T-cell help was not required for the maintenance of B-cell memory, it was required for effective Ab responses following a secondary challenge with soluble antigen. Should $CD4^+$ T-cell help be lacking in such a case, the near lack of an Ab response could be partially recovered by the use of a larger dose of antigen paired with an adjuvant. Circumventing an absolute requirement for T-cell help may allow for rapid recall responses against foreign antigens in the correct context, without waiting for cognate cell-to-cell interactions with antigen-specific $CD4^+$ T-cells (24, 205).

It is unclear whether priming or periodic re-exposure to antigen is needed to maintain high frequencies of memory T-cells, though antigenic re-exposure is highly unlikely in the case of *Vaccinia* virus (11). It is known that the initial size of the $CD8^+$ effector compartment is correlated with the magnitude of the long term memory response (188). Another factor that contributes to the difference in $CD4^+$ and $CD8^+$ T-cell frequency is the difference between $CD8^+$ and $CD4^+$ T-cell proliferation. Naïve $CD8^+$ T-cells require much smaller amount of antigen in order to start proliferating and they proliferate at a much more accelerated rate than $CD4^+$ T-cells (207). Finally, survival of memory T-cells is regulated by complex homeostatic mechanisms, and is possibly influenced by cross-reactivity with other pathogens, which contribute to the differences in $CD4^+$ and $CD8^+$ T-cell frequencies (188, 208). The mechanisms that regulate memory T-cell survival depend on prior antigen exposure, cytokines such as IL-15 and IL-7, and the regulation of apoptosis (209-211).

Recently, IL-21 has been indicated as a mediator of $CD4^+$ T-cell help to the $CD8^+$ T-cell response. The study showed that direct action of IL-21 on $CD8^+$ T-cells was essential for the *in vivo* vaccinia-specific $CD8^+$ T-cell response, intrinsic IL-21 signalling being critical for the survival of activated $CD8^+$ T-cells and the generation of long-lived memory cells (212). Meanwhile, the TNF receptor molecule OX40 (CD134) has been shown to be a crucial player when it comes to primary vaccinia-specific $CD8^+$ T-cell expansion and antiviral cytokine production, also dictating the development of strong memory to both dominant and subdominant *Vaccinia* virus epitopes (213).

After differentiating into memory cells, neither $CD4^+$ or $CD8^+$ T-cells need to be stimulated again with either their specific antigen or a cross-reactive antigen in order to persist (214, 215). However mouse studies have shown that after a peak during acute viral infection, $CD4^+$ memory T-cells decreased slowly in the absence of antigen, while $CD8^+$ T-cell memory was maintained for life via homeostatic proliferation mechanisms (216, 217). Similarly, it has been observed in human studies on vaccinia-specific memory that $CD4^+$ memory T-cell responses decline gradually, with a half life of 8 to 12 years (Figure 4). Unlike in the mouse study, it was also observed that $CD8^+$ memory T-cell numbers declined over time, but unexpectedly they would drop below detection in approximately half the vaccinees sometime before 20 years postvaccination. As mentioned, it seems that for human vaccinia-specific immune responses, $CD4^+$ T-cell memory preferentially survives over $CD8^+$ T-cell memory (30, 31, 201).

It is interesting that while memory T-cell numbers slowly decline, memory B-cell numbers remain relatively stable (Figure 4). It is possible that they do undergo a similar decline, but the rate is too slow for current methods to detect. It is also possible that memory B-cells possess more vigorous DNA

repair systems, allowing them to repair damage from environmental irritation and maintain their proliferative potential. This idea, albeit speculative, could be tied to a developmental difference between mature B- and T-cells. Mature B-cells maintain the ability to upregulate extensive DNA repair programs as they are able to undergo somatic hypermutation, while T-cells do not (24).

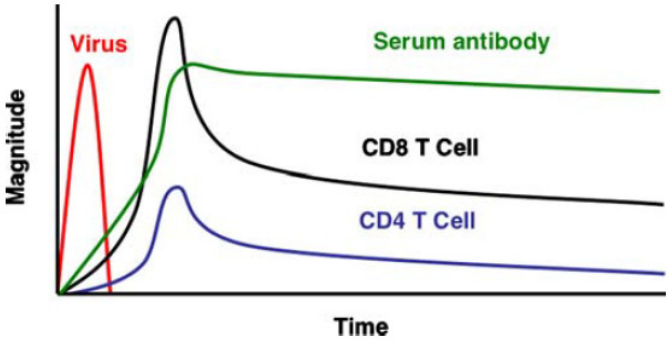

Figure 4. T- and B-cell mediated immune responses after acute viral infection (204). With kind permission from Springer Science + Business Media: Journal of Clinical Immunology, Human Immune Memory to Yellow Fever and Smallpox Vaccination, Vol. 29, 2009, page 152, Jens Wrammert, Joe Miller, Rama Akondy and Rafi Ahmed, Figure 1. © Springer Science + Business Media, LLC 2008.

These speculations aside, there are four proposed mechanisms for the maintenance of human memory B-cells:

The antigenic depot mechanism.
The stimulation by cross-reaction mechanism.
The bystander polyclonal activation mechanism.
The programmed homeostatic proliferation mechanism.

The antigenic depot mechanism is the classic model of the maintenance of antigen-dependent immunological memory. It has been suggested that after the generation of memory B-cells, they are maintained by periodic re-encounters with antigen presented long-term by follicular dendritic cells (FDCs) (218). Since essentially all proteins have a restricted lifespan however, it seems unlikely that biologically relevant levels of antigen could be retained by FDCs for more than a few months at most before the exhaustion of the antigen depot.

The second mechanism for the maintenance of human memory B-cells is stimulation by cross-reactive environmental or self-antigens. B-cells are stimulated by direct interaction of the BCR with antigen, but as the specificity of a BCR is not absolute, cross-reactivity is observed. It is therefore possible that memory B-cells are maintained long-term by intermittent interaction with environmental antigens. This hypothesis is extremely difficult to test (24).

The bystander polyclonal activation mechanism is a model that is based on the observation that memory B-cells stimulated *in vitro* with TLR ligands or select polyclonal activators will proliferate and differentiate into plasma cells while naïve B-cells will not. Based on these findings it was suggested that memory B-cells proliferate *in vivo* in response to unrelated infections (58). This model seems unlikely, as mice living in pathogen-free conditions maintain long-term serum Ab responses (219, 220).

Programmed homeostatic proliferation is the fourth mechanism which has been suggested for the maintenance of human memory B-cells. It draws from known mechanisms of $CD8^+$ T-cell memory maintenance in mice (188, 210). Murine memory $CD8^+$ T-cells are maintained for long periods of time mainly via IL-15 signals. It is, therefore, hypothesised that antigen-specific memory B-cells may preserve themselves by a programmed homeostatic maintenance involving sporadic proliferation triggered by autocrine or paracrine cytokines, or other as yet unknown factors (24).

The mechanisms behind the maintenance of plasma cells, the effector cells of B-cell immunity, have not been determined conclusively either. Long-lived plasma cells are a vital component of immunological memory, as they are largely responsible for the long term secretion of Abs. They are terminally differentiated, unlike memory B-cells, and cannot be stimulated by antigen to divide or increase their rate of Ab production. Despite these restrictions, it has been shown that Ab responses after *Vaccinia* virus immunisation were maintained for up to 88 years (195). There are two main theories for how long-lived antigen-specific plasma cells are sustained for such lengths of time. The first theory suggests that long-lived plasma cells are regularly replenished from memory B-cells and the second theory proposes intrinsic plasma cell longevity.

The replenishment theory suggests that memory B-cells intermittently differentiate into long-lived plasma cells and replace those that have perished. Each variation of this model proposes that the replenishment of the plasma cell compartment occurs at the same time as the replenishment of the memory B-cells. Therefore, the mechanisms suggested for triggering intermittent memory B-cell to plasma cell differentiations are the same as already discussed for memory B-cell maintenance. Should this model be correct, it would follow that memory B-cell numbers and plasma cell numbers (or serum Ab levels) should be correlated. Such a correlation has been found in some studies, but not in others (31, 55, 221).

The intrinsic plasma cell longevity model hypothesises that once a long-lived plasma cell is produced and homes to a specific site such as the bone marrow, the plasma cell may survive for decades without requiring replenishments from the memory B-cell pool. Memory B-cell numbers could correlate with plasma cell numbers in this model, but this correlation would not be a requirement for sustaining stable serum Ab levels. The two cell populations would be distinct and independently regulated. If plasma cells and memory B-cells are indeed two independently regulated B-cell populations, it is possible that the duration of Ab production by plasma cells depends on the two different populations competing for space in immunological niches like the bone marrow. Amanna et al. discussed these models in a recent review where they also suggested the ‘Imprinted Lifespan’ model of plasma cell longevity (222). In essentials, the model proposes that plasma cells are imprinted with a predetermined lifespan which is based on the amount of B-cell signalling that occurs at the

induction of the antigen-specific humoral immune response. This model would explain why antigen-specific Ab responses sometimes fade over time, while sometimes they are maintained for life.

A recent case control study by Hammarlund et al. has showed that the duration of immunity following smallpox infection was remarkably similar to that observed after smallpox vaccination (Figure 4). The same antiviral T-cell responses that declined slowly over time and antiviral Ab responses that remained stable for decades after recovery from infection were seen. The levels of immunity induced after natural *Variola* virus infections were comparable to the levels induced following vaccination. Happily, this indicates that potentially life-threatening disease is not required for the development of sustainable long-term immunity. This may explain the outstanding success of vaccination in eradicating smallpox (223).

2 AIM OF THE STUDY

The overall aim of this study was to investigate long term immunity to *Vaccinia* virus and how it relates to the original responses to vaccinia vaccination over three decades ago.

Many studies have shown that B- and T-cell memory to *Vaccinia* virus can persist for more than half a century. This study aims to take a more nuanced look at long term memory. The accurate vaccination records kept in Iceland have provided the unique opportunity to study long term memory in those rare individuals who had adverse reactions to the vaccine or did not respond to the vaccine ("no take").

2.1 Specific Aims

The specific aims of this study were to perform extensive measurements of memory T and B cell responses in three groups of individuals, with about thirty individuals per group. The first group includes individuals who responded normally to the vaccinia vaccine when they were vaccinated more than thirty years ago. The second group consists of individuals who did not respond to repeated vaccination attempts, and the third group is comprised of individuals who experienced adverse events.

More precisely:

1. **To assess the frequency of vaccinia virus specific memory B-cells over 30 years after vaccination in individuals with extreme primary responses, i.e. lack of response or adverse events compared to those with normal response to the vaccination.**

2. **To assess the frequency of vaccinia specific memory T-cells over 30 years after vaccination in individuals with extreme primary responses, i.e. lack of response or adverse events compared to those with normal response to the vaccination. To assess major T-cell subpopulations: $CD4^+$ Th1, Th2, Th17 cells, and the profile of cytokines, chemokines and inflammatory mediators.**

3. **To assess the relationship between strength and type of memory B- and memory T-cell responses within and across the vaccine response groups.**

3 METHODS AND MATERIALS

3.1 Study subjects

Before the study was initiated, an approval from the National Bioethics Committee was obtained [VSN-04-172].

Study subjects were participants in deCODE's Population Genetics Analysis Program: "Immunity to Vaccines/ Infections". They were all vaccinated with vaccinia virus as children (0-14 years old) before 1978. They have previously been genotyped for 370,000 single-nucleotide polymorphisms (SNPs) on the Illumina platform to identify sequence variants that associate with lack of response or adverse events by genome wide association analysis. Participants in deCODE's Population Genetics Analysis Program who had been genotyped, and who agreed to be recontacted for additional blood samples, were invited to participate in this study. They were selected from the 21,313 individuals with consent in the project (out of 58,477 individuals with records).

Prospective participants were recruited from the following three groups: **1. Normal responders** or those who reacted with a "take" to the smallpox vaccination at the time it was administered (29 out of n > 7000 were recruited). **2. Nonresponders** or individuals who had "no take" to repeated vaccination attempts (30 out of n = 495 were recruited). **3. Adverse reaction group** or individuals who experienced adverse events (defined as prolonged absence from school after vaccination) following their vaccination (30 out of n = 888 were recruited).

Participants were recruited at the Patient Recruitment Centre (PRC), their identity was coded and their blood samples sent to the Department of Immunology, Landspitali – The University Hospital of Iceland, where the experiments were performed. The key to the identity code was kept at the PRC.

3.2 Study design

Fresh whole blood from 90 study participants was obtained and peripheral blood mononuclear cells (PBMCs) were isolated from it using density centrifugation. The PBMCs were subsequently stimulated *in vitro* and phenotyped using flow cytometric technology. The relative sizes of the main lymphocyte subpopulations were estimated, $CD3^+$ T-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, B-cells, naïve B-cells, memory B-cells and plasma cells.

PBMCs were stimulated by heat inactivated *Vaccinia* Virus (VV) to measure VV specific memory T-cell responses. They were also stimulated with anti-CD3 and anti-CD28 to measure total T-cell responses, or left unstimulated so that background levels could be evaluated. After 48 hours, supernatants were collected and frozen. T-cell responses were estimated based on cytokine levels in the supernatant, measured with enzyme-linked immunosorbent assay (ELISA) for IL-2, IFN-γ and IL-17, and with a 27 plex Luminex assay for an array of cytokines, chemokines and inflammatory mediators (Figure 5). The PBMCs were stimulated non-specifically as well, with a mitogen cocktail designed to differentiate memory B-cells into AbSCs. Following six days of stimulation, the enzyme-

linked immunospot (ELISPOT) assay was employed to measure the frequency of VV specific IgG^+ AbSCs derived from memory B-cells (Figure 5).

