Chapter 1

Review of Literature
1.1. Introduction

The host immune system is under constant alert to keep control on the invading pathogens. It is broadly divided into innate immunity (‘natural immunity’) and adaptive immunity (‘acquired immunity’). The complement system is an integral part of innate immunity and has an ability to protect the host from existing and newly emerging pathogens, including viruses [1]. It is composed of more than 30 soluble and membrane bound proteins which are involved in activation and regulation of the complement pathways. Upon entry of the pathogen, the complement system is activated by three major pathways viz, classical (CP), alternative (AP) and lectin (LP) pathways. These pathways are activated in the extracellular milieu. Recent evidences however show that complement is also activated intracellularly by non-canonical proteases [2, 3].

The three major complement pathways differ from each other in their initiation molecules. The classical pathway is majorly activated by the antigen-antibody complex [4] or pattern recognition molecules like serum amyloid protein (SAP) [5], C-reactive protein (CRP) [6] and C1q itself [7, 8]. The lectin pathway is initiated by recognition of the sugar moieties present on the pathogen surface by mannose binding lectin (MBL) and ficolins (L, M and H). Interestingly, the alternative pathway (AP) does not need any specific recognition molecule and is activated by a slow, continuous hydrolysis of C3 to C3(H2O) that forms the initial fluid phase C3-convertase [9]. All the three complement pathways converge at the C3 activation step. The C3-convertase (C4b2a for the CP and LP and C3bBb for AP) cleaves the C3 molecule into anaphylatoxin C3a and opsonin C3b. The C3-convertase then interacts with newly generated C3b to form the C5 convertase that cleaves C5 into anaphylatoxin C5a and C5b. The C5b molecule initiates the assembly of membrane attack complex (MAC; C5b-9) which causes lysis of pathogen. Apart from lysis of the pathogen, the byproducts of complement activation (C3a and C5a) help in shaping the acquired immune responses against the pathogens [10].

The complement activation pathways possess the ability to directly neutralize viruses. The mechanism of virus neutralization includes: i) coating or opsonisation of viral particles by complement opsonin proteins like C3b or C4b, ii) aggregation of viruses by complement proteins C1q or ficolins, iii) formation of membrane attack complex
(MAC) on the surface of pathogen which results in virolysis, iv) recruitment of inflammatory cells at the site of infection which participate in controlling the infection.

Since complement is a major threat to the survival of viruses, it is requisite for viruses to develop strategies against the host complement attack. And this is indeed the case - successful viruses have evolved multiple strategies to subvert complement assault to ensure their survival [11, 12]. The various strategies employed by viruses are: i) encoding structural and functional homologue of complement regulatory proteins, ii) acquiring host complement regulatory protein on their surface and iii) use of membrane bound complement proteins as a receptor for entry inside the cells [13, 14], iv) inhibition of complement synthesis, and v) upregulation of complement regulators on the infected cells [14, 15].

Vaccinia virus (VACV) is the prototypic member of the family *Poxviridae*. It was used as a vaccine to eradicate the highly contagious and deadly smallpox disease from the globe. VACV contains linear double-stranded DNA genome (approximately 190 kb) with a cluster of immunomodulatory genes at the terminal region of its genome [16]. It has two morphologically distinct infectious forms: an intracellular mature virion (MV), and an extracellular enveloped virion (EV). Studies on VACV neutralization have shown that infectivity of MV as well as EV is destroyed by the classical complement pathway when anti-vaccinia antibodies are present [17, 18]. However, role of other complement pathways in VACV neutralization and also the mechanism of MV neutralization is not clearly understood.

To counteract the complement attack, VACV C21L ORF encodes a 27 kDa complement regulatory protein named vaccinia virus complement control protein (VCP) [19, 20]. Structurally, it is entirely formed by four short consensus repeats (SCR) or complement control protein (CCP) domains which is a characteristic feature of the human regulators of complement activation (RCA) family. *In vitro* studies have revealed that VCP possess the ability to inactivate the classical/lectin and alternative pathway C3-convertases by accelerating the decay of C3-convertase and by supporting the factor I-mediated inactivation of C3b and C4b [21-23] which are the subunits of C3-convertases. Further, *in vitro* studies have shown that VCP inhibits the complement-mediated neutralization of MV as well as EV [24]. Similar to VCP, a
homologue of RCA is also encoded by the smallpox virus (Variola virus). This regulator is also formed by four CCP domains and is dubbed as SPICE (smallpox inhibitor of complement enzymes). VCP and SPICE differ from each other only by 11 amino acids, which are distributed in CCP domains 2, 3 and 4. Interestingly, using purified complement proteins, it has been shown that SPICE is much more potent in inactivating human complement compared to VCP [25, 26]. However, whether SPICE is more potent than VCP in rescuing poxvirus from human complement-mediated neutralization is not clear. And if such is the case, it would be interesting to determine which amongst the 11 variant amino acids of SPICE that differ from VCP play crucial role for enhancing its rescuing ability.

Earlier studies using VCP-null VACV have demonstrated that VCP acts as a virulence factor in intradermal infection model [18, 27]. But whether VCP also serves as a virulence factor during natural VACV infection i.e., during infection via intranasal route, has not yet been established. As VCP inhibit complement at the C3-convertase step, expression of VCP during VACV infection is expected to inhibit generation of complement anaphylatoxins C3a and C5a which are expected to participate in generation of the optimal immune response against the virus. We now know that VCP inhibits complement at high nanomolar concentration [28] which can be achieved only at the site of infection. This raises the question: what role complement activation products (C3a and C5a generated in the viral habitat play in controlling VACV infection?

Hence, in the present study, I aimed to investigate role of the individual complement pathways in VACV (MV) neutralization and the mechanism involved in complement-mediated neutralization of VACV (MV). Efforts were also made to understand the role of VCP in subversion of host complement and that of the locally generated complement anaphylatoxins C3a and C5a in controlling VACV infection.

1.2. The complement system:

The primary function of the immune system is to protect us from the invading pathogens. It is broadly classified into two types: innate immunity and adaptive immunity. The innate immune responses act at a very early stage of infection but are not specific. The adaptive immune responses on the other hand are pathogen-specific but take longer to set in with a lag of 4-7 days after the exposure. The complement
system is a vital arm of the innate immune responses and can act against existing as well as newly emerging pathogens. The system was discovered in late 18th century by Prof. Jules Bordet and was termed as ‘alexin’ – a heat labile factor in normal human serum that complements heat stable antibody to bring about bacteriolysis. Later, it was named as complement for complementing the function of antibody. Apart from its classical function of direct killing of pathogen, it was also shown to be involved in the clearance of apoptotic cells [29] and more recently, a large body of literature has shown that it plays a key role in shaping the acquired immune responses [14, 30-32]. Additionally, it was recognized to be important in tissue regeneration and angiogenesis [33, 34].

1.2.1. Complement activation:

The complement system encompasses membrane bound and soluble proteins which participate in initiation, activation and regulation of complement cascades. On the basis of initiation molecules involved, the complement activation pathways divided into three major pathways – the classical (CP), alternative (AP) and lectin pathways (LP). Apart from these three major complement activation pathways, the complement system is also known to be activated by other ways: i) direct cleavage of C3 and C5, ii) C2-bypass pathway, and iii) properdin mediated activation of AP. For example, human coagulation system proteases such as thrombin, factor IX, XI, Xa and plasmin are known to cleave C3 [35] and C5 [36, 37] that can result in activation of complement cascade. The cleavage site of C3 and C5 is different for coagulation system proteases, therefore the cleavage products generated are not exactly identical to the fragments generated by complement cascade but are shown to possess their biological activity [35]. In C2 bypass pathway, MASP-1 has been shown to cleave and activate C3 [38]. Recently, properdin and FHR4 have also been shown to activate complement cascade by providing platform for AP [39-42]. However, the proposition that properdin serves as a platform for activation of AP has recently been challenged [43].

1.2.1.1 The classical pathway:

The classical pathway majorly depends on immune complexes for its activation but it can also be activated in an immune complex independent manner. The IgG and IgM immunoglobulins activate CP in immune complex dependent manner, whereas pattern
recognition molecules like SIGN-DC [44], serum amyloid protein (SAP) [5], C-reactive protein (CRP) [6] and C1q itself [7, 8] can initiate the CP in an immune complex independent manner. The C1q molecule, which is a part of C1 complex (a 790-kDa assembly), recognizes the pathogens coated with immunoglobulins or the pathogen recognition molecules. According to a recent model, such binding results in conformational changes in C1q collagen stem that reorganizes the C1r2s2 in such a way that the two C1r serine protease domains come close and activate each other. Subsequently, the activated C1r activates C1s molecules, which then cleave C4 molecule into C4a and C4b and C2 molecule into C2a and C2b. The cleavage of C4 molecule into C4b leads to exposure of its thioester bond which forms amide or ester linkage with the pathogen surface. C4b along with C2a forms C4b2a, known as classical pathway C3-convertase (Fig. 1).

1.2.1.2. The lectin pathway:

In the lectin pathway (LP), pattern recognition molecules (PRMs) such as mannose binding lectins (MBL), ficolins (H, L and M), and collectin (CL)-11 recognize the sugar moieties present on pathogen surface. These pattern recognition molecules are associated with serine protease family members known as MBL associated serine-protease (MASP). There are three types of MASP s – MASP-1, MASP-2 and MASP-3 – that are associated with MBL/ficolins/CL-11. Earlier it was thought that MASP-2 is autoactivated and this then cleaves C4 and C2 to form C3 convertase C4b2a. A study using MASP-1 and MASP-2 specific inhibitors however showed that under physiological conditions, MASP-1 is the exclusive activator of MASP-2 [45, 46]. Later, this activation of MASP-2 by MASP-1 was shown to be due to clustering of MASP-1 and MASP-2 carrying PRMs i.e, MASP-1 associated with one PRM was shown to activate MASP-2 that was associated with another PRM and this was solely due to juxta-positioning of these complexes [47]. Activation of MASP-1 however was shown to be due to its autoactivation [46]. Thus, recognition of pathogens by PRMs results in auto-activation of MASP-1 which subsequently activates MASP-2 [46]. Both MASP-1 and MASP-2 can cleave C2 molecule whereas only MASP-2 cleaves C4 molecule. This cleavage of C4 and C2 molecules leads to the formation of LP C3-convertase, C4b2a. The role of MASP-3 is not yet clear but in mouse model, it has been suggested to have role in activation of factor D [48] (Fig. 1).
Figure 1: Activation of the classical and lectin pathways of the complement system.

