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Leukocyte trafficking during infection with Modified Vaccinia virus Ankara: the role of chemokine receptor 1 and complement activation

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Dedicated to my incredibly patient wife

“L'amour sans complications”

List of abbreviations

A

APC - Antigen presenting cells 27

ATRA - All-*trans* retinoic acid 35

B

BAC - Bacterial artificial chromosome 10

BAL - Bronchoalveolar lavage 37

C

CCL12 - Chemokine (C-C motif) ligand 12 16

CCL2 - chemokine (C-C motif) ligand 2 2

CCL3 - Chemokine (C-C motif) ligand 3 26

CCL5 - Chemokine (C-C motif) ligand 5 30

CCL7 - Chemokine (C-C motif) ligand 7 16

CCR1 - Chemokine (C-C motif) receptor 1 2

CCR2 - Chemokine (C-C motif) receptor 2 29

CCR5 - Chemokine (C-C motif) receptor 5 30

CEF - Chicken embryo fibroblast 8

CEV - Cell associated enveloped virus 4

CTL - CD8 cytotoxic lymphocyte 13

CVA - Chorioallantoic vaccinia virus Ankara 8

CX_3CL1 - Chemokine (C-X_3-C motif) ligand 1 30

CX_3CR1 - Chemokine (C-X_3-C-motif) receptor 1 29

CXCL2- Chemokine (C-X-C motif) ligand 2 26

CXCR2 - Chemokine (C-X-C motif) receptor 2 25

CXCR4 - Chemokine (C-X-C motif) receptor 4 25

D

DARC - Duffy antigen receptor for chemokines 30

DC - Dendritic cells 15

E

EEV - Extracellular enveloped viron 3

ER - Endoplasmic reticulum 4

F

FACS buffer - PBS + 1% v/v FCS + 0.01% w/v sodium azide 38

FCS - Foetal calf serum 35

G

G-CSF - Granulocyte colony-stimulating factor 25

GM-CSF - Granulocyte macrophage colony stimulating factor 35

H

HSV - Herpes simplex virus 16

I

IEV - Intracellular enveloped virus 4

IFN - Type I interferon 12

IFNAR - Type I IFN receptor 15

IFNγ - Interferon-γ 32

IM - Inflammatory monocytes 15

J

L

M

N

P

R

S

T

V

W

Table of Contents

1 Introduction

Poxviridae are a large family of enveloped viruses with double stranded, linear DNA genomes that replicate in the cytoplasm and exhibit a broad host range. Vaccinia virus (VACV) is a prototypical poxvirus that was used in the successful vaccination campaign which led to the eradication of the closely related variola virus, the etiologic agent of smallpox. Despite the eradication of smallpox the need for an effective vaccine has not entirely disappeared as there remains a small but significant threat of outbreaks of smallpox-like diseases. The latest generations of vaccine strains such as the highly attenuated modified vaccinia virus Ankara (MVA) would likely fill this gap if the need were to arise again.

Although unable to propagate in human cells, MVA efficiently expresses viral and recombinant genes. Coupled with its intrinsic adjuvant properties MVA is a promising viral vector, which, along with a number of other VACV strains are being developed for use as viral vector vaccines. VACV has shown promise in these applications, however, there is little understanding of the mechanisms underlying the immune response to VACV. In view of this and the complex regulatory questions associated with the use of recombinant viral vectors it is imperative that we develop a better understanding of the immune responses elicited by VACV and decipher the role of host and viral derived factors in the mechanisms behind the potent immunity induced by VACV.

MVA is unique amongst VACV strains in its ability to trigger the rapid release of inflammatory cytokines and recruitment of leukocytes. We believe that this potent induction of innate immunity may play an important role in the efficacy of MVA vaccination. Previous work by our group has demonstrated the importance of chemokine (C-C motif)

ligand 2 (CCL2) in the recruitment of monocytes and lymphocytes to the site of infection, however a large proportion of the infiltrating leukocytes are neutrophils which are recruited independently of CCL2. Despite being one of the most abundant cell types recruited during the early stages of infection little is known about the role of neutrophils in poxvirus immunity. The aim of this study was to investigate MVA induction of the innate immune response by assessing the contribution of signalling through chemokine (C-C motif) receptor 1 (CCR1) and activation of the complement system to the recruitment of neutrophils and monocytes.

Cell migration assays were employed to assess the contribution of CCR1 to MVA induced migration of monocytes and neutrophils. Infiltrating leukocytes in MVA infected *Ccr1*$^{-/-}$ mice were quantified by flow cytometry to show the important role of CCR1 for neutrophil and monocyte recruitment. Similarly the role of complement activation in the induction of leukocyte migration was assessed by infection studies in mice deficient in the central complement component C3. Investigations into complement component C5 showed that MVA infection activates C5 independently of C3 and that C5 contributes to the recruitment of neutrophils and $CD8^+$ T-cells. These studies demonstrated an important role for CCR1 and complement activation in the recruitment of leukocyte subpopulations during MVA infection, advancing our knowledge of the innate immune response to MVA.

2 Literature review

2.1 Poxviruses

2.1.1 Taxonomy

Poxviruses, from the *Poxviridae* family, are divided into two subfamilies, *Entomopoxvirinae* which infect insects and the *Chordopoxvirinae* which infect vertebrates. Within the *Chordopoxvirinae* family there are 8 genera, of which the *Orthopoxviruses*, *Parapoxviruses*, *Molluscipoxviruses* and *Yatapoxviruses* contain species that can infect humans. Generally most poxviruses exhibit a specific host range however some strains such as cowpox are known to infect various hosts including humans, others are acquired as rare zoonotic infections [1].

2.1.2 Structure & lifecycle

Poxviruses particles are large (240 by 300 nm) with a distinct brick like shape. Virus is released from the cell in two major infectious forms, the extracellular enveloped viron (EEV) which buds from the cell acquiring an additional envelope, and the intracellular mature viron (IMV) which is released upon cell lysis and only has a single viral envelope. The two forms differ in antigenicity and, due to different entry mechanisms, also in infectivity [2]. Poxviruses have large linear double stranded DNA genomes with inverted terminal repeats and contain several hundred open reading frames. Genes involved in replication and morphogenesis are largely conserved, whilst many of the non-structural genes which are involved with host interaction are adaptively evolved to specific species, and so are more divergent [3].

Poxvirus entry into cells is a complex process involving many viral and host factors that trigger the activation of signalling pathways and actin remodelling leading to internalisation, possibly by macropinocytosis [4]. Though the exact cell surface receptors involved have not been determined it is thought that the virus binds to ubiquitously expressed glycosaminoglycans [5]. As the macropinosome matures the change in pH triggers release of the virus core into the cytosol [6]. The first wave of early viral transcription is then initiated by the viral transcriptome at early promoters leading to the synthesis of early viral mRNA [7] (Fig. 2.1). In the second step the viral core is uncoated releasing the viral genome into the cytosol, where host factors are recruited for genome replication and the subsequent synthesis of intermediate and late genes in cytosolic membrane wrapped virus factories [8]. An important feature of poxvirus infection is the synthesis of a vast array of host immunomodulatory factors which subvert important host pathways including apoptosis, antigen presentation and immune signalling. These factors act both intracellularly and extracellularly, allowing the virus to avoid detection by the host immune response [9].

Assembly and release of progeny virus is a complex process, whereby virons bud through the smooth endoplasmic reticulum (ER) acquiring a double membraned cisterna to form IMV [10], the majority of which remain inside the cell until it is ultimately lysed. Other types of virus particles are formed by subsequent budding of IMV through the *trans*-golgi network. This leads to the further acquisition of another double cisterna membrane forming intracellular enveloped virus (IEV) [11]. The outer membrane of IEV can fuse with the cell membrane, leaving a cell associated enveloped virus (CEV) exposed on the cell surface. Under the control of several host proteins, CEV polymerises actin leading to the release of

EEV or extrusion of CEV from the cell surface, propelling them towards neighbouring cells [12].

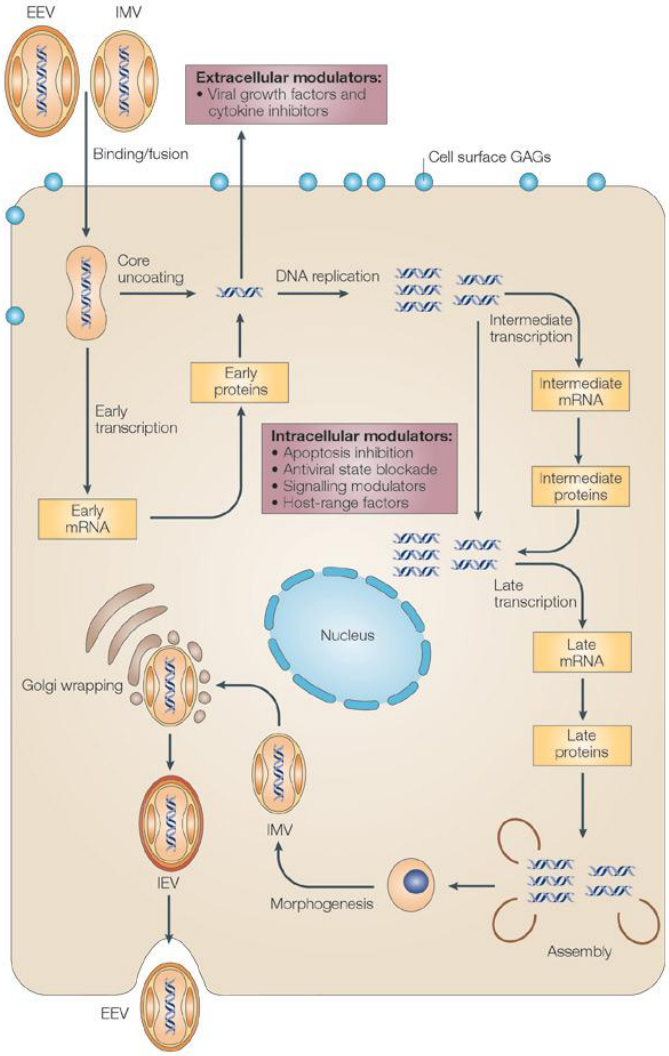

Fig. 2.1: Life cycle of VACV. See main text for details. Adapted from McFadden 2005 [13], with permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, copyright 2005.

2.1.3 Disease

A main characteristic feature of poxvirus infection is the development of skin lesions, sometimes solely at the primary site of infection, which can then spread further due to self-inoculation. Lesions can be haemorrhagic as seen with some orthopoxviruses, tumor-like as

with parapox or leporipox viruses, or less innocuous fleshy nodules as seen with molluscum contagiosum. Other poxviruses are capable of establishing generalised infections [1].

Historically, the orthopoxviruses are perhaps the most infamous of the poxviruses due to variola virus, the etiologic agent of smallpox. Smallpox infections have been traced back to antiquity and with an overall fatality rate of approximately 20-40%, smallpox was one of the most deadly diseases to affect mankind [14]. Variola was likely transmitted by close contact with lesions, material or respiratory secretions of infected persons. In generalised infections such as smallpox the virus first replicates locally, for example in the respiratory tract, before spreading to the draining lymphoid organs and finally throughout the body to peripheral sites. The prodromal symptoms are similar to many other viral infections and included fever, malaise, vomiting, headache and muscle pain. After an incubation period of approximately 12 days the first enanthema appear on the mucus membranes, followed by the characteristic macules. Once they appear the macules spread rapidly across the body and then in the majority of cases develop into the characteristic pustules (though not usually full of pus) which would dry up into scabs and begin to resolve after some 20 days often leaving disfiguring scars [14].

It was in the effort to combat smallpox that the concept of vaccination was invented, firstly with the slightly precarious practice of variolation which was then superseded when Edward Jenner demonstrated the effectiveness of vaccination with cowpox in 1796 [15]. One of the enigmas of the smallpox eradication campaign is that whilst it is widely thought that the original vaccinations were carried out with cowpox, the vaccine stocks that were eventually used to eradicate the disease were actually the closely related vaccinia virus (VACV). To this day, smallpox is the only disease that has been completely eradicated by vaccination.

Since the WHO declared the world free of smallpox in 1979, modern research has predominantly focused on the development of VACV for viral vector vaccines and oncolytic therapy, applications in which they show much promise. However since the advent of globalised terrorism, interest in poxvirus vaccines has been somewhat renewed. In 1763, variola virus was allegedly used as a bioweapon when it was distributed on contaminated blankets by the British during the Pontiac's uprising [16, 17], and there remain fears that it could yet be deliberately released into the now mostly immunologically naïve population. However, perhaps the most immediate threat to human health is posed by the emergence of zoonotic strains such as cowpox [18] or the more virulent monkeypox. The potential threat of monkeypox was demonstrated by an outbreak in 2003 when it was inadvertently imported to the United States in rodents from West Africa [19, 20]. This particular variant was relatively benign and monkeypox remains a relatively rare disease, however little is known about the natural host of monkeypox, and if it were able to establish a reservoir in heterologous animal species the public health consequences could be more severe.

More recently, the spread of avian pox in wild bird populations throughout Europe but particularly in the United Kingdom has caused some alarm [21]. Though in many species avian pox is a relatively mild self-limiting disease, in the much beloved garden bird *Parus major* (common name: Great tit), lesions are significantly larger. This can lead to visual impairment and difficulties feeding, which can negatively impact survival through increased predation and starvation, particularly in juveniles. Though infection rates have not reached a threshold where they are expected to cause a decline in *Parus* populations, it is thought that avian pox may hinder the recovery of some rarer *Parus* species [22]. Other poxvirus

diseases such as ecthyma orf, sheep pox, goat pox, lumpy skin disease and myxomatosis continue to be important causes of morbidity in wild and domesticated animals.

Viruses are incredibly resilient and are constantly evolving and adapting as we have seen with the emergence of diseases such as HIV and SARS. Despite their rather cumbersome DNA genomes, poxviruses are able to adapt relatively quickly [23] and though the threat of smallpox has been vanquished, many other poxvirus species have natural reservoirs that would make control considerably harder if a more virulent species was to emerge. Therefore it would be wise to be prepared should the situation arise where a poxvirus vaccine is again needed, whether it is to stop the spread of an emerging disease or save a species from extinction.

2.2 MVA

2.2.1 Development

During the last decades of the smallpox vaccination campaign, chorioallantoic vaccinia virus Ankara (CVA) was attenuated by serial passage in chicken embryo fibroblast (CEF) cells at the Institute for Infectious Diseases and Zoonoses (formerly the Institute of Medical Microbiology, Infectious and Epidemic Diseases) at the Ludwig-Maximilians-University of Munich. The resulting strain designated modified vaccinia virus Ankara (MVA), had lost approximately 15% of its genome through 6 large deletions and acquired a number of point mutations [24]. These changes affected many genes involved in host interaction [25], as a result MVA displays a greatly restricted host range and is unable to productively infect human and many other mammalian cells. Despite the inability to replicate, MVA can still enter and efficiently express viral genes in many cell types [26] triggering robust immune responses. The loss of its replicative capacity greatly improved the safety profile of MVA

and as a result, it was licensed by the Paul-Ehrlich-Institute in Germany for immunization against smallpox. Though the vaccination campaign was almost at an end, over 100,000 individuals were vaccinated with MVA without any documentation of the severe adverse reactions that were associated with previously used vaccine strains [27, 28]. Given its improved safety profile MVA is an excellent candidate should a poxvirus vaccine be required in the future. Currently, interest is predominately focused on developing MVA and other VACV strains as viral vectors, in which they appear to show promise as vaccine platforms [29] and for oncolytic viral therapy [30].

Being non replicative MVA cannot be used for oncolytic therapy, however MVA has many desirable characteristics that make it a suitable candidate for development as a vector vaccine platform [31, 32]: i) The inability to replicate in most mammalian cells not only minimises the risk to the patient, but also the potential for cross infection, which is particularly important given the ever increasing number of immunocompromised individuals in the population. ii) MVA has a large genome and can take large and even multiple inserts, thus it could potentially be developed as a multivalent vaccine against multiple diseases. iii) VACV are easy to manipulate, relatively stable genetically and easy to store. iv) MVA induces robust immune responses to recombinant and even co-administered antigens [33] and VACV vaccination produces long lasting immunity [34]. v) MVA has already been used in humans with a proven safety record, and is routinely used to vaccinate elephants [35].