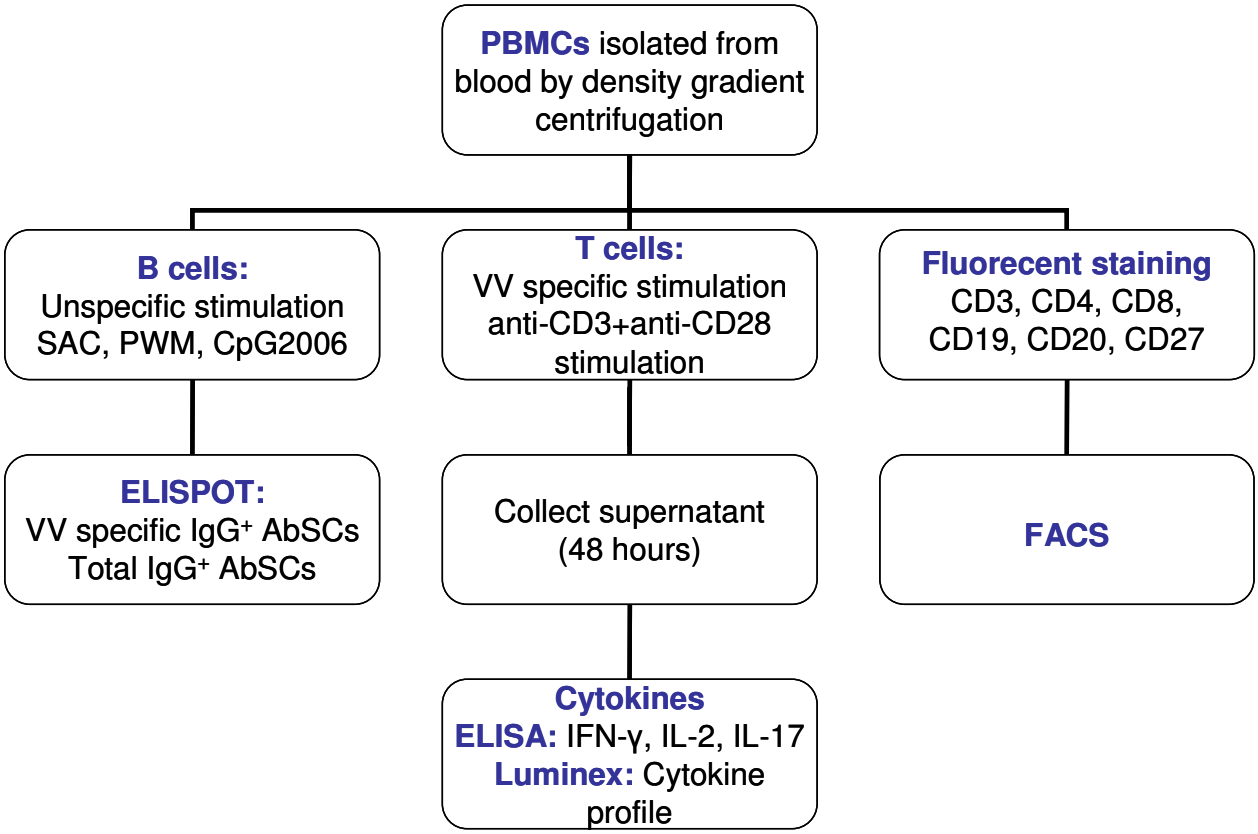

Figure 5. Study design flow chart. The methods and work flow used in this study.

The results of this study will later be analysed in relation to the genetic variants deCODE has found to associate with non-response ("no take") or adverse event. In addition, an assessment of whether any of the immune parameters of memory responses associate with sequence variants in a large set (>1000) of genes involved in innate or adaptive immune response mechanisms, or any of the 370,000 SNPs that the individuals have been genotyped for, will be performed.

3.3 Virus

The VV strain used in this study was kindly supplied by Prof. Rick Lyons, University of New Mexico, USA. Concentration of the stock was $2x10^8$ plaque forming units (pfu)/mL. Different dilutions were tested for each assay. The optimal concentration for T-cell stimulation was $1x10^6$ pfu per $1x10^6$ PBMCs. ELISPOT plates were coated with $1x10^5$ pfu/well.

3.4 Isolation of peripheral blood mononuclear cells

Two to three sodium heparin tubes (Terumo®, Leuven, Belgium) containing 9 mL of blood were obtained from each subject. Samples were loaded on to 12.5 mL of Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) in sterile tubes (Sarsted, Nuernberg, Germany) and centrifuged for 20 min at 2400 rotations per minute (rpm) without brakes at room temperature (RT). PBMCs were collected from the interphase. The PBMCs were transferred to sterile tubes (Sarsted) and washed with 15 - 25 mL phosphate buffered saline (PBS). The supernatant was discarded after centrifugation for 5 min at 2400 rpm (RT). Cells were resuspended in 20 mL PBS and counted. After counting, the cells were centrifuged for 10 min at 1400 rpm (4°C). Supernatant was discarded and the cells resuspended in 1 – 4 mL of Complete media (RPMI 1640 medium, supplemented with L-glutamine (200 mM), 5% foetal calf serum (FCS), Penicillin (10.000 unit/mL) and Streptomycin (10.000 µg/mL) (all from Gibco®, Invitrogen, Paisley, UK)). Cells were then kept on ice until B- and T-cell stimulations were set up.

3.5 Phenotyping of PBMCs

Flow cytometry was used to evaluate the relative sizes of the main lymphocyte subpopulations: $CD3^+$ T-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, B-cells, naïve B-cells, memory B-cells and plasma cells.

Tubes containing 10^6 PBMCs in FACS buffer (PBS, 0.5% bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany), 4mM EDTA (ethylene diamine tetraacetic acid, Sigma)) were centrifuged for 5 min at 300xg (4°C). Cells were resuspended in 100 µL FACS buffer and stained with appropriate concentration of fluorochrome-conjugated monoclonal Abs specific for T-cells (CD3 PerCP, CD4 FITC, CD8 PE (BD Biosciences, San Diego, CA)), specific for B-cells (CD19 APC, CD20 PerCPCy5.5, CD27 PE (BD Biosciences)) or isotype control (clone 40X PE, FITC, PerCP, APC (BD Biosciences)). The mixtures were incubated for 30 min at 4°C in the dark and the cells were washed by adding 500 µL of FACS buffer and centrifuged at 300 g for 5 min (4°C). Supernatants were discarded and cells resuspended in 500 µL FACS buffer. Samples were stored at 2°C – 8°C in the dark until analysed within 2 hours. Cells were collected using FACSCalibur flow cytometer (BD Biosciences). Results were expressed as percentage of T-cells ($CD3^+$), helper T-cells ($CD3^+$, $CD4^+$, $CD8^-$), cytotoxic T-cells ($CD3^+$, $CD4^-$, $CD8^+$), B-cells ($CD19^+$), naïve B-cells ($CD19^+$, $CD20^+$, $CD27^-$), memory B-cells ($CD19^+$, $CD20^+$, $CD27^+$) and plasma cells ($CD19^+$, $CD20^-$, $CD27^{high}$) of all lymphocytes. Computer analysis was done with Cell Quest Pro (BD Biosciences).

3.6 B-cell stimulation

PBMCs were stimulated with a mitogen mixture containing $5.x10^{-8}$ mg/mL pokeweed mitogen (PWM, Sigma-Aldrich), 2 µg/mL CpG 2006 (Oligos etc., Wilsonville, OR) and 10% *Staphylococcus aureus* (SAC, Sigma-Aldrich) diluted to 1/10,000 in complete medium supplemented with 50 µM β-Mercaptoethanol (Sigma-Aldrich). The stimulation was performed with $0.5x10^6$ PBMC/mL in two U-bottomed tissue culture tubes (BD Biosciences), 2 mL per tube. Complete medium without mitogen was used as control. Cultures were incubated at 37°C, 5% CO_2 and 95% humidity for 6 days.

Cells were collected by centrifugation for 8 min at 1200 rpm RT, washed once with complete media and resuspended in 1 mL of complete media, counted and diluted to $2.5x10^4$ cells/100 µL for the ELISPOT assay.

3.7 Antibody secreting cells

Sterile MultiScreen filter plates (Millipore, city, Ireland) were prewet with 70% ethanol (200 µL/well) and washed twice with PBS (200 µL/well). To measure total IgG^+ AbSCs, the plate was coated with rabbit anti-human IgG (Dako Denmark A/S, Glostrup, Denmark) 5 µg/mL in PBS. To measure VV specific AbSCs the plate was coated with a solution of $1x10^6$ pfu/mL VV in PBS or $1x10^5$ pfu/well. The plates were incubated overnight at 37°C, 5% CO_2 and 95% humidity wrapped in aluminium foil. Plates were washed 3 times with 200 µL PBS/well and blocked with 200 µL/well of RPMI containing 10% FCS for 1 - 2 hours at 37°C, 5% CO_2 and 95% humidity wrapped in aluminium foil. Plates were washed again 3 times with 200 µL PBS/well. Mitogen stimulated cells (100,000 PBMCs) were added to each well coated with $1x10^5$ VV (100 µL/well, duplicates) and 50,000 mitogen stimulated PBMCs were added to wells coated with anti-IgG (100 µL/well, duplicates). Plates were incubated for 5 hours at 37°C, 5% CO_2 and 95% humidity. Plates were washed 5 times with 200 µL PBS-T (PBS containing 0.05% Tween20)/well. Detection of bound Abs was done by adding 100 µL goat anti-human alkaline phosphatase (AP) conjugated IgG (Sigma-Aldrich) 1:500 diluted in PBS-T containing 1% FCS to each well. Plates were incubated at 4°C overnight, wrapped in aluminium foil. Plates were then washed 5 times with PBS-T (200 µL/well) and development by adding 100 µL of enzyme substrate solution (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate solution (10 µL of NBT and BCIP in 1 mL AP colour development buffer, AP buffer stock 25x (BioRad Labs, Hercules, CA)). The reaction was allowed to proceed for 10-15 minutes in the dark. Plates were washed with tap water and allowed to dry in the dark overnight. Spots were then counted and the results expressed as the mean value of the duplicates for each subject. Spots were analyzed with Zeiss Axio imaging system (Birkerod, Denmark) and KL ELISpot reader software (KL, New York, NY). The results are presented as number of VV specific IgG^+ AbSCs per 100,000 PBMCs and total IgG^+ AbSCs per 100,000 PBMCs, or the percentage of VV specific IgG^+ AbSCs out of total IgG^+ AbSCs. Number of spots in unstimulated cultures (background) was subtracted from stimulated AbSCs numbers (224).

3.8 T-cell stimulation

PBMCs from each individual were stimulated with VV ($1x10^6$ pfu/mL), medium alone as negative control and as positive controls the cells were stimulated with anti-CD3 (0.025 µg/mL) and anti-CD28 (0.025 µg/mL) (BD Biosciences). For all stimulations and negative controls, $1x10^6$ PBMCs/mL in a total volume of 1.5 mL were added to each U-bottomed tissue culture tube (BD Biosciences). The cells were incubated for 48 hours at 37°C, 5% CO_2 and 95% humidity. Supernatants were harvested and frozen in aliquots at -70°C for measurement of cytokines by ELISA and Luminex.

3.9 Cytokines in lymphocyte culture supernatant

ELISA and Luminex methods were used to measure the concentration of cytokines in supernatant secreted by memory T-cells after *in vitro* stimulation with VV.

For ELISA, microtiter plates (MaxiSorp; Nunc AS, Roskilde, Denmark) were coated with capture mouse anti-human IFN-γ Abs (BD Biosciences) in 0.1 M carbonate buffer pH 9.5, according to the manufacturer's protocol. Plates were incubated over night at 4°C. After blocking with PBS containing 10% FCS for 1 hour at RT, T-cell culture supernatants serially diluted were added in duplicates. Recombinant human IFN-γ (2.35 - 300 pg/mL) was used as a standard and was used in seven twofold dilutions. Plates were incubated for 2 hours at RT. For detection of IFN-γ, biotinylated anti-human IFN-γ with avidin-labelled horseradish peroxidase (HRP) was added 100 µL/well and incubated for 1 hour at RT. The reaction was developed with 100 µL 3,3',5,5'-tetramethylbenzidine peroxidase substrate (TMB, Kirkegaard Kem-En-Tec, Taastrup, Denmark), stopped with 50 µL 1M H_2SO_4 and the optical density read at 450 nm. Concentration of IFN-γ was calculated from the standard curve and expressed in pg/mL using the ELISA software Titri. IL-2 was measured in the same way using the anti-human IL-2 ELISA Kit (BD Biosciences). The standard was at a concentration range of 3.9 - 500 pg/mL.

IL-17 was measured using the anti-human IL-17 DuoSet® ELISA Development system (R&D Systems, Minneapolis, MN). Microtiter plates (MaxiSorp) were coated with capture mouse anti-human IL-17 Abs in PBS according the manufacturer's protocol. Plates were incubated overnight at RT. After blocking with PBS containing 1% BSA for 1 hour at RT, T-cell culture supernatants were added, undiluted in duplicates. Recombinant human IL-17 (7.8 - 1000 pg/mL) was used as a standard and was used in seven twofold dilutions. Plates were incubated for 2 hours at RT. For detection of IL-17, biotinylated goat anti-human IL-17 was added 100 µL/well and incubated for 2 hours at RT. After 2 hours, streptavidin-HRP was added 100 µL/well and incubated at RT for 20 minutes. The reaction was then developed with 100 µL TMB, stopped with 50 µL 1 M H_2SO_4 and the optical density read at 450 nm. Concentration of IL-17 were calculated from the standard curve and expressed in pg/mL using Titri.

For Luminex, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, GM-CSF, IFN-γ and TNF-α were measured using a human Th1/Th2 9-plex panel (BioRad) and IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, Eotaxin, Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF were measured using a human 27-plex panel (BioRad). All regents and buffers used were included in the kits.

After prewetting the wells of a 96-well filter plate with 100 µL/well of assay buffer at RT for 15 – 30 seconds, the assay buffer was aspirated using a filtration vacuum manifold for bead washing (BioRad). Following the aspiration, 50 µL of diluted coupled bead solution was added to each well. Washing buffer was then added, 100 µL per well, and aspirated using the filtration vacuum manifold as before. This washing step was performed twice. Next, 50 µL of appropriate standard dilutions were put into the wells designated for the standard curve and 50 µL of the diluted samples were placed in the wells designated for samples. The standard and the samples were then protected from light with aluminium foil and incubated at RT for 30 minutes on an orbital shaker. After 30 minutes the liquid was aspirated with the vacuum manifold and the washing step described before was repeated three times.

After washing, 25 µL of diluted detection Abs were added to each well and incubated for 30 minutes at RT on an orbital shaker as before. Washing was done three times as before and then 50 µL of appropriately diluted streptavidin-PE was added to each well and incubated for 10 minutes at RT on an orbital shaker. Washing was again repeated three times and finally 125 µL of assay buffer were added to the wells and the plate was placed on the orbital shaker for a minute to resuspend the beads. The plate was then read on a Luminex 200™ instrument (BioRad) and the data analysed In Bio-Plex Manager™ Software (BioRad).

3.10 Statistical analysis

Most measurements were not normally distributed and therefore the median and interquartile ranges were calculated, and the non-parametric Mann-Whitney Rank Sum test and Spearman Rank Order Correlation were used for statistical analysis. A P value of <0.05 was considered statistically significant. All graphs and calculations were done with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

4 RESULTS

4.1 Study participants

To ascertain whether the study participants in the three groups with different primary responses to the smallpox vaccine were comparable, data regarding their age, gender, and age at the time when they were first vaccinated was analysed. The results are summarised in Table 3.

The number of years since vaccination was comparable between the groups (Table 3). The gender ratios were also comparable, with slightly more males in all three groups (Table 3). However, the subjects of the nonresponder group were significantly younger than the subjects of the normal responder group (P = 0.0446) and the adverse reaction group (P = 0.0091).