The classical pathway is activated by recognition of antigen-antibody complex by C1 (and also by antigen-antibody independent pathways) whereas the lectin pathway is initiated through recognition of sugar moieties present on the pathogen surface by MBL, ficolins and CL-11. This recognition leads to activation of C1 complex or MASP1/2 (associated with MBL and ficolins) which then cleave C4 and C2. The cleavage product of C4, C4b, is deposited on the pathogen surface, whereas the cleavage product of C2, C2a with C4b forms the CP/LP C3-convertase C4b2a. C3 convertase cleaves C3 molecule to C3a and C3b. The newly formed C3b molecule attaches to the surface or C4b2a to form CP/LP C5-convertase i.e. C4b2a3b.

1.2.1.3. The alternative pathway:

Unlike the activation of CP and LP, for the alternative pathway (AP), there is no requirement of the initiation molecule. AP is activated due to spontaneous hydrolysis of C3 molecule [i.e., conversion of C3 to C3(H₂O)] which occurs at a very low level. It is believed the C3(H₂O) molecule undergoes huge conformational change like C3b leading to exposure of factor B binding site. Thus, C3(H₂O) binds to factor B in the presence of Mg²⁺ ions forming C3(H₂O).B complex. This complex is susceptible to factor D mediated cleavage of factor B and forms the initial C3-convertase C3(H₂O)Bb which is stabilized by properdin (Fig.2). This initial C3(H₂O)Bb convertase in fluid phase cleaves C3 into C3a and C3b. It is thus obvious that unlike CP and LP, initiation of AP occurs in the fluid phase and not on the surface. The C3b generated by initial C3-convertase gets covalently linked to the surface by ester or amide linkages owing to thioester bond present in the TED domain of C3. The surface
deposited C3b then binds to factor B forming C3bB complex which gets cleaved by a serine protease factor D leading to generation of AP C3-convertase, C3bBb.

**Figure 2:** Activation of the alternative pathway of the complement system. C3 is spontaneously hydrolyzed at a low rate leading to formation of C3(H2O) which resembles C3b and gets bound to serine protease factor B (FB). C3(H2O)B formed gets cleaved by protease factor D (FD), generating initial AP convertase, C3(H2O)Bb in fluid phase. The initial AP convertase cleaves C3 into C3a and C3b. C3b gets deposited on the activator/pathogen surface through ester or amide linkages. FB binds to the deposited C3b molecule and gets cleaved into Ba and Bb by FD and consequentially generates AP C3-convertase, C3bBb. The newly generated C3 convertase cleaves additional C3 molecules to C3a and C3b (known as amplification loop and indicated in dashed arrow). The C3b molecules generated binds to the surface C3 convertase and forms AP C5 convertase, C3b3bBb. Properdin binds to the C3 and C5 convertase and increases the half-life of these convertases.

### 1.2.1.4 The terminal pathway:

All the three complement pathways converge at the C3 activation step i.e., cleavage of C3 into C3a and C3b by C3 convertase. The newly generated C3b binds to the C3-convertase (C3bBb or C4b2a) to form C5-convertase (C4b2a3b in case of CP and LP, and C3bBb3b in AP) as shown in Fig 1 and 2. The attachment of C3b to the C3-
convertase increases the affinity of the convertase towards C5 molecule leading to binding and cleavage of C5 into C5a and C5b. The cleaved C5b molecule undergoes conformational change and commences assembly of membrane attack complex (MAC) through its binding with C6. The binding of C6 to C5b facilitates binding of C7 to the C5bC6 complex and forms C5b-7 complex which is lipophilic in the nature. Binding of C8 to C5b-7 directs the complex to the pathogen surface and penetrates into the lipid bilayer. C9 binds to C5b-8 and undergoes polymerization resulting ultimately in the formation of pore in split-washer shape, also known as membrane attack complex, on the pathogen surface. The MAC forms large, 101 Å wide pore on the pathogen membrane [49].

Figure 3: Activation of terminal pathway. The C5 convertase generated during complement activation cleaves C5 into C5a and C5b. C5b initiates the process of membrane attack complex formation along with C6 and C7 molecules. C7 is lipophilic in nature and directs complex towards pathogen surface followed by binding of C8 and C9, which results in the formation of membrane attack complex leading to lysis of the pathogen.

1.2.2. Complement anaphylatoxins and their receptors:

The complement cascade activation results in the cleavage of C3, C4 and C5 proteins and generation of anaphylatoxins C3a, C4a and C5a which are 9 kDa, 8.7 kDa and 11 kDa peptides, respectively [50]. These anaphylatoxins generated are proinflammatory in nature and their activity is dependent on the cell type [51-54]. They activate immune cells and non-immune cells through the anaphylatoxins receptors present on these cells. Anaphylatoxins also regulates smooth muscle contraction, permeability of blood vessel and vasodilation. The activity of these anaphylatoxins is regulated by the
carboxypeptidase enzyme which cleave terminal arginine residue of these anaphylatoxins and generates a less potent desArg form of anaphylatoxins.

Anaphylatoxin C3a has been shown to bind its receptor C3aR. The C3aR receptor is 54 kDa glycoprotein and expressed on both immune and non-immune cells. Immune cells like eosinophils, neutrophils, mast cells, dendritic cells, basophils (myeloid origin) astrocytes, T cells, smooth muscles cells, and endothelial and epithelial cells (non myeloid origin) express C3aR receptor. Expression of C3aR receptor is seen in lung, liver, kidney, brain, heart and testis. C3a has been shown to have chemotactic activity for eosinophils [55], dendritic cells [56] and mast cells [57]. C3a-C3aR interaction on macrophages [58], neutrophils [59] and eosinophils [60] triggers oxidative burst, whereas it induces degranulation in basophils [61] and mast cells [62] which results in histamine release.

The C4a receptor is still unidentified. C4a however has been shown to induce smooth muscle contraction, increase vascular permeability, and induce generation of generation of oxygen free radicals from macrophages and monocytes [63-66].

C5a is very potent chemoattractant compared to C3a and C4a. Two receptors are known for the anaphylatoxin C5a – C5aR1 (CD88) and C5aR2 (earlier known as C5L2). C5aR1 is a 42 kDa G-protein coupled glycosylated receptor [67] that has a strong affinity for C5a (1 nM) compared to C5a desArg, which binds with 10- to 100-fold lower affinity [68]. Expression of C5aR1 is seen on the surface of myeloid cells such as eosinophils, neutrophils, basophils, monocytes, mast cells and dendritic cells and also on cells of non-myeloid origin such as astrocytes, plasmacytoid DC, endothelial and epithelial cells, hepatocytes, keratinocyte and smooth muscle cell [68]. It acts as chemoattractant for neutrophils [69], eosinophils, basophils [70], mast cells [71], memory and naive tonsillar B cells [72] and T cells [73]. C5a-C5aR1 interaction in granulocytes stimulates oxidative burst whereas in neutrophils it stimulates reactive oxygen species production [74]. The engagement of C5aR1 on mast cells promotes mast cell degranulation on the other hand in monocytes and eosinophils it leads to secretion of proinflammatory cytokine [75].

Another receptor for C5a is C5aR2, 37kDa receptor which has high affinity for C5a and C5a desArg [76]. C3a and C3a desArg has been shown to bind to C5aR2 but the functional role for these interactions are not known [77]. C5aR2 expression is seen in
tissues of myeloid and non myeloid origin. C5aR2 does not have any cytoplasmic tail, therefore for a long time it was thought as a C5a decoy receptor [78]. Recent studies using C5aR2 deficient mice though have suggested C5a-C5aR2 signalling is functional and exhibit anti-inflammatory activity [79, 80].

1.2.3. Role of complement anaphylatoxins in immune response:

The first evidence that complement influences/modulates adaptive immune response was demonstrated more than 40 years ago. A study by Pepys et al. (1975) showed complement C3 depleted (CVF treated) mice show impaired antibody response [81, 82]. Later studies suggested that the antigen bound cleavage product of C3b, C3d, is essential for the optimal antibody response [83-86]. The role of complement has also been studied in T cell mediated immune response. The first evidence for this was reported by Kopf et al. in 2002 where influenza virus infected C3−/− mice showed hampered B and T cell response. Both CD4+ and CD8+ T cell proliferation and interferon gamma production was drastically reduced in C3−/− mice compared to the WT mice [87]. On the other hand, influenza virus infected CR1/CR2−/− mice did not show any altered response suggesting hampered CD4+ and CD8+ T cell response in C3−/− mice is CR1/CR2 independent [87]. Thereafter, several studies suggested role of complement in T cell activation, differentiation and survival [31, 88-90].