There have been a number of attempts to determine which viral genes, and the loss thereof, account for the attenuation of MVA. This has led to the discovery of a number of important virulence factors [36-39]. In a comprehensive study, the six major deletions in the MVA genome were introduced, in various combinations, into the parent CVA strain using a

bacterial artificial chromosome (BAC) system. Interestingly, the loss of three regions actually increased virulence in mice, indicating the possibility for a gain in fitness. Mutant strains with five or six deletions retained virulence and replicative capacity with only partial attenuation, which was subsequently traced to region V [40]. There are some important technical caveats with the technology employed in this study, namely the possibility for complementation and recombination between the viruses that were used. Despite this, it does raise some important points regarding the attenuation of MVA: Firstly, it appears that mutations in genes outside of the deleted regions have had profound effects on viral fitness, particularly regarding replication. Secondly, it indicates that the attenuation of MVA was a stepwise process involving multiple genes. The interactions of VACV with the host are complex and viral genes can have overlapping and redundant functions, there is also the consideration of the different mechanisms that may be required for infection of different hosts. Therefore a virulence factor in one host species or cell type, does not necessarily apply globally to all others making it difficult to account for what is required during different stages of the infection cycle *in vivo*. The traditional approach of deleting viral genes and testing mutant phenotypes undoubtedly has its place, and has produced interesting results [39]. However, assuming that multiple mutations are required to achieve the attenuation of MVA, this would require an enormous number of possible combinations to reproduce the observed phenotype of MVA. Therefore, as an alternative approach, characterising the immune response to MVA gives us an indication as to the host mechanisms that the virus needs to avoid, and thus may eventually enable the reconstruction of the major virulence factors that are required, based on knowledge acquired from testing of single gene mutants.

2.2.2 Efficacy of MVA

There are two main murine models for assessing the protective efficacy of orthopoxvirus vaccines. The first is based on infection with a virulent laboratory strain, Western Reserve (WR), and the second is infection with ectromelia virus the etiologic agent of mousepox. Mousepox is arguably the better model, as ectromelia has evolved to evade murine immune responses and some of these interactions have a high degree of species specificity [41].Therefore, mousepox shares similarities with human smallpox, namely the low infectious dose, respiratory transmission [42], long incubation period, generalised infection and exanthematous rash [43, 44]. However, there are some important differences: the disease course of mousepox is shorter, with fatalities occurring between 7 - 14 days post infection (p.i.) whereas fatalities from smallpox occur after some 18 - 22 days. During mousepox infection the major lesions occur in the liver and spleen, the extent of replication in these organs determines the outcome of the infection. Extensive replication in the liver ultimately leads to death due to acute hepatitis in the absence of skin lesions. Alternatively in resistant strains, skin lesions occur during the secondary viremia caused by the systemic release of the virus from the liver and spleen [45, 46]. This differs from smallpox where death occurs probably due to hypotension after the appearance of skin lesions. Thus mousepox is the better of the two models, however like all models there are some limitations.

MVA has been tested alongside other conventional VACV vaccine strains, all of which protect from lethal infection in both disease models. Vaccination with MVA shortly before exposure or even therapeutically after infection protects from lethal disease [47, 48]. The potential for therapeutic vaccination is a greatly desirable trait for any vaccine; therefore

the mechanisms underlying the post exposure protection provided by MVA are of great interest and could potentially boost future efforts in vaccine development.

2.3 Innate Immune response to MVA

2.3.1 Induction of the innate immune response

Typically, when it comes to vaccination, the development of the adaptive immune response and long lasting immunological memory is the primary requirement. In this respect, MVA is no different from other VACV strains as evidenced by the success of the smallpox vaccination campaign. With the adaptive immune response typically taking 7 - 14 days to develop [49] it is unlikely that any divergence in the adaptive immune response to the different strains would explain the superior short-term protection provided by MVA vaccination in VACV challenge models. One feature that distinguishes MVA from other VACV strains is its ability to activate robust innate immune responses.

To this extent, MVA vaccination of *Rag1*$^{-/-}$ mice, which lack an adaptive immune response, provides a transient survival benefit in poxvirus disease models, though ultimately the mice eventually succumb to lethal infection [47, 48]. This short-term protection is associated with an infiltration of cells of the innate immune system to the site of vaccination. Therefore, it is possible that the induction of the innate immune response plays a key role providing protection in the early stages, buying time for the adaptive immune response to develop.

2.3.2 Signal activation by pathogen recognition receptors

Activation of pathogen recognition receptors (PRR) is an important step in the induction of innate antiviral immunity, which leads to activation of immune cells and the production of type I interferons (herein referred to as IFN, Fig 2.2). VACV are known to trigger multiple cytosolic and membrane associated PRR. A prevailing theory is that recognition of an as yet

undetermined viral ligand by cell membrane associated Toll-like receptor (TLR) 2 is the crucial event preceding IFN production [50]. One premise of this study, that TLR2 discriminates between bacterial and viral ligands, was largely disproven shortly after publication [51], and definitive proof of the crucial role of TLR2 that has been claimed, is still lacking. Indeed prior studies whilst noting a role for TLR2 recognition, reported that IFN induction occurs independently of TLR2 [52]. These studies are inherently complicated, and we recently raised our concerns regarding the handling of technical factors and the interpretation of results in a review [53].

Despite the controversy, TLR2 has been the target of much investigation with others also proposing a role for TLR2 in the induction of IFN [51]. However, studies in disease models have ultimately shown that TLR2 has a minor influence over the course of disease [54]. Viral loads in WR infected *Tlr2*$^{-/-}$ mice only differ in a small time window between days 5 - 10 with the infections resolving in a similar time frame, and TLR2 is completely dispensable for resistance to ectromelia infection [54]. This coincides well with the observation that whilst the CD8 T-cell response is dependent on the MyD88 signal adaptor molecule, *Tlr2*$^{-/-}$ mice still produce a robust antiviral immune response as TLR2 is not the upstream receptor [55]. As infection with recombinant IFNβ expressing VACV restores CD8 cytotoxic lymphocyte (CTL) activation in MyD88 knockout mice, it appears that this effect on T-cell expansion is indirect through the induction of IFN [56].

As intracellular pathogens, recognition of VACV ligands by cytosolic PRR is thought to be a major pathway of innate immune activation, and was noted in many of the studies that implicate TLR2. Sensing of viral DNA by TLR9 has been shown to be important in ectromelia infection, with *Tlr9*$^{-/-}$ mice showing increased susceptibility to infection. Crucially, MVA was

shown to induce other TLR9 independent pathways, and provided protection from disease in *Tlr9*$^{-/-}$ mice [47]. Other cytosolic factors of importance are protein kinase R (PKR) and RNase L, which are involved with the host interferon response and have been demonstrated to be important in signal induction and poxvirus immunity [57, 58].

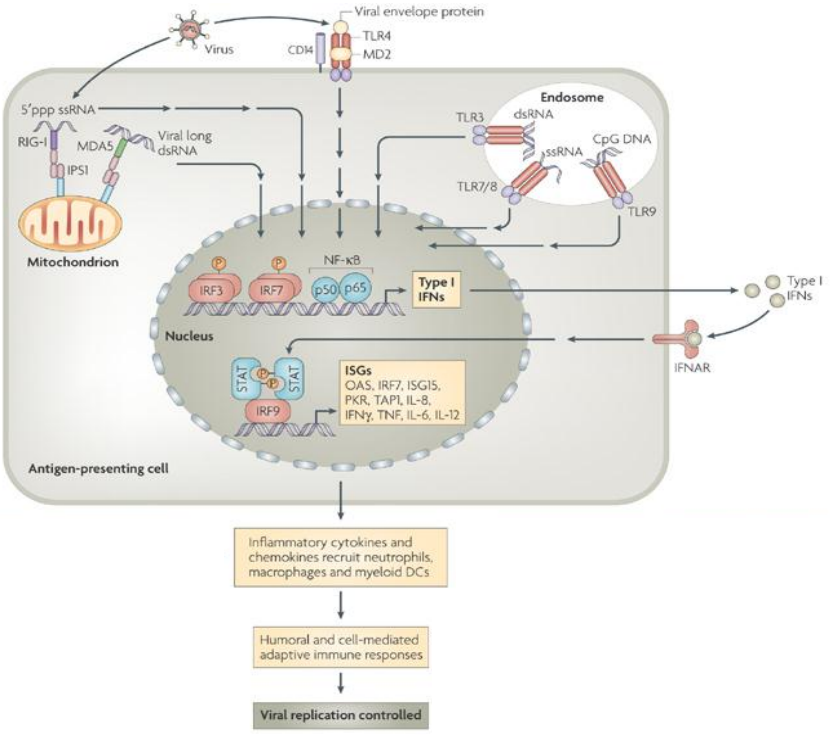

Fig. 2.2: Recognition of viral products by PRR. (Left) Rig-I and MDA5 recognition of cytosolic viral RNA. (Middle) TLR4 recognition of viral surface antigens at the cell membrane. (Right) Recognition of viral antigens in endocytotic compartments by TLR. Recognition of viral antigens by PRR leads to the induction of cell signalling and the production of type I IFN. IFN works in an autocrine and paracrine fashion to induce inflammatory cytokines leading to the recruitment of leukocytes and the induction of the immune response. Adapted from Katze *et al* 2008 [59], with permission from Macmillian Publishers Ltd: Nature Reviews Immunology, copyright 2008.

2.3.3 Type I interferon

IFN is a key mediator of antiviral immune responses. The heterodimeric type I IFN receptor (IFNAR) is ubiquitously expressed and binding of IFN to IFNAR leads to the transcription of hundreds of IFN stimulated genes (ISG). Many of these ISG encode additional PRR for the detection of viral molecules, and transcription factors that serve to amplify IFN production. Others such as RNaseL, PKR and the Mx GTPases interfere with the virus life cycle at various stages, serving to make the host cell inhospitable and unproductive for progeny virus [60]. This induction of an antiviral state is particularly important for cells of the immune system which will be recruited to peripheral infection sites where they will invariably encounter virus. Exposure to IFN makes these cells resistant to viral infection, allowing them to carry out their intended functions, rather than providing more potential hosts for virus production (Fig 2.3) [61].

The effects of IFN are not limited to the induction of ISG; it has recently become clear that IFN has a profound influence over the innate and adaptive immune responses [62-64]. Natural killer (NK) cells are important effectors of the innate immune system, particularly with regard to poxvirus infection [62]. IFN signalling not only mediates the migration of NK cells, it also plays an important role in eliciting their crucial cytotoxic functions [63]. Likewise, in IFNAR deficient mice the recruitment of inflammatory monocytes (IM) is diminished, and although still capable of differentiating into inflammatory dendritic cells (DC), they do not functionally mature [65]. IFN has also been implicated to play a role in the adaptive immune response as it is required for the clonal expansion and memory formation of virus specific $CD8^+$ CTL [66].

The main mechanism of IFN mediated recruitment appears to be by the induction of chemokines (Fig 2.3). Chemokines are chemoattractant cytokines that mediate the migration of leukocytes during the host immune response, and play a particularly important role in inflammation [67]. Based on the arrangements of disulphide bridges between cysteine residues at the N' terminus, chemokines are classified into 4 groups C, C-C, C-X-C, C-X_3-C [68]. Chemokine receptors are differentially expressed on leukocyte subpopulations, which along with the chemokine milieu, determines which populations are recruited to sites of inflammation. IFN induces chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 7 (CCL7) and chemokine (C-C motif) ligand 12 (CCL12) expression in bone marrow macrophages during murine cytomegalovirus (MCMV) infection, which was shown to induce monocyte egress and migration to peripheral infection sites [69]. These recruited cells themselves further enrich the cytokine milieu, which contributes to the development of the local immune response. Herpes simplex virus (HSV) infection of IFNAR deficient mice is associated with abhorrent chemokine production, resulting in defective lymphocyte recruitment and an enhanced sensitivity to viral infection, despite the development of adaptive immune responses in the lymph node [64].

IFN is known to play an important role in poxvirus immunity, as demonstrated by the protection from lethal WR infection afforded by IFN therapy [70], and increased susceptibility of IFNAR deficient mice to VACV infection [44, 47]. However, one baffling observation is that MVA vaccination can still protect IFNAR knockout mice from a lethal infection [44, 47], highlighting that we still have much to learn about the immune response to MVA.

Poxviruses are masters of immune evasion, so given the critical role of IFN in the immune response it is unsurprising that VACV has evolved mechanisms to evade IFN signalling. Viral proteins such as B18R, N1L and E3L are known virulence factors that interfere with IFN signalling [71-73]. Many of these factors were lost during the attenuation of MVA. The result, is that MVA, unlike other VACV strains, induces rapid local immune responses [53] and IFN dependent expansion of virus specific $CD8^+$ CTL [74].

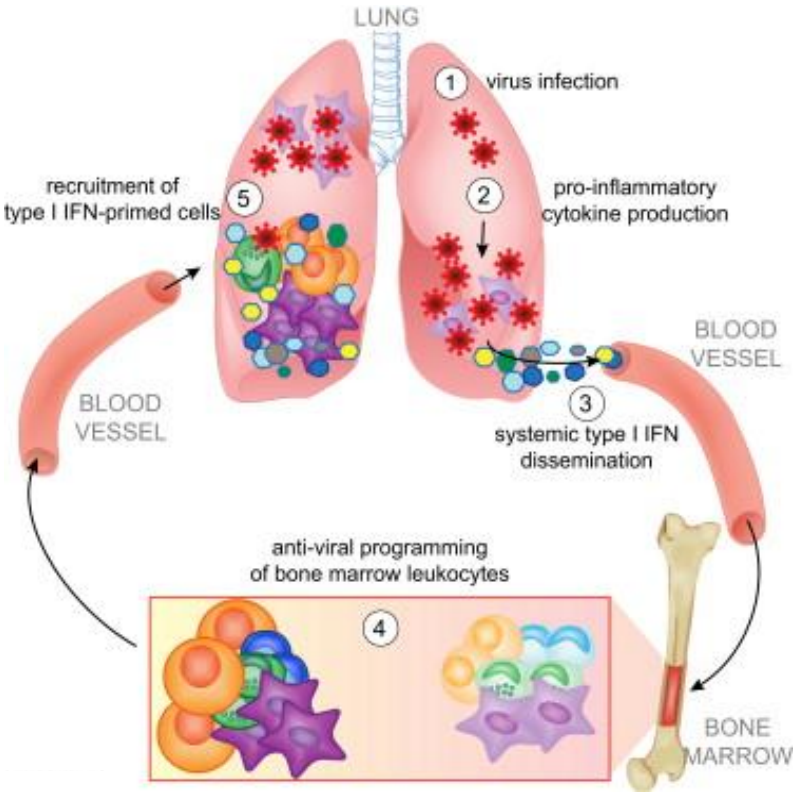

Fig.2.3: IFN primes and mediates the release of leukocytes. (1-2) Virus infection leads to the activation of PRR and the local release of pro-inflammatory cytokines. (3) IFN is transported into the circulation where it reaches the bone marrow. (4) IFN induces an antiviral state, and mediates the production of CCL2 and CCL7 stimulating the release of monocytes into the circulation. (5) Primed monocytes are guided to infected tissue by chemokine gradients and locally released inflammatory mediators. Adapted from Hermesh *et al* 2010 [61] with permission from Elsevier, copyright 2010.

2.3.4 Complement activation

Many PRR are cell associated, triggering cellular signalling and gene expression. However, there are other PRR that initially function independently of host cells, recognising conserved pathogen associated molecular patterns (PAMPs), inducing rapid antimicrobial immune responses. One of the best characterised is the complement system.

Complement is a key constituent of innate immunity that helps to bridge the innate and adaptive immune responses [75]. The complement system comprises over 30 serum proteins and cell surface receptors that elicit rapid inflammatory and cytolytic responses. Activation of complement sets off a proteolytic cascade resulting in the generation of inflammatory mediators, opsonins and membrane penetrating lytic components [76]. The complement cascade is activated by three main pathways: classical, lectin and alternative (Fig 2.4) [76]. All three pathways converge on the activation of the central C3 component, leading to opsonisation of the target, and activation of the terminal C5 component. Proteolytic cleavage of C5 releases C5a, an anaphylatoxin, which is a potent chemoattractant for neutrophils, and C5b which mediates the formation of the lytic membrane attack complex (MAC).