Table 3. Number of subjects, gender ratios, age, and years since first smallpox vaccination, grouped by different primary responses to the vaccine.

Subject group	No.	Males [%]	Females [%]	Age ± Std. Dev. [Years]	Years since vaccination ± Std. Dev.
Adverse reaction	30	60	40	56.5 ± 5.0	45.1 ± 5.0
Nonresponders	30	60	40	51.7 ± 6.9	44.0 ± 5.6
Normal responders	29	59	41	56.8 ± 10.5	46.7 ± 7.9

4.2 Phenotyping of PBMCs

The PBMCs were phenotyped by FACS analysis using monoclonal Abs for the cell surface markers CD3, CD4, CD8, CD19, CD20 and CD27.

The percentage of B-cells ($CD19^+$), and B-cell subpopulations: naïve B-cells ($CD20^+/CD27^-$), memory B-cells ($CD20^+/CD27^+$) and plasma cells ($CD20^-/CD27^{high}$) was within normal range for most study subjects (Table 4). The percentage of T-cells ($CD3^+$), and T-cell subpopulations: T-helper cells ($CD4^+$) and cytotoxic T-cells ($CD8^+$) was also within normal range for most study subjects (Table 4). However, the size of the T-cell subpopulation tended to be slightly on the low side compared with reference values (225).

Eight individuals were found to have slightly larger subpopulations of cytotoxic T-cells than Th cells (Table 6, appendix). But for one subject (no. 31) the percentage of cytotoxic T-cells was more than double the percentage of the Th cells.

No significant differences were found between the lymphocyte subpopulations of subjects with different primary responses to the smallpox vaccine, except in the case of plasma cells. Subjects who suffered adverse reactions to the smallpox vaccine had a significantly higher proportion of plasma cells than normal responders. However, no significant difference was found between the size of the plasma cell fraction of the adverse reaction group and the nonresponder group. Neither was there a significant difference between the normal responder group and the nonresponder group, although a trend towards significance was observed (Figure 6).

Table 4. The average size of lymphocyte subpopulations for the study participants.

Lymphocyte Subtype	Marker	Mean ± Std. Dev. [%]	Median (5^{th} to 95^{th} percentile) [%]
T-cells	%$CD3^+$ of lymphocytes	55.2 ± 7.1	72 (55 – 83)
Th-cells	%$CD4^+$ out of $CD3^+$	34.3 ± 6.8	44 (28 – 57)
Tcx-cells	%$CD8^+$ out of $CD3^+$	19.6 ± 5.8	24 (10 – 39)
B-cells	%$CD19^+$ of lymphocytes	8.0 ± 2.7	12 (6 – 19)
Naïve B-cells	%$CD20^+/CD27^-$ out of $CD19^+$	69.9 ± 11.3	
Memory B-cells	%$CD20+/CD27^+$ out of $CD19^+$	26.5 ± 10.9	
Plasma cells	%$CD20/CD27^{high}$ out of $CD19^+$	1.2 ± 1.0	

The standard deviation within each subpopulation is shown, and median reference values with percentiles for lymphocyte subpopulations are also shown when available (225).

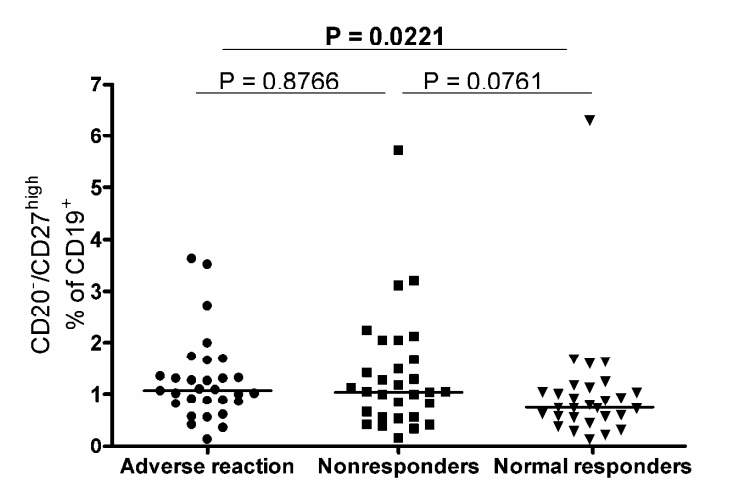

Figure 6. Plasma cells in subjects with different primary responses to the smallpox vaccine. Plasma cells are shown as a percentage of B-cells. P values comparing groups are bolded when statistically significant.

4.3 B-cell Memory

To assess the frequency of VV specific memory B-cells over 30 years after vaccination in individuals with extreme primary responses, the frequency of AbSCs was analysed following *in vitro* stimulation of their PBMCs with a mitogen cocktail (SAC, PWM and CpG2006). To stabilise the solution, β-Mercaptoethanol was also added. As a negative control, PBMCs were cultured in β-Mercaptoethanol supplemented medium lacking in mitogens. ELISPOT assays were performed to detect the frequency of VV specific memory cells that were induced to differentiate and secrete Abs (IgG^+) by the stimulation, as well as the total frequency of IgG^+ AbSCs. Negative controls revealed little or no background in most cases. Number of spots in unstimulated cultures (background) was subtracted from stimulated AbSCs numbers.

Subjects that experienced adverse reactions when receiving the smallpox vaccine over 30 years ago showed significantly lower numbers of VV specific IgG^+ AbSCs per 100,000 PBMCs than normal responders and nonresponders (Figure 7;A). No significant difference was found between normal responders and nonresponders. The number of total IgG^+ AbSCs per 100,000 PBMCs did not vary significantly between the three groups (Figure 7;B). Since results on AbSCs induced by stimulation as a measure of memory are expressed in various ways (30, 31, 224) we also show the results as the percentage of VV specific IgG^+ AbSCs out of the total number of IgG^+ AbSCs. The percentage was significantly lower for subjects that experienced adverse reactions than for normal responders and nonresponders (Figure 7;C). No significant difference was found between the percentage of nonresponders and normal responders (Figure 7;C). Thus, there was good agreement between the results obtained by the two different ways of calculation. A significant positive correlation was found to exist between the number of VV specific IgG^+ AbSCs and the number of total IgG^+ AbSCs (Figure 7;D).

The ELISPOT assay revealed extremely low numbers of IgG^+ AbSCs for six individuals. The number of VV specific IgG^+ AbSCs was not unusually high or low for these individuals, but the assay failed to detect a normal amount of total IgG^+ AbSCs. Four subjects had no total IgG^+ AbSC above background, and the number of VV specific IgG^+ cells was therefore divided by 1 in order to visualise the data. The subjects with measureable VV specific IgG^+ AbSCs, but almost no total IgG^+ AbSC numbers have astonishingly high percentages of VV specific IgG^+ AbSCs as seen in Figure 7;C. Statistic analysis revealed that the significant difference between the adverse reaction and the nonresponder groups was maintained whether the subjects with extremely low total IgG^+ AbSC were included or not (Figure 7;C).

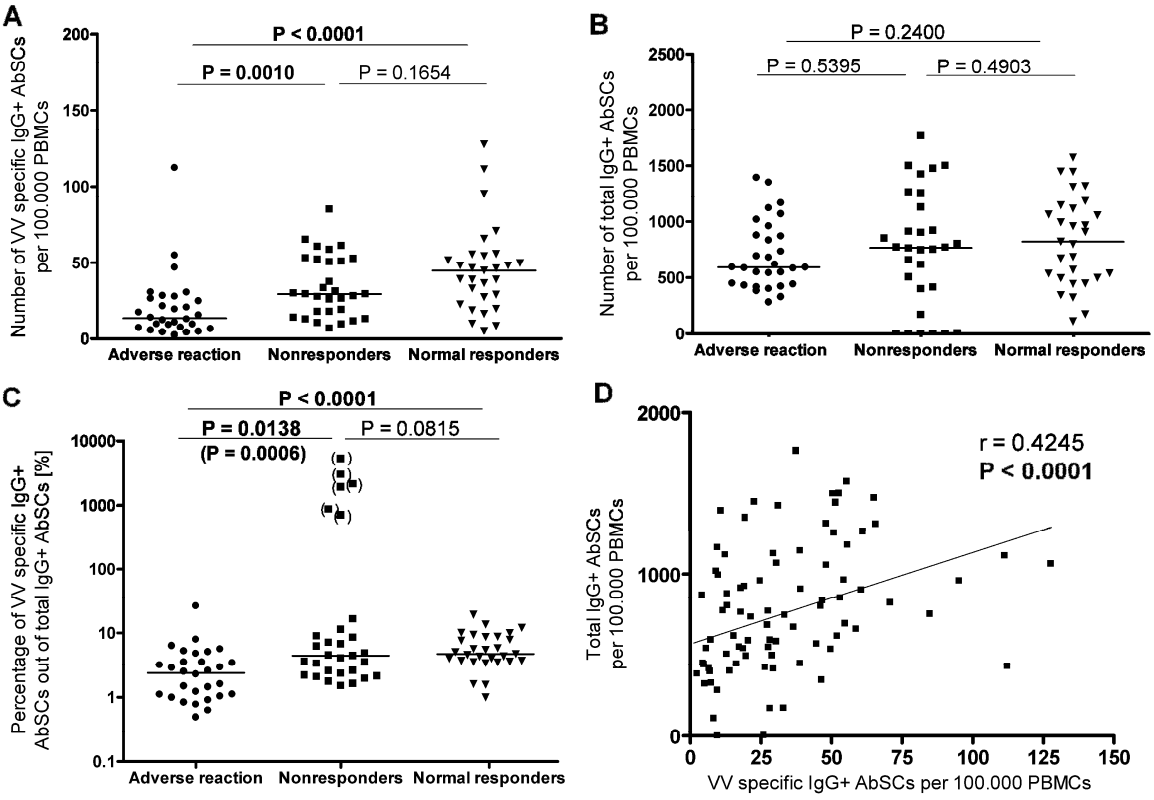

Figure 7. Antibody secreting cells in subjects with different responses to the smallpox vaccine. A) VV specific IgG^+ AbSCs per 100,000 PBMCs. B) Total IgG^+ AbSCs per 100,000 PBMCs. C) Percentage of VV specific IgG^+ AbSCs out of all IgG^+ AbSCs shown on a log scale. Outliers and P value when outliers are included are shown in parentheses. D) Relationship between VV specific and total IgG^+ AbSCs in all subjects with linear regression, Spearman r and P values shown. P values comparing groups are bolded when statistically significant.

4.4 T-cell Memory

To assess vaccine-induced, memory persisting T-cell responses in individuals with extreme primary reactions, cytokine production was analysed following *in vitro* stimulation of their PBMCs with VV, or anti-CD3 and anti-CD28 as a positive control.

4.4.1 Cytokine secretion measured by ELISA

The cytokines IFN-γ, IL-2 and IL-17 were measured in supernatants from VV or anti-CD3/anti-CD28 stimulated PBMCs, collected at 48 hours. Supernatants from unstimulated PBMCs were also measured to assess the baseline or constitutive cytokine secretion.

Subjects that experienced adverse reactions when receiving the smallpox vaccine over 30 years ago showed significantly higher VV induced IL-2 (Figure 8;A) and IFN-γ (Figure 8;B) responses than normal responders and nonresponders. Furthermore, VV induced IFN-γ and IL-2 levels show significant positive correlation (Figure 9). Meanwhile, the total IL-2 response, induced by anti-CD3 and anti-CD28, was significantly lower for nonresponders than for normal responders and subjects in the adverse reaction group (Figure 8;C).The total IFN-γ levels induced by the anti-CD3 and anti-CD28 cocktail were extremely high, often over the sensitivity range of the ELISA assay. When this occurred

the IFN-γ levels were arbitrarily assigned a value equalling the least diluted consentration of the standard multiplied with the highest dilution factor of the sample in question. Total IFN-γ levels were significantly lower in the nonresponder and adverse reaction groups than in the normal responders. The levels detected in supernatants from subjects in the adverse reaction group were the lowest, being significantly lower than the ones that were observed for the nonresponder and normal responder groups (Figure 8;D).

Baseline levels of IFN-γ and IL-2 measured in supernatant from unstimulated PBMCs were generally low, although the some samples, mainly in the the adverse reaction group, were observed to have surprisingly high IFN-γ levels. The baseline cytokine levels did not vary significantly between the three groups (Figure 12, appendix).

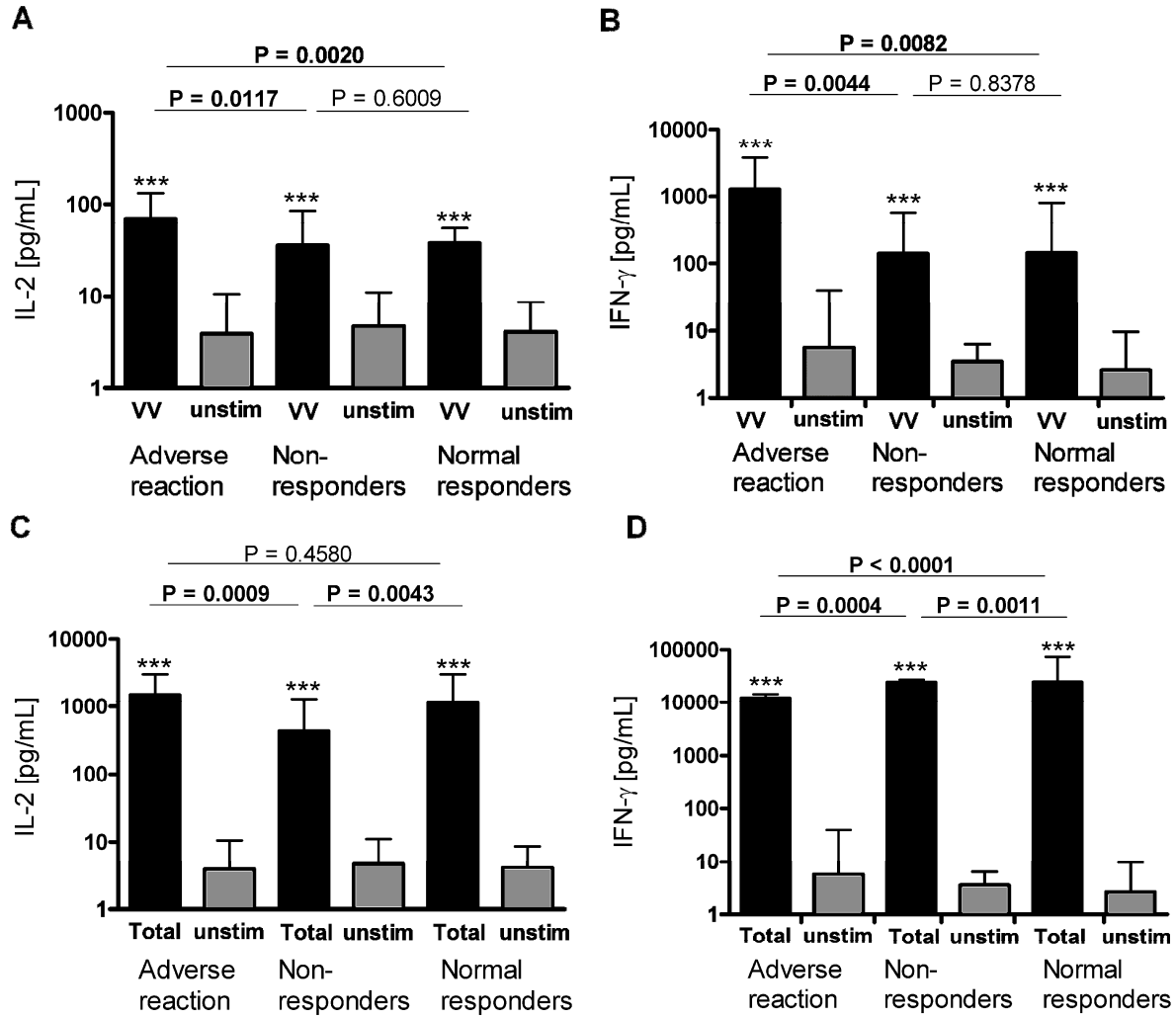

Figure 8. Cytokine levels in supernatant of stimulated PBMCs from subjects with different responses to smallpox vaccine. A) VV-induced and baseline IL-2 levels. B) VV-induced and baseline IFN-γ levels. C) anti-CD3 and anti-CD28-induced and baseline IL-2 levels. D) anti-CD3 and anti-CD28-induced and baseline IFN-γ levels. All graphs show median and interquartile range. P values comparing cytokine levels between groups are bolded when statistically significant. *** P < 0.001 compared with the unstimulated cytokine levels. Cytokine levels are shown on a log scale.

