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Role of the Myxoma Virus Soluble CC-Chemokine Inhibitor Glycoprotein, M-T1, during Myxoma Virus Pathogenesis

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Myxoma virus is a poxvirus that causes a virulent systemic disease called myxomatosis in European rabbits. Like many poxviruses, myxoma virus encodes a variety of secreted proteins that subvert the antiviral activities of host cytokines. It was recently demonstrated that the myxoma virus M-T1 glycoprotein is a member of a large poxvirus family of secreted proteins that bind CC-chemokines and inhibit their chemoattractant activities *in vitro*. To determine the biological role of M-T1 in contributing to myxoma virus virulence, we constructed a recombinant M-T1-deletion mutant virus that was defective in M-T1 expression. Here, we demonstrate that M-T1 is expressed continuously during the course of myxoma virus infection as a highly stable 43-kDa glycoprotein and is dispensable for virus replication *in vitro*. Deletion of M-T1 had no significant effects on disease progression or in the overall mortality rate of infected European rabbits but heightened the localized cellular inflammation in primary tissue sites during the initial 2 to 3 days of infection. In the absence of M-T1 expression, deep dermal tissues surrounding the primary site of virus inoculation showed a dramatic increase in infiltrating leukocytes, particularly monocytes/macrophages, but these phagocytes remained relatively ineffective at clearing virus infection, likely due to the concerted properties of other secreted myxoma virus proteins. We conclude that M-T1 inhibits the chemotactic signals required for the influx of monocytes/macrophages during the acute-phase response of myxoma virus infection *in vivo*, as predicted by its ability to bind and inhibit CC-chemokines *in vitro*. © 1999 Academic Press

INTRODUCTION

It is now evident that viruses use multiple mechanisms to oppose host defenses to ensure their survival within an immunocompetent host organism (Kotwal, 1997; McFadden, 1995; Smith, 1994; Spriggs, 1996). Recent investigations have revealed a myriad of proteins expressed by large DNA viruses that function to evade, block, or modulate key effector molecules of the innate or acquired arms of the immune system. Such viral strategies for immune evasion include interference with antigen presentation, inhibition of apoptosis, blockade of the complement cascade, and modulation of the cytokine networks (Barry and McFadden, 1998a; McFadden *et al.,* 1998; Ploegh, 1998)

Poxviruses, the largest known DNA viruses, are particularly adept at subverting the host inflammatory response by expressing a collective arsenal of proteins that alter host cytokines (Alcamí *et al.,* 1998b; McFadden, 1994; McFadden *et al.,* 1998; Pickup, 1994). Many poxviruses express proteins that bear significant amino acid similarity to the extracellular ligand-binding portion of cellular cytokine receptors. These viral cytokine receptor mimics, or viroceptors, are often secreted glycoproteins that function by inhibiting competitively the cytokine ligands from binding to their cognate cellular receptors (McFadden, 1995). To date, functional poxviral cytokine receptor homologues have been identified for receptors of tumor necrosis factors (TNFs) (Hu *et al.,* 1994; Loparev *et al.,* 1998; Schreiber *et al.,* 1996), interferon- (IFN-)α/β (Colamonici *et al.,* 1995; Symons *et al.,* 1995), IFN-γ (Mossman *et al.,* 1995a; Upton *et al.,* 1992), and interleukin-1β (IL-1β) (Alcamí and Smith, 1996; Spriggs *et al.,* 1992).

Myxoma virus, a member of the *Leporipoxvirus* genus, causes a highly lethal systemic disease known as myxomatosis in the European rabbit, *Oryctolagus cuniculus* (Fenner, 1994; Fenner and Ratcliffe, 1965; McFadden, 1988). Given the well-documented natural history and pathogenic profile of myxoma virus and its rabbit host, myxomatosis provides an excellent model for the study of virus–host interactions. The pathogenic features of myxomatosis include extensive hemorrhagic lesions at the primary site of virus inoculation that is characterized by tissue degeneration and necrosis. By 7–10 days postinfection (p.i.), the virus effectively disseminates through the host lymphoreticular system and induces numerous internal and external secondary lesions that become visibly evident around the ears, eyelids, lips, nares, and genitalia. Furthermore, severe dysfunction in cell-mediated immunity facilitates supervening gram-

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negative bacterial infections of the nasal and conjunctival mucosa and respiratory tract; within 10–14 days, infected rabbits invariably experience dyspnea and die (Fenner, 1994; Fenner and Ratcliffe, 1965).