During the T cell activation, the interaction of T cells with antigen presenting cells (APC) upregulates the synthesis of complement components. Both APCs and T cells secrete components of the alternative pathway like C3, FB, and FD as well as C5. Along with the increased secretion of complement proteins, expression of complement receptors, C3aR and C5aR, is also upregulated on T cells and APCs [91, 92]. The costimulatory signalling between T cell and APC via CD28/CD80/CD86 and CD154/CD40 leads to downregulation of complement regulator CD55 (DAF). Upregulation of AP components and downregulation of DAF leads to activation of complement cascade in local milieu leading to generation of C3a and C5a anaphylatoxins. These generated anaphylatoxins bind to their respective receptors (C3aR and C5aR) and stimulate APCs and T cells in an autocrine manner. Stimulation of APC and T cell leads to induction and secretion of different cytokines such as IFN-γ, IL-2 and IL-6, IL-23 [91-93]. These cytokines generated in local milieu determines the fate of CD4+ T cell response towards T_H1 or T_H17 [94, 95]. Further, it is also
known that C5a-C5aR signalling in T cells downregulates pro-apoptotic molecule Fas and induces anti-apoptotic protein Bcl-2 which enhance T cell proliferation in complement dependent manner [96]. Interaction of T cell with APC in the absence of C3a-C3aR and C5a-C5aR signalling results in upregulation and enhanced secretion of TGF-β and IL-10 in both APC and T cells [88]. Synthesis of TGF β and IL10 in the local milieu leads to generation of forkhead box P3 (FOXP3) expressing T regulatory cells [88, 97], which have suppressive function. These cells have also been shown to express the receptor for anaphylatoxins C3a and C5a and signalling through C3aR and C5aR in Treg cells diminish their suppressive activity [98, 99].

1.2.4. Regulation of complement system:

Complement is the key component of innate immunity which gets activated as soon as it encounters pathogens. Further, it is capable of acting on almost all the pathogens and it does so by recognizing ‘missing self’ on the pathogens. These ‘missing self’ are complement regulators. And consequently, the complement proteins that are involved in the activation process do not discriminate between self and non-self and have the ability to damage even the host cells. Thus, any uncontrolled activation of complement system leads to damage of host tissues. To inhibit damage of host tissues by complement system, the complement system is tightly regulated by a series of complement regulators, which are called regulators of complement activation (RCA). In addition, regulation is also achieved by non-RCA regulators. The RCA genes are located on the long arm of chromosome 1. The RCA proteins are present in soluble as well as membrane bound forms. The soluble RCA regulators are factor H (FH), factor H-like protein 1 (FHL-1), and C4b-binding protein (C4BP) whereas the membrane bound RCA regulators are membrane cofactor protein (MCP; CD46), decay-accelerating factor (DAF; CD55) and complement receptor 1 (CR1). Although factor H is a soluble regulator, it gets associated with the polyanions present on the host cell surface to protect host cells from the alternative pathway-mediated damage.

The RCA proteins are formed by characteristic bead like domains known as short consensus repeats (SCR) or complement control proteins (CCP). Typically, these CCP domains are composed of approximately 60 amino acids with 4 invariant cysteines, and these domains are linked together by 3-7 amino acid linkers. The numbers of CCP domains in RCA proteins vary from 4 to 56.
Two different mechanisms are employed by RCA proteins to regulate complement activation: i) decay-acceleration activity and ii) cofactor activity. The decay-acceleration activity of RCA protein involves irreversible dissociation of the protease subunit from C3/C5-convertase by the RCA proteins. The decay activity is present in DAF, CR1, FH and C4BP. Among these, CR1 and DAF dissociate CP, LP as well as AP C3 convertase, whereas FH and C4BP are specific for AP and CP/LP C3- and C5-convertases, respectively [100].

In cofactor activity, the RCA protein acts as a cofactor for the factor I-mediated cleavage of C3b or C4b that leads to generation of inactivated C3b (iC3b) or inactivated C4b (C4c and C4d). The RCA proteins which possess cofactor activity include MCP, CR1, C4BP and FH. Among these, CR1 and MCP serve as the cofactor for both C3b and C4b whereas FH and C4BP are specific for C3b and C4b, respectively. The iC3b which is generated can get further cleaved by factor I only in presence of CR1 and to a lesser extent FH [101].

The regulation of complement activation is also achieved by non-RCA regulators. The non-RCA regulators are C1-inhibitor, CD59, vitronectin, clusterin and carboxypeptidase-N. These non-RCA inhibitors act at different steps of complement pathway. C1-inhibitor binds to the classical pathway initiation complex C1 and inactivates its protease subunits C1r and C1s [102, 103]. C1-inhibitor can also inhibit MASP-1 and MASP-2 activity and thereby inhibit the lectin pathway activation [104, 105]. CD59 is GPI anchored protein which binds to the C9 subunit of MAC complex (C5b-9) and thus inhibits the lysis of host cell [106, 107]. Vitronectin and clusterins binds to the lipid binding site of C5b-7 complex which ultimately inhibit formation of MAC [108]. Carboxypeptidase N inhibits the activity of the potent anaphylatoxins (C3a, C4a and C5a) by removing the C-terminal arginine residue which decreases the affinity of these anaphylatoxins to their receptor upto 1000-fold and inhibit their inflammatory activity [109, 110].

1.3. Poxviruses:

Poxviruses are mysterious and fascinating DNA viruses as they differ from other DNA viruses in their ability to replicate in cytoplasm. Poxviruses infect various animal genera from insects to mammals. Poxvirus members, in particular molluscum contagiosum and variola virus, display strict human tropism. However, members of at
least four genera namely orthopoxviruses, parapoxviruses, molluscipoxvirus and yatapoxviruses can infect humans. Intriguingly, vaccinia virus, which was used as vaccine against smallpox, has become feral in India and Brazil and is known to cause sporadic outbreaks in dairy cattle [111, 112].

1.3.1. Classification:

The family Poxviridae is divided into two subfamilies based on the host range. The viruses which infect mammals are classified under the subfamily Chordopoxvirinae and those which infect insects are classified under Entomopoxvirinae. The subfamily Chordopoxvirinae consists of nine genera - Avipoxvirus, Capripoxvirus, Cervidopoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suipoxvirus, and Yatapoxvirus. The subfamily Entomopoxvirinae on the other hand consists of three genera - Alphaentomopoxvirus, Betaentomopoxvirus, and Gammaentomopoxvirus. Each genus comprises of members which are genetically and antigenically related and shares similar morphology and host range. Members belonging to different genera and their characteristic features are summarized in Table 1.1. The two viruses squirrel pox and crocodile poxviruses are unassigned members of chordopoxvirinae.
### Table 1.1: Classification of poxviruses

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Genus</th>
<th>Member</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chordopovirinae</strong></td>
<td><strong>Avipoxvirus</strong></td>
<td>Canarypox, Fowlpox, Junco, Mynahpox, Pigeonpox, Quailpox, Turkeypox, Starlingpox, Sparrowpox, Psittacinepox</td>
<td>Brick shaped, Genome - ~ 260 kbp DNA</td>
</tr>
<tr>
<td></td>
<td><strong>Orthopoxvirus</strong></td>
<td>Camelpox, Cowpox, Ectromelia, Variola, Vaccinia, Taterapox, Monkeypox, Raccoonpox, Skunkpox, Volepox</td>
<td>Brick shaped, Genome~200 kbp DNA, approx 36% G+C content</td>
</tr>
<tr>
<td></td>
<td><strong>Molluscipoxvirus</strong></td>
<td>Molluscum contagiosum</td>
<td>Brick shaped, Genome~190 kbp, G+C content more than 60%</td>
</tr>
<tr>
<td></td>
<td><strong>Cervidopoxvirus</strong></td>
<td>Deerpox</td>
<td>Genome - ~170 kbp</td>
</tr>
<tr>
<td></td>
<td><strong>Leporipoxvirus</strong></td>
<td>Hare fibroma, Myxoma, Rabbit fibroma, Squirrel fibroma</td>
<td>Brick shaped, Genome~160 kbp, 40% G+C content</td>
</tr>
<tr>
<td></td>
<td><strong>Capripoxvirus</strong></td>
<td>Goatpox, Sheeppox, Lamp sheep disease</td>
<td>Brick shaped, Genome~155 kbp</td>
</tr>
<tr>
<td></td>
<td><strong>Parapoxvirus</strong></td>
<td>Bovine papular stomatitis, Orf, Parapoxvirus of red deer, Pseudocowpox</td>
<td>Ovoid in shape, Genome~ ~140 kbp, 36% G+C content</td>
</tr>
<tr>
<td></td>
<td><strong>Suipoxvirus</strong></td>
<td>Swinepox</td>
<td>Brick shaped, Genome ~170 kbp</td>
</tr>
<tr>
<td></td>
<td><strong>Yatapoxvirus</strong></td>
<td>Tanapox, Yaba monkey tumor</td>
<td>Brick shaped, Genome ~145 kbp</td>
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<td></td>
<td><strong>Entomopoxvirinae</strong></td>
<td>Alphaentomopoxvirus</td>
<td>Ovoid in shape, Genome ~260-370 kbp</td>
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<tr>
<td></td>
<td></td>
<td>Melolontha melolontha, Aphodius tasmaniae</td>
<td>Ovoid in shape, Genome ~230 kbp, 18% G+C</td>
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<tr>
<td></td>
<td><strong>Betaentomopoxvirus</strong></td>
<td>Acrobasis zelleri, Amsacta moorei</td>
<td>Brick shaped, Genome ~250-380 kbp</td>
</tr>
<tr>
<td></td>
<td><strong>Gammaentomopoxvirus</strong></td>
<td>Chironomus attenuatus, C. luridus</td>
<td>Brick shaped, Genome ~250-380 kbp</td>
</tr>
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</table>
1.3.2. Structure:

Using cryoelectron microscopy, poxviruses were seen as a brick shaped with a dimension of approximately $360 \times 270 \times 250$ nm. The internal structure of virus contains dumbbell shaped core which consist of viral genome, lateral bodies and the outer envelope. The poxviruses exist in two forms during their replication cycle: the intracellular mature virus (MV) and extracellular enveloped virus (EV). MV consists of dumbbell shaped core with the DNA genome, lateral bodies and lipoprotein membrane whereas EV is a mature virus with an envelope acquired during budding from the infected cell (Fig. 4). The major components of VACV virion is protein (90%) followed by lipid (5%) and DNA (3.2%).