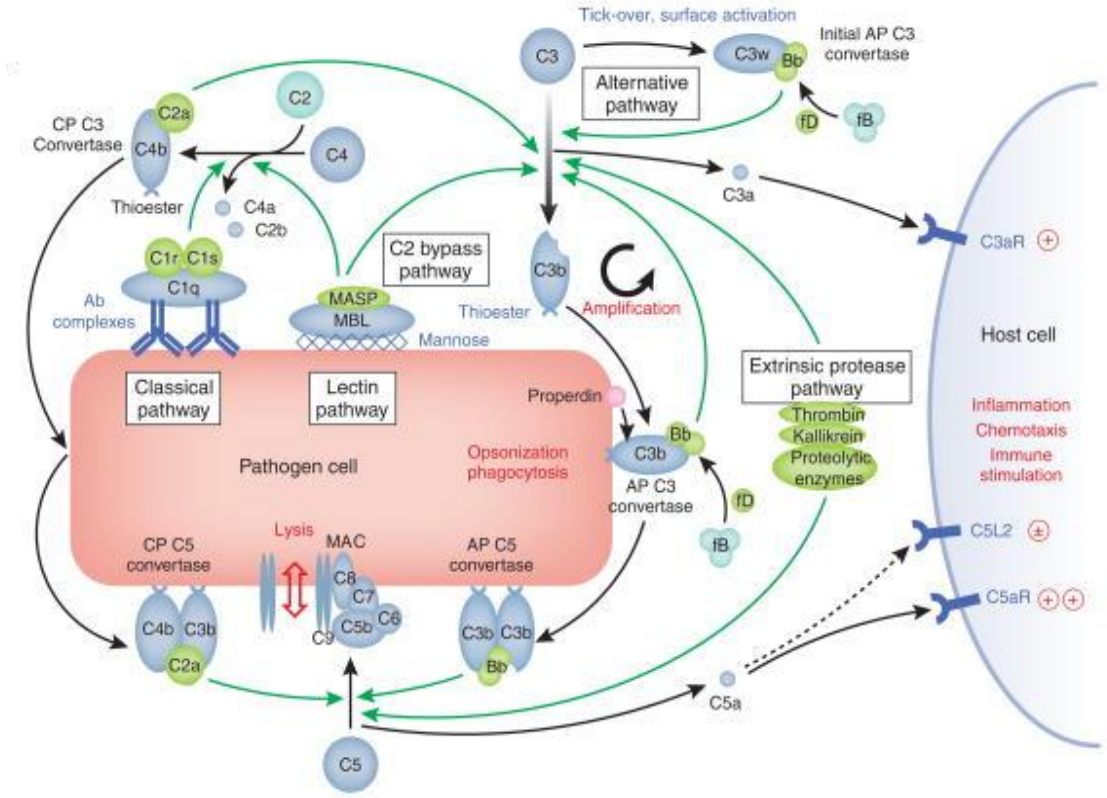

Fig 2.4: Complement activation pathways. See main text for details. Adapted from Ricklin & Lambris 2007 [77], with permission from Macmillian Publishers Ltd: Nature Biotechnology, copyright 2007.

Initiation of the classical pathway requires pathogen bound complement fixing antibodies (IgG1 and IgM), which are bound by the C1 complex resulting in autocatalytic activation of C1. Activated C1 then recruits and cleaves C4 and C2, which associate to form a C3 convertase (C4bC2a). Cleavage of C3 releases the C3a anaphylatoxin, and C3b which forms stable covalent bonds with hydroxyl groups on proximate carbohydrates and proteins. C3b is recognised by complement receptors, so deposition of C3b on the surface of microbes not only leads to further activation of the terminal pathway, but also opsonises the target for phagocytosis. The lectin pathway is analogous to the classical pathway, utilising lectin proteins as PRR rather than antibody complexes. Binding of lectins to PAMPs activates serine proteases which then cleave C2 and C4 to generate the C3 convertase.

The alternative pathway unlike the classical and lectin pathways does not utilise PRR. Instead the alternative pathway is constitutively active with surface expressed complement regulatory proteins preventing activation on host cells. Low level spontaneous hydrolysis of C3 generates C3b which binds promiscuously to a wide range of targets. Bound C3b recruits factors B and D to create the alternative pathway C3 convertase, which cleaves C3 to form a feedback amplification loop.

Viral infection can trigger complement activation on the surface of infected cells and virus particles. Deposition of complement and the formation of the MAC on infected cells can lead to premature lysis of the host cell thus disrupting virus replication. Direct activation of complement on the surface of virons can lyse virus membranes, enhance phagocytosis, and may interfere with receptor interaction, virus entry and uncoating [78]. The second consequence of complement activation is the release of the C3a and C5a anaphylatoxins which induce local pro-inflammatory responses recruiting leukocytes and stimulating the release of lysosomal enzymes and vasoactive amines.

Complement is known to play an important role in poxvirus immunity as a functioning complement system is required to survive mousepox infection [79]. The complement system directly attacks foreign microbes inducing rapid local immune responses, and therefore exerts a strong selective pressure on pathogens. VACV activates both the alternative [80] and classical pathways [81], however two strategies are employed to avoid the consequences of this activation of the complement system. During the budding process, VACV incorporates host complement regulatory factors into the viral membrane [82], which limits complement activation on the viral surface. The second strategy is to release a virally encoded complement control protein (VCP), which is secreted in large amounts, and is

bound back to the surface of the infected cell by viral anchor proteins [83, 84]. VCP binds to and accelerates the decay of C3b and C4b [85] thus interfering with both the classical and alternative pathways of complement activation [81, 86]. As complement plays a role in the induction of both B and T-cell responses during viral infection [87, 88], blocking complement in this way is highly advantageous, as it not only provides direct protection for virons and infected cells, but also modulates downstream immune responses. This has been demonstrated *in vivo* where infection of mice with mutant viruses lacking VCP leads to increased infiltration of $CD8^+$ T-cells and enhanced neutralising antibody titres, resulting in reduced viral titres and reduced pathogenicity [89, 90].

2.4 Neutrophils

2.4.1 Role

Neutrophils are polymorphonuclear (PMN) leukocytes that are classically characterised as professional phagocytes. During infection, neutrophils are often the primary responders recruited in large numbers and thus constitute the first line of defence against invading microorganisms. Neutrophils are highly destructive cells, well equipped to deal with pathogens. Traditionally, their primary role is to contain the initial infection until the adaptive immune response can take over. To execute this primary function, neutrophils employ three main mechanisms of killing, targeting both intracellular and extracellular pathogens (Fig 2.5).

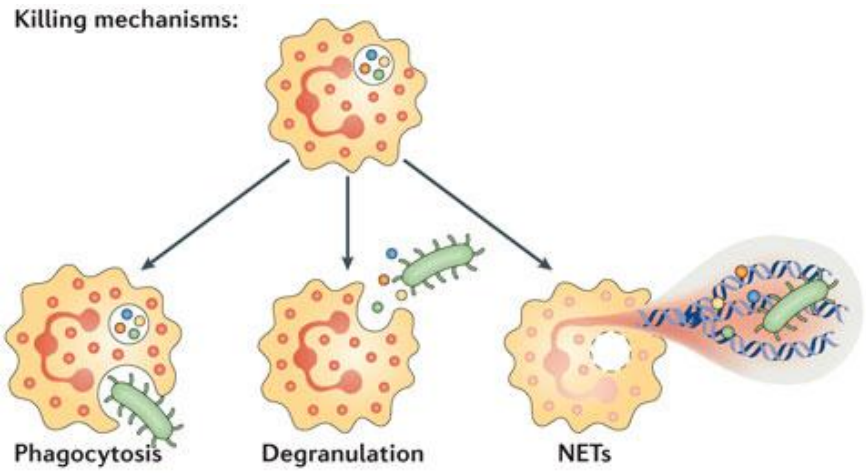

Fig. 2.5 Neutrophil killing mechanisms. (left) Uptake of extracellular pathogens by phagocytosis. (middle) Activation of neutrophils causes degranulation and production of ROS. (Right) NETs are used to ensnare extracellular pathogens which are inactivated by antimicrobial enzymes preventing further spread of microbes. Adapted from Kolaczkowska & Kubes 2013 [91], with permission from Macmillian Publishers Ltd: Nature Reviews Immunology, copyright 2013.

Neutrophils have four types of granules, each containing a repertoire of receptors and inflammatory mediators as well as antimicrobial peptides and enzymes. These granules are released in a strict hierarchical order after activation, creating an inflammatory environment that ultimately damages both pathogen and host [92]. The binding of PAMPs to neutrophil PRR, or of complement opsonised microbes to neutrophil complement receptors, initiates phagocytosis. The phagosome is then fused with granules leading to the destruction of the ingested microbe [93]. A final antimicrobial mechanism of neutrophils is the condensation and release of cellular DNA to form neutrophil extracellular traps (NETs). The release of NETs is generally considered to be an alternative pathway of programmed cell death, termed NETosis. However, recent evidence has shown that NETting neutrophils can continue to carry out other antimicrobial functions [94]. NETs serve to immobilise pathogens and are studded with myeloperoxidase, neutrophil elastase and other

antimicrobial proteins, which inactivate microorganisms, including viruses that become ensnared in the NET [95]. Neutrophil NETs can also be found at peripheral sites in the circulatory system, such as the liver [96]. Thus, NETs are thought to be an important immunological mechanism that prevents the spread of pathogens from the primary site of infection, and filters pathogens out of the circulatory system [97].

2.4.2 Subsets and new functions

Neutrophils were long regarded as a uniform population of short-lived end stage effector cells, with little influence over the progression of the immune response. It was recognised some time ago that neutrophil populations display differences in phagocytosis, protein synthesis, receptor expression and oxygen metabolism [98], however, this received little attention at the time. It is only relatively recently, that the wider scientific community has started to regard neutrophils as a heterogeneous population. There has been somewhat of a renaissance in neutrophil biology, with many new exciting discoveries as people begin to appreciate the previously overlooked roles that neutrophils play in the immune response. The renewed interest in neutrophils has revealed that they display a level of plasticity, and has highlighted the previously unappreciated immunoregulatory role neutrophils play in both arms of the immune response [99].

Many of the previously held conceptions about neutrophils have recently been challenged, with reports of distinct neutrophil subsets [100, 101], and that neutrophils may have a considerably longer half-life than previously reported [102]. Possibly the most significant developments have come from studies investigating the influence of neutrophils in the progression of the immune response. Neutrophils can detect cytosolic DNA inducing the production of IFNβ [103], and it is now apparent that neutrophils synthesise a vast array of

other immune mediators including pro and anti-inflammatory cytokines [99]. Many of the pro-inflammatory mediators that neutrophils produce serve to recruit other neutrophils. Though these mediators are not produced to the same levels as seen with other master coordinator cell types, during the early stages of the inflammatory response neutrophils make up the majority of the cells that are recruited and therefore sheer numbers presumably compensate for this. In addition to perpetuating their own recruitment neutrophils produce chemotactic factors for monocytes, which then eventually limit the further recruitment of neutrophils thus contributing to the maturation of the immune response [99].

Contrary to previously established neutrophil dogma, several studies have reported that tissue emigrated neutrophils can re-enter the circulation [104, 105]. These functionally primed “veteran” neutrophils display distinct patterns of surface receptor expression that differs from naïve resting and classically activated neutrophils [106] and may contribute to pathology *via* induction of systemic inflammation [107]. During infection, reverse transmigrated neutrophils emigrate to the lymph nodes following antigen capture [108], and have been shown to cross prime naive $CD8^+$ T-cells [109]. One of the most striking advances is the essential co-operation of neutrophils in the development of NK cells. Neutrophils are essential for NK cell maturation [110] and have been shown to modulate all major NK cell functions including cytotoxic activity and IFNγ production [111].

Previously the importance of neutrophils has mostly only been appreciated for extracellular pathogens. Though many viral infections induce neutrophil recruitment, until relatively recently their role has gone largely unaddressed and it has often been assumed that neutrophils are a major contributor to pathology and thus may in fact be detrimental.

The development of antibodies against Ly6G, which is thought to be exclusively expressed on neutrophils, has provided a useful tool to specifically deplete neutrophils, which has allowed scientists to start addressing the contribution of neutrophils to antiviral immunity. Depletion of neutrophils has been shown to exacerbate viral diseases including influenza [112] and respiratory syncytial virus infection [113]. However, their contribution is not always straight forward, as early on neutrophils may serve as a viral reservoir, whilst also playing a role in virus clearance at later stages, as has been observed during infection of mice with West Nile virus [114].

2.4.3 Trafficking

The essential role that neutrophils play in the immune response is counterbalanced with the need to control their destructive capacities, which are also potentially damaging to the host. The primary mechanism is to control the recruitment and activation of neutrophils with signals that guide them directly to where they are needed. Neutrophils respond to a diverse range of chemoattractants, including chemokines, peptides and lipid mediators, any combination of which are often present at sites of inflammation. Previously, it was considered that these different chemoattractants served overlapping, redundant functions. However, recent studies suggest that *in vivo* these signals work in overlapping, non-redundant cascades [115, 116].

Under physiological conditions neutrophils are retained in the bone marrow *via* locally produced signals acting on chemokine (C-X-C motif) receptor 4 (CXCR4) [115]. During inflammation, granulocyte colony-stimulating factor (G-CSF) alters the balance of signals in the bone marrow favouring production of chemokine (C-X-C motif) receptor 2 (CXCR2) ligands. The increased local production likely works in conjunction with circulating CXCR2

ligands derived from the primary site of inflammation, to facilitate the rapid release of neutrophils into the bloodstream *via* desensitisation and down regulation of CXCR4 [117].

Once in the circulation, neutrophils must then be guided to the site of inflammation. This requires arrest on the endothelium, allowing transmigration into the affected tissue. The release of pro-inflammatory mediators, especially chemokines and lipid mediators, from local sentinel cells, serves a dual purpose [118]. The first of which is to prepare the local endothelium by upregulating adhesion molecules. The second is to provide a graduated signal, through chemokines immobilised on glycosaminoglycans [119], which guides neutrophils towards the site of inflammation, eventually reaching a critical concentration inducing arrest and extravasation through a complex process involving a cascade of adhesion molecules [120]. Interaction with these immobilised chemokines activates neutrophils leading to altered expression of adhesion molecules and cytokine receptors, thus fine tuning their effector functions [121, 122]. Expression of proteases, such as matrix metalloproteinase 9 (MMP9), is thought to be a crucial mechanism by which neutrophils penetrate the basement membrane gaining access to tissue during infection [123]. Inside the tissue, activated neutrophils carry out their antimicrobial functions and perpetuate the further recruitment of neutrophils *via* the release of inflammatory mediators, including chemokine (C-X-C motif) ligand 2 (CXCL2) and chemokine (C-C motif) ligand 3 (CCL3) [115].

2.4.4 Roles in poxvirus infection

Neutrophils are an important part of the immune response, as *Cxcr2*$^{-/-}$ mice, which are unable to recruit neutrophils, have impaired innate and adaptive immune responses to parasites [124] and show increased susceptibility to viral infection [125]. Neutrophils make up a large proportion of the cells recruited to the infection site during VACV infection, however very little is known about the role that they play.

Incubation of neutrophils with VACV induces activation and functional priming of neutrophils [126], which have been shown to take up and destroy VACV [127]. The inactivation of VACV by neutrophils appears to require antibody recognition, and is dependent on reactive oxygen species (ROS) [128]. Based on these studies it was postulated that neutrophils may play a role in poxvirus immunity, this has been confirmed by exciting new *in vivo* studies that have implicated important roles for neutrophils in virus clearance, T-cell induction and in the regulation of the inflammatory response to poxvirus infection.