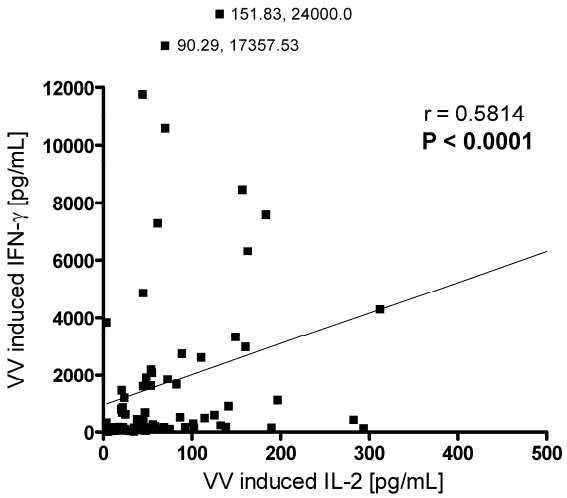

Figure 9. Relationship between VV induced IFN-γ and IL-2 levels. Linear regression, outliers, Spearman r and P values shown.

The anti-CD3 and anti-CD28 induced IL-2 and IFN-γ levels were not correlated (Spearman r = -0.023, P = 0.830), neither were VV induced IL-2 levels correlated with anti-CD3 and anti-CD28 induced IL-2 levels (data not shown). However, a significant negative correlation was observed for VV induced IFN-γ levels and anti-CD3 and anti-CD28 induced IFN-γ levels (data not shown).

VV induced IL-17 levels were under the detection limit in the ELISA for most subjects (data not shown), which was in agreement with the results from the Luminex measurements (chapter 4.4.2).

4.4.2 Cytokine profile measured by Luminex

Cytokines, chemokines and growth factors were measured by Luminex in supernatants from VV stimulated PBMCs, unstimulated PBMCs and PBMCs stimulated with anti-CD3 and anti-CD28, collected at 48 hours. The Luminex assay was chosen because it is a method that allows for the measurement of multiple cytokines in the same small sample volume. The cytokines, chemokines and growth factors included in the assay were IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, Eotaxin (CCL11), Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), PDGF-BB, RANTES (CCL5), TNF-α and VEGF (Table 7, appendix).

Cytokine levels in supernatants from VV stimulated PBMCs were compared with levels in unstimulated cell supernatantant from all three groups together (n = 87). The following cytokines had significantly higher levels in the VV stimulated supernatants than in the unstimulated and were selected for further analysis: IL-1β, IL-1ra, IL-2, IL-4, IL-9, IL-13, IL-15, Eotaxin, Basic FGF, GM-CSF, IFN-γ, IP-10, MIP-1α, MIP-1β, PDGF-BB and TNF-α. Additionally, IL-5 and VEGF had significantly lower levels in the VV stimulated supernatant than in the unstimulated (Table 8, appendix). For each of cytokine with a significant increase, comparisons were made to ascertain whether there were significant differences between VV induced levels and baseline levels within each of the three groups.

This was not done for IL-5 as the measurements were all very close to the detection limit and unlikely to show true differences. VEGF was not analysed further as it was not considered to be important to the memory responses under investigation. Significant differences between VV induced levels and baseline levels were found to exist within at least one of the three groups for all cytokines except PDGF-BB (Table 9, appendix).

To determine whether PBMCs from subjects with different primary responses to the smallpox vaccine showed different responses to VV stimulation, VV induced cytokine levels were compared between the three groups. For IL-2, IL-4, IL-9, Basic FGF, IFN-γ, IP-10, and PDGF-BB there were significant differences between groups. Additionally, a trend towards significance was observed for the inflammatory mediators IL-1β and TNF-α (Table 10, appendix). The same analysis was performed to establish whether the groups had significantly different baseline levels, or if their response to stimulation with anti-CD3 and anti-CD28 varied significantly (Tables 11 and 12, appendix).

The VV induced Th1 cytokines IL-2 and IFN-γ had significantly higher levels in the adverse reaction group than in normal responders (Figure 10;A and B). Additionally, the mean fold increase from baseline IFN-γ to VV-induced levels was significantly higher in the adverse reaction group than in the normal responders (Table 13, appendix). VV induced IL-2 levels were also significantly higher in the adverse reaction group than in nonresponders (Figure 10;A). No differences were observed between groups for background cytokine levels, or anti-CD3 and anti-CD28 induced cytokine levels (Tables 11 and 12, appendix). Although the VV induced IL-2 and IFN-γ levels detected with the Luminex method were lower than detected by ELISA, the results were highly positively correlated (Figure 13, appendix) and therefore in line with previous results.

The VV induced Th2 cytokine IL-4, much like IL-2, was observed in significantly higher levels in the adverse reaction group than in normal responders and nonresponders (Figure 10;C). No differences were observed between groups for background cytokine levels, or anti-CD3 and anti-CD28 induced cytokine levels (Tables 11 and 12, appendix). The other Th2 cytokines, IL-5, IL-9 and IL-13, did not show the same pattern. The VV induced levels of IL-5 were often close to the detection limit and not significantly higher than background levels. VV induced IL-13 responses were not significantly different between the three groups (Table 10, appendix). However, the background levels of IL-13 were significantly lower in the nonresponder and adverse reaction groups than in the normal responders (Table 11, appendix). VV induced levels of IL-9 were significantly lower in the adverse reaction and nonresponder groups than in the normal responders (Figure 10;D). No differences were observed between groups for anti-CD3 and anti-CD28 induced cytokine levels, but the subjects of the adverse reaction group had significantly lower background levels than normal responders and nonresponders (Tables 11 and 12, appendix).

Although IL-15 is not produced by T-cells it is important for T-cell responses because of its IL-2-like qualities and its role in maintaining $CD8^+$ T-cell memory. Baseline levels of IL-15 were significantly lower in the adverse reaction group than normal responders and nonresponders (Table 11, appendix), but no significant differences were observed between groups following VV stimulation (Figure 10;E) or anti-CD3 and anti-CD28 stimulation (Table 12, appendix).

The main inflammatory mediators detected were IL-1β and TNF-α. Neither showed significant differences in VV induced levels between the three groups (Figure 11;A and B), although trends were observed. The VV induced IL-1β levels in the adverse reaction group were almost significantly lower ($P = 0.0525$) than in the normal responders, and VV induced TNF-α levels were close to being significantly lower for nonresponders than for the normal responder ($P = 0.0737$) and the adverse reaction groups ($P = 0.0798$). Significant differences were found between groups for both baseline levels of IL-1β and TNF-α, and for anti-CD3 and anti-CD28 induced levels of TNF-α. Baseline levels of IL-1β were significantly lower in both the adverse reaction and nonresponder groups than in normal responders (Table 11, appendix). For TNF-α, the baseline levels were also significantly lower in nonresponders than in normal responders, and nearly significantly lower ($P = 0.0575$) in the adverse reaction group than in normal responders (Table 11, appendix). No significant differences were found between groups for either IL-1β or TNF-α when the mean fold increase from unstimulated levels to VV-induced levels was calculated (Table 13, appendix). No significant differences were found for anti-CD3 and anti-CD28 induced IL-1β between groups, but the levels of anti-CD3 and anti-CD28 induced TNF-α were significantly higher in the adverse reaction group than in normal responders (Table 12, appendix).

MIP-1α and MIP-1β, chemokines that are also important in inflammatory processes, did not show significant differences between the three groups when stimulated with VV (Figure 11;C and D) or anti-CD3 and anti-CD28 (Table 12, appendix). However, the baseline levels of MIP-1α were significantly lower for the adverse reaction and nonresponder groups than for the normal responders. Meanwhile, the baseline levels of MIP-1β were significantly lower for the adverse reaction group than normal responders but similar for nonresponders and normal responders (Table 11, appendix). The mean fold increase for MIP-1α was significantly lower for the adverse reaction group than the normal responders, but no significant difference was found between the mean fold difference of the nonresponder and normal responder groups. However, there was a trend toward nonresponders having a higher mean fold increase than normal responders ($P = 0.0969$). For MIP-1β the mean fold increase was significantly higher in the adverse reaction and nonresponder groups than in the normal responders (Table 13, appendix).

IP-10, a chemokine associated with chemotaxis, apoptosis, cell growth inhibition and angiostasis, but also with inflammation, was induced by VV in significantly higher amounts in the adverse reaction and nonresponder groups than in normal responders (Figure 11;E). The same pattern was observed for anti-CD3 and anti-CD28 induced IP-10 levels (Table 12, appendix), but no significant differences were seen in baseline levels between the three groups (Table 11, appendix). The mean fold increase was significantly higher in nonresponders than in normal responders, but no significant difference was found between the mean fold increase of the adverse reaction and normal responder groups (Table 13, appendix).

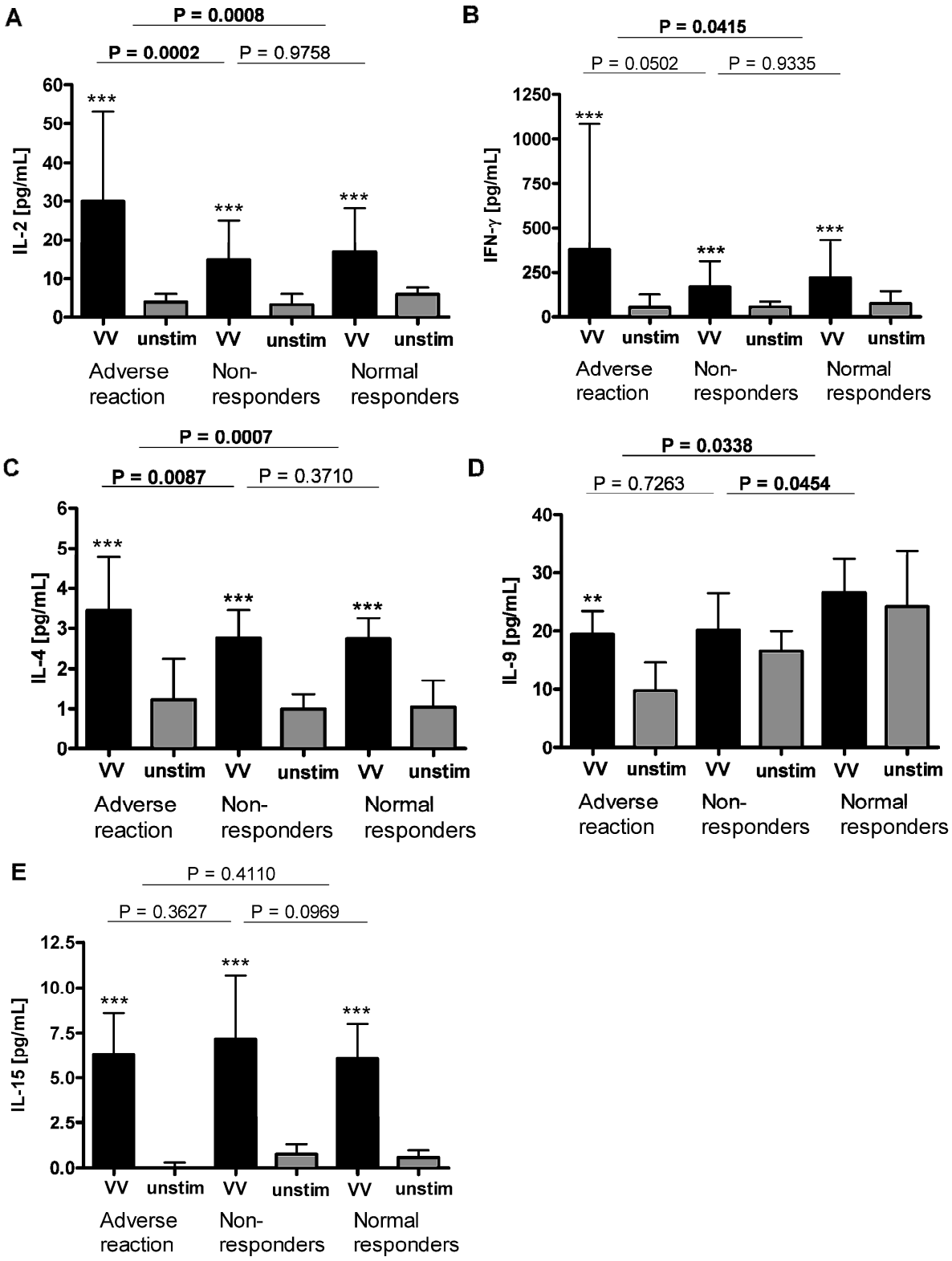

Figure 10. Key T-cell cytokine levels in supernatant of VV stimulated and unstimulated PBMCs from subjects with different responses to smallpox vaccine. A) IL-2 levels. B) IFN-γ levels. C) IL-4 levels. D) IL-9 levels. E) IL-15 levels. All graphs show median and interquartile range. P values comparing cytokine levels between groups are bolded when statistically significant. ** P < 0.01, *** P < 0.001 compared with the unstimulated cytokine levels.