![Figure 4: Structure of VACV virion.](http://www.lacasamorett.com/foxgallery/small-pox-virus-structure.html)

1.3.3. Genome:

Poxviruses are enveloped viruses with large double-stranded DNA as genome. The genome size of poxviruses ranges from 140-300 kb. Avipoxviruses has the largest genome (300 kb) in the *poxviridae* family. Poxviruses have inverted terminal repetitions (ITR) at both the ends of genome which connect the two DNA strands.
These are basically A+T rich, incompletely base-paired and form hairpin loop. The VACV genome contains approximately 10 kbp ITR [113]. The complete genome sequences are available for at least one member of *Chordopoxviridae* genera and it suggest that nearly 100 genes are conserved in all chordopoxvirus genera of which 50 genes are present in the *Entomopoxviridae* [114]. The highly conserved genes are located centrally and these genes are involved in the transcription and maturation of the virus whereas genes located at both the ends are variable and are usually involved in the immune evasion [114-116]. The ORFs from the variable region are unique for different members of poxviruses and therefore are critical for host range dictation. The poxvirus genes are non-overlapping and show temporal expression: early, intermediate and late. The VACV ORFs are named using complete sequence of VACV Copenhagen strain, digested with *HindIII* endonuclease fragment letter (letters A to P), followed by ORF number (assigned sequentially from left to right) and transcription direction (left (L) or right (R) depending on the transcription direction of the gene).

Poxvirus proteomics analysis performed with VACV virion suggested around 80 viral proteins are present in the mature virus which are necessary for replication of the virus [117-119]. The EV has 6 additional membrane proteins among which 5 proteins are glycosylated (A33, A34, A56, B5 and K2) and F13 is non-glycosylated. The A34 and F13 proteins are conserved in all chordopoxviruses. Studies with A34 gene deletion virus indicated that this protein is involved in the entry of the virus in host whereas the F13 gene is required for efficient wrapping of the virus.

1.3.4. Life cycle or replication of poxviruses:

As mentioned earlier, the uniqueness of the poxvirus is despite being a DNA virus, it replicates entirely in the cytoplasm with no involvement of nucleus for replication. The transcription, translation and replication of viral genome occur in a discrete region in the cytoplasm known as ‘virus factories’. These factories are enclosed by endoplasmic reticulum membrane and are devoid of other cellular organelles [120]. The life cycle of poxvirus can be divided into following steps: entry, transcription, translation, DNA replication, virion assembly and morphogenesis.
1.3.4.1. Poxvirus entry:

As mentioned earlier, poxviruses have two distinct forms and the proteins which are expressed on these two are different from each other. Therefore, MV and EV are antigenically different and use different mechanism for their entry into the cells [121, 122]. However, till date, not much is known about the cellular receptor for poxviruses and how the virus entry exactly happens inside the cell.

**MV entry:** MV has been shown to enter host cells by fusing with plasma membrane (at neutral pH) or by endocytosis (at low pH) [123, 124]. Endosomal acidification facilitates entry of virus into the cell cytoplasm. MV attachment is mediated by 4 different viral proteins, while its entry is mediated by 12 proteins. The attachment protein A26L binds to laminin, while A27L and H3L interact with heparan sulfate and D8L with chondrotin sulfate [122].

**EV entry:** For EVs, the viral proteins involved in attachment are not identified but studies have suggested that the virus binds to cell membrane glycosaminoglycans (GAG). The main route for EV entry is endocytosis. EV utilizes actin tailing for efficient spread of virus and the A33, A34 and A36 play crucial role in actin polymerization [125-127]. Therefore, deletion of these proteins results in reduction in virus spread [128]. Though EV contains additional membrane, the attachment is different in MV and EV but this additional membrane of EV is discarded in the endosome and entry of EV is mediated by fusion similar to that of MV [129]. At the plasma membrane, fusion of MV membrane with host cell membrane is mediated by entry-fusion complex (EFC) [130]. Mutational studies suggested that 12 different viral proteins (A16, A21, A28, F9, G3, G9, H2, I2, J5, L1, L5 and O3) are a part of fusion entry complex and are involved in the entry of virus into the cells [12] and these proteins do not participate in the attachment of virus to the cells [130]. The spread of EV is enhanced by inhibiting re-infection of the already infected cells. This is achieved by expression of A33 and A36 on the plasma membrane of infected cells that caused virion repulsion and directs the viruses to uninfected cells [128].

1.3.4.2. Transcription and translation:

Poxvirus genes transcription and translation is temporal and fall into early, intermediate and late classes based on their time of expression [131]. The fusion
process leads to release of virus core into the cell cytoplasm and then it is transported to the site of transcription through microtubules [132, 133].

A. Early gene expression: The released core contains early transcription system which initializes transcription of the early genes. The early genes are detected as early as 20 minutes after infection [134]. During early genes transcription, half of the genome gets transcribed and they majorly encode for the proteins which are involved in the DNA replication [135-137], transcription of intermediate genes [138-140], nucleotide biosynthesis [137, 141] and immune evasion. The cessation of early genes expression leads to disruption of viral core known as uncoating. The uncoating of virus core leads to transition of early gene expression to the DNA replication (discussed in detail in separate section). Poxvirus DNA replication starts at 1-2 hr after infection and is essential for the intermediate and late gene expression. Inhibition of DNA replication leads to prolonged expression of the early genes and inhibit intermediate and late gene expression [142].

B. Intermediate gene expression: Intermediate gene expression is seen shortly after the initiation of DNA replication [143]. The intermediate genes are detectable after 90-120 min after infection [134] and till date 53 genes in VACV genome are known to belong to intermediate class [144]. Intermediate genes encode for late transcription factors [134].

C. Late gene expression: The late gene expression follows intermediate gene expression and continues till the end of virus life cycle. The late genes are mostly clustered on the central region of the genome. Recent studies have identified 38 genes in the VACV genome that belong to the late class and these genes encode proteins which are involved in morphogenesis as well as proteins belonging to mature virion membrane, entry and early transcription factors [144].

1.3.4.3. DNA replication:

As mentioned earlier, replication of poxviruses is entirely cytoplasmic unlike other DNA viruses. Most of the DNA viruses utilize DNA replication enzymes from the host which are found in the nucleus, whereas poxviruses encode their own replication machinery. DNA replication of poxviruses starts 1-2 hr after infection in the viral
factory. The enzymes required for the nucleotide biosynthesis and DNA replication are synthesized during early gene transcription [135, 141].

The exact mechanism of poxviral DNA replication is not clear. But several models have been proposed for the poxviral DNA replication. A self priming model is proposed for the poxviral replication, wherein the hypothetical nick occurs at hairpin loop in one or both the stands of poxviral genome which generates free 3’ -OH in the genome, for priming replication. The nuclease responsible for nicking though is not yet identified. The replicated DNA then folds back which allows the replication complex to synthesize the remaining part of the genome. The first formed concatemers can form a very large, joint and branched concetemers due to several new rounds of DNA replication [145]. The large concatemers are resolved in the beginning of late gene expression. The inverted complementary loops of genome are regenerated which leads to formation of duplicate poxvirus genome. Another conventional discontinuous model is hypothesized on the basis of early reports which showed that VACV DNAs are covalently linked with RNA. This covalently linked RNA serves as a primer for the replication of the poxviruses DNA leading to formation of lagging DNA strand [146-148]. Next it is hypothesized that the lagging strand produced is then filled using virus-encoded DNA ligase. But till date no virus-encoded DNA ligase is identified [12]. Many of the poxvirus replication enzymes are produced early during infection. The viral DNA polymerase is comprised of several proteins including E9L (DNA polymerase enzyme with 3’ exonuclease activity) [149-151], D5R (helicase and primase activity) [152], D4R (DNA repair) [153] and A20R (processivity factor) [154]. VACV A50R encodes a 63 kDa protein which functions as ATP-dependent DNA ligase enzyme. The H5R protein is known to play roles in transcription, mRNA processing and morphogenesis [155-159] (Fig.5).

1.3.4.4. Virion assembly and morphogenesis:

Upon synthesis of late protein synthesis, the virus assembly starts within the viral factories. The virus assembly beginning is characterized by formation of crescent-shaped structures in the viral factories [160, 161]. The crescents are formed with single lipid bilayer membrane and external honeycomb lattice layer formed by viral proteins [162]. These crescent structures are surrounded by newly formed viral genome. Till date, the mechanism of crescent membrane formation is not clear but
recent studies suggest that the endoplasmic reticulum can be a source of membrane [163]. D13, A14 and A17 gene products have been shown to be crucial for the crescent formation along with 6 regulatory proteins which include A6, A11, F10, H7, L2 and A30. D13 trimer arranged in hexagon forms a lattice which binds ER to form a crescent precursor [164], whereas A14 and A17 proteins span the viral membrane [165, 166].

During the extension of crescent to immature virus (IV), the dense viroplasm containing core proteins are integrated into the crescent. Next, viral DNA along with transcription apparatus are enclosed before the crescent spheres are sealed [162, 167] to form IV. The detailed mechanism of viroplasm and DNA incorporation into crescent is unknown, but various studies suggested that a complex of 7 viral core proteins is essential for the incorporation of viroplasm during IV formation. This complex includes A15, A30, F10, J1, D2, D3 and G7. The mutant virus of any protein in the complex accumulates empty IV with no defect in the viral membrane [168-170]. The mechanism and viral proteins involved in enclosing DNA into IV is not yet known. The gene product of A32 is predicted to be involved in DNA packaging because it shares sequence similarity with the adenovirus DNA packaging enzyme IVa2 [171].