In one study, systemic poxvirus infection induced the recruitment of neutrophils to the liver microvasculature. Adherent neutrophils interacted with platelets forming large aggregates inducing the release of NETs, which filtered out circulating virus protecting host cells from infection [129]. In a second study, it was shown that after intradermal vaccination with MVA, neutrophils rapidly transport antigen from the dermis to the bone marrow in a CCR1 dependent manner. This antigen transport mechanism led to the induction of a functionally distinct lineage of virus specific $CD8^+$ memory T-cells. The induction of the bone marrow $CD8^+$ T-cell response was dependent on myeloid antigen presenting cells (APC), and as the migrating neutrophils expressed annexin V, the apoptotic “eat me” signal, it seems the migrating neutrophils were ingested by myeloid APC upon arrival [130]. Finally a ground

breaking but slightly perplexing study, showed the infiltration of ROS and IFNβ producing $CD11b^{+}Ly6G^{+}$ cells during the later stages of VACV infection (>5 days), a time point that is not normally associated with neutrophil infiltration. In addition to the neutrophil marker Ly6G, these cells were mononuclear and also expressed monocytic markers. However, origin studies demonstrated that these cells were not derived from traditional monocytic lineages. In terms of functionality, these cells played only a minor role in limiting viral spread. Instead, they appeared to have a regulatory function *via* induction of ROS, as depletion of these cells led to enhanced tissue pathology [131].

2.5 Monocytes

2.5.1 Subsets and functions

Monocytes are myeloid cells, derived from common progenitor cells shared with granulocytes [132]. A complex signalling network controls their release into the blood, where they circulate as non-dividing cells [133]. Monocytes are themselves precursor cells and as such are not yet terminally differentiated. It is thought that in steady state conditions, circulating monocytes may act as a reservoir migrating into tissues where they differentiate to replenish populations of long-lived resident macrophages and dendritic cells [134]. During inflammation, large numbers of monocytes are mobilised from reservoirs in the bone marrow and spleen. Locally produced chemotactic signals released from the site of inflammation recruit monocytes from the circulation into tissues where they differentiate into macrophages and dendritic cells (Fig 2.6) [135].

In most mammals circulating monocytes in the blood are a heterogeneous population, whether these represent distinct lineages or different states of maturation is still a matter of debate. Murine monocytes are broadly classified into 2 subsets, “inflammatory“ or

"resident", based on the expression of cell surface markers CD11b, chemokine (C-X_3-C-motif) receptor 1 (CX_3CR1), chemokine (C-C motif) receptor 2 (CCR2) and Ly6C. Inflammatory monocytes (IM) are $CD11b^+$ $CCR2^+$ CX_3CR1^{low} $Ly6C^{high}$, and resident monocytes are $CD11b^+$ $CCR2^-$ CX_3CR1^{high} $Ly6C^{low}$. Human monocytes also fall into 2 major categories based on the expression of CD14 and CD16. The so called "classical" monocytes, which are an approximate counterpart to murine IM, are defined as $CD14^{high}$ $CD16^-$. Non-classical monocytes are $CD14^+$ $CD16^+$, and are similar to murine resident monocytes. Despite some physiological differences, human and murine subsets are thought to be similar in terms of their differentiation and contribution to immune defence [136].

2.5.2 Trafficking

IM make up some 2-5% of white blood cells in steady state conditions, and are rapidly recruited to peripheral sites during the inflammatory response [137]. At sites of infection IM differentiate into macrophages and dendritic cells. Both of these cell types are professional phagocytes that present antigen to cells of the adaptive immune response, however both fulfil distinct roles. Macrophages are primarily charged with clearing debris, killing invading microbes, and regulating local immune responses [138]. Dendritic cells are arbitrators of the immune response, producing large amounts of immune mediators, capturing antigen and migrating to peripheral immune sites where they are potent activators of the adaptive immune response [139].

The migration of IM is mediated in part by CCR2 and its ligands CCL2 and CCL7 [140]. Infection with MVA, but not other VACV strains, leads to the production of large amounts of CCL2 that recruits monocytes into the lung after intranasal infection [141]. At the primary site of inflammation, CCL2 is transported across endothelial cells *via* Duffy antigen receptor

for chemokines (DARC) and so reaches high levels in the circulation. In one proposed model for CCL2 mediated trafficking, CCL2 dimerises and binds to glycosaminoglycans establishing a gradient which directs monocytes to the site of inflammation [142, 143]. Additionally, the systemic release of IFN during viral infection drives expression of CCL2 in bone marrow cells, promoting monocyte egress into the bloodstream [69]

Other receptors, namely CCR1 and chemokine (C-C motif) receptor 5 (CCR5), which are expressed on monocytes [144], have been shown to play a role in monocyte recruitment [145, 146]. However, ascertaining the role of CCR1 and CCR5 has been more complicated as CCR1 and CCR5 are widely expressed on a variety of cell subpopulations [147, 148] and share some important ligands including CCL3 and chemokine (C-C motif) ligand 5 (CCL5) [149], so it is not always clear if observed effects are directly related.

The role of resident monocytes is less clear and has only recently been proposed. Intravital microscopy studies showed that resident monocytes appear to patrol tissues by attaching to and migrating along blood vessels [150] entering non-inflamed tissue [151]. These patrolling monocytes can also sense foreign antigens with PRR, triggering rapid recruitment into tissues where they differentiate into macrophages and elaborate inflammatory cytokines including TNFα, IL-1β and CCL3 [152]. Patrolling of resident monocytes appears to be at least partially mediated by CX_3CR1, as patrolling is diminished in the absence of this receptor [150]. During bacterial infection the recruitment of resident monocytes to splenic sites of infection is mediated by chemokine (C-X_3-C motif) ligand 1 (CX_3CL1), which also provides an additional survival signal [153]. Thus unlike IM, resident monocytes appear to be recruited before neutrophils and may be an important primary responder.

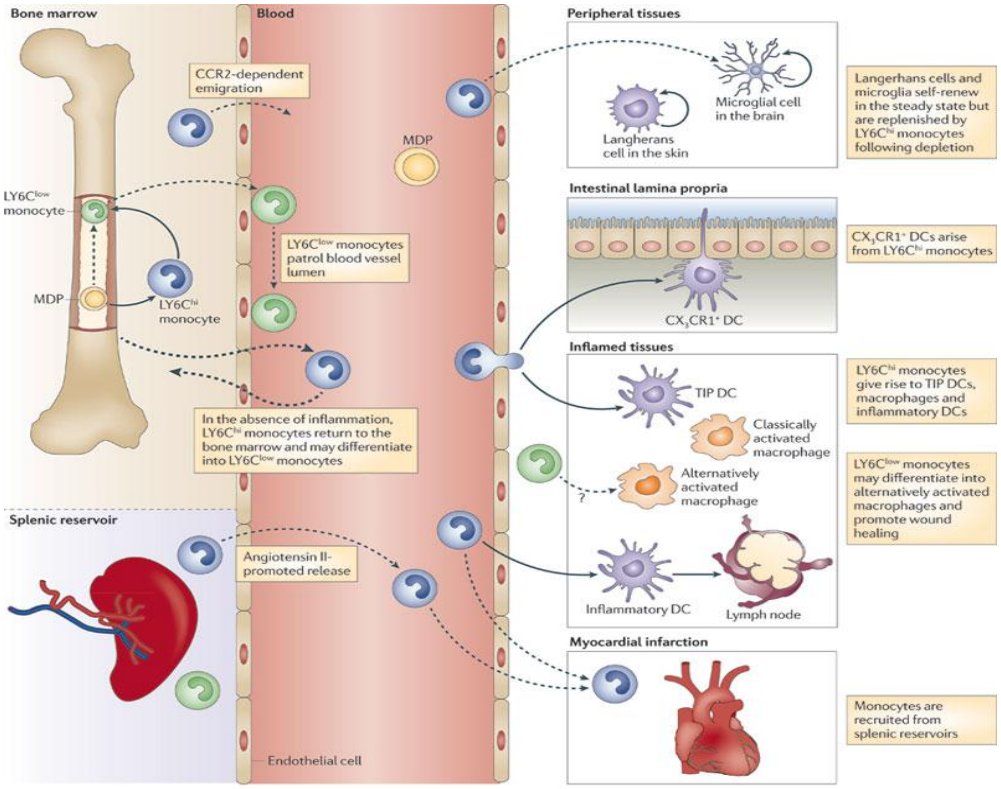

Fig 2.6: Monocyte subsets and functions. Ly6C^{hi} monocytes are derived from macrophage and DC precursor cells in the bone marrow. Exit and recruitment of Ly6C^{hi} monocytes is mediated by CCR2, and once inside tissues they differentiate into inflammatory macrophages or DC. Some Ly6C^{hi} monocytes may return to the bone marrow where it is thought they could differentiate into ly6C^{low} monocytes. Ly6C^{low} monocytes "patrol" blood vessels and are rapidly recruited to sites of tissue damage where they differentiate into macrophages. In addition to the bone marrow, the spleen acts as an additional monocyte reservoir. Adapted from Shi & Pamer 2011 [140], with permission from Macmillian Publishers Ltd: Nature Reviews Immunology, copyright 2011.

2.5.3 Role in poxvirus infection

Monocytes, and their terminally differentiated counterparts, have been studied quite extensively and have been shown to play an important role in poxvirus immunity. *In vitro* infectivity assays demonstrated that VACV preferentially infects monocytic cells [154]. The productivity of this infection appears to be related to the activation state of the cell, as VACV replicates in macrophages from naïve but not immune animals [155]. This is mirrored *in vivo*, where macrophages in rabbits are initially amenable, however as the immune response develops permissiveness is lost, with activation occurring faster after secondary challenge [156]. Virus specific antibodies may be partially responsible for this effect [157], however abortive replication in macrophages can also be induced by treatment with interferon-γ (IFNγ) [158]. Infected macrophages readily undergo apoptosis [159] and in activated macrophages VACV replication appears to be blocked at later stages [160, 161], indicating direct roles for cell mediated effects.

Monocytic cells appear to have an important role in limiting virus replication and dissemination. Macrophages collected late after VACV infection have been shown to have selective cytotoxicity towards virus infected cells [162]. In co-culture experiments, alveolar macrophages limit the spread of VACV, and depletion of these cells *in vivo* increases the severity of disease, due to increased virus replication and dissemination. Interestingly, depletion of alveolar macrophages does not appear to affect chemokine expression, but none the less, leads to increased numbers of neutrophils, natural killer cells and dendritic cells at the site of infection, indicating that alveolar macrophages may play a role in limiting the local immune response [163].

It has been proposed that IM are the most important source of IFN production during VACV infection [50]. This conclusion was reached using a CD11b conditional depletion model, however as we [53] and others have pointed out [51] CD11b is widely expressed on a number of immunologically important cell subsets including dendritic cells, and therefore is not a reliable model to test inflammatory macrophage function.

Besides the direct role played by monocytic cells in controlling poxvirus infection, there is evidence that these cells may also play a critical role in the development and execution of the adaptive immune response by augmenting the activity of $CD8^+$ CTL's [164]. Indeed, in ectromelia disease models, depletion of macrophages has dire consequences on survival, resulting in 100% mortality which is associated with an impaired antiviral $CD8^+$ CTL response [165]. Therefore monocytic cells are key players in both arms of the immune response, and ultimately in the induction of poxvirus immunity.

3 Objectives

Unlike other VACV strains, MVA induces rapid cytokine release and early migration of leukocytes. This inability to avoid the innate immune response may play a key role in the superior early protection provided by MVA vaccination in disease models, and is therefore of great interest. Additionally, exploring the mechanisms underlying this immune response may provide some insight into dominant virus host interactions.

These studies examine the signals that lead to the early recruitment of leukocytes during MVA infection. Previous work has shown that recruitment of monocytes and lymphocytes during MVA infection is dependent on CCL2 [141]. However, deficiency of CCL2 does not affect the recruitment of neutrophils, which make up a large proportion of the cells that are recruited during the early stages. MVA infection activates complement and induces secretion of ligands for CCR1, both of which have been shown to play important roles in the recruitment of neutrophils [166-168] and monocytes [169, 170]. *In vitro* migration assays, and *in vivo* infection models were used to examine the impact of CCR1 and complement activation on the early MVA induced migration of monocytes and neutrophils.

4 Methods

4.1 Virus and Cell lines

All VACV strains (MVA, WR and Wyeth) were propagated in chicken embryo fibroblasts (CEF) and purified by sucrose density centrifugation using standard methodology [171]. Titres of concentrated virus stocks were determined by standard plaque assay in CEF and corroborated by tissue culture infectious dose 50 (TCID50) assay performed in 96 well plates using BHK and DF1 cells. Infectious dose 50 was calculated using Spearman Karber methodology, and infectious units per ml estimated by applying Poisson distribution.

MH-S murine alveolar macrophages and human monocytic THP-1 cells were maintained in RPMI 1640 (Sigma) + 10% v/v foetal calf serum (FCS, Biochrom). Murine NIH3T3 fibroblasts and MLE-12 lung epithelial cells were maintained in DMEM (Biochrom) + 10% v/v FCS (Biochrom).

Murine MPRO cells were maintained in DMEM (Sigma) + 10% v/v FCS (Biochrom) + 10% v/v granulocyte macrophage colony stimulating factor (GM-CSF) conditioned medium, at a density < 1×10^6 cells/ml. To prepare GM-CSF conditioned medium, GM-CSF expressing X6310 cells were grown in DMEM (Sigma) + 10% v/v FCS (Biochrom) until confluent (>3 days of growth), cells were removed from the medium by centrifugation at 400 × g for 5 min then the culture supernatant was filtered through a 0.2 µm filter (BD Biosciences), aliquoted and frozen until use. MPRO cells were differentiated into neutrophils (nMPRO) by addition of 10 µM all-*trans* retinoic acid (ATRA, dissolved in ethanol) to the growth medium, and incubated for 72 h.

4.2 Harvesting of supernatants

NIH3T3, MLE-12, and MH-S cells: 1×10^6 cells were seeded into 6 well plates in appropriate medium and incubated overnight. Medium was removed and cells were infected at the indicated multiplicity of infection (MOI) in medium containing 0.5% v/v FCS. At the indicated time points cell culture supernatant was removed and centrifuged for 5 min at 450 × g then at 4000 × g, the supernatants were then treated with 800 mJ of UV light (Stratalinker 1800, Stratagene) to inactivate residual virus, aliquoted and frozen at -80 °C until use.

THP-1 cells: 2×10^6 cells were added to 6 well plates in RPMI 1640 + 2% v/v FCS and infected at the indicated MOI. After 6 h supernatants were harvested by centrifugation at 400 × g, treated with 800 mJ of UV light to inactivate residual virus then frozen at -80 °C until use.

4.3 Chemotaxis assay

nMPRO cells: MPRO cells were differentiated as described above, and resuspended at a density of 1×10^6 cells/ml in DMEM + 0.5% v/v FCS, where indicated 10 nM of CCR1 or CXCR2 antagonists, J113863 and SB265610 (Tocris), or equivalent amount of ethanol solvent was added. Test culture supernatant (150 µl) was placed in the bottom well of a 96 well Multi-Screen-MIC plate equipped with hydrophilic polycarbonate filters (5 µm pore size; Millipore Corp), and 75 µl of nMPRO cell suspension was added to the top. Cells were left to migrate at 37 °C for 2 h, before quantification on a FACScan (BD).

THP-1 cells: Cells were resuspended to a density of 1×10^6 cells/ml in RPMI 1640 + 0.5% v/v FCS, the procedure was carried out as above using a Multi-Screen-MIC plate with a 8µm filter, migrating cells were quantified on a MACSQuant VYB (Miltenyi). Where indicated 5

nM of CCR1 or CXCR2 antagonists, J113863 and SB265610 (Tocris), or equivalent amount of ethanol solvent was added.

4.4 Mice

C57BL/6 mice and FVB mice were purchased from Charles River. *C3*$^{-/-}$ mice were kindly provided by Dr. Stoiber (Division of Virology, Innsbruck Medical University, Innsbruck, Austria) and *Ccr1*$^{-/-}$ mice were provided by Dr. Luckow (Klinikum der Universität München, Medizinische Klinik und Poliklinik IV, Arbeitsgruppe Klinische Biochemie, München, Deutschland). All mice were maintained under specific pathogen-free conditions. Mice were housed in a temperature and light controlled room (21 – 23 °C, 55 ± 3% relative humidity, 12 h light : 12 h dark cycle) and were fed a standard rodent diet with sterilised water ad libitum. All experiments were licensed under the Regierung von Oberbayern and planned using resource equation methodology or power analysis (when standard deviation could be estimated) so as to limit the number of animals used.