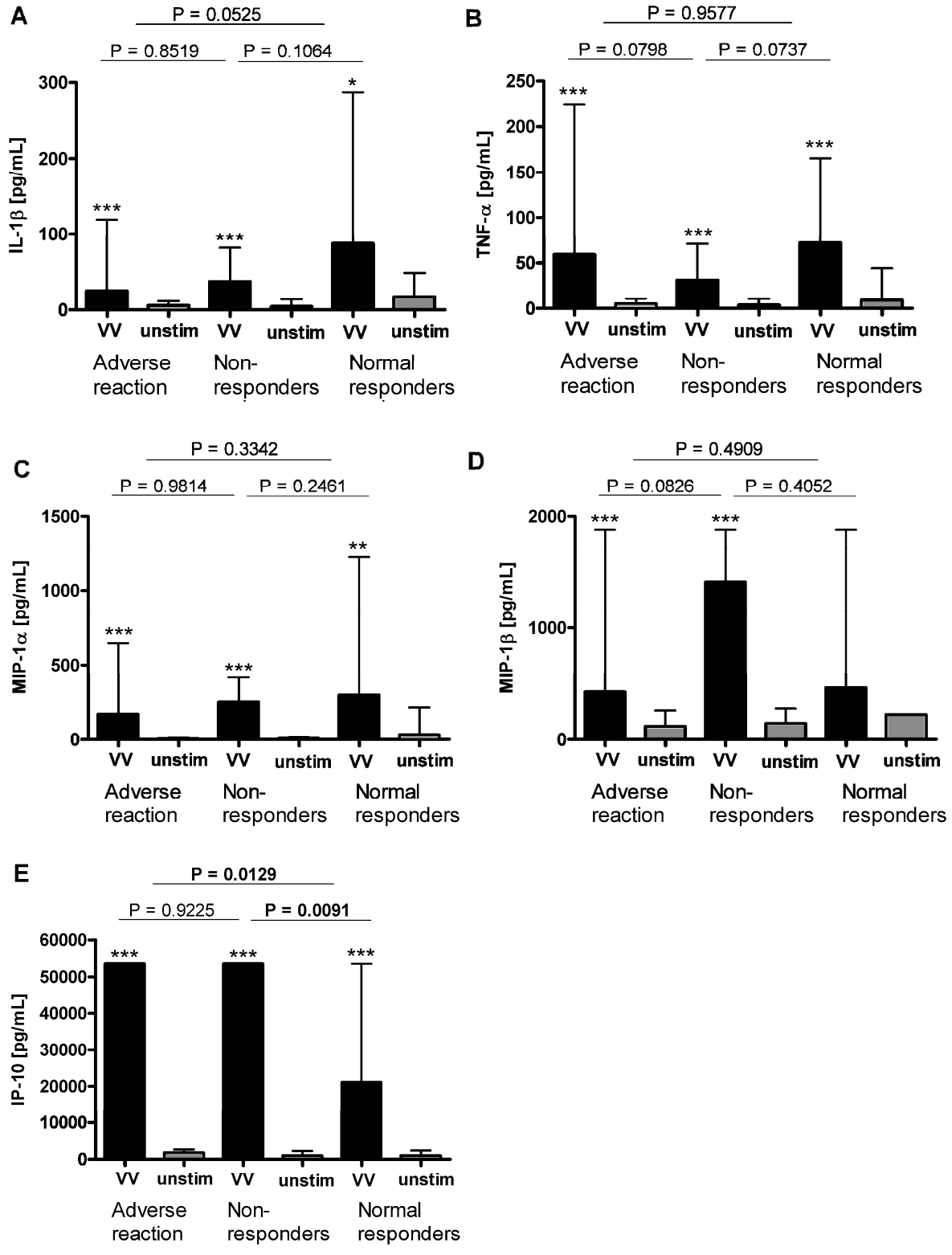

Figure 11. Key chemokines and inflammatory mediators in supernatant of VV stimulated and unstimulated PBMCs from subjects with different responses to smallpox vaccine. A) IL-1β levels. B) TNF-α levels. C) MIP-1α levels. D) MIP-1β levels. E) IP-10 levels. All graphs show median and interquartile range, except graph D where column for unstimulated normal responders does not show interquartile range. P values comparing cytokine levels between groups are bolded when statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the unstimulated cytokine levels.

4.5 Relationship between B- and T-cell memory responses

The VV induced T-cell responses, represented by Th1 and Th2 cytokine levels detected at 48 hours via ELISA and Luminex, were analysed in relation to the VV specific B-cell responses, represented by the frequency of VV specific IgG^+ AbSCs per 100,000 PBMCs measured by ELISPOT assay.

In general there was no significant correlation found between the levels of any of the VV induced cytokines and the frequency of VV specific IgG^+ AbSCs, except for a negative correlation for IL-2, IL-4, PDGF-BB and RANTES (Table 5).

When this relationship was analysed based on the primary response to the smallpox vaccine, significant correlation was found between VV induced RANTES levels and the number of VV specific IgG^+ AbSCs in the group of normal responders, between VV induced IL-7, GM-CSF, MCP-1, TNF-α, and VEGF levels and the number of VV specific IgG^+ AbSCs in the group of nonresponders, but no significant correlation was found between the levels of any of the VV induced cytokines and the number of IgG^+ AbSCs in the adverse reaction group (Table 5). It is important to note that the correlation coefficient does not indicate a very strong negative correlation for any of the cytokines mentioned.

Table 5. Correlation between VV induced cytokine levels and frequency of VV specific IgG^+ AbSCs.

	Frequency of VV specific IgG^+ AbSCs			
	All subjects	**Adverse reaction**	**Nonresponders**	**Normal responders**
Cytokine	Spearman r (P value)	Spearman r (P value)	Spearman r (P value)	Spearman r (P value)
IL-2	-0.2094 **(0.0489)**	0.1978 (0.2947)	-0.0541 (0.7765)	0.0037 (0.9848)
IL-4	-0.2622 **(0.0131)**	-0.2459 (0.1902)	0.1329 (0.4839)	-0.1545 (0.4236)
IL-7	0.0672 (0.5364)	-0.1807 (0.3573)	-0.4446 **(0.0138)**	-0.0626 (0.7471)
GM-CSF	-0.1818 (0.0881)	0.0369 (0.8465)	-0.3667 **(0.0462)**	-0.0567 (0.7702)
MCP-1	-0.1246 (0.2503)	-0.0005 (0.9978)	-0.4405 **(0.0148)**	-0.2429 (0.2042)
PDGF-BB	-0.2709 **(0.0112)**	-0.2299 (0.2393)	-0.1818 (0.3363)	0.0793 (0.6826)
RANTES	-0.2465 **(0.0214)**	-0.0576 (0.7708)	0.0898 (0.6371)	-0.5050 **(0.0052)**
TNF-α	-0.0683 (0.5250)	0.2038 (0.2800)	-0.3845 **(0.0359)**	-0.0030 (0.9879)
VEGF	-0.1668 (0.1226)	-0.2184 (0.2642)	-0.4559 **(0.0113)**	-0.1064 (0.5828)

Cytokine levels as measured by Luminex were used in this comparison. Spearman r and P values for cytokines where significant correlations were found. Correlation for all subjects and for subjects grouped by response to the smallpox vaccine is shown. Statistically significant P values are bolded.

5 DISCUSSION

In the study presented in this thesis, B- and T-cell memory responses to VV were characterised in healthy adults that were divided into three groups based on their primary responses to the smallpox vaccine over 30 years ago. The groups were comprised of individuals who suffered adverse reactions to the vaccine (adverse reaction group), those who had “no take” following vaccination (nonresponder group) and those who responded with a normal “take” (normal responder group). This is the first study to examine long term B- and T-cell responses to VV in subjects with different primary responses to the smallpox vaccine.

5.1 Study participants

In deCODE’s study on the genetics of vaccine responses against smallpox, approximately 1.5% of the vaccinees were non-responders, defined as a failure to show a positive response or “take” to two or more vaccination attempts. The nonresponders show significant familial aggregation. Following vaccination there was more than threefold increase in absence from school, peaking at 8-12 days postvaccination, which coincides with the time of known adverse events. Prolonged absence from school within one month after vaccination was used as a surrogate for adverse events to vaccination (defined as absence for more than four consecutive days during the month after vaccination and no absences for more than a day during the two weeks before vaccination). Using these criteria close to 2% of the vaccinees experienced adverse events, and these also showed significant familial aggregation compared with vaccinees matched for school, age and sex, not absent following vaccination. The significant familiality of both nonresponders and those suffering adverse events strongly indicates that genetic factors play a role in the immune response and outcome of vaccination by vaccinia virus vaccine (Jonsdottir I., unpublished data).

The subjects who donated blood to this study, 30 in the adverse reaction group, 30 nonresponders and 29 normal responders were comparable with regard to gender ratios and number of years that had passed since their first vaccination. As these are two variables that have been shown to have an effect on neutralising antibody titres (199), it is important to note that they should not skew the results of this study.

5.2 Phenotyping of PBMCs

Phenotyping of their PBMCs revealed that most subjects had lymphocyte subpopulations that were within normal size range, and that the different groups did not have significantly different PBMC profiles. The only significant difference that was observed was with the plasma cell subpopulations of the adverse reaction and the normal responder groups. There was also a trend towards nonresponders having higher plasma cell levels than normal responders ($P = 0.0761$). If the subjects of the adverse reaction group consistently have a higher plasma cell percentage than normal responders, it might suggest that they have stronger antibody responses in general. However, it is

impossible to know whether this difference was present at the time of smallpox vaccination, or if it was true for VV-specific plasma cells.

Most of the 89 subjects that donated blood to the study had a higher percentage of Th cells than cytotoxic T-cells. For the eight subjects that deviated, the percentage of cytotoxic T-cells was usually only slightly higher than for Th cells, but for one subject (no. 31) the percentage of cytotoxic T-cells was more than twice the percentage of the Th cell subpopulation. A viral infection may cause cytotoxic T-cells to expand and subsequently become a relatively higher percentage of the total T-cell numbers than Th cells (226). The staining for the surface markers may have been less than optimal for this subject due to technical issues. It is extremely unlikely that subject no. 31 is suffering from acquired immune deficiency syndrome (AIDS), although it is one theoretical explanation for a low Th cell percentage (227).

5.3 B- and T-cell memory

Both VV specific B- and T-cell responses were efficiently elicited in all three groups, which is in line with results published by different researchers (30, 31, 195). However, in this study we showed for the first time that subjects with different primary responses to the smallpox vaccine present with differences in both B- and T-cell memory cell responses to VV decades after the vaccination. Due to the novelty of these results there is a shortage of studies to compare them to. No comparable research has been published. However, a few studies with some similarities bear mention.

The production of IFN-γ, TNF-α, IL–2, IL-4, IL-5, and IL-10 has been measured in 107 subjects, some of which experienced adverse reactions in response to the Aventis Pasteur smallpox vaccine, and some who did not experience adverse reactions. The subjects with adverse reactions were found to have significantly increased levels of all of these cytokines when compared with subjects who did not experience adverse reactions (228). Additional cytokines and chemokines were quantified in a subset of the same serum samples from the same individuals; 22 that experienced adverse events after primary immunisation with the Aventis Pasteur smallpox vaccine and compared with 52 who did not experience adverse events. Six cytokines, G-CSF, stem cell factor (SCF), CXCL9, intercellular adhesion molecule-1 (ICAM-1), Eotaxin and Tissue inhibitor of metalloproteinase 2 (TIMP-2) were found that discriminated between the two groups (229). However, neither of the two research papers mentioned studied memory responses. A small study by Ovsyannikova et. al. compared cellular immune responses for subjects with extreme reactions to the measles-mumps-rubella II vaccine. They studied 15 subjects (aged 15 to 25 years) who were seronegative or highly seropositive for measles vaccine IgG-specific antibody following immunisation with two doses of measles-mumps-rubella II vaccine. They showed that proliferation of T-cells in seronegative subjects was significantly lower than in highly seropositive subjects and that both groups responded to the measles virus with more IFN-γ than IL-4 secretion (230). While this study evaluated memory responses, it focused on a different virus and did not address long term memory.

5.3.1 B-cell memory

B-cell memory was demonstrated to be weaker in the adverse reaction group, both with significantly fewer numbers of VV specific IgG^+ AbSCs per 100,000 PBMCs than normal responders and nonresponders, and significantly lower percentage of VV specific IgG^+ AbSCs out of total IgG^+ AbSCs in the adverse reaction group than in normal responders and nonresponders, a difference which was not affected by the exclusion of outliers.

The outliers had measurable VV specific IgG^+ response which were neither unusually high nor low, but very low total IgG^+ responses, which caused the percentage of VV specific IgG^+ AbSCs out of total IgG^+ AbSCs to be very high. The lack of total IgG response is likely due to technical issues such as a failed stimulation. However, other subjects who were stimulated with exactly the same mitogen mixture on the same day did not reveal any deficiency in IgG^+ AbSCs numbers. This indicates that the mitogen mixture worked, and that experimental conditions were unlikely to be the cause. Another explanation could be that some of the wells on the ELISPOT plate may have dried out or they may have been faulty. It is interesting that the subjects that failed to respond to the assay were all members of the nonresponder group. Ideally the stimulation should be attempted again on the same individuals in order to observe whether they repeatedly fail to respond. Regrettably, such repeats were not within the scope of this project. It is highly unlikely that the subjects had a defective IgG response as they produced VV specific IgG^+ AbSCs.

As a significant positive correlation was observed between the numbers of VV specific IgG^+ AbSCs per 100,000 PBMCs and total IgG^+ AbSCs per 100,000 PBMCs, it would seem that the weak vaccinia specific B-cell response of the adverse reaction group should go hand in hand with a generally meagre B-cell response. However, there were no significant differences in the numbers of total IgG^+ AbSCs between groups, making it unlikely that the weak vaccinia specific B-cell response in the adverse reaction group was entirely due to a largely feeble B-cell response. Additionally, although the correlation was highly significant ($P < 0.0001$), the Spearman r was only at 0.407 which is not very high. It is of note that the frequencies of vaccinia specific B-cell responses out of total B-cell responses in this study were 10 fold higher than those observed by Crotty et. al. and Mamani-Matsuda et. al., indicating that the total IgG^+ AbSC numbers may have been underestimated (31, 196). However, this could be due to assay differences and dissimilar presentation of results as frequency of vaccine specific AbSCs is not always presented in the same way. It can be expressed as a percentage out of PBMCs, out of B-cells, out of IgG^+ AbSCs, etc. and this can have an impact. In this thesis the results are consistently presented in the same way and can therefore reliably be used to compare the memory B-cell responses of the different groups in this study.