The following step in poxvirus morphogenesis is transition of spherical IV to the barrel shaped mature virus (MV). This transition is achieved by loss of D13 scaffold from IV [172] and also by proteolysis of viral proteins. Five core proteins A3, A10, A12, G7, L4 and membrane protein A17 undergo proteolytic cleavage during IV to MV transition [163]. The MVs are non-enveloped and have the ability to infect cells but they are released upon lysis of the infected cells. Subsequently, some MV particles undergo wrapping with endosome or trans-golgi membrane to form the wrapped virus (WV) [173, 174]. The process of wrapping incorporates A33, A34, A36, A56, E2, B5, F12, F13 and K2 viral proteins [164]. Then, WV moves on microtubules towards the cell membrane and this result in fusion of WV membrane with the cell membrane. The fusion of WV to the cell membrane leads to generation of virus particle with two membranes known as EV which is released by exocytosis [175] (Fig.5).
Figure 5. Steps involved in VACV replication: (1) attachment, entry of virus and release of core into the cytoplasm; (2), transcription and translation of early mRNA; (3), uncoating of core; (4), replication of DNA; (5), transcription and translation of intermediate gene; (6), late gene transcription and translation; (7), assembly of membrane structure; (8), formation of immature virus; (9), mature virus formation; (10), golgi wrapping of mature virus (11) enveloped virus for transportation to cell periphery; (11), release of EV form of VACV (adapted with modification from Grant McFadden, Nature Reviews Microbiology, 2005).
EV remains attached to the cell membrane till formation of actin microvilli under the cell-associated EV (CEV). The viral proteins A33, A34 and A36 are essential for formation of actin tail. The actin tail in CEV directs the virus to infect the neighbouring cell and deletion of A33, A34 or A36 results in hampered cell to cell transmission [128] (Fig.5).

1.3.5. Poxvirus pathogenesis:

In Chordopoxviridae subfamily, only the members of Parapoxvirus, Molluscipoxvirus, Yatapoxvirus and Orthopoxvirus infect humans. After infection, pathogenesis is dependent on the mode of transmission, infecting species and immune system of the host.

A. Parapoxvirus: The parapoxvirus usually infects goat, sheep and cattle, but human infection occurs due to contact with the infected animals. Parapoxvirus infection in humans occurs via cuts and scratches. The infection remains localized and produces lesion in 1-2 weeks after infection. The lesion is characterized by formation of red centre which is surrounded by a white circle. The lesion grows to nodular stage followed by papillomatous stage. Parapoxvirus infection does not have long term effect and resolves within 6-8 weeks.

B. Yatapoxvirus: The yatapoxvirus infection in humans occurs owing to infection by its member tanapox virus. The first evidence was documented in 1957 in Kenya [176]. Yatapox pathogenesis is not clearly understood. The infection in monkeys and human is thought to be mediated by an insect vector [177]. The clinical manifestation includes short (2-4 days) febrile illness. The lesions appear within 1-2 weeks and are characterized as a hyperpigmented macule with central elevation. The infection in human is rare and resolves within 6 weeks [178].

C. Molluscipoxvirus: Molluscipoxvirus species Molluscum contagiosum virus is an obligate human pathogen which does not lead to systemic infection but causes a localized disease. The virus is transmitted to humans by contact with infected skin or contaminated surface. About 2 to 5 mm diameter dome-shaped lesions are formed. The disease usually resolves 6-9 months post infection [12].

D. Orthopoxvirus: Among family Poxviridae, the orthopoxviruses have a major impact on humans primarily because of their most notorious member variola virus
which causes smallpox in humans. Consequently, the pathogenesis of orthopoxviruses is also studied extensively. Apart from variola virus, other members like monkeypox, cowpox and vaccinia virus can also infect humans. The orthopoxvirus infection can be localized or systemic.

i) **Variola virus (VARV):** VARV is the causative agent of the smallpox disease. The entry of the virus is majorly through aerosol or through the contact with contaminated surface. After infection, there is a period of 12-14 days of latent asymptomatic incubation period, which follows influenza like symptoms. These symptoms include fever (upto 103°F), headache, backache and prostration, which is followed by appearance of pustular rash and spreading of rash to the entire body [179]. During this period the inhaled virus migrates towards nearest lymph nodes. The lymph nodes serve as primary replication site for the virus and by day 13 the virus enters into the blood stream and infects and multiplies at secondary sites of infection which include spleen, bone marrow and lymph nodes. After 10-13 days, the pustules attain their maximum size and gradually get dried to form scrubs. The dried scrubs fall off by 30-40 days after infection. There are numerous complications associated with smallpox disease which include keratitis, corneal ulcers (which may lead to blindness), hearing impairment and secondary infections. The virus transmission can occur throughout the disease course, but it is most contagious from development of skin lesions till lesions turns into scabs. The severe form of smallpox disease is caused by variola major which has ~30-40% mortality. The variola minor however exhibits ~1% mortality [180]. The majority of smallpox disease survivors are scarred for life with a large number of blinds. WHO lists VARV as a top bioterrorism agent due to its high mortality rate, unvaccinated population and no effective treatments [181].

ii) **Monkeypox virus:** The primary host of the monkeypox is monkeys and ground squirrels are considered as the reservoir host. The entry of the virus is believed to be through aerosol route or through contact with infected animals, and the disease pattern is similar to that of smallpox disease. The disease mortality is approximately 10% of infected individuals.

iii) **Cowpox virus (CPXV):** Cowpox virus is endemic in rodent population in Europe and Asia [182, 183]. Human infection of cowpox is through close contact with the infected domestic animals [184]. In humans, the cowpox disease is localized and
mostly single lesion is seen, which is restricted to hands and face. In immunocompromised persons however, the virus causes severe infection that may result in death. In normal individuals, the cowpox disease is resolved by 6-8 weeks after infection.

iv) Vaccinia virus (VACV): The origin and the natural host for the VACV are still unknown. VACV is the most studied member of the poxvirus family and is known to have a broad host range. Till date, several variants of VACV have been described. In India, a variant of VACV buffalopox has been shown to cause disease to their caretakers [111]. As mentioned earlier, as vaccinia virus was used for vaccination against the smallpox disease the pathogenesis is studied extensively. VACV usually cause mild or asymptomatic infection in the healthy individual. Within 3-5 days post vaccination a papule is developed at the site of vaccination with swelling and tenderness in the draining lymph node and papules becomes vesicle which reach to their maximum size by day 8-9 and become pustular. The lesions formed then get dried from day 10 and by day 14-21 typical vaccination scar is formed at the site of vaccination [185]. Major complications caused by vaccinia during smallpox vaccination were relatively low. The administration of VACV in the immunocompromised persons can cause severe complications. These complications include progressive vaccinia, eczema vaccinatum, generalized vaccinia, postvaccinial encephalitis and carditis [12]. In progressive vaccinia complication, the virus spreads to the other body sites and leads to necrosis. Eczema vaccinatum is a clinical complication which usually occurs in persons with a history of atopic dermatitis. The clinical symptoms include localized or generalized papular, vesicular, or pustular rash or localized to previous eczematous lesions [12].

1.3.6 Animal models of orthopoxvirus infection:

Though variola virus, the causative agent of smallpox disease, has been eradicated, the concern is that it can be used as a bioterrorism agent. Hence, there is increased interest in understanding poxvirus pathogenesis [186, 187]. Moreover, zoonotic infections of monkeypox and cowpox viruses are on the rise which also renewed the interest in understanding poxvirus pathogenesis. Animal models that have been developed to study virus pathogenesis are described below.
A. VACV infection models: VACV is the most extensively studied virus for the poxvirus pathogenesis. The majority of studies have been done in mouse and rabbit animal model. In mouse, a variety of infection routes have been studied. These include intranasal (IN), intradermal (ID), intravenous (IV), intraperitoneal (IP) and footpad scarification. Among the various routes of infection in mouse, IN route is most studied as it mimics the natural route of disease. The IN administration of VACV was shown to cause lethal infection in BALB/c mice and the viral load correlated with the body weight loss [188, 189]. Studies using ID route of infection in mouse ear pinnae demonstrated that lesions formed at site of infection are localized and do not spread to other body parts. The onset of lesion takes about 5 days, peaks at day 8-10, and resolves by day 22 [27, 190]. The lesion size in mouse ID infection model was shown to be affected by the age and strain of the mice [191].

In rabbit ID infection model, VACV was demonstrated to cause localized skin lesion at the site of infection and a mild disease [192, 193]. However, the disease course was found to be affected by the age of rabbit as ID infection of VACV caused systemic infection and higher mortality in 9 weeks old rabbit compared to 6 months old rabbits [194].

B. Rabbitpox virus infection models: Rabbitpox virus is considered as a subspecies of the VACV which is virulent in the rabbits [195]. Studies with intradermal infection model of rabbits showed appearance of lesion within 1-2 days post inoculation of virus which then grow to pustular with black and necrotic centre. The disease course is dependent on the age of the rabbits. Older rabbits (9 weeks old) developed systemic disease with 100% mortality whereas 6 months old rabbits showed 80% mortality along with similar disease sign [194].

C. Ectomelia virus infection models: Ectomelia virus (ECTV) has been studied extensively in mouse model using IN, ID, IV, subcutaneous (SC), IP, footpad scarification and intracerebral (IC) route [196]. The pathogenesis of ECTV is similar to the VARV pathogenesis with localized and systemic infection [196, 197]. The most commonly studied method used for ECTV infection is footpad scarification which causes primarily localized infection in regional lymph node which is followed by systemic spread of virus into other organs [196, 198]. The susceptibility of mouse for ECTV differs with the strain and immune status of the animal; BALB/c and DBA/2
have been shown to be highly susceptible whereas C57BL/6 and AKR are relatively more resistant to ECTV infection [196, 198].

D. Monkeypox virus infection models: Monkeypox virus (MPXV) has been studied extensively in different animal models like mouse, prairie dogs, squirrels and non-human primates with different routes of infection. Infection in mice was studied using intranasal [199] and intraperitoneal [199, 200] infection route in 8 days old mice and found them to be susceptible to the MPXV infection. The infected mice showed disease symptoms which included weight loss, fever malaise, rash and lymphadenopathy. Mice with intact immune systems showed resistance to the MPXV infection when inoculated through with IN and IP route [201, 202].