4.5 Infection

For infection studies mice were anesthetised with a weight adjusted dose of ketamine/xylazine, (100 mg/Kg and 10 mg/Kg of body weight respectively) administered by intraperitoneal injection and infected intranasally with 1×10^7 PFU of MVA diluted in endotoxin free PBS (Biochrom) to a total volume of 30 µl, control mice were dosed with the equivalent volume of endotoxin free PBS. After 48 h mice were euthanized by overdose of ketamine/xylazine, and bronchoalveolar lavage (BAL) was performed using RPMI 1640 supplemented with 10% v/v FCS and 10 mM EDTA (Sigma). The first wash was retained in a separate 1.5 ml tube, and the 3 subsequent washes were combined into 15 ml tubes. Samples were centrifuged at 400 × g, cells were combined into V-bottomed 96 well plate

(Corning), and supernatant from the first wash was aliquoted and frozen at -80 °C until use. For C5 ELISA and Western blot, BAL fluid was collected 16 h after infection.

4.6 Flow cytometry

Analysis of CCR1 expression on THP-1 cells: THP-1 cells were incubated with non-specific human IgG (0.1 µg/ml) and live/dead fixable red dye (Invitrogen) in PBS 1% v/v FCS for 30 min on ice. Cells were washed three times with PBS 1% v/v FCS then cells were fixed and permeabilised using a Cytofix/Cytoperm kit (BD biosciences) according to the manufacturer's instructions. Cells were then incubated with Alexa fluor-405 conjugated anti-CCR1 (Santa Cruz Biotechnology) for 30 min, washed 3 times then analysed on a MACSQuant VYB (Miltenyi).

Analysis of cells collected from BAL: Cells were distributed on a 96 well pate (Corning Life Sciences) and non-specific binding was blocked by incubation of cells with anti-CD14/16 in PBS + 1% v/v FCS + 0.01% w/v sodium azide (FACS buffer), for 30 min on ice. Antibody cocktail containing anti-murine CD11b –FITC, Ly6G-APC, Ly6C-APC-Cy7, CD3-PE and CD8a-PE-Cy7 or CD4-PE-Cy7 (all from Biolegend) was added and incubated for a further 30 min on ice. Cells were washed 3 times in FACS buffer, and then transferred into tubes for analysis on a FACS Calibur II (BD biosciences). Alternatively, cells were stained with anti-Ly6C-Alexa Fluor 488, Ly6G-APC (Biolegend) and CD11b-VioGreen (Miltenyi) and analysed on a MACSQuant VYB (Miltenyi).

Analysis was performed using FlowJo software (Tristar); cells were gated off the forward/side scatter and dead cells were excluded using 7-Aminoactinomycin D (BD Biosciences) or live/dead fixable violet stain (Invitrogen).

4.7 ELISA

ELISA for C5a in BAL fluid was performed using a murine C5a ELISA Duo set from R&D Systems. The assay was carried out according to the manufacturer's instructions. Capture antibody was diluted to 4 µg/ml in PBS, 100 µl was added to each well of a 96 well plate (Nunc Maxisorp) sealed and incubated overnight at room temperature. The plate was then washed 3× with excess PBS containing 0.01% Tween20 (wash buffer, Sigma) and blotted dry on absorbent towel. Non-specific binding was blocked by adding 200 µl of PBS + 1 % BSA (Sigma) and incubated at room temperature for 1 h. A two fold serial dilution of recombinant C5a (1000 pg/ml – 15.625 pg/ml) in PBS + 1% BSA was prepared and was added to the plate along with BAL samples (100 µl each) and incubated for 2 h at room temperature. The plate was washed 3× prior to addition of biotinylated secondary detection antibody (200 ng). After 1 h incubation at room temperature, the plate was washed 3× and 100 µl of Streptavidin-HRP (1 µg/ml) diluted in PBS + 1% BSA was added to each well then incubated for 20 min at room temperature. The plate was washed 4x then developed by addition of 100 µl of 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma). The reaction was stopped by addition of 100 µl of 1 M sulphuric acid and optical density measured at 450 nm on a Tecan Sunrise microplate reader. Concentrations of C5a in BAL samples were determined by interpolation from the standard curve using non-linear regression.

4.8 Western blot

For western blot of C5, 4 µl of 4× laemmli sample buffer (Bio-Rad), containing β-mercaptoethanol (100 µl/ml) was added to 12 µl of BAL fluid and held on ice. 15 µl of each sample was run on a 4 - 20% Criterion TGX stain free gel (Bio-Rad), which allows rapid

fluorescent detection of total proteins before and after protein transfer on the basis of incorporated trihalo compounds. For determination of size, 5 µl of colour plus P7711S prestained protein ladder (New England Biolabs) was used as a marker and the gel was run for 40 min at 120 V in the provided Tris-Glycine buffer (Bio-Rad). Protein was transferred onto a 0.2 µm nitrocellulose membrane using Bio-Rad Trans Blot Turbo system, and protein transfer was verified using the Bio-Rad stain free UV activation protocol on the ChemiDoc MP (Bio-Rad). Staining was carried out using the ECL Advance western blotting kit (GE Healthcare) according to the manufacturer's instructions. Non-specific binding was blocked by incubation in the provided block solution, 2% w/v dissolved in Tris buffered saline containing 0.1% Tween20 (TBS, see appendix for composition) for 1 h, before staining with 0.2 µg/ml of rat, anti-mouse C5a (taken from ELISA kit, R&D systems) in blocking solution for 1 h. Membrane was washed 6 times with TBS, then incubated for 1 h with 8 ng/ml of horseradish peroxidise conjugated anti-rat IgG antibody (Biolegend) in block solution. Membrane was washed 6 times with TBS then incubated in the substrate solutions for 5 min. Positive signals were visualised on a ChemiDoc Imager (Bio-Rad).

4.9 Statistical analysis

All data was assembled using Prism 5 (Graphpad Software), significance was determined by non-parametric Mann-Whitney U test, or two-way ANOVA using Bonferroni post-hoc test, with P value of ≤ 0.05 deemed to be statistically significant.

5 Results

5.1 CCR1

5.1.1 Chemotaxis of differentiated MPRO neutrophils towards supernatants from MVA infected cells is mediated by CXCR2 not CCR1

CCR1 has been shown to play an important role in the recruitment of neutrophils in disease various models [172, 173]. Neutrophils express CCR1 and respond to CCR1 ligands [121, 174] however, due to the expression of CCR1 on different leukocyte subsets, determining whether the effects observed *in vivo* are direct or indirect can be difficult. Therefore, an *in vitro* chemotaxis assay was used to access the role of CCR1 in the recruitment of neutrophils during MVA infection. The mouse fibroblast cell line NIH3T3 was infected with MVA at different multiplicities of infection (MOI) and compared to WR for the ability of cell culture supernatants to induce the chemotaxis of promyelocytic MPRO cells differentiated into neutrophils (nMPRO). Cell culture supernatants from MVA but not WR infected cells induced chemotaxis of nMPRO cells, and this induction was dependent on the MOI used (Fig. 5.1a). This MVA specific induction of nMPRO chemotaxis was also observed from supernatants of other murine cell lines, namely MLE-12 lung epithelial cells and MH-S alveolar macrophages, which did not induce chemotaxis of nMPRO cells when infected with VACV Wyeth (Fig. 5.1b & c). As the strongest induction was seen by supernatants from MVA infected MH-S cells, these cells were selected for further testing.

The contributions of two chemokine receptors CCR1 and CXCR2 were assessed by the addition of non-peptide receptor antagonists J113863 and SB265610 (CCR1i and CXCR2i respectively) to nMPRO cells. This assay revealed that induction of nMPRO chemotaxis by

supernatants from MVA infected cells was mediated by CXCR2, whereas inhibition of CCR1 had no effect on the level of chemotaxis (Fig. 5.1d).

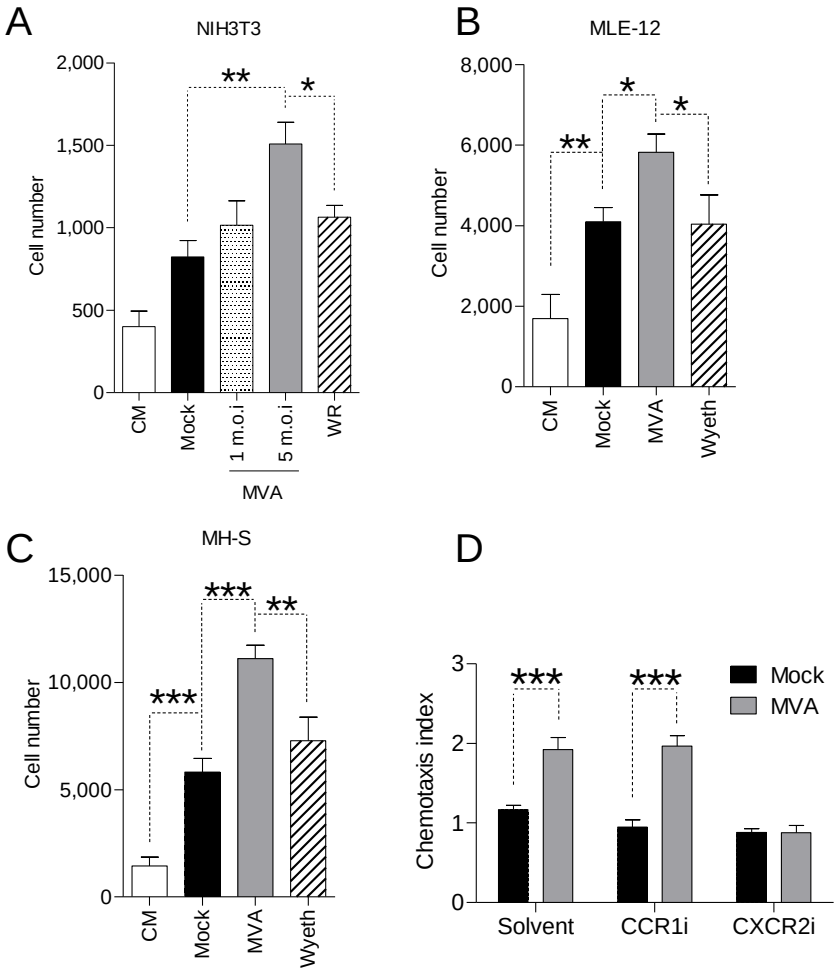

Fig. 5.1: MVA infected cells produce a chemotactic factor for MPRO cells differentiated into neutrophils (nMPRO) which acts on CXCR2 and not CCR1. (**A**) Number of nMPRO cells that migrated towards cell culture supernatants of mock, MVA or VACV WR (5 MOI, 16 h) infected NIH3T3 cells or cell culture medium (CM). (**B**) MLE-12 cells were infected with MVA or VACV Wyeth at MOI of 5 for 16 h, (**C**) MH-S cells for 8 h at a MOI of 1, and supernatants used for chemotaxis assay of nMPRO cells. (**D**) Chemotaxis of nMPRO cells towards supernatants of MH-S cells infected with MVA for 8 h at a MOI of 1. Where indicated, nMPRO cells were treated with 10 nM of CCR1 inhibitor J113863 (CCR1i) or CXCR2 inhibitor SB265610 (CXCR2i) or the equivalent amount of solvent. The chemotaxis index was calculated as the ratio of the number of cells that migrated in response to cell culture supernatants from MVA infected cells, to cells that migrated towards cell culture supernatants of mock treated cells. Data are means ± SEM, ($n \geq 4$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Mann-Whitney-U test (a - c); 2-way ANOVA with Bonferroni post-hoc test (d). Results are from at least 2 independent experiments.

5.1.2 Chemotaxis of THP-1 cells is mediated by CCR1

Previously it was shown that cell culture supernatants from MVA infected human monocytic THP-1 cells induce chemotaxis of non-infected THP-1 cells by production of CCL2 [141]. Extravasation is a complex process, involving multiple chemokine receptors fulfilling non-redundant overlapping functions, with CCR1 playing a critical role in arrest of monocytes [169]. Analysis of CCR1 expression showed that THP-1 cells express CCR1 (Fig. 5.2a). Subsequent tests of THP-1 migration towards FCS, which contains CCR1 ligands [175, 176], showed that CCR1 plays an important role in the migration of THP-1 cells (Fig. 5.2b). Having confirmed the expression and functionality of CCR1 on THP-1 cells, the role of CCR1 in the chemotaxis of THP-1 cells towards cell culture supernatants from MVA infected cells was assessed. This clearly demonstrated that CCR1 plays a critical role in the chemotaxis of THP-1 cells, as addition of the CCR1 antagonist not only blocked chemotaxis towards supernatants of MVA infected cells, but also reduced the level of migration towards supernatants from the mock infected control group (Fig. 5.2c). Additionally the induction of THP-1 chemotaxis was shown to be specific to MVA, as supernatants from WR infected cells failed to induce chemotaxis of THP-1 cells above the levels seen in supernatants from mock infected cells (Fig. 5.2d) which is in line with previously published *in vivo* data [141].

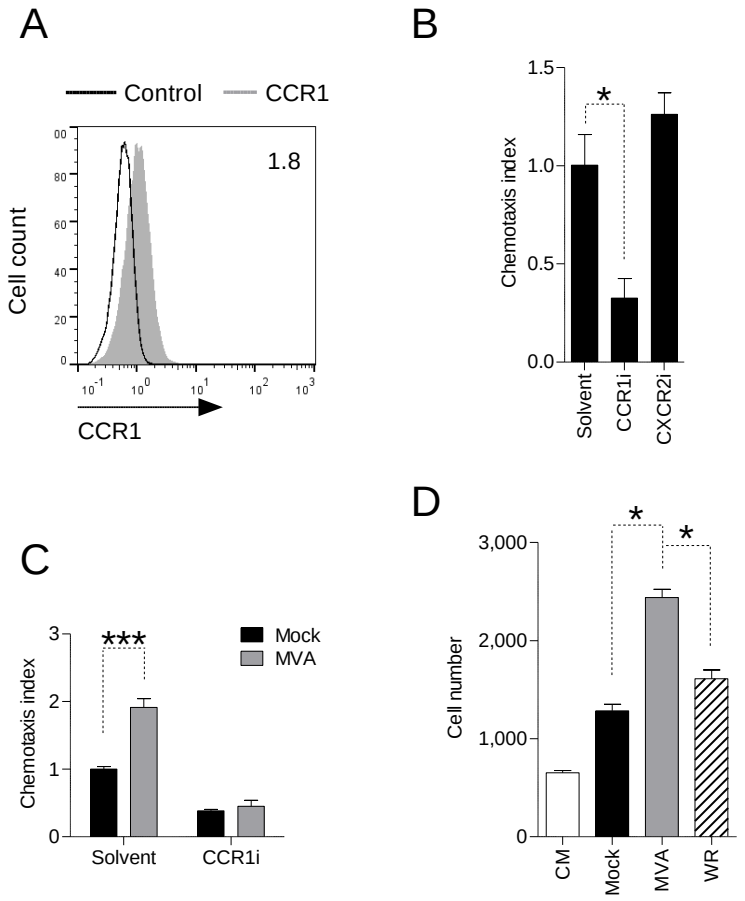

Fig. 5.2. Chemotaxis of THP-1 cells towards supernatants of MVA infected THP-1 cells is dependent on CCR1. (**A**) Flow cytometric staining of THP-1 cells with an antibody directed against CCR1. The ratio of the mean fluorescence intensities of THP-1 cells stained with anti-CCR1 antibody to the negative control is indicated in the upper right-hand corner of the histogram. (**B**) Chemotaxis index; the ratio of the number of THP-1 cells treated with 5 nM of J113863 (CCR1i) or SB265610 (CXCR2i) that migrated in response to 5% FCS, to migrating solvent treated THP-1 cells. (**C**) Chemotaxis of THP-1 cells towards supernatant of THP-1 cells infected with MVA for 6 h at a MOI of 4. Where indicated, THP-1 cells were treated with 5 nM of CCR1 inhibitor J113863 (CCR1i) or the equivalent amount of solvent. The chemotaxis index was calculated as described before. (**B**) Chemotaxis of THP-1 cells towards supernatants from mock, MVA or VACV WR infected (6 h, 4 MOI) THP-1 cells, or cell culture medium (CM). All data are means ± SEM (n = 4). *, $P < 0.05$; ***, $P < 0.001$; Mann-Whitney-U test (b) and (d); 2-way ANOVA with Bonferroni post-hoc test (c). Results are from 2 independent experiments.