Like all poxviruses, myxoma virus contains a large (~160-kb) double-stranded DNA genome with covalently closed hairpins and terminal sequences that are inverted and repeated (McFadden *et al.,* 1995). Although the central portion of most poxvirus genomes are typically conserved and encodes for proteins essential for virus replication and morphogenesis, genes that map toward the termini have been shown to be important for determining virus virulence and host range (Moss, 1996). The deletion or disruption of these virulence genes usually has no effect on virus replication *in vitro* but can exert a demonstrable effect on influencing the pathogenic profile within an infected host. To date, the biological roles of many distinct myxoma virus genes in contributing to virus virulence in infected laboratory rabbits has been characterized (Barry *et al.,* 1997; Graham *et al.,* 1992; Macen *et al.,* 1993; Messud-Petit *et al.,* 1998; Mossman *et al.,* 1996a, 1996b; Opgenorth *et al.,* 1992b; Upton *et al.,* 1991). For example, the M-T2 and M-T7 myxoma gene products have previously been shown to be functional soluble viral cytokine receptors that competitively inhibit the proinflammatory activities of TNF and IFN-γ, respectively (Mossman *et al.,* 1995b; Schreiber *et al.,* 1996). Disruption of these genes alters pathogenesis and/or markedly attenuates the virus, suggesting that these proteins function in disrupting the host antiviral cytokine circuitry during infection (Mossman *et al.,* 1996b; Upton *et al.,* 1991).

The mobilization and activation of immune cells to sites of viral infection are pivotal components of the early host inflammatory response (Nathanson, 1997). Chemoattractant cytokines, or chemokines, are crucial in orchestrating this process (Baggiolini, 1998; Luster, 1998). Chemokines comprise a large superfamily of proinflammatory peptides that mediate the activation and transmigration of diverse leukocytes by binding to cell surface seven-transmembrane spanning receptors (Murphy, 1996; Premack and Schall, 1996). The chemokine superfamily is further subdivided into four subfamilies (C, CC, CXC, and CX_3C) based on structural and functional characteristics. Members belonging to the CC-chemokine subfamily are particularly important in the activation and recruitment of monocytes, T lymphocytes, dendritic cells, and natural killer cells during inflammation processes (Luster, 1998). Given the central role of these immune effectors in virus clearance, it is not surprising that certain viruses may have adopted mechanisms for abrogating the biological activities of CC-chemokines (Lalani and McFadden, 1997; Murphy and Pease, 1998; Nelson and Krensky, 1998; Wells and Schwartz, 1998).

We have recently shown that the first open reading frame (ORF) from the termini of the myxoma virus genome, termed M-T1, encodes a 43-kDa secreted glycoprotein that binds to a broad range of CC-chemokines with high affinity in a non-species-specific fashion (Graham *et al.,* 1997; Lalani *et al.,* 1998). Homologous relatives of M-T1 have also been detected in some strains of vaccinia, rabbitpox, cowpox, ectromelia, raccoonpox, Shope fibroma, camelpox, and variola (smallpox) viruses (Alcamí *et al.,* 1998a; Graham *et al.,* 1997; Lalani *et al.,* 1998; Smith *et al.,* 1997). This family of related secreted poxvirus 35- to 40-kDa glycoproteins is collectively known as the T1/35kDa family of poxvirus chemokine binding proteins. Although members of the T1/35kDa family show variable homology among each another, sequence analyses predict no identifiable relationship to currently known cellular proteins, including chemokine receptors (Alcamí *et al.,* 1998a; Graham *et al.,* 1997; Smith *et al.,* 1997). Recently, purified M-T1 protein from myxoma virus was demonstrated to block human CC-chemokines such as MIP-1α and MCP-1 from binding to their cognate receptors with an inhibitory constant (K_i) of ~1.0 nM (Lalani *et al.,* 1998). Moreover, purified M-T1 potently inhibited chemokine-dependent migration of human monocytes *in vitro* (Lalani *et al.,* 1998). In contrast, neither M-T1 nor its homologous 35-kDa counterparts were capable of neutralizing CXC-chemokine activity, indicating that the T1/35kDa viral proteins are specific biological inhibitors for the CC, but not the CXC, subfamily of chemokines (Alcamí *et al.,* 1998a; Lalani *et al.,* 1998; Smith *et al.,* 1997).