Monkeypox is most extensively studied in nonhuman primate animal model. Both rhesus and cynomolgus macaques are sensitive to the monkeypox virus infection. Cynomolgus macaques display severe disease and mortality compared to the rhesus macaques [203, 204]. Several different infection routes were studied for MPXV infection which included IN, IV and intrabronchial [205-207]. In intranasal infection route, macaques showed symptoms which included mild anorexia, depression, fever, and lymphadenopathy on day 6 post exposure [205]. Intravenous infection of MPXV in rhesus macaques leads to widespread vesiculopastular rash along with lymphadenopathy [208].

The pathogenesis of MPXV is also studied in prairie dogs using IP [209], IN [209, 210] and ID route [210]. IP inoculation of MPXV resulted in 100% mortality whereas in IN infection model, MXPV showed 60% mortality [209]. Animals infected with IN route showed severe haemorrhage, pulmonary edema and necrosis compared to the IP route of infection [209].

1.4. Complement and Viruses

As discussed in the earlier section, complement also acts as a first line of defence against viruses. The entry of viruses in the host results in activation of the complement system owing to deposition of complement components on their surface. Such virus-mediated complement activation has been shown to limit virus infection by two ways: i) by direct neutralization of the viruses by complement, and ii) by enhancement and mobilization of virus-specific adaptive immune response. This
section describes various examples where complement has been shown to either directly neutralize viruses and/or induce virus-specific adaptive immune response.

### 1.4.1. Direct neutralization of viruses by complement:

Complement-assisted neutralization of virus was reported as early as 1930. In this report, Douglas and Smith [211] showed that the thermolabile constituent of the rabbit serum has viricidal activity. Later many other reports implicated the role of complement in virus neutralization. In a nutshell, all the three major complement pathways have been shown to neutralize viruses. These neutralizations are either direct or indirect. The direct neutralization of viruses is achieved through aggregation, opsonisation or virolysis whereas the indirect neutralization is due to phagocytosis of opsonised virion [13, 20] (Fig. 6).

**Figure 6: Mechanism of virus neutralization by the complement system.** A. coating or opsonisation of viral particles by complement opsonin proteins like C3b or C4b, B. aggregation of viruses by C1q or ficolins, C. loss of viral integrity by membrane attack complex, D. phagocytosis of opsonised viruses by phagocytes.

A. Aggregation: The antibodies generated during the viral infection have ability to aggregate virus particles because of their ability to bind to two or more particles. Such property is also shown by complement proteins. For example, complement-dependent aggregation of viruses has been shown for simian virus 5 [212], and influenza virus.
Although the molecules responsible for this have not yet been identified. Additionally, C1q has been shown to aggregate polyoma virus [214] and more recently serum ficolin H has been shown to aggregate Influenza A and prevent its entry [215].

**B. Opsonisation:** Activation of complement on the viral surface leads to generation of the opsonin molecules such as C3b and C4b. Both C3b and C4b molecules contain a highly reactive internal thioester bond formed owing to intramolecular transacylation reaction between the thiol group of cysteine and the $\gamma$-amide group of the glutamine. This bond allows the formation of ester or amide linkage with the viral surface resulting in coating of viruses with C3b and C4b. Such coating with complement proteins can create steric hindrance during attachment, entry or it can hamper uncoating of the virus. The viruses which gets neutralized due to opsonisation includes influenza [213, 216, 217], new castle disease virus [218], HTLV-1 [219], HIV-1 [220], gc-Null herpes simplex virus (HSV) and West Nile virus [221]. The opsonising molecules required for neutralization varies for different viruses. HTLV-1 [219] and influenza viruses [216] get neutralized with C1q alone, while HIV-1 [220] and West Nile virus [221], require complement proteins C1 to C3. Neutralization of gc-Null HSV on the other hand requires coating with components C1-C5 [222, 223].

**C. Virolysis:** Apart from opsonisation, complement activation on the viral surface also leads to formation of membrane attack complex (MAC) that disrupts the viral membrane integrity. Thus, virolysis results in loss of infectivity. The viruses which are known to be neutralized by virolysis are coronavirus [224], alphavirus [225] and herpesviruses [226]. However, many enveloped viruses acquire CD59 from the host membrane to inhibit MAC-mediated virolysis. Direct binding of C1q to the surface proteins of coronavirus [4] leads to activation of classical pathway and results in lysis of the virus.

**D. Phagocytosis:** Viruses coated with complement proteins C3b or C4b or their degradation products (iC3b and C3dg) are recognized by the complement receptors CR1, CR2, CR3, CR4 and CR1g (complement receptor of the immunoglobulin superfamily) of the phagocytic cells. For example, CR1 and CR2 present on the phagocytic cells can recognize C3b/ C4b and C3dg/iC3b coated viruses, respectively. The iC3b coated viruses can also be recognized by CR3 and CR4. CR1g however can
recognize C3b as well as iC3b coated viruses. The interaction of opsonins with their respective complement receptors facilitates phagocytosis. This type of neutralization is seen in case of HSV [227, 228] and Japanese encephalitis virus [229].

1.4.2 Role of complement in enhancement and mobilization of virus-specific adaptive immune response

A number of in vivo studies have been performed to study the role of complement in viral infection. The effects seen in these studies can be attributed to direct neutralization of the virus by complement as well as the crosstalk between the complement system and the adaptive immune system, which is crucial for efficient generation of the virus-specific immune response.

Before the availability of complement knockout mice, the in vivo role of complement was studied either by utilizing genetically complement-deficient animals or by depleting complement using cobra venom factor (CVF). When CVF treated mice were infected with rabies virus they showed increase in viral load compared to the wild-type mice [230]. On a similar line, C5 deficient mice and CVF treated mice showed prolonged viremia after infection with influenza virus [231] and sindbis virus [232, 233]. Although these studies pointed out the role of complement in clearance of virus, the exact mechanism involved in clearance was not clear.

Role of complement on B cell activation was first demonstrated in 1996 by Dempsey et al. [83] though the indirect evidences were available as early as 1974 [81]. In this study, the authors showed that attachment of antigen to C3d, the cleaved product of C3b, results in 1000-10,000-fold increased humoral immune response compared to the antigen alone suggesting C3d is essential for the optimal antibody response [83]. Further, they also showed that binding of C3d to B cell expressed CR2 receptor facilitates antigen presentation to the B cells and induce B cell signalling leading to activation of B cells [83-85]. Later, studies also illustrated the role of C3d-CR2 interaction in induction and maintenance of memory B cells in the germinal centre. It was observed that during development of memory B cell in the germinal centre, B cell encounters C3d tagged antigen complex on follicular dendritic cells [86] and this result in generation of effector and antigen specific memory B cell [86].
Examination of role of complement in augmentation antibody response to viruses was not studied until 1999. The first study in this direction was performed by Carroll and his colleagues [234]. During HSV infection in C3, C4 or CR1/CR2 (CD21/CD35) deficient mice, they observed significant reduction in IgG response compared to the wild-type mice [234]. Later, the same group [235] performed reconstitution of bone marrow from wild-type mice to the C3-deficient mice and showed that the C3-deficient mice developed normal antibody response when reconstituted with bone marrow from wild-type mice following HSV infection suggesting critical role of complement proteins synthesized by non-hepatic cells [235]. Interestingly, during LCMV and VSV infection in C3−/−, C4−/− and CD21/CD35−/− mice did not show any alteration in the B-cell response [236].

During West-Nile virus infection, the C3 and CR2 deficient mice showed higher viral load and mortality compared to wild type mice. C3 and CR2 deficient mice showed impaired WNV specific IgM and IgG response suggesting role of complement dependent B cell response in controlling WNV infection [237]. Further study in C1q, C4, FB and FD deficient mice showed increased viral titer and mortality in these mice suggesting all the three complement pathways are crucial for conferring protection against WNV infection. FB and FD deficient mice showed reduced CD8+ T cell response and normal IgM and IgG response compared to wild-type mice, whereas C1q and C4 deficient mice showed hampered IgM and IgG response suggesting T cell response is dependent on alternative pathway whereas development of WNV-specific antibody response is dependent on classical pathway [238].

Using influenza virus infection model, Fernandez GS et al. studied role of complement in generation of B cell response in mice deficient in C3, CR1/CR2, IgM and C3/IgM. They observed that complement and natural antibodies perform a prerequisite role in generation of long term B-cell memory response [239]. Using complement deficient mice Kopf et al. studied the role of complement in cell mediated immune response and showed delayed virus clearance in C3−/− mice. This delayed virus clearance was due to impaired T cell priming, and migration of effector T cells to the lung [87]. Kandasamy et al. recently confirmed that impaired priming of T cells in C3−/− mice was a result of defect in dendritic cells-mediated transport of viral antigen to the dLNs [240]. Recently, our laboratory studied the relative role all the
three pathways during pandemic influenza A (H1N1) 2009 virus infection. We observed that synergy between the classical and alternative pathway is a requisite for efficient protection against the virus [241] and complement synthesized by B cell is critical for protection. Further, our results also pointed out that C3a-C3aR signalling is essential for optimal generation of the protective immune response [241].

Similar to influenza virus, ectomelia virus also shows requirement of natural antibodies and complement for protection against infection. Infection of ectomelia virus in C3, C4, μMT and factor B deficient mice showed higher mortality suggesting complement and antibodies are required for the protection. The C3 deficiency leads to higher dissemination of virus to the other organs. Further in vitro analysis using depleted sera demonstrated that the primary mechanism for neutralization of virus was opsonisation of virus with C3b and C4b [242].

1.4.3. Complement evasion by various viruses:

It is apparent from the above mentioned studies that complement exerts a strong selection pressure on viruses. This pressure therefore must drive adaptation in viruses to improve their fitness in the host. Adaptations that have been identified in viruses can be broadly divided into: i) encoding the structural and functional homologues of host complement regulators or molecular mimicry of host complement regulators, ii) use of complement regulators and receptors for cellular entry and iii) acquisition of host complement regulators. Apart from the above, viruses are also known to encode proteases which can cleave complement components and inactivate them [14, 243].