5.1.3 CCR1 mediates recruitment of neutrophils and inflammatory monocytes during MVA infection

The results from the *in vitro* migration assays indicated that CCR1 plays a role in the MVA induced recruitment of monocytes but does not directly influence the MVA induced chemotaxis of neutrophils. To gain a better understanding of how CCR1 signalling influences leukocyte recruitment, the infiltration of leukocytes to the lung after intranasal infection with MVA was examined in *Ccr1*$^{-/-}$ mice. As has been shown previously MVA infection of WT mice triggers the recruitment of a substantial number of neutrophils and monocytes to the lung [141]. Comparison of WT and *Ccr1*$^{-/-}$ mice showed that levels of recruited neutrophils ($CD11b^{+}Ly6C^{+}Ly6G^{+}$) were significantly reduced in *Ccr1*$^{-/-}$ mice (Fig 5.3). This effect, however, was not as pronounced as the dramatic reduction seen in the inflammatory monocyte ($CD11b^{+}Ly6C^{hi}Ly6G^{-}$) population.

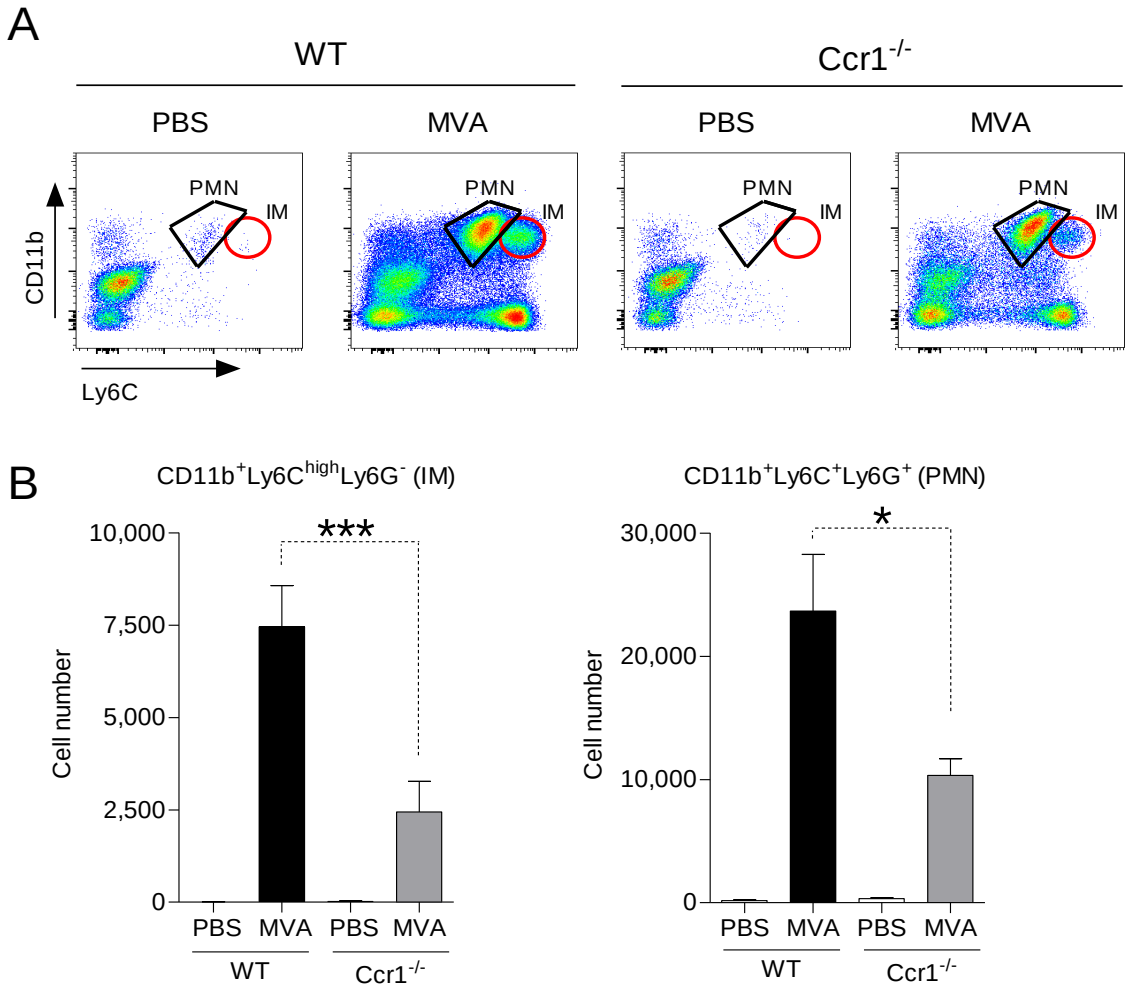

Fig. 5.3. CCR1 plays a critical role in the recruitment of inflammatory monocytes to the lungs of MVA infected mice. C57BL/6 (WT) and *Ccr1*$^{-/-}$ mice (C57BL/6) were intranasally infected with 1 × 10^7 PFU of MVA resuspended in PBS. An equal volume of PBS was used for control animals. Cells in the lung were recovered at 48 h p.i. by BAL and analysed by flow cytometry. Inflammatory monocytes (IM) were gated as CD11b$^+$ Ly6C^{high}, and neutrophils (PMN) were gated as CD11b$^+$ Ly6C$^+$ Ly6G$^+$. (**A**) Representative dot plots from each group. (**B**) Summary of the analysis. For groups of MVA infected mice n ≥ 11; for groups of PBS dosed mice n ≥ 8. Columns represent the mean number of gated cell populations ± SEM. *, P < 0.05; ***, P < 0.001; Mann-Whitney-U test. Data are from 3 independent experiments

5.2 Complement

5.2.1 Complement component C3 is not required for the early migration of leukocytes during MVA infection

VACV is capable of activating both the classical and alternative complement pathways; however the consequences of this activation are partly ameliorated by VCP. MVA no longer encodes VCP, thus complement activation by MVA potentially generates inflammatory mediators, such as C5a that induce leukocyte chemotaxis. The contribution of the central pathways of complement activation to the recruitment of leukocytes during MVA infection was assessed in an intranasal infection model. This study showed that activation of complement *via* C3 dependent mechanisms did not play a significant role in the recruitment of neutrophils, monocytes or lymphocytes 48 h after infection (Fig. 5.4).

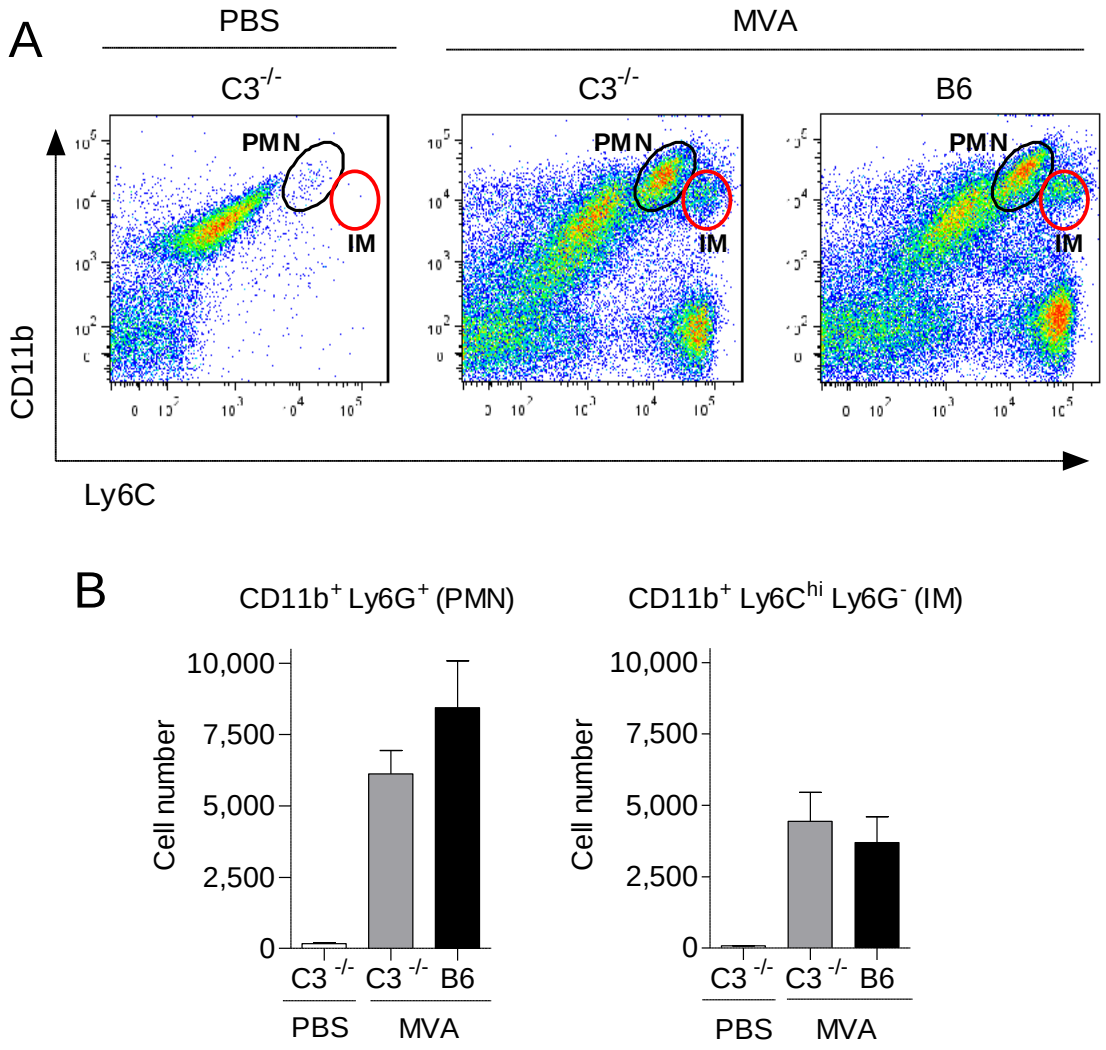

Fig. 5.4. Recruitment of neutrophils and inflammatory monocytes to the lung after intranasal infection with MVA is unaffected in *C3*$^{-/-}$ mice. B6 and *C3*$^{-/-}$ mice were intranasally inoculated with MVA or the equivalent volume of PBS. Cells collected by BAL, at 48 h p.i., were analyzed by flow cytometry. (**A**) Representative dot plots from flow cytometric analysis showing levels of infiltrating CD11b^{+}Ly6G^{+} neutrophils (PMN) and CD11b^{+}Ly6C^{hi}Ly6G^{-} inflammatory monocytes (IM). (**B**) Summary of the analysis. Columns represent mean cell number of each gated cell population ± SEM of 3 independent experiments (PBS, n = 2; B6, n = 7; C3$^{-/-}$, n = 8).

5.2.2 C5 is activated independently of C3 in *C3*$^{-/-}$ mice

Though C3 did not play a role in the recruitment of leukocytes after MVA infection, this did not rule out the possibility that other components of the complement system were activated independently of C3 [177]. To test this, levels of C5a in the BAL fluid were assayed by ELISA. As has been shown previously C5 was found in the lungs [178], which was increased by MVA infection. Surprisingly, the levels of C5a in the BAL fluid of MVA infected *C3*$^{-/-}$ mice were comparable to those of MVA infected wild type mice (Fig. 5.5A) which indicated that C5 may still be activated in *C3*$^{-/-}$ mice.

To verify the C3 independent activation of C5 in MVA infected *C3*$^{-/-}$ mice, C5 collected in BAL fluid 16 h after infection was visualised by Western blot using a C5 specific antibody. This antibody was tested prior to use and was shown not to bind to any FCS components in the wash solution (appendix Fig. 11.1). Immunoblot for C5 (Fig. 5.5B) detected the C5 alpha chain at the expected size of approximately 115 kDa in all groups. Proteolytic cleavage of C5 was confirmed in MVA infected groups by the detection of low molecular weight cleavage fragments. An additional band of approximately 80 kDa (marked C5*) was also detected in MVA infected groups and is presumed to be a C–terminal truncated proteolytic fragment.

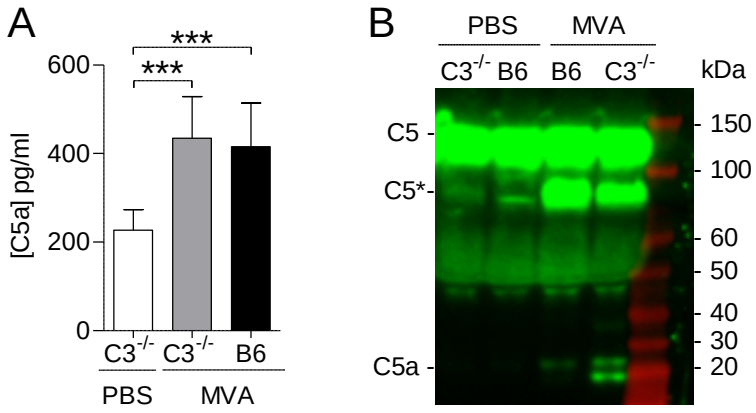

Fig. 5.5. **C5 is equally activated in the lungs of MVA infected B6 and *C3*$^{-/-}$ mice**. B6 and *C3*$^{-/-}$ mice were intranasally inoculated with MVA or the equivalent volume of PBS and BAL was performed 16 h p.i. (**A**) C5a concentrations in BAL fluids were determined by ELISA. Data are group means ± SEM of 3 independent experiments (PBS, n = 2; B6, n = 10; C3$^{-/-}$, n = 8), *** $p < 0.001$. (**B**) BAL fluids were analyzed with C5 specific Western blot. ColorPlus Prestained Protein Ladder, Broad Range (NEB) was visualized using the red LED module of the ChemiDoc MP Imaging System. The 2 images were overlaid using the multichannel image tool of the Image Lab 4.1 software (Bio-RAD).

5.2.3 C5 activation plays a role in the recruitment of neutrophils during MVA infection

C5a is a potent neutrophil chemoattractant, therefore the C3 independent activation of C5 potentially makes a significant contribution to leukocyte recruitment. To address this, the contribution of C5 to the recruitment of leukocytes during intranasal infection with MVA was assessed in the C5 deficient FVB strain. The recruitment of monocytes and CD4^{+} lymphocytes in FVB mice was no different from levels seen in C57BL/6 mice. However levels of CD11b^{+}Ly6C^{+}Ly6G^{+} neutrophils were significantly decreased in FVB mice (Fig. 5.6). As expected the levels of CD8^{+} lymphocytes also appeared to be impacted, however, this difference was not quite significant (Fig. 5.6C & D).