It has already been mentioned that genetic factors are likely to play a role in the immune responses and vaccination outcome in subjects immunised with the smallpox vaccine. A study by Haralambieva et. al. has shown that common SNPs or haplotypes in the *IL18* and *IL18R1* genes may be partly responsible for the variation in the amount of virus-specific neutralising Abs following primary immunisation with the VV vaccine in both Caucasians and African Americans (231). Such results lend credence to the idea that the subjects of the adverse reaction group may be genetically predisposed to

weak humoral responses, and for that reason show a diminished frequency of VV specific IgG^+ AbSCs when compared with subjects from other groups which may have different genetic profiles.

Taken together, the results show that the memory B-cells of subjects from all three groups can be induced to differentiate into AbSCs that produce VV specific IgG^+, more than three decades after the administration of the smallpox vaccine. However, subjects from the adverse reaction group showed significantly lower frequencies of AbSCs than the other two groups, which may indicate that they have a less robust memory B-cell response than normal responders and nonresponders.

5.3.2 T-cell memory

Many of the T-cell memory responses were observed to be stronger in subjects from the adverse reaction group. Their PBMCs responded to VV stimulation by producing significantly larger amounts of the Th1 cytokines IL-2 and IFN-γ than both normal responders and nonresponders as measured by cytokine ELISA. Luminex data showed a similar pattern, although the difference found between VV induced IFN-γ levels in the adverse reaction and nonresponder groups was not significant. However, the cytokine levels measured by ELISA correlated significantly with levels measured by Luminex, and the Luminex results show a trend toward significance between the adverse reaction and nonresponder groups ($P = 0.0502$), Both the pattern and main results were comparable. In general, IL-2 and IFN-γ levels were lower when measured by Luminex than when measured by ELISA. This is most likely due to the fact that sensitivity ranges differ between the assays with different experimental conditions, including different standards. For both assays new aliquots of tissue culture supernatant that had not been thawed were used.

The anti-CD3 and anti-CD28 stimulated cultures showed significant differences in IL-2 and IFN-γ levels between the three groups when measured by ELISA, but no significant differences were found between groups when measured by Luminex. As the anti-CD3 and anti-CD28 induced cytokine levels were mostly outside the sensitivity range of the ELISA assay, and mainly inside the sensitivity range of the Luminex assay, the Luminex measurement is likely to be more accurate. This implies that since there were no significant differences between anti-CD3 and anti-CD28 induced IL-2 and IFN-γ levels between groups, the VV induced IL-2 and IFN-γ increase in the adverse reaction group was not a result of a general Th1 bias within the group. The results of the ELISA assays do no contradict this, as no significant difference was found between the anti-CD3 and anti-CD28 induced IL-2 levels of the adverse reaction and normal responder groups, and the anti-CD3 and anti-CD28 induced IFN-γ levels were significantly lower in the adverse reaction group than in the normal responder and nonresponder groups – not higher.

All groups had very low levels of the Th2 cytokines IL-4, IL-5, and IL-13, with significantly higher VV induced IL-4 levels in the adverse reaction group than in the normal responder and nonresponder groups. There were no significant differences in baseline levels or anti-CD3 and anti-CD28 induced levels of IL-4 between groups, indicating that the high VV induced IL-4 levels in the adverse reaction group were not the result of a Th2 bias, or due to naturally high baseline levels.

These results indicate that the stimulation of PBMCs from previously vaccinated individuals with VV induces mainly Th1 cytokine production. They also indicate that the adverse reaction group showed an exaggerated VV induced Th1 response, as well as an increased VV induced IL-4 response, when compared with normal responders and nonresponders. It can be surmised that increased Th1 and IL-4 responses play a role in the immune memory of the adverse reaction group.

The VV induced levels of IL-9 were higher than levels of the other Th2 cytokines, and unlike the pattern for IL-4, the adverse reaction and nonresponder groups had significantly lower IL-9 levels than the group of normal responders. In addition, the baseline IL-9 levels were significantly lower for the adverse reaction group than for the normal responders and nonresponders. However, as the adverse reaction group was the only group to have significantly higher VV induced levels than baseline levels, it looks as though the adverse reaction group was the only one to increase IL-9 production in response to VV stimulation and therefore it seems that they have both increased Th1 and Th2 responses when compared with normal responders and nonresponders.

VV induced IL-15 levels were not significantly different between the three groups, but the baseline levels were significantly lower for the adverse reaction group than for the normal responders and nonresponders. IL-15 is the cytokine important to the maintenance of $CD8^+$ T-cells and the fact that all groups respond to VV stimulation with a marked increase in IL-15 production is indicative of healthy cytotoxic memory responses in all groups (210).

Neither of the main inflammatory mediators, IL-1β and TNF-α, showed significant differences in VV induced levels between the three groups. All groups responded to VV induction by significantly increased IL-1β production, but the adverse reaction and nonresponder groups had significantly lower baseline levels than the normal responders. No differences were found between the mean fold increase of the adverse reaction and normal responder groups or the nonresponders and normal responders. The VV induced increase in TNF-α levels was not different between groups. Interestingly for TNF-α, the total response induced by anti-CD3 and anti-CD28 was significantly higher for the adverse reaction group than normal responders, indicating that the group might be biased in favour of a greater proinflammatory response. However, this bias was not observed following VV stimulation. There was a trend towards significantly higher VV induced levels of TNF-α in the adverse reaction group than in nonresponders ($P = 0.0798$), but no such trend was observed between the adverse reaction and normal responder groups. No differences were found between the mean fold increase of the adverse reaction and normal responder groups or the nonresponders and normal responders for TNF-α.

The levels of the VV induced chemokines MIP-1α and MIP-1β did not show significant differences between the three groups. However, MIP-1α showed a similar pattern to IL-1β. All groups responded to VV stimulation by increasing MIP-1α production significantly, but the adverse reaction and nonresponder groups had significantly lower baseline levels than normal responders. The mean fold increase was also significantly lower in the adverse reaction group than in the normal responders, but trended towards being higher in nonresponders than in normal responders. On the other hand, the adverse reaction group had significantly lower baseline levels for MIP-1β than both normal responders and nonresponders, and a significantly higher mean fold increase from baseline MIP-1β levels to VV-

induced levels. Nonresponders also had a significantly higher mean fold increase from baseline to VV-induced MIP-1β levels than normal responders.

Finally, all groups showed a significant increase in IP-10 levels after VV stimulation, with significantly higher levels produced by the adverse reaction and nonresponder groups than by the normal responders. The total response induced by anti-CD3 and anti-CD28 followed the same pattern, indicating that the adverse reaction and nonresponder groups may simply be biased in favour of producing high levels of IP-10 in response to stimulation. This means that the pattern of IP-10 response is not restricted to VV stimulation. The mean fold increase from baseline IP-10 levels to VV-induced levels was significantly higher in nonresponders than in normal responders, but the adverse reaction and normal responder groups had a similar mean fold increase.

Taken together, these results indicate that the subjects of the adverse reaction and nonresponder groups do not react differently to VV stimulation than normal responders for the most part. The adverse reaction and nonresponder groups respond with a more prominent increase of MIP-1β than normal responders, partly due to having lower baseline levels than normal responders, and nonresponders also show a more prominent increase in IP-10 than normal responders.

Rock et. al. have shown that subjects with adverse reactions exhibited significantly increased levels of IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10 compared with subjects who did not, following primary smallpox immunisation (228). Although cytokine responses following primary immunisation may not be comparable to memory responses more than three decades after vaccination, it is interesting to see that the patterns for these cytokines are not dissimilar.

MicKinney et. al. showed that the role of inflammatory cytokines in adverse event development, coupled with their previous work demonstrating the role of T-cell derived factors and the similarities of systemic adverse events observed after smallpox vaccination with the clinical presentation of macrophage activation syndrome (232), suggest that systemic adverse events following smallpox vaccination may be consistent with low-grade macrophage activation syndrome caused by virus replication and serious tissue injury and repair (229). Their model proposes that during the inflammatory response, IL-17 is secreted by T-cells that are recruited by IFN-γ induced monokine (MIG) and ICAM-1. Fibroblasts stimulated by IL-17 proceed to secrete the inflammatory and hematopoietic G-CSF and SCF and increase the surface expression of ICAM-1 and the production of eotaxin. Eotaxin and MIG then stimulate macrophage activation (229). In the present study no significant increase was seen in IL-17 or G-CSF levels following VV stimulation. However, the model applies to cytokines observed after primary immunisation and the same patterns may not appear in the responses of long lived memory cells.

Ovsyannikova et. al. compared cellular immune responses of subjects with extreme reactions to the measles-mumps-rubella II vaccine. They studied the frequencies of measles virus-specific memory Th1 and Th2 cells by an ELISPOT assay. They demonstrated that proliferation of T-cells in seronegative subjects was significantly lower than in highly seropositive subjects and that both groups responded to the measles virus with more IFN-γ than IL-4 secretion. IFN-γ secreting $CD8^+$ T-cells reactive to the measles virus were fewer in seronegative subjects than in highly seropositive subjects. IL-4 secreting $CD4^+$ T-cells reactive to the measles virus were also fewer in seronegative subjects than

in highly seropositive subjects (230). These results indicate that seronegative subjects have weaker cellular immune responses, which is in contrast to the results of the present study, which shows that subjects that experienced adverse reactions to the smallpox vaccine had weak B-cell memory responses, but strong T-cell memory responses. However, these studies are not directly comparable as Ovsyannikova et. al. studied responses to a different viral vaccine and did not address long term memory.

Genetic factors play an important role in the host immune response to vaccination. Because HLA gene polymorphisms associate with discrepancy in *Vaccinia* virus antigen presentation, it has been speculated that variations in immune response to smallpox vaccine are influenced by the polymorphisms of HLA genes (233). It has previously been shown that there are associations between HLA polymorphisms and variations in both humoral and cell-mediated immune responses in other viral vaccines, such as measles, rubella, mumps, and influenza (234, 235).

Research by Ovsyannikova et. al. has demonstrated significant associations between the HLA-B and HLA - DQB1 loci and vaccinia-induced antibodies, with the HLA-B*1302, B*3802, DQB1*0302, and DQB1*0604 alleles being associated with higher levels. Furthermore, significant global associations between vaccinia-specific IFN-γ and DQA1, IL–1β and HLA-B, TNF–α and HLA-B, and IL-6 and HLA-B locus for secreted cytokines have been reported, as well as between $CD8\alpha^{+}$ IFN-γ responses and DQB1. It has been demonstrated that subjects carrying B*3906 and B*5701 secreted higher levels of IL-1β than subject lacking the alleles and that subjects carrying the B*5301 and B*5601 alleles secreted less IL-1β than those who did not. The B*3502, B*5601, and B*5701 alleles were all significantly associated with variations in TNF-α secretion. These findings suggest that variations in antibody and cellular IFN-γ, IL-1β, TNF-α, and IL-6 immune responses after smallpox vaccination are genetically controlled by HLA genes or genes in close linkage disequilibrium to these alleles (233). The variation in IFN-γ levels, and the trend toward differences in IL-1β and TNF-α levels, between the adverse reaction group and the other groups might therefore partly be due to HLA differences between the subjects. However, analysis of HLA association with cytokine and B-cell responses remains to be done. The present study did not observe a significant VV induced increase in IL-6 levels compared with baseline levels (Table 8), although a trend was observed ($P = 0.0568$).

If differences in HLA alleles and haplotypes are the reason for the different responses to the smallpox vaccine it will be important to take them into account for future vaccine development. As they seem to influence variation in inflammatory mediator levels (233), they may also be risk factors when it comes to chronic inflammatory diseases.

When the results for B- and T-cell memory responses are taken together, it seems as if it is possible that individuals who suffered adverse reactions to the smallpox vaccine may have been induced to produce stronger memory Th1 and Th2 cells at the cost of a less robust memory B-cell response.

5.4 Relationship between B- and T-cell memory responses

Comparing B- and T-cell memory responses across all subjects revealed a negative correlation between VV induced IL-2, IL-4, PDGF-BB, RANTES and the number of VV specific IgG^+ AbSCs. Dividing the subjects based on their primary responses to the smallpox vaccine made the negative correlations found for IL-2, IL-4 and PDGF-BB disappear, but showed negative correlations for IL-7, GM-CSF, MCP-1, TNF-α, and VEGF within the nonresponder group, and a negative correlation between VV induced RANTES and VV specific IgG^+ AbSCs within the normal responder group.

These results could indicate that nonresponders, and normal responders where RANTES is concerned, could have less robust cytokine responses when B-cell responses are strong and vice versa. However, the Spearman r was never lower than -0.5050, and the P values did not indicate a high degree of significance. Additionally, IL-7, MCP-1, RANTES and VEGF did not show a significant increase from baseline levels after VV induction. Therefore, the negative correlations found for these cytokines cannot convincingly be related to VV memory responses. For these reasons it is difficult to confidently draw conclusions from these results.

Our results are in agreement with what others have shown, that no correlation exists between long term B- and T-cell memory, and that these two arms of adaptive immunity are independently regulated (30, 31, 205, 206). Negative correlations between Ag specific B- and T-cell memory responses have not been reported for *Vaccinia* virus before.

6 SUMMARY AND CONCLUSION

This is the first study to examine long term B- and T-cell responses to VV in subjects with different primary responses to the smallpox vaccine. The participants in this study consisted of subjects with adverse reactions to the smallpox vaccine, subjects with “no take” at the time of vaccination, and subjects with “take” at the time of vaccination. They were comparable with regard to gender ratios and number of years that had passed since their first vaccination and phenotyping of their PBMCs revealed that most subjects had lymphocyte subpopulations that were within normal size range, and that the different groups did not have significantly different PBMC profiles.

Both B- and T-cell responses were efficiently elicited in all three groups, which is in agreement with published data. This study went on to demonstrate for the first time that subjects with different primary responses to the smallpox vaccine present with differences in both B- and T-cell memory cell reactions to VV.

B-cell memory was found to be weaker in the adverse reaction group, both with significantly fewer numbers of vaccinia specific IgG^+ AbSCs per 100,000 PBMCs than nonresponders and normal responders, and significantly lower percentage of VV specific IgG^+ AbSCs out of total IgG^+ AbSCs in the adverse reaction group than in normal responders and nonresponders.

The subjects of the adverse reaction group were found to have increased Th1 and Th2 responses when compared with nonresponders and normal responders. Subjects with extreme reactions to the smallpox vaccine did not respond to VV stimulation with different levels of inflammatory mediators and chemokines than normal responders for the most part.

Although a negative correlation was found between cytokine levels and numbers of VV specific IgG^+ AbSCs, it was of low level and could, therefore, not be convincingly said to be related to VV memory responses.

In conclusion, it seems as if individuals who suffered adverse reactions to the smallpox vaccine may have been induced to produce stronger memory Th1 and Th2 cells at the cost of a less robust memory B-cell response. In addition, nonresponders produce B- and T-cell responses that are equivalent to the responses of normal responders despite showing “no take” at the time of vaccination.