1.4.3.1. Encoding of structural and functional homologues of host complement regulators: Large DNA viruses encode proteins that are structural and functional mimics of human complement regulators. It is believed that during evolution, viruses acquire these genes from the host by horizontal gene transfer. These homologs primarily belong to a gene family termed “regulators of complement activation” (RCA) and only two virus families namely poxviridae and herpesviridae are known to encode RCA homologs. Apart from RCA homologs, herpesvirus viruses are also known to encode a homolog of CD59 which prevents MAC formation.

The viral RCA regulators mimic human RCA proteins in that they are also composed of bead like structures termed complement control protein (CCP) domains. Typically,
these CCP domains are composed of approximately 60 amino acids with 4 invariant cysteines which form disulfide bonds in a 1-3, 2-4 pattern and are linked together by 4 amino acid linkers. The number of CCP domains in viral RCA proteins vary from 3-8, whereas in human they vary from 4 to 56. Functionally, viral RCA resembles human RCA in that they also exhibit: i) decay accelerating activity for CP and AP, and ii) cofactor activity for complement proteins C3b as well as C4b.

1.4.3.1.1. Poxviral complement regulators:

The Orthopoxvirus genera in the *Poxviridae* family encode complement regulators which exhibits approximately 35% homology with host RCA proteins, but amongst them, they exhibit more than 90% homology. This high homology and phylogenetic analysis between poxviral complement regulators suggests recent split in their lineage [244]. The poxviral complement regulators from vaccinia virus (VCP), variola virus (SPICE), ectomelia virus (EMICE) and monkeypox virus (MOPICE) are studied very well and amongst these, vaccinia virus complement control protein (VCP) was the first to be identified [19].

The discovery of VCP was accidental. While characterizing an attenuated vaccinia virus it was observed that left end of its genome had a major deletion. Sequence analysis of the deleted region revealed that one of the ORFs (C21L; 21st open reading frame from left end of Hind III C-fragment) encodes for a CCP domains containing protein. Structurally, the encoded protein was entirely formed by four short consensus repeats (SCR) or complement control protein (CCP) domains and exhibited sequence similarity with human complement regulators MCP (35%), DAF (38%), CR1 (37%), C4b-binding protein (37%) and factor H (26%) (Kotwal and Moss, 1988). Later, functional characterization of this protein purified from the culture supernatant of VACV infected cells revealed that it possesses the ability to bind to complement proteins C3b and C4b and inactivate the classical/lectin and alternative pathway C3-convertases by accelerating the decay of C3-convertase and by the factor I-mediated inactivation/cleavage of C3b and C4b [11, 21, 22], the subunits of C3-convertases. Our laboratory further characterized the domain level functional activity of VCP using domain deletion and domain swapping strategies and demonstrated that VCP domain 1 is essential for the CP C3-convertase decay activity whereas domains 1, 2 and 3 are necessary for imparting AP C3-convertase decay [23, 28]. The study also
revealed that domains 2 and 3 are critical for the cofactor activity [23, 28]. In vitro studies also showed that VCP inhibits the complement-mediated neutralization of MV as well as EV [18, 245].

Similar to VCP, a homologue of RCA is also encoded by the smallpox virus (variola virus). This regulator is also formed by four CCP domains and is dubbed as SPICE (smallpox inhibitor of complement enzymes). VCP and SPICE differ from each other only by 11 amino acids, which are distributed in CCP domains 2, 3 and 4. Rosengard et al. in 2002 demonstrated that SPICE is 100-fold and 6-fold more potent in inactivation of human complement components C3b and C4b respectively, compared to VCP [25]. Another study by Sfyroera et al. showed SPICE inhibits CP and AP, 75- and 1000-fold more efficiently compared to VCP [246]. Further, using point mutagenesis approach our laboratory identified the residues which are important for its enhanced activity against human complement and demonstrated that the enhanced activity of SPICE towards human complement proteins is governed by the its 4 variant amino acids [247]. Next, our laboratory also showed that VCP and SPICE display species specificity in inactivation of alternative pathway - SPICE efficiently inactivates human alternative pathway, while VCP is potent towards bovine alternative pathway and the difference in their activity is due to the charge reversal in domain 2 and 3 [26]. Very recently we have shown that VCP also displays specificity towards bovine classical pathway and this is mediated primarily by its acidic residues situated in domains 2 and 3 [248].

Another complement regulator EMICE is encoded by ectomelia virus. Like VCP and SPICE, it has 4 CCP domains. It was found that it possesses both C3b and C4b cofactor activity and CP C3 convertase decay activity. It regulates complement activation on MV as well as on infected cells [249].

Monkeypox virus also encodes a complement regulator termed as MOPICE. Unlike the other poxviral regulators MOPICE is composed of only three CCP domains and truncated fourth domain due to frame shift mutation in CCP4. In vitro studies with MOPICE exhibited only C3b and C4b cofactor ability with no decay acceleration activity for both AP and CP [250]. The MOPICE protein is however only present in more virulent virus i.e., Congo Basin strain [251]. Recently Hudson et al. showed that incorporation of MOPICE in less virulent strain like West African strain increased
clinical manifestation but did not increase mortality, suggesting MOPICE plays only a moderate role in monkeypox pathogenesis [252].

1.4.3.1.2. Herpesviral complement regulators:

Another family of large DNA viruses known as *Herpesviridae* also encodes for complement regulators. The members of subfamily *Alphaherpesvirinae* encode for the non-RCA complement regulators (e.g., Herpes simplex virus 1 and 2) whereas the members of subfamily *Gammaherpesvirinae* encode for RCA complement regulators. Unlike poxviruses, RCA regulators which show 90% homology with each other, herpesviruses RCA regulators display only 43-89% homology with each other and also exhibit sites for glycosylation. The gammaherpesviruses that encode homologs of RCA are Kaposi’s sarcoma associated herpesvirus (KSHV), herpesvirus saimiri (HVS), rhesus rhadinovirus (RRV), murine γ-herpesvirus 68 (γ-HV68).

The complement regulator of Herpesvirus saimiri (HVS) known as HVS-CCPH was the first identified and studied RCA in herpesviruses [253]. The ORF4 of HVS encodes for RCA homologue which can be membrane bound or soluble form due to differential splicing [254]. Initial studies with CCPH indicated that it inhibits complement activation and deposition of C3d on the infected cells [253]. Later our laboratory showed that CCPH possesses cofactor activity for both C3b and C4b and decay acceleration activity against CP C3-convertase [255]. The mutational analysis to dissect the functionally important sites in CCPH suggested that the functional sites for C3b and C4b are different and ionic interactions play important role in its functionality [256].

Kaposi sarcoma associated herpesvirus (KSHV) ORF4 encodes for a RCA protein. This protein was studied simultaneously by two different groups and named by our group as Kaposi’s sarcoma-associated herpesvirus inhibitor of complement activation (Kaposica) [257] and others as Kaposi’s sarcoma-associated herpesvirus complement control protein (KCP) [258]. The full length protein contains four CCP domains followed by a serine/threonine rich region and a transmembrane domain [254, 258]. The protein contains three putative N-linked glycosylation sites that are located in CCP1, 2 and 4. Initial characterization of Kaposica suggested that it has both C3b and C4b cofactor activity and also C3-convertase decay acceleration activity for CP and
Our group further characterized these activities at the domain level and showed that domain 2 and 3 are essential for the cofactor activity while domain 1 and 2 are crucial for the decay acceleration activity [259]. Recently, our group identified that two patches of residues present in CCP2 and 3 are critical for its cofactor activity against C3b and C4b. The 1st patch present in CCP2 is critical for interaction with factor I, while the 2nd patch present in CCP3 is crucial for bridging CUB with TED domain [260].

1.4.3.2. Use of complement regulators and receptor for cellular entry:

To evade host’s complement attack efficiently, viruses also exploit complement regulators and receptors for their entry. Complement regulators like CD46 (MCP) and CD55 (DAF) are expressed ubiquitously on the host cells, and hence use of these regulators for entry can be dubbed as an efficient subversion mechanism. The entry of the viruses through the complement regulators/receptors is either direct or indirect. In direct entry, viral proteins directly interact with complement regulators/receptors whereas in the indirect entry, the complement fragments deposited on the viral surface are recognized by the complement regulators/receptors present on the host cells. Viruses belonging to adenoviridae, flaviridae, herpesviridae, paramyxoviridae, picornaviridae and retroviridae family are known to use complement receptors for their entry [14].

Complement regulator MCP is used as receptor by four human specific viruses (and five human specific bacteria) including adenovirus group B and D [261, 262], human herpesvirus-6 (HHV-6) [263] and measles virus [264]. Additionally, bovine viral diarrhoea viruses utilize bovine CD46 as a receptor for viral entry. For attachment and entry, different viruses target different CCP domains of MCP. Specifically, HHV-6 glycoprotein H binds to the CCP2 and 3 of MCP for their entry whereas, adenovirus protein fiberknob [265] and measles virus envelope protein hemagglutinin H [266, 267] have been shown to interact with CCP 1 and 2 [268, 269]. Binding of adenoviruses with MCP is followed by clathrin mediated-endocytosis [270].

Another complement regulator, DAF, is targeted by the members of family picornaviridae which include coxsackievirus, echoviruses and enterovirus 70. It has been observed that the DAF cannot trigger cellular entry; instead, it acts as a co-
receptor and facilitates viral attachment. Similar to MCP, for attachment and entry, different viruses target different domains of DAF [271-274]. Unlike the above viruses, Epstein-Barr virus (EBV) facilitates its entry into B cells through CR2 as well as CR1 receptors; its entry via CR1 however requires the co-expression of HLA Class II molecules [275]. The EBV protein required for its interaction with CR2 is a glycoprotein (gp350) which shares significant homology with C3d [276].