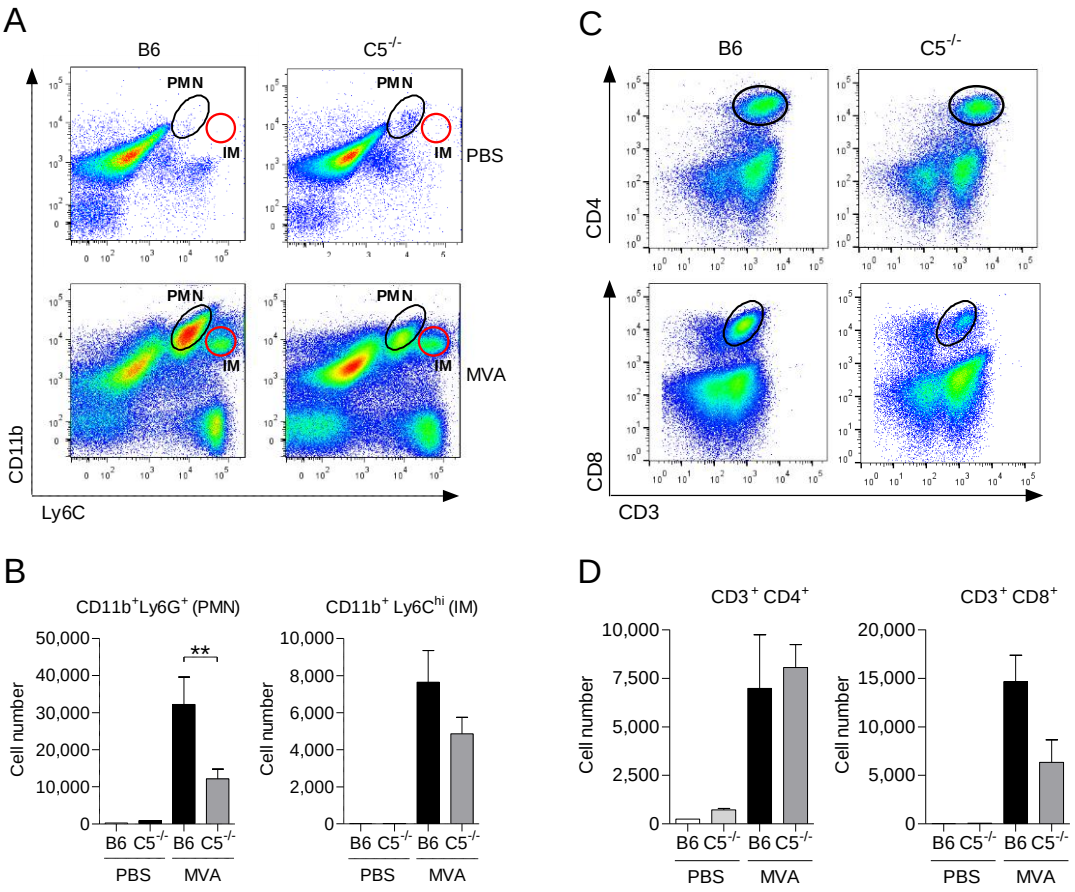

Fig. 5.6. **Neutrophil recruitment to the lung after infection with MVA is impaired in C5 deficient mice**. B6 and *C5*$^{-/-}$ mice were intranasally inoculated with MVA or the equivalent volume of PBS. Cells collected by BAL, at 48 h p.i. were analysed by flow cytometry. (**A**) Representative dot plots from flow cytometric analysis showing levels of infiltrating CD11b^{+}Ly6G^{+} neutrophils (PMN) and CD11b^{+}Ly6C^{hi}Ly6G^{-} inflammatory monocytes (IM). (**B**) Summary of the analysis. Columns represent mean cell number of each gated cell population ± SEM of 2 independent experiments (PBS groups n = 4, MVA groups n = 8), ** $p < 0.01$. (**C**) Representative dot plots from flow cytometric analysis showing levels of infiltrating CD3^{+}CD4^{+} and CD3^{+}CD8^{+} lymphocytes in MVA mice. (**D**) Summary of the analysis. Columns represent mean cell number of each gated cell population ± SEM (MVA groups n ≥ 4).

6 Discussion

6.1 CCR1: Role in the recruitment of neutrophils and inflammatory monocytes

There is an increasing appreciation of the role played by both monocytes and neutrophils not only in innate immunity but also in the development and even the execution of the adaptive immune response [164]. The two arms of the immune response are not mutually exclusive; innate immune activation precedes the development of adaptive immunity and during the adaptive immune response elements of innate immunity are required for optimal function [75, 164]. A large proportion of the non-structural genes of VACV are targeted at the innate immune response [179] underlining its importance in poxvirus immunity. Having lost many of these genes MVA potently activates the innate immune response, which may play an important role in its efficacy as a vaccine and a viral vector. The emigration of effector cells is a key step in the execution of the immune response that is tightly controlled by intricate cascades of inflammatory mediators serving to guide cells to exactly where they are needed.

Previous work has shown that CCL2 plays a critical role in the recruitment of monocytes and lymphocytes to the lung after intranasal infection with MVA [141]. To further dissect the mechanisms involved in leukocyte recruitment during MVA infection, the chemotaxis of neutrophils and monocytes towards culture supernatants of MVA infected cells was investigated. Culture supernatants from MVA but not VACV infected cells, induced migration of differentiated MPRO neutrophils in a MOI dependent manner. All cell types tested produced neutrophil chemotactic factors after MVA infection, unsurprisingly the

strongest induction was seen by supernatants from MH-S alveolar macrophages. Similar results were seen using monocytic THP-1 cells; with only supernatants from MVA infected cells inducing THP-1 chemotaxis. A study comparing cowpox virus to VACV produced similar results showing that supernatants from cowpox virus, but not VACV virus, infected cells induced the chemotaxis of monocytes [180]. These *in vitro* observations are in line with previous *in vivo* findings that only MVA induces chemokine production which leads to the recruitment of leukocytes [141].

CCR1 has been shown to be important for the recruitment of neutrophils [166-168] and monocytes [169, 170]. However CCR1 is expressed on a wide range of immune cell subpopulations making it difficult to determine if the effects observed are directly related to CCR1. The involvement of CCR1 in the migration of neutrophils towards supernatants from infected cells was tested using specific non-peptide antagonists. These studies revealed that the chemotaxis of nMPRO cells towards supernatants from MVA infected cells was independent of CCR1, requiring CXCR2 instead.

CXCR2 is well established as a key mediator in the mobilisation and recruitment of neutrophils. *In vitro* assays of neutrophil chemoattractants often point to overlapping redundant functions; however it is now thought that chemokines exert complex special and temporal relationships *in vivo*. Many of these mediators are linked in inflammatory cascades, with leukotriene B4 (LTB4) inducing IL-1β which then induces C-C and C-X-C chemokines, all of which are required for the recruitment of neutrophils [181, 182]. Different mediators may play important roles at different phases of recruitment. Chemokines take longer to produce but have greater half-lives than other rapidly produced inflammatory mediators, making them better suited to act at later stages. There is also the

possibility of spatial signalling whereby chemokines provide the long range signals which mobilise neutrophils from reservoirs, and as neutrophils approach they “home in” by prioritising shorter lived end target signals such as C5a or LTB4 [183], as has been seen in a model of liver necrosis [116]. Therefore whilst these results indicate an important role for CXCR2, they do not necessarily rule out a contribution from other important mediators such as complement, arachidonic acid derived lipid mediators or even CCR1 ligands *in vivo*.

Levels of recruited neutrophils were reduced in MVA infected *Ccr1*$^{-/-}$ mice indicating that CCR1 signalling did indeed play an important role in neutrophil recruitment during MVA infection. Others have reported that CCR1 deficiency does not affect the recruitment of neutrophils after MVA infection [130]. This does not conflict with our results as this was 2 hours after infection where recruitment is likely due to rapidly produced short-lived inflammatory mediators such as C5a and LTB4, whereas our studies looked after 48 hours where chemokines presumably play a much greater role. Though levels of neutrophils were reduced, there was no gross deficiency, which when taken together with our *in vitro* findings and those of Duffy *et al* [130], indicates that the role of CCR1 on neutrophil recruitment is indirect. This fits well with observations that CCR1 ligands induce neutrophil recruitment through the induction of other mediators including LTB4 [166, 184]. This study only examined the recruitment into infected tissue and did not look at the effects of CCR1 on recirculation or migration within infected tissue. Expression of CCR1 on neutrophils is up-regulated upon activation [121, 185], and CCR1 is required for the recirculation of neutrophils and induction of memory $CD8^+$ T-cell responses [130]. Thus the effects of CCR1 deficiency go beyond the recruitment from the circulation and may significantly impact

downstream processes such as migration within infected tissue, and the development of virus specific T-cell responses.

The effect of CCR1 on the recruitment of monocytes during MVA infection was much more striking. Inhibition of CCR1 dramatically reduced chemotaxis of THP-1 cells towards both mock and MVA infected supernatants. The role for CCR1 was confirmed by infection studies, whereby *Ccr1*$^{-/-}$ mice presented with greatly reduced levels of infiltrating inflammatory monocytes giving a strong indication that CCR1 plays an important direct role in the recruitment of inflammatory monocytes during MVA infection. This coincides well with the importance of CCR1 for the recruitment of inflammatory monocytes in other infection models [186].

Taken together with other studies, the work presented here suggests overlapping, non-redundant functions of CCR1 and CCR2 in the recruitment of monocytes during MVA infection. MVA induces expression of CCL3 [141] a CCR1 ligand which is known to play a role in monocyte recruitment [187]. Therefore it seems highly likely that CCL3 is involved in the CCR1 dependent recruitment of monocytes that we observed.

Based on these findings and currently available literature it is possible to put together a hypothetical model of monocyte recruitment during MVA infection. In the first step detection of MVA by PRR leads to the induction of inflammatory cytokines including TNFα, IL-1β and IFN and the recruitment of neutrophils. IFN induces local expression of chemokines including CCL2 [188] and CCL3 [189] which is further supplemented by expression from infiltrating neutrophils [190]. CCL2 is transported into the circulation where working in conjunction with systemic IFN, it reaches a critical concentration inducing egress of monocytes from reservoirs in the bone marrow and spleen. Local production of

inflammatory cytokines induces up-regulation of adhesion molecules on endothelial cells, allowing interaction with circulating inflammatory monocytes which are guided towards the site of infection by a gradient of immobilised CCL2. As they approach the infected tissue, signalling through CCR1, possibly by interaction with immobilised CCL3, induces arrest triggering extravasation into infected tissue. The local cytokine milieu induces infiltrating monocytes to differentiate, altering the expression of cell surface receptors as they do so [144] which presumably affects how signals are prioritised allowing monocytes to "home in" to sites of infection.

These studies did not address the role of Ly6C^{low} patrolling monocytes which are likely recruited before neutrophils, in response to different signals. These non-classical monocytes differentiate into macrophages and produce inflammatory chemokines including IL-6 and CCL2 [191] which are abundant in the lung after MVA infection. Non-classical monocytes are therefore of great interest as they may play an important role in the initial production of inflammatory mediators immediately after infection which leads to the recruitment of neutrophils.

Another important aspect that is not touched upon is regarding the consequences of immune cell recruitment during poxvirus infection. This seemingly simple question is not as straight forward as it may appear, as has been demonstrated by studies investigating poxvirus chemokine binding proteins. Poxviruses encode a number of chemokine binding proteins, of which two are found in VACV [192]. One such chemokine binding protein, A41, which is expressed at early and late stages of infection, is conserved in many VACV strains indicating its importance [193, 194]. Interestingly, A41 has a fairly limited range of targets, and does not directly block receptor binding. Instead, A41 interferes with chemokine

glycosaminoglycan binding sites presumably preventing the establishment of chemokine gradients [194, 195]. Thus A41 reduces inflammatory cell infiltration, and deletion of A41 from WR enhances virus clearance [193]. MVA still retains a functional A41, deletion of which leads to stronger induction of $CD8^+$ T-cell responses and confers better protection after VACV challenge [196] giving an indication that limiting the infiltration of host immune cells is beneficial to the virus.

The second chemokine binding protein found in VACV is the 35 kDa, viral C-C chemokine inhibitor (vCCI) which shows a high affinity for many human and murine C-C chemokines including CCL2 and CCL3 [197]. Due to the lack of homology with host chemokine receptors vCCI may have evolved separately [198-200]. vCCI acts as a competitive inhibitor, binding to C-C chemokines, preventing receptor interaction [198], inhibiting chemotaxis and extravasation of monocytes but not neutrophils [201]. Interference of chemokine receptor binding invariably alters the inflammatory immune response leading to decreased overall levels of cytokines and reduced cell infiltration [199, 202]. Despite this, the virulent WR strain does not encode a functional vCCI [203], and somewhat paradoxically expression of vCCI actually decreases the virulence of the virus [204]. This is not entirely dissimilar to A41, deletion of which was also associated with increased pathology [193]. The decreased virulence associated with expression of vCCI is likely due to its ability to reduce self-inflicted damage by the host immune system. However, decreased access to cells that could potentially enable further dissemination, such as monocytic cells, along with reduced inflammation, and the accompanying vascular permeability which may allow escape, could potentially explain why vCCI expression reduces virus replication and spread *in vivo*. This highlights the fine balance between virus and host; arguably the ultimate goal for the virus

is long-term survival in a host population, and vCCI appears to nicely illustrate that a gain of virulence is not always desirable as it may impact long-term spread and survival. The apparently different effects of the two poxvirus chemokine binding proteins on virulence could potentially be explained by their diverging target specificities and mechanisms of action. However it is also worth bearing in mind variations that arise from routes of infection. To this extent, whilst the loss of A41 altered the immunopathology of intradermal infection, there was no effect on the outcome of intranasal infection [193] which was the same route used to assess the recombinant vCCI expressing WR. Therefore, it would be interesting to investigate the impact of vCCI on the pathology of other routes of infection.

The results presented here suggest that MVA induced recruitment of neutrophils is mediated by CXCR2 and that CCR1 also plays an important role, possibly *via* induction of downstream processes. On the other hand CCR1 strongly influenced monocyte recruitment, suggesting a direct involvement. This study builds on previous work adding a further level of complexity by implicating an important role for CCR1 in the recruitment of neutrophils and particularly inflammatory monocytes, expanding our knowledge of the mechanisms involved in the recruitment of leukocytes during the inflammatory response to MVA infection.

6.2 Complement: MVA triggers C3 independent activation of C5 contributing to respiratory migration of neutrophils

Neutrophils play an important role in host defence, responding to a large number of chemotactic signals which are produced after infection. Deciphering the role of these inflammatory mediators has proved difficult as multiple signals are elaborated from infected tissue, which seemingly fulfil overlapping redundant roles in migration assays. Complement plays an important role in neutrophil biology, augmenting recruitment, and effector functions. Recent studies have shown that cell surface activation of complement on neutrophils leads to a self-perpetuating amplification loop [205]. Complement components are present in the serum and tissues in high concentrations, making the complement system uniquely placed to rapidly activate immune responses independently of host gene expression. VACV are known to activate complement however, incorporation of host regulatory proteins and the secretion of VCP presumably limits the activation of the complement system by VACV. MVA does not have these important regulators of complement activation, raising the possibility that complement activation may play a role in the induction of the immune response.

This study addressed the role of complement activation in the recruitment of leukocytes to the lung after infection with MVA. Recruitment of leukocytes during MVA infection was not affected by the absence of the central C3 component. A surprising observation was that the levels of C5a in the BAL fluid of MVA infected WT and *C3*$^{-/-}$ mice were similar which suggested that C5 was activated independently of the canonical C3 component. Further analysis of C5 cleavage products in the BAL fluid revealed that this was indeed the case.

Activation of C5 *via* C3 independent mechanisms has been shown before *in vitro* [177,206, 207] and was observed during cerebral malaria infection [208]. However, the relevance of these C3 independent pathways of C5 activation *in vivo* is not well studied and has not yet been observed during viral infection. Immunoblot of C5 showed that C5 is cleaved in the lungs of MVA infected mice, including those deficient in the central C3 component. The proposed mechanism of C5 activation is through direct cleavage of C5 by serine proteases produced from phagocytic cells [209] (Fig. 6.1) rather than by oxidation [206] though the possibility of oxidation occurring as an additional mechanism was not ruled out. Activation of C5 in this way could serve to immediately amplify the local immune response by rapidly creating a high local concentration of inflammatory mediators. Alveolar macrophages appear to be particularly efficient at activating C5 in this way, which raises a possibility, that this mechanism may be specific to the lung; therefore it would be interesting to investigate if this occurs after infection by other routes.

As C5a is a potent inflammatory mediator, particularly with regard to neutrophil recruitment, the contribution of C5 activation to MVA induced leukocyte recruitment was examined. Recruitment of neutrophils was significantly lower in the C5 deficient FVB strain, giving a strong indication that the activation of C5 plays a role in the recruitment of neutrophils during MVA infection. *In vitro* migration assays with differentiated MPRO cells indicated a role for CXCR2 in MVA induced neutrophil migration. This does not conflict with the observations implicating a role for C5, as C5a works in conjunction with CXCR2 ligands to induce inflammation [210] and is known to induce expression of CXCR2 ligands [211] and lipid mediators [212] in addition to being directly chemotactic.