The results of this study have increased our understanding of long term T- and B-cell memory to VV and how it relates to the primary response to vaccination with the *Vaccinia* virus. They have also provided the unique opportunity to overlay the immunological results for the two groups of extreme vaccine responders onto the extensive genotype results available. This will help to identify genetic factors and molecular pathways that contribute to the nature of the immune response and outcome of vaccination. Understanding the mechanisms that determine effective response, lack of response or adverse events to *Vaccinia* and other vaccines will facilitate the development of novel safe and effective vaccine formulation and vaccination and intervention strategies.

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APPENDIX

Table 6. Relative size of PBMC lymphocyte subpopulations of all subjects.

	T-cells	Helper T-cells	Cytotoxic T-cells	B-cells	Naïve B-cells	Memory B-cells	Plasma cells
Surface marker:	$CD3^+$	$CD4^+/CD3^+$	$CD8^+/CD3^+$	$CD19^+$	$CD20^+/CD27^-$	$CD20^+/CD27^+$	$CD20/CD27^{high}$
Subject	%Lymphct.	%Lymphct.	%Lymphct.	%Lymphct.	% CD19+	%CD19+	%CD19+
1	58.17	37.88	18.27	11.53	65.53	30.95	1.26
2	58.61	36.17	19.11	5.73	51.31	45.29	1.65
3	47.43	28.22	18.46	11.82	69.37	29.2	0.55
4	48.15	30.19	16.63	8.55	76.35	21.44	1.3
5	55.05	35.56	17.71	10.48	76.57	21.41	1.01
6	60.8	45.67	7.94	8.97	88.08	9.58	1.31
7	43.33	26.05	16.44	10.49	36.04	58.48	3.63
8	58.79	**25.63**	**30.18**	6.55	83.54	14.25	0.9
9	52.67	29.16	17.91	11.24	64.82	32.5	0.99
10	57.42	39.25	18.12	7.74	82.13	15.3	1.72
11	57.2	30.78	24.19	4.25	64.46	34.61	0.56
12	59.89	36.17	22.94	5.46	62.22	34.36	1.25
13	61.31	45.59	13.8	5.78	51.05	26.98	0.41
14	49.66	**21.95**	**26.99**	7.53	57.16	27.93	1.06
15	56.5	33.29	18.99	10.38	69.1	39.34	0.81
16	61.37	44.78	16.81	5.54	74.14	24.29	0.85
17	61.31	48.01	12.94	7.1	69.99	6.28	0.35
18	39.93	27.55	10.9	6.06	76.76	19.89	1.68
19	53.68	39.58	13.13	7.53	71.2	25.62	1.09
20	39.33	23.87	12.44	9.05	59.81	33.5	2.72
21	60.67	34.27	26.88	4.43	77.35	20.7	1.08
22	42.87	34.06	11.53	6.67	80.93	15	1.99
23	55.04	30.34	25.77	9.13	74.14	24.03	0.6
24	64.03	43.83	18.51	10.83	57.8	41.33	0.13
25	52.75	32.72	19.45	8.71	80.94	16.28	1.34
26	57.29	34.1	21.63	4.76	67.96	29.1	0.88
27	60.63	38.12	27.16	8.22	60.01	36.67	1.3
28	59.41	42.35	15.68	6.46	85.75	12.28	1.01
29	72.47	46.93	24.88	3.98	79.95	18.14	0.87
30	63	40.59	22.45	8.79	78.22	18.18	0.65
31	57.45	**15.43**	**35.04**	3.77	84.02	12.65	1.48
32	53.29	33.01	18.31	7.89	51.33	41.59	3.52
33	54.85	**23.67**	**27.3**	9.12	78.34	18.62	1.17
34	58.64	46.69	11.37	12.27	88.14	9.38	0.33
35	39.71	25.1	14.67	17.13	67.14	30.03	0.52
36	42.19	25.06	17.03	5.38	60.72	34.26	2.04
37	54.51	31.17	21.59	9.25	74.62	22.15	1.28
38	52	32.91	18	8.97	71.85	25.41	1.41
39	44.13	28.93	14.98	8.36	73.42	24.73	0.55
40	41.37	26.88	13.87	6.16	73.5	24.01	0.54
41	57.99	43.97	14.19	8.41	69.2	26.54	2.11
42	51.62	30.81	20.53	5.07	68.76	24.58	3.19
43	55.17	26.28	24.82	5.25	59.74	37.28	0.4
44	48.42	25.61	17	5.79	74.94	20.23	2.23

45	49.03	36.5	15.43	9.81	90.42	7.24	0.82
46	67.57	42.4	25.64	7.6	72.99	25.53	0.38
47	58.41	41.82	16.47	7.23	72.86	24.65	0.99
48	52.8	**23.19**	**25.18**	9	55.49	36.89	3.1
49	61.56	38.89	20.73	9.51	68.28	28.36	1.04
50	58.89	34.49	28.09	5.96	82.4	15.16	0.84
51	64.23	36.8	22.74	5.81	77.95	19.92	1.04
52	59.25	32.51	23.73	10.3	46.37	51.19	0.99
53	65.7	**31.42**	**32.15**	4.73	75.81	22.29	0.41
54	52.8	34.71	17.3	8.37	58.07	32.84	5.71
55	62.29	36.03	26.3	10.53	51.25	46.53	1.03
56	44.68	26.85	14.04	7.95	74.96	23.35	0.15
57	45.03	32.51	12.1	6.27	88.63	8.32	1.11
58	57.16	32.81	21.89	6.64	64.35	30.69	2.04
59	62.13	38.1	21.98	7.86	79.61	17.91	1.27
60	44.55	36.14	7.36	4.89	82.78	13.31	1.66
61	54.76	36	17.73	12.82	79.93	18.51	0.22
62	49.08	26.63	21.46	11.21	44.48	51.84	1.04
63	38.35	23.49	10.13	12.63	77.22	19.26	0.79
64	53.88	37.13	15.8	18.12	88.87	7.21	0.61
65	57.63	32.73	23.41	6.44	73.25	23.94	1.62
66	57.91	43.93	14.4	6.46	75.65	23.12	0.37
67	43.3	32.12	11.79	11.41	59.56	39.9	0.13
68	53.42	35.65	19.1	8.41	58.01	39.58	0.54
69	54.95	**23.14**	**32.63**	5.49	76.39	20.05	1.03
70	57.31	44.66	13.72	9.94	64.67	33.14	0.28
71	59.37	40.32	16.01	7.92	72.69	25.31	0.59
72	64.74	34.25	27.22	8.47	70.25	20.06	6.3
73	59.13	42.57	16.73	8.7	74	24.13	0.57
74	54.45	33.14	22.94	7.11	65.33	31.24	1
75	55.4	35.08	20.43	8.18	47.68	47.11	1.58
76	65.07	37.16	27.02	3.77	56.94	39.64	0.91
77	60.25	41.16	18.88	7.38	72.75	23.02	0.72
78	53.88	33.13	20.71	7.31	76.21	21.09	0.56
79	55.85	31.53	22.54	3.99	60.88	35	0.71
80	48.32	33.16	15.22	9.34	82.72	16.5	0.31
81	66.92	36.88	30.29	3.69	71.18	26.25	1.12
90	51.68	32.66	18.36	9.58	72.9	23.78	1.17
91	61.47	37.57	22.33	9	72.57	24.03	0.92
92	58.49	**22.64**	**23.13**	9.89	54.02	42.9	1.24
93	51.27	29.06	21.47	5.99	57.46	38.28	0.87
94	61.05	44.19	15.67	8.5	80.39	17.3	0.73
95	57.44	40.51	16.13	6.35	72.82	22.42	1.67
96	63.1	35.18	27.23	5.93	54.27	43.08	0.74
97	56.83	41.03	13.99	12.18	80.26	17.98	0.44

T-cell, Th cell, cytotoxic T-cell, B-cell, naïve B-cell, memory B-cell and plasma cell populations are identified based on surface markers and their relative size shown. Subjects with a larger cytotoxic T-cell population than a Th cell population are bolded.

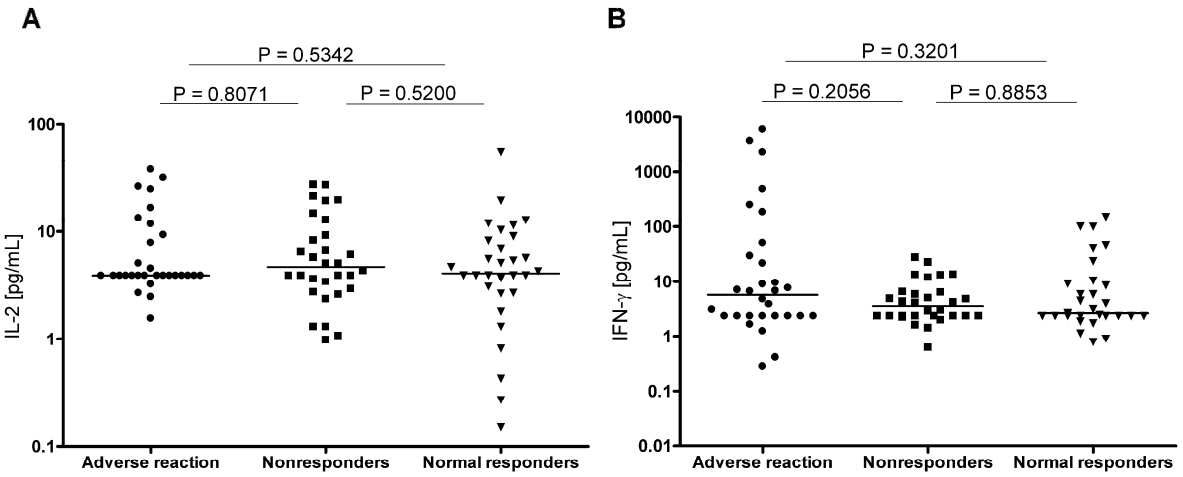

Figure 12. Cytokine levels in supernatant of unstimulated PBMCs from subjects with different responses to smallpox vaccine. A) Baseline IL-2 levels. B) Baseline IFN-γ levels. P values comparing cytokine levels between groups are shown.

Table 7. Median cytokine levels detected via Luminex.

	Adverse reaction			Nonresponders			Normal responders		
Cytokine:	VV [pg/mL]	anti-CD3/ anti-CD28 [pg/mL]	unstim [pg/mL]	VV [pg/mL]	anti-CD3/ anti-CD28 [pg/mL]	unstim [pg/mL]	VV [pg/mL]	anti-CD3/ anti-CD28 [pg/mL]	unstim [pg/mL]
IL-1β	24.85	751.9	5.405	37.04	496.9	4.185	87.27	711.67	17.66
IL-1ra	284.6	487	36.69	262.7	570.4	31.85	220	543.2	51.14
IL-2	29.94	2963	4.01	14.69	2941	3.265	16.76	2817	5.94
IL-4	3.455	9.605	1.22	2.75	8.48	0.99	2.73	9.75	1.04
IL-5	0.71	211.5	0.73	0.675	152.9	0.845	0.49	203.6	0.89
IL-6	159.2	9706	90.31	304.3	9706	162.1	1447	9706	632.9
IL-7	4.35	17.09	3.895	9.17	19	6.045	8.63	15.07	12.05
IL-8	2501	13983	2453	2999	13983	2718	4995	13983	2818
IL-9	19.37	382.1	9.68	20.1	339.2	16.52	26.61	310.1	24.21
IL-10	4.515	266.3	2.935	2.32	355.7	2.705	5.17	333.6	4.68
IL-12p70	8.89	46.39	7.545	10.48	47	9.08	9.97	41.74	11.4
IL-13	8.54	385.2	4.21	9.765	265.6	4.055	8.69	321.7	8.69
IL-15	6.32	2.91	0.0005	7.175	2.105	0.745	6.07	2.22	0.57
IL-17A	19.45	451.7	16.48	18.93	489.8	18	19.52	499.9	23.65
Eotaxin	72.67	106.6	4.93	68.37	101.1	2.303	71.58	101.5	6.82
Basic FGF	4.72	17.1	0.035	11.16	14.96	2.79	10.16	14.53	8.4
G-CSF	21.86	53.02	18.06	17.36	50.17	14.04	37.13	74.74	22.97
GM-CSF	0.52	179.4	0.01	0.15	146.7	0.01	0.055	166.9	0.01
IFN-γ	375.4	18390	52.15	168.4	17191	53.96	219.4	17693	77.48
IP-10	53556	26701	1737	53556	21882	891.7	21048	16442	925.9
MCP-1	538.1	1048	452.3	672.7	999.2	772.3	604.5	1082	643.1
MIP-1α	169.9	450.9	4.76	251.5	459.2	6.23	298.1	471.4	32.78
MIP-1β	370.1	2500	119.3	1880	738.7	145.4	462.8	2500	223.9
PDGF bb	629.9	529.6	488.3	549.2	446	407.9	446.5	418	311
RANTES	445.9	5913	545.5	456.2	1943	425.2	398.6	3149	402.1
TNF-α	58.96	8084	4.865	30.88	6431	3.57	71.68	5973	8.96
VEGF	14.07	187	19.65	16.59	186.5	25.58	21.87	177.3	29.92

Medians of VV induced, anti-CD3 and anti-CD28 induced, and unstimulated cytokine levels for subjects with different primary responses to the smallpox vaccine.

Table 8. VV induced and unstimulated cytokine levels compared across all subjects.

Cytokine	VV stim Median [pg/mL]	Unstim Median [pg/mL]	VV stim vs. Unstim P value
IL-1β	38.84	6.7	**< 0.0001**
IL-1ra	277.12	39.54	**< 0.0001**
IL-2	18.3	4.07	**< 0.0001**
IL-4	2.94	1.1	**< 0.0001**
IL-5	0.64	0.83	**0.0022**
IL-6	278.74	185.36	0.0568
IL-7	7.55	5.93	0.2310
IL-8	3134.87	2685.9	0.9856
IL-9	21.02	15.01	**0.0015**
IL-10	3.56	3.43	0.9422
IL-12p70	9.81	9.04	0.4313
IL-13	8.98	5.3	**< 0.0001**
IL-15	6.28	0.29	**< 0.0001**
IL-17A	19.2	20.09	0.5372
Eotaxin	71.58	4.26	**< 0.0001**
Basic FGF	9.52	3.31	**< 0.0001**
G-CSF	18.52	17.07	0.3828
GM-CSF	0.15	0.01	**< 0.0001**
IFN-γ	220.83	57.61	**< 0.0001**
IP-10	53556	1093.6	**< 0.0001**
MCP-1	589.84	680.67	0.2310
MIP-1α	256.16	7.76	**< 0.0001**
MIP-1β	508.17	134.46	**< 0.0001**
PDGF bb	509.09	385.39	**0.0040**
RANTES	416.31	463.15	0.6344
TNF-α	46.49	5.64	**< 0.0001**
VEGF	17.33	24.93	**0.0014**

Medians of VV induced and unstimulated cytokine levels shown. P values comparing VV induced levels to unstimulated levels are bolded when significant. Names of cytokines with significant differences are also bolded.