The entry of members of family Retroviridae and Flaviviridae is promoted by the deposition of complement on their surface. In particular, retroviruses such as HTLV-1 and HIV use this strategy for their entry - Complement opsonized HIV has been shown to enhance infection in macrophages, dendritic cells and T cells using CR3 and CR4 as a receptor [277-279]. Among flaviviruses, West Nile virus utilizes CR3 for its entry into macrophages [280].

1.4.3.3. Acquisition of host complement regulators:

Most viruses have smaller genome and therefore they do not have the luxury to encode complement regulators and hence they resort to acquisition of complement regulators from the host. This however does not mean that this property is exclusively shown by the small genome viruses. The most common way of acquiring host complement regulator is while budding. Thus, enveloped viruses while budding acquire host-derived membrane which contains complement regulators along with viral proteins. Viruses belonging to herpesviridae, orthomyxoviridae, poxviridae, paramyxoviridae and retroviridae family are known to acquire complement regulators from host cells. CD55/DAF and CD59 are acquired during budding by VACV [24], HIV-1 [281, 282], human cytomegalovirus (HCMV) [283], HTLV-1 [283] and Simian immunodeficiency virus (SIV) [284]. Incorporation of CD46/MCP in the viral envelope has been demonstrated in VACV [24], HIV [281], SIV [284], HCMV [283], SV5 and mumps virus (Johnson JB 2009). Recent studies suggest conflicting results for hepatitis C virus. A study by Ejaz et al. suggested selective recruitment of CD59, but not CD55 and CD46 on the HCV membrane [285] whereas Mazumdar et al. demonstrated the presence of CD55 on its membrane [286].

Another mechanism of acquiring complement regulator involves interaction of viral protein with the soluble complement regulators like FH and C4BP. West Nile virus
has been demonstrated to recruit factor H through interaction with nonstructural-1 (NS-1) protein [287]. Likewise, HIV-1 protects itself from complement mediated attack by recruitment of factor H which interacts with gp41 and gp120 proteins of HIV-1 [288, 289]. In contrast to WNV and HIV, sindbis virus recruits factor H through sialic acid present on the viral surface which is acquired from host though budding [225]. The NS-1 proteins of WNV also confer protection to virus by interacting with C4BP [290].

Apart from acquiring complement regulatory proteins on viral surface, viruses also up-regulate the expression of the complement regulators on virus infected cells to avoid complement mediated lysis of cell. Human herpesvirus 7 infected cells show up-regulation of CD46 and CD59 [15] whereas HCMV infection leads to up-regulation of CD55 and CD46 on virus infected cells [291]. Interestingly, a recent study with Nipah virus has demonstrated a novel mechanism of protection which is achieved by recruitment of factor I protease on the viral membrane [292].

1.5. Immune evasion by poxviruses

Beside protection from the complement system (described above), poxviruses have also developed several other immune evasion mechanisms for their efficient survival in the host which include other innate immune mechanisms as well as adaptive immune mechanisms. As mentioned earlier, poxviruses have large DNA genome (ranges from 140-300 kb). It thus allows encoding of multiple genes for immune evasion and such genes are located in the terminal regions of the genome [12]. Apart from evading the immune modulators of host, poxviruses also target various intercellular signal transductions e. g., apoptosis [293], ubiquitination [294] etc.

PRR-induced signal transduction pathways ultimately result in the activation of gene expression and synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules, and immunoreceptors [295]. These together orchestrate the early host response to infection and at the same time represent an important link to the adaptive immune response.

1.5.1. Cytokines regulation by poxviruses: Entry of the pathogen into the host leads to their recognition by immune and non-immune cells and triggering of innate immune response that includes generation of cytokines, chemokines and interferons.
Cytokines are key regulators of immune response. Consequently, poxviruses target cytokines by encoding homologues of cytokines and their receptors [296, 297]. For the tumor necrosis factor (TNF) superfamily, cowpox virus encodes most number of TNF binding proteins. These proteins are named as cytokine response modifier (Crm). Cowpox virus encodes CrmB, CrmC, CrmD, CrmE and vCD30 [298, 299]. VACV genome encodes for CrmB (B28R) and CrmC (A53R), whereas VARV genome encodes only for CrmB (G2R). These proteins block the binding site of TNF molecules to their receptor and thereby inhibit their function [300]. Orf virus encodes a secretary protein which inhibits granulocyte macrophage colony stimulating factor (GM-CSF) and IL2 [301].

Type I interferons are the important molecules for containing virus infections. Poxviruses have developed variety of secretary molecules to evade interferon response. VACV Western Reserve strain encode B18R (B19R in VACV Copenhagen strain) gene which contains immunoglobulin domains that binds and inhibit type I interferons [302]. Similarly, cowpox virus and VARV encodes B17R and B20R, respectively, to inhibit type I interferon response. VACV deficient in B18R genes showed attenuated disease in mouse intranasal infection model [303]. These proteins compete with host IFN receptor for the binding of type I interferons. VACV E3L and K3L also reduce the activation of interferon effector proteins (Chang HW 1992). VACV also blocks INF-γ secretion by C12 gene product that binds to IL-18 [304, 305].

1.5.2. Chemokine regulation by poxviruses: Poxviruses modulate chemokine response to avoid influx of immune cells at the site of infection. They encode chemokine binding proteins and/or chemokine homologues [306, 307]. Poxviral chemokine binding proteins are further divided into Class I and Class II. Class I protein binds to a wide range of C, CC and CXC chemokines. The only example of class I is rabbit myxoma virus protein M-T7. M-T7 targets C-terminal heparin-binding site present on the chemokines and probably inhibit the binding of chemokines to the glycosaminoglycan (GAGs) [308, 309]. Class II include M7 protein encoded by VACV C23 gene. This 35 kDa protein binds to the chemokines [310]. Apart from VACV, cowpoxvirus, myxomavirus, rabbitpoxvirus, variola virus (G5R) and ectomelia virus (chemokine binding protein type II) encode for the
chemokine evasion proteins. Apart from these, fowlpox virus and Molluscum contagiosum virus show the presence of proteins which are homologous to the host chemokines [311].

Recently, DNA-PK, a DNA dependent protein kinase (formed by Ku70/Ku80 heterodimer and the catalytic subunit DNA-PKcs) is identified as a cytoplasmic pattern recognition receptor for DNA. VACV protein C16 has ability to bind to the Ku heterodimer and inhibit innate response – Production of cytokines and chemokines [312].

1.5.3. Cell mediated immune response regulation by poxviruses: Myxoma virus encodes two proteins M128L and M141R which share homology with CD47 and CD200, respectively. Infection in rabbit model using M128- and M141-deficient virus showed reduced pathogenesis [313, 314]. Most of poxviruses down regulate expression of MHC class I molecules to avoid detection from cytotoxic T cells [315, 316]. The most extensively studied protein which down regulate MHC class I molecules is myxoma virus leukemia-associated protein (MV-LAP) [317]. Apart from myxoma virus, malignant rabbit fibroma virus shown to downregulate MHC class I molecules [318] whereas A35 gene of VACV has been shown to inhibit MHC class II mediated antigen presentation [319].
1.6. Aims and objective:

The notorious member of the family Poxviridae, variola virus (VARV), which causes smallpox disease, is now listed as the bioterrorism agent by the Centers for Disease Control and Prevention (CDC), USA. This is because the vaccination against smallpox was stopped about 40 years ago, and as a result, the current human population has no immunity against the virus. The cessation of smallpox vaccination has also increased the zoonotic orthopoxvirus infections such as those caused by monkeypox and cowpox viruses [320, 321]. Together, these elements have renewed interest in understanding poxvirus pathogenesis.

Vaccinia virus (VACV), the prototype poxvirus, has been extensively studied for understanding the poxvirus pathogenesis and hence was also utilized in the present study to probe host complement-poxvirus interactions. Previous studies signify the role of neutralizing antibodies in controlling the smallpox infection [322-324] and vaccinia immune globulin (VIG) has been shown to reduce smallpox cases amongst the exposed individuals by 80% [325, 326]. Additionally, complement has been shown to enhance antibody-dependent neutralization (i.e., classical pathway (CP)-mediated neutralization) of both the infectious forms of VACV – MV as well as EV [18, 24, 327]. But till date, the role of other two major pathways – the alternative and lectin pathways – in the neutralization of the VACV remains unanswered. Also, the mechanism of complement-mediated neutralization of VACV has remained undefined. VACV infects various domestic animals, and yet, it is still not clear if the complement system of these species is capable of neutralizing VACV.

VACV encodes complement regulator VCP. Previous studies with VCP suggest that it inhibits the CP-mediated neutralization of VACV [18]. Moreover, using purified complement proteins, our laboratory has demonstrated that VCP and SPICE (complement regulator of VARV) show functional selectivity in inactivation of human complement [26, 247]. But the question that remained unanswered is, whether SPICE is potent than VCP in rescuing poxvirus from the human CP-mediated neutralization and which of the variant residues of SPICE are important for rescuing VACV from the CP-mediated neutralization. Further, expression of VCP during VACV infection is expected to inhibit complement activation, which is necessary for generation of the optimal immune response against the virus. Because VCP inhibits
complement at high nanomolar range [28], such concentrations are expected to be achieved only at the site of infection. This raises the question what role complement activation products (e.g., C3a and C5a) generated in the viral habitat play in controlling VACV infection?

It is thus apparent that multiple questions related to the role of VCP in subversion of host complement and that of the locally generated complement anaphylatoxins C3a and C5a generated during VACV infection are not clear. Hence, the objectives of the study were as follows:

1. To investigate the mechanism of complement-mediated neutralization of vaccinia virus and its rescue by the virus encoded complement regulator VCP.

2. To study in vivo role of VCP-complement interaction in vaccinia virus infection and role of locally produced complement C3a and C5a anaphylatoxins in controlling the infection.