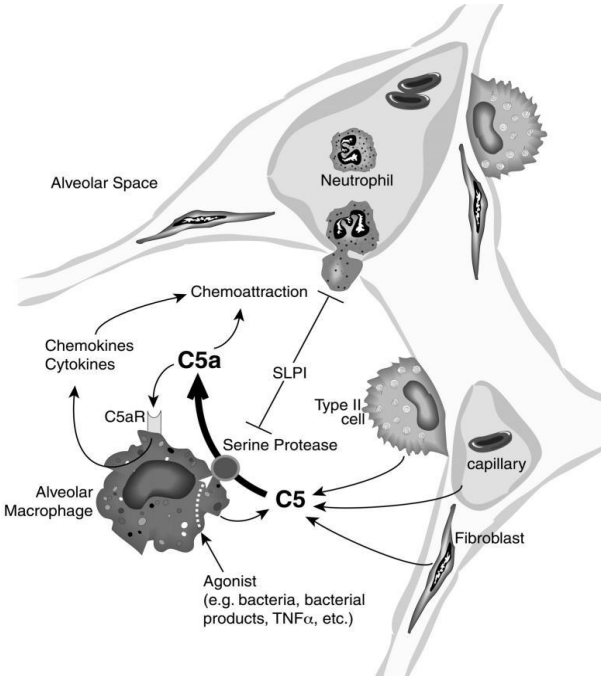

Fig 6.1 Proposed model for C3 independent activation of C5 by resident macrophages. Activation of resident macrophages leads to the production of serine proteases that cleave locally synthesised C5. Liberated C5a acts in an autocrine manner, stimulating chemokine production in macrophages and acts directly to induce recruitment of neutrophils from the circulation. Figure adapted from Huber-Lang et al 2002 [209], with permission from Elsevier, copyright 2002.

Bearing in mind current thinking regarding the spatial and temporal interactions that lead to neutrophil recruitment, it is possible that CXCR2 ligands and C5a are fulfilling distinct roles, similar to those proposed for CCL2 and CCL3. In this hypothetical situation CXCR2 ligands, such as CXCL1 and CXCL2 which are induced by MVA infection, provide the long range signals to mobilise neutrophils from reservoirs. C5a on the other hand has a shorter half-life as it is rapidly inactivated [213], thus C5a may provide the “end point” signal which is prioritised over CXCR2 as neutrophils approach the infected tissue. This would potentially explain why C5 deficiency does not grossly affect the recruitment of neutrophils, as a likely

possibility is that C5a serves to amplify CXCR2 ligands by inducing expression in non-infected cells, and may play a role in guidance within infected tissue.

In addition to its role in the recruitment of neutrophils, a reduction in the levels of infiltrating $CD8^+$ T-cells was also seen in *C5*$^{-/-}$ mice. Though this difference was not statistically significant, due primarily to the low statistical power of the lymphocyte analysis, a reduction of infiltrating $CD8^+$ T-cells in virally infected *C5*$^{-/-}$ mice has been previously reported by others [214]. Indeed C5 appears to play an important role in the development of antiviral immunity as C5 deficient mice are more susceptible to influenza infection [215]. Complement is required for development of $CD8^+$ CTL responses [214, 216] and appears to be important for the induction of type I (TH1) immune responses [217]. Cells of the innate immune system induce up-regulation of the C5a receptor on T-cells [218] which provides additional costimulatory and survival signals [219], allowing the expansion of antiviral effector cells [220]. These properties of C5a have recently been harnessed by the development of the C5a agonist, EP67, which retains the immune enhancing properties without the accompanied inflammation associated with C5a. This synthetic peptide has been used for its adjuvant properties, and has been shown to induce CTL responses [221]. More importantly, it has also been used alone to boost host immune responses where it has been shown to provide protection from lethal influenza infection when used for prophylactic or therapeutic treatment [222]. In light of these studies the rapid activation of C5 by MVA is particularly interesting as C5 appears to play a role in boosting the host immune response and aids the development of antiviral CTL responses. Additionally in the context of poxvirus infection, C5 has recently been shown to be critical for neutralisation of VACV [223]. Therefore the rapid induction of C5 activation by MVA infection could

potentially have important consequences, and may be a mechanism underlying the potent immunity induced by MVA infection particularly regarding post-exposure protection provided by MVA vaccination.

The role of complement in poxvirus immunity does not appear to be straight forward, as currently available literature points to divergent roles of the different complement components. In line with our studies, it was observed that deficiency of C5 affects the primary immune response to cowpox infection, which was associated with uncontrolled inflammation and increased pathology. The potential role of C5 in the clearance of VACV [223] and in the expansion of virus specific CTL [214, 216] may account for this exacerbation of disease. However these differences were no longer apparent upon re-infection indicating that the long-term development and effectiveness of the adaptive immune response was ultimately not grossly affected by C5 deficiency [224]. Conversely, we observed that deficiency of C3 does not affect the infiltration of leukocytes during MVA infection as C5 appears to be activated independently of C3. However with its role in opsonisation and complement dependent antibody neutralisation complement plays an important role in the adaptive immune response as deficiency of C3 impairs humoral [225] and T-cell [87] responses. Thus central complement components are required to survive mousepox infection as deficiency of components of the classical or alternative complement activation pathways impairs the humoral immune response to poxvirus infection [79]. The seeming roles for C5 in early immune response and C3 in the late immune response raises interesting questions as to the roles of the complement components in the development of protective immunity. The first is if prophylactic vaccination of C3 deficient mice protects

from lethal challenge, the second is whether C5 plays an important role in protection afforded by therapeutic vaccination with MVA.

The C3 independent activation of C5 by MVA raises another important issue regarding poxvirus VCP. VCP is a virulence factor [90] that binds to and accelerates the decay of complement activation products of C3 and C4 [85, 226], thus it is not capable of directly preventing complement activation but serves to limit further activation and avoid the consequences. As C3 and C4 are critical components of the classical and alternative pathways VCP provides protection from both but is particularly efficient at inhibiting the antibody dependent, classical pathway [227]. This indicates that the main function of VCP may be to aid virus dissemination and to protect from the adaptive immune response [89] particularly by avoiding complement dependent antibody neutralisation [228]. MVA does not possess VCP, however as MVA is non-replicative and as activation of C5 occurs independently of C3, it seems unlikely that VCP would provide any additional benefit in this instance. Complement products are synthesised locally by phagocytic cells [178, 229], and local activation by MVA infection leads to the secretion of proteases which likely directly activate C5 [209]. Therefore it would seem that as a consequence, the viral mechanisms that block cellular activation prevent direct cleavage of C5 rather than those that interfere with the complement system itself. None the less, it is worth bearing in mind that VCP can modulate inflammatory immune responses [230], and that complement plays an important role in the development and execution of the adaptive immune response. Therefore it would be interesting to assess the influence of VCP on leukocyte migration and particularly whether VCP affects the development of the adaptive immune response to MVA. Additionally this study did not address the second effector function of C5 which is the

formation of the MAC. It is not clear if C5 activation by C3 independent pathways preserves the ability of C5 to form the MAC which could potentially lead to widespread lysis. The difficulties of working with murine complement, which is highly unstable, would likely make addressing this issue rather complicated, therefore the development of an *in vitro* system using human cells and complement would be preferable.

This study investigated the role of complement activation during the inflammatory immune response to MVA and revealed that the central C3 component does not impact the early recruitment of leukocytes. Despite the absence of C3, it was observed that levels of C5a in MVA infected *C3*$^{-/-}$ mice were similar to WT controls, as C5 appeared to be activated independently of C3. Analysis of leukocyte recruitment in C5 deficient FVB mice showed that levels of neutrophil recruitment were reduced compared to a C5 sufficient strain, implicating a role for C5 in the recruitment of neutrophils during MVA infection.

7 Summary

The triggering of the immune response and induction of immune cell trafficking is a critical step in the induction of antiviral immunity. Modified vaccinia virus Ankara (MVA) is a promising viral vector vaccine, however little is known about the mechanisms underlying its potent induction of the host immune response. Therefore, this work was undertaken to investigate the mechanisms of leukocyte recruitment during infection with MVA.

In vitro chemotaxis assays demonstrated that MVA, but not other Vaccinia virus infected cells produce chemotactic factors for neutrophils and monocytes. Addition of chemokine receptor inhibitors indicated that neutrophil and monocyte chemotaxis was mediated by CXCR2 and CCR1 respectively. The important role for CCR1 in the recruitment of inflammatory monocytes was then confirmed by infection studies in *Ccr1*$^{-/-}$ mice.

To further evaluate the mechanisms of neutrophil recruitment the role of complement activation was examined. Leukocyte recruitment was unaffected in C3 deficient mice suggesting that activation of central C3 dependent pathways is not required. Further investigation of C5 activation products showed that MVA induces C5 activation in C3 deficient mice, and that C5 appears to play a role in neutrophil recruitment.

In conclusion, these results suggest an important role for CCR1 signalling in the recruitment of inflammatory monocytes and implicate a role for C3 independent activation of C5 in neutrophil recruitment to the lung during MVA infection. These findings extend our understanding of the mechanisms triggered by MVA to induce cell migration, which presumably plays a role in its efficacy as a viral vector vaccine.

8 Zusammenfassung

Die Wanderung von Immunzellen an den Ort einer Infektion oder Impfung ist ein wichtiger Schritt bei der Induktion von spezifischer Immunität gegen Krankheitserreger. Das Modifizierte Vaccinavirus Ankara (MVA) ist ein hoch attenuiertes Orthopockenvirus, das bereits häufig für die Entwicklung von neuartigen rekombinanten Impfstoffen gegen Infektionserreger eingesetzt wird. Jedoch ist noch wenig über die immunologischen und zellbiologischen Abläufe bekannt, die der Anregung einer durch die Verabreichung von MVA Impfstoff erzielten Immunantwort zugrunde liegen. Diese Arbeit untersucht daher die Mechanismen der Leukozytenrekrutierung nach Immunisierung mit dem MVA.

In-vitro-Untersuchungen zur Chemotaxis zeigten, dass mit MVA infizierte Zellen, jedoch nicht mit anderen Vaccinavirus infizierte Zellen chemotaktische Faktoren für neutrophile Granulozyten und Monozyten produzieren. Der Einsatz von spezifischen Inhibitoren der Funktion von Chemokinrezeptoren legte nahe, dass die Chemotaxis von neutrophilen Granulozyten und Monozyten durch die Rezeptormoleküle CXCR2 beziehungsweise CCR1 vermittelt wurde. Die Bedeutung von CCR1 bei der Rekrutierung von inflammatorischen Monozyten wurde anschließend durch Impfstudien in Rezeptor-defizienten (*Ccr1*$^{-/-}$) Mäusen bestätigt.

Um die Mechanismen der Rekrutierung von neutrophilen Granulozyten besser zu verstehen, wurde auch die Bedeutung der Komplementaktivierung nach intranasaler Verabreichung von MVA im Mausmodell untersucht. In Mäusen, denen der Komplementbestandteil C3 fehlt, war die Leukozytenrekrutierung nicht beeinträchtigt. Dies legte nahe, dass die Aktivierung des zentralen Komplementbestandteils C3 für die Einwanderung von Immunzellen nicht erforderlich ist. Die weitere Erforschung von C5-Aktivierungsprodukten

zeigte, dass MVA die Aktivierung von C5 in C3-defizienten Mäusen induzieren kann und anscheinend C5 eine Rolle bei der Rekrutierung von neutrophilen Granulozyten spielt.

Diese Ergebnisse weisen auf eine wichtige Rolle des Chemokinrezeptors CCR1 bei der Rekrutierung von inflammatorischen Monozyten hin und implizieren bei der Einwanderung von neutrophilen Granulozyten eine Aktivierung des Komplementfaktors C5 durch MVA unabhängig von der Funktion der zentralen Komplementkomponente C3.

Diese Ergebnisse vertiefen das grundlegende Verständnis der durch MVA Immunisierung ausgelösten Einwanderung von neutrophilen Granulozyten und inflammatorischen Monozyten, welche vermutlich die Wirksamkeit von MVA als Impfstoff beeinflusst.

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11 Appendix

11.1 List of materials

11.1.1 Cell culture and Chemotaxis

Reagent/Equipment	**Supplier**
All *trans*-retinoic acid	Sigma
J113863 (dissolved in ethanol 50 mM)	Tocris
FCS (VLE)	Biochrom
FACScan	BD Biosciences
TrypZean Solution (Trypsin)	Sigma
Ethanol (96%)	Roth
Filter 0.2µm	BD Biosciences
SB265610 (dissolved in ethanol 10 mM)	Tocris
96 well Multi-Screen-MIC plate	Millipore
MACSQuant VYB	Miltenyi
RPMI (Endotoxin free)	Sigma
DMEM (Endotoxin free)	Biochrom

11.1.2 Infection and BAL

PBS (Endotoxin free)	Biochrom
EDTA	Sigma
Ketamine	Bela-Pharm
Xylazine	Bayer Vital
RPMI (Endotoxin free)	Sigma

11.1.3 Flow cytometry

96 well (V bottom for FACS)	Corning Life Sciences
Cytofix/Cytoperm kit	BD biosciences
FCS (VLE)	Biochrom
FACSCanto II	BD Biosciences
MACSQuant VYB	Miltenyi
Microplate Centrifuge 5810R	Eppendorf

11.1.4 Antibodies and Live dead exclusion

Reagent	**Conjugate**	**Clone**	**Supplier**	**Concentration**
CD11b	FITC	M1/70	Biolegend	2.5 µg/ml
CD11b	VioGreen	M1/70	Miltenyi	2.5 µg/ml
CD3	PE	17A2	Biolegend	2 µg/ml
CD4	PE-Cy7	GK1.5	Biolegend	1 µg/ml
CD8	PE-Cy7	53.6.7	Biolegend	1 µg/ml

Ly6C	APC-Cy7	HK1.4	Biolegend	1 µg/ml
Ly6C	AlexaFluor-488	HK1.4	Biolegend	1 µg/ml
Ly6G	APC	1AB	Biolegend	0.4 µg/ml
CCR1	AlexaFluor 405	C-20	Santa Cruz	10 µg/ml
C5a	N/A	N/A	R&D Systems	0.2 µg/ml
Anti-rat IgG	HRP	Poly4054	Biolegend	8 ng/ml
Fixable Live/Dead	Violet or Red	N/A	Invitrogen	2 µl/ml
7AAD	N/A	N/A	BD Biosciences	2.5 µg/ml

11.1.5 ELISA

Reagent/Equipment	**Supplier**
C5a ELISA Duo set	R&D Systems
BSA	Sigma
Sunrise microplate reader	Tecan
Sulphuric acid 1 M (2 N)	Roth
Tween20	Sigma
96 well (Maxisorp, flat bottom for ELISA)	Nunc

11.1.6 Western blot

Reagent/Equipment	**Supplier**
4-20% Criteron TGX stain free gel	Bio-Rad
ChemiDoc MP imager	Bio-Rad

Colour plus protein ladder	New England Biolabs
ECL Advance western blotting kit	GE healthcare
laemmli buffer (4×)	Bio-Rad
Mini PROTEAN tetra cell (electrophoresis tank)	Bio-Rad
TBS (10×) (0.2 M Tris base, 1.5 M NaCl)	Sigma
Trans-Blot Turbo Mini Nitrocellulose transfer pack	Bio-Rad
Trans-Blot Turbo Transfer system	Bio-Rad
Tris Glycine solution (10×)	Bio-Rad
Tween20	Sigma
β-Mercaptoethanol	AppliChem

11.2 C5a Western blot controls

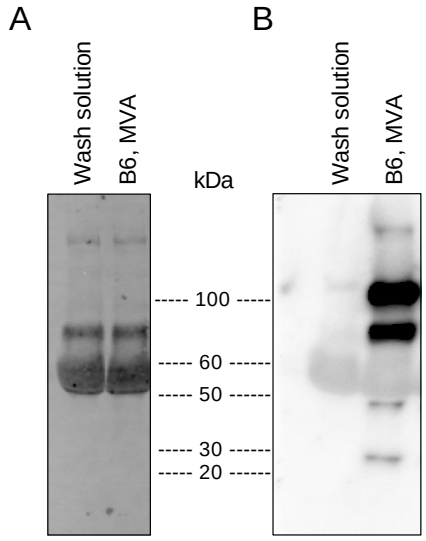

Fig. 11.1. Specificity of the C5a antibody. A B6 mouse was intranasally inoculated with MVA and subjected to BAL 48 h p.i. RPMI 1640 containing 10% FCS was used as wash solution. Protein in the BAL fluid and wash solution was separated by SDS-PAGE. The gel was subsequently treated with UV according to the manufacturer's instructions (Bio-Rad) to activate the trihalo compounds. Protein was then transferred onto a nitrocellulose membrane as described in materials and methods. (**A**) Fluorescence detection of total protein. (**B**) C5 specific Western blot.