Table 9. VV induced and unstimulated cytokine levels compared for subjects with different responses to the smallpox vaccine.

	Vaccinia induced vs. unstimulated within group		
	Adverse reaction	**Nonresponder**	**Normal**
IL-1β	**0.0008**	**0.0009**	**0.0118**
IL-1ra	**< 0.0001**	**< 0.0001**	**< 0.0001**
IL-2	**< 0.0001**	**< 0.0001**	**< 0.0001**
IL-4	**< 0.0001**	**< 0.0001**	**< 0.0001**
IL-9	**0.0002**	0.1393	0.4841
IL-13	**0.0236**	**0.0003**	0.4889
IL-15	**< 0.0001**	**< 0.0001**	**< 0.0001**
Eotaxin	**< 0.0001**	**< 0.0001**	**< 0.0001**
Basic FGF	**< 0.0001**	**0.0008**	0.1335
GM-CSF	**< 0.0001**	**< 0.0001**	**< 0.0001**
IFN-γ	**< 0.0001**	**< 0.0001**	**0.0009**
IP-10	**< 0.0001**	**< 0.0001**	**< 0.0001**
MIP-1α	**< 0.0001**	**< 0.0001**	**0.0031**
MIP-1β	**< 0.0001**	**< 0.0001**	0.1571
PDGF bb	0.0728	0.1154	0.0713
TNF-α	**< 0.0001**	**< 0.0001**	**< 0.0001**

VV induced cytokine levels in the adverse reaction group (Adverse reaction) are compared with unstimulated cytokine levels in the adverse reaction group. The same is done for nonresponders (Nonresponder) and normal responders (Normal). P values comparing VV induced levels to unstimulated levels are bolded when significant. Names of cytokines with significant differences are also bolded.

Table 10. VV induced cytokine levels compared between subjects with different responses to the smallpox vaccine.

	Adverse reaction VV stimulated		Nonresponders VV stimulated		Normal responders VV stimulated	
Cytokine	Median [pg/mL] (25% - 75%)	P value: vs.Non	Median [pg/mL] (25% - 75%)	P value: vs. Norm	Median [pg/mL] (25% - 75%)	P value: vs. Advs
IL-1β	24.85 (5.3 - 119.1)	0.8519	37.04 (8.7 - 81.9)	0.1064	87.27 (10.8 - 287.3)	0.0525
IL-1ra	284.6 (153.1- 596.3)	0.8155	262.7 (181.4 - 520.1)	0.4714	220 (78.5 - 515.0)	0.3423
IL-2	29.94 (18.9 - 53.2)	**0.0002**	14.69 (12.6 - 25.0)	0.9758	16.76 (9.8 - 28.2)	**0.0008**
IL-4	3.455 (2.8 - 4.8)	**0.0087**	2.75 (2.3 - 3.5)	0.3710	2.73 (1.8 - 3.3)	**0.0007**
IL-9	19.37 (15.1 - 23.5)	0.7263	20.1 (12.9 - 26.5)	**0.0454**	26.61 (19.2 - 32.3)	**0.0338**
IL-13	8.54 (3.1 - 12.9)	0.2838	9.765 (7.5 - 11.9)	0.7559	8.69 (6.2 - 13.8)	0.3792
IL-15	6.32 (3.7 - 8.6)	0.3627	7.175 (4.4 - 10.7)	0.0969	6.07 (1.6 - 8.0)	0.4110
Eotaxin	72.67 (49.7 - 87.4)	0.6744	68.37 (49.9 - 89.6)	0.8260	71.58 (35.7 - 93.7)	0.7678
Basic FGF	4.72 (3.7 - 6.3)	**0.0002**	11.16 (7.5 - 14.8)	0.6657	10.16 (8.5 - 12.7)	**0.0003**
GM-CSF	0.52 (0.1 - 3.4)	0.4662	0.15 (0.1 - 1.5)	0.9939	0.055 (0.1 - 3.7)	0.5976
IFN-γ	375.4 (146.9 -1084.0)	0.0502	168.4 (139.3 - 311.4)	0.9335	219.4 (88.5 - 433.3)	**0.0415**
IP-10	53556 (53556 - 53556)	0.9225	53556 (53556 - 53556)	**0.0091**	21048 (5846 - 53556)	**0.0129**
MIP-1α	169.9 (34.2 - 646.3)	0.9814	251.5 (50.4 - 416.8)	0.2461	298.1 (35.2 - 1223.0)	0.3342
MIP-1β	423.8 (313.7 - 1880)	0.0826	1410 (353.5 - 1880)	0.4052	462.8 (314.7 - 1880)	0.4909
PDGF-BB	629.9 (504.4 - 837.2)	0.2279	549.2 (379.7 - 749.6)	**0.0345**	446.5 (304.9 - 511.9)	**0.0008**
TNF-α	58.96 (26.9 - 224.6)	0.0798	30.88 (21.9 - 70.7)	0.0737	71.68 (24.2 - 165.5)	0.9577

Median VV induced cytokine levels and interquartile ranges are shown for the adverse reaction, nonresponder and normal responder groups. P values comparing VV induced levels between groups are bolded when statistically significant. Names of cytokines with significant differences are also bolded.

Table 11. Unstimulated cytokine levels compared between subjects with different responses to the smallpox vaccine.

	Adverse reaction Unstimulated		Nonresponders Unstimulated		Normal responders Unstimulated	
Cytokine	Median [pg/mL] (25% - 75%)	P value: vs.Non	Median [pg/mL] (25% - 75%)	P value: vs. Norm	Median [pg/mL] (25% - 75%)	P value: vs. Adv
IL-1β	5.405 (0.8 - 11.59)	0.4185	4.185 (2.615 - 13.96)	**0.0192**	20.08 (5.73 - 61.81)	**0.0019**
IL-1ra	36.69 (23.17 - 72.07)	0.5754	31.85 (20.85 - 49.89)	**0.0371**	51.56 (29.06 - 120.2)	0.1275
IL-2	4.01 (2.3 - 6.12)	0.9814	3.265 (2.66 - 6.1)	0.1374	6.22 (2.785 - 8.05)	0.1602
IL-4	1.22 (0.875 - 2.245)	0.1332	0.99 (0.71 - 1.355)	0.7966	1.04 (0.69 - 1.8)	0.1906
IL-9	9.68 (5.235 - 14.69)	**0.0009**	16.52 (12.41 - 19.96)	0.4086	24.43 (10.88 - 37.04)	**0.001**
IL-13	4.21 (2.63 - 6.82)	0.6801	4.055 (2.745 - 8.38)	**0.0039**	8.775 (5.255 - 11.92)	**0.0012**
IL-15	0.0005 (0.0005 - 0.3)	**0.0096**	0.745 (0.0005 - 1.3)	0.4847	0.575 (0.0005 - 1.03)	**0.0219**
Eotaxin	4.93 (0.345 - 14.82)	0.9937	2.303 (0.345 - 17.38)	0.5057	8.46 (0.345 - 40.69)	0.4619
Basic FGF	0.035 (0.035 - 2.54)	**0.0152**	2.79 (0.035 - 10.74)	**0.0344**	8.725 (4.79 - 13.78)	**< 0.0001**
GM-CSF	0.01 (0.01 - 0.01)	1.0000	0.01 (0.01 - 0.01)	0.2483	0.01 (0.01 - 0.01)	0.2568
IFN-γ	52.15 (29.17 - 128.7)	0.9938	53.96 (37.85 - 88.27)	0.1032	77.48 (48.52 - 164.4)	0.1699
IP-10	1737 (723.5 - 2610)	0.0828	891.7 (313.8 - 2271)	0.9819	1008 (322.8 - 2870)	0.0726
MIP-1α	4.76 (1.505 - 11.46)	0.1415	6.23 (3.43 - 12.44)	**0.0116**	37.86 (6.4 - 216.9)	**0.0017**
MIP-1β	119.3 (47.05 - 262.1)	**0.0378**	145.4 (91.38 - 278.2)	0.2280	213.5 (108.9 - 45576)	**0.0065**
PDGF-BB	488.3 (297.4 - 765.6)	0.6351	407.9 (291.4 - 605.7)	**0.0144**	330.2 (245.9 - 469.8)	**0.0202**
TNF-α	4.865 (2.275 - 11.01)	0.5701	3.57 (1.045 - 10.72)	**0.0471**	11.85 (3.745 - 54.8)	0.0575

Median unstimulated cytokine levels and interquartile ranges are shown for the adverse reaction, nonresponder and normal responder groups. P values comparing VV induced levels between groups are bolded when statistically significant. Names of cytokines with significant differences are also bolded.

Table 12. Anti-CD3 and anti-CD28 induced cytokine levels compared between subjects with different responses to the smallpox vaccine.

	Adverse reaction anti-CD3 and anti-CD28 stimulated		Nonresponders anti-CD3 and anti-CD28 stimulated		Normal responders anti-CD3 and anti-CD28 stimulated	
Cytokine	Median [pg/mL] (25% - 75%)	P value: vs.Non	Median [pg/mL] (25% - 75%)	P value: vs. Norm	Median [pg/mL] (25% - 75%)	P value: vs. Advs
IL-1β	751.9 (410.9 - 1513)	0.2795	496.9 (418.9 - 843.6)	0.3244	711.7 (440.9 - 1049)	0.7195
IL-1ra	487 (400.1 - 822.5)	0.8458	570.4 (394 - 701.1)	0.6934	543.2 (336.2 - 652.7)	0.7739
IL-2	2963 (1414 - 3797)	0.4320	2941 (281.2 - 3695)	0.7216	2817 (420.5 - 3728)	0.6264
IL-4	9.605 (7.165 - 15.32)	0.2432	8.48 (6.52 - 13.81)	0.7617	9.75 (6.18 - 12.57)	0.4875
IL-9	382.1 (263.1 - 854.6)	0.4694	339.2 (235.5 - 614.6)	0.4622	310.1 (239.8 - 515.6)	0.1775
IL-13	385.2 (219 - 623.7)	0.1235	265.6 (197 - 445.2)	0.3589	321.7 (209.4 - 449.1)	0.3108
IL-15	2.91 (2.285 - 3.79)	0.0885	2.105 (1.26 - 3.55)	0.5748	2.22 (0.795 - 3.955)	0.0630
Eotaxin	106.6 (96.63 - 122.3)	0.8260	101.1 (89.28 - 115)	0.6767	101.5 (86.23 - 116.5)	0.1464
Basic FGF	17.1 (11.55 - 23.02)	0.4096	14.96 (12.28 - 18.49)	0.4394	14.53 (13.48 - 16.31)	0.1853
GM-CSF	179.4 (137.9 - 207.9)	0.1834	146.7 (124 - 204.5)	0.3026	166.9 (139.3 - 198.3)	0.6264
IFN-γ	18390 (14537 - 23643)	0.4886	17191 (15748 - 18208)	0.7329	17693 (15320 - 22469)	0.9936
IP-10	26701 (19847 - 31510)	0.0630	21882 (17065 - 27882)	**0.0332**	16442 (13065 - 23006)	**0.0005**
MIP-1α	450.9 (368.1 - 716.2)	0.7615	459.2 (346 - 1054)	0.9456	471.4 (339.6 - 804.2)	0.7678
MIP-1β	500 (512.6 - 2500)	0.3926	738.7 (463.8 - 2500)	0.4959	2500 (475 - 2500)	0.8971
PDGF-BB	529.6 (370 - 683.4)	0.4959	446 (381.8 - 539.6)	0.5493	418 (348 - 536.4)	**0.0331**
TNF-α	8084 (5667 - 9941)	0.1546	6431 (4476 - 8966)	0.4173	5973 (4663 - 7931)	**0.0373**

Median anti-CD3 and anti-CD28 induced cytokine levels and interquartile ranges are shown for the adverse reaction, nonresponder and normal responder groups. P values comparing VV induced levels between groups are bolded when statistically significant. Names of cytokines with significant differences are also bolded.

Table 13. Mean fold increase from unstimulated cytokine levels to VV-induced.

Cytokine	Mean fold increase				
	Adverse reaction	Adv vs Norm P value	Non-responders	Non vs Norm P value	Normal responders
IL-1β	11	0.1197	14	0.4903	7
IFN-γ	10	**0.0104**	9	0.2110	4
IP-10	45	0.3505	82	**0.0430**	51
MIP-1α	75	**0.0239**	159	0.0969	105
MIP-1β	9	**0.0435**	8	**0.0371**	5
TNF-α	32	0.0805	44	0.2002	32

Mean fold increase: mean of cytokine level (pg/ml) in VV-stimulated cultures divided by baseline cytokine level (pg/ml) in unstimulated cultures. P values comparing adverse reaction and nonresponder groups to the normal responders shown. Statistically significant P values are bolded.

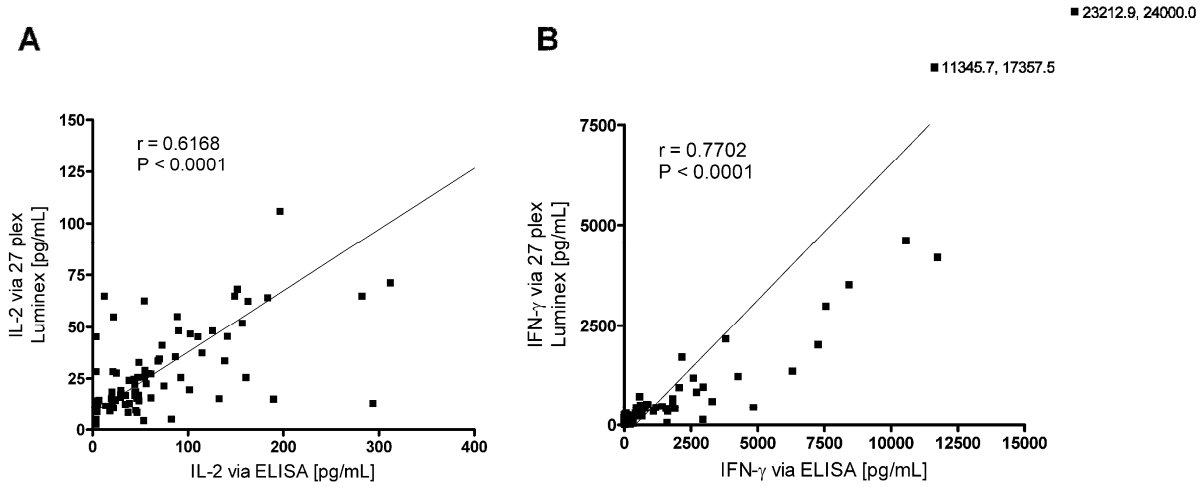

Figure 13. Relationship between cytokine levels measured via ELISA and Luminex. A) IL-2 levels. B) IFN-γ levels. Linear correlation, outliers, Spearman r and P values shown.