The biological role of one T1/35kDa family member has previously been studied in a rabbitpox virus (RPV) model of pathogenesis (Martinez-Pomares *et al.,* 1995). RPV, a member of the *Orthopoxvirus* genus, has a broader host range and has a distinct disease phenotype to myxoma virus in infected European rabbits. Sequence comparisons between the RPV-35kDa and myxoma virus M-T1 proteins reveal significant sequence divergence (40% amino acid identity) between the two family members (Graham *et al.,* 1997). Mice infected intranasally with an RPV 35-kDa null mutant displayed an exacerbated illness in comparison to a wild-type RPV virus infection, suggesting that this T1/35kDa family member may modulate the host inflammatory response *in vivo* (Martinez-Pomares *et al.,* 1995). Detailed histological analyses of RPV-infected rabbit lesions in subsequent studies demonstrated that RPV-35kDa alters leukocyte trafficking significantly during the early phases of RPV infection (Graham *et al.,* 1997).

Given the potent *in vitro* CC-chemokine inhibitory properties of the T1/35kDa family members demonstrated recently, we decided to investigate the biological role of the M-T1 glycoprotein during myxoma virus pathogenesis in the European rabbit. European rabbits were infected with a recombinant myxoma M-T1-deletion mutant virus and assessed for differences in pathological profiles. Here, we demonstrate that M-T1 markedly influ-

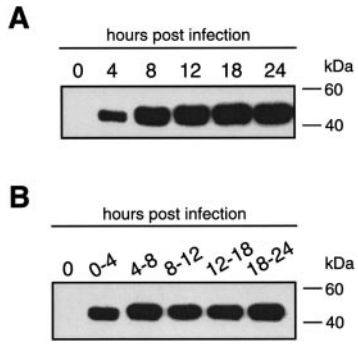

FIG. 1. Immunoblotting analysis of M-T1 secretion from myxoma virus-infected cells. (A) Accumulation of secreted M-T1 glycoprotein detected from supernatants of vMyxlac-infected BGMK cells harvested at various times p.i. (B) Infected cells were washed at 4-h intervals and replaced with fresh serum-free medium. Newly synthesized soluble M-T1 in the medium of infected cells during the indicated times p.i. was detected by Western blotting analysis as outlined in Materials and Methods.

ences the chemotaxis of inflammatory cells, particularly macrophages, into infected tissue sites during the initial phases of virus infection. However, the increased number of phagocytes are relatively ineffective at clearing the M-T1 deletion mutant virus, suggesting that other myxoma viral proteins are able to neutralize the antiviral activities of the infiltrating macrophages.

RESULTS

Kinetics of M-T1 expression

Previous transcriptional analysis of homologous M-T1 genes in other poxviruses, namely S-T1 of Shope fibroma virus and 35 kDa of vaccinia virus (strain Lister), have demonstrated that at least these two members of the T1/35kDa family are transcribed at both early and late times (Macaulay *et al.,* 1987; Patel *et al.,* 1990). To determine the kinetics of M-T1 expression during high multiplicity (m.o.i. = 10) myxoma virus infection, we immunoblotted myxoma virus-infected cellular supernatants that were harvested at various times p.i. As shown in Fig. 1A, M-T1 protein was detected in the medium of infected cells as early as 2–4 h p.i. and accumulated increasingly during the late times of virus replication. By 12 h, $>1 \times 10^7$ molecules/cell of M-T1 was routinely observed in the medium of infected cultures (Table 1). An analysis of the levels of accumulated proteins during infection demonstrated no significant reduction in the amounts of soluble M-T1 even after 24 h p.i., indicating that the secreted M-T1 polypeptide accumulates as a highly stable ~40- to 45-kDa species (Fig. 1A and Table 1). To determine whether the mature M-T1 glycoprotein was secreted continuously during the course of infection, infected cell cultures were washed thoroughly at various times, replaced with fresh medium at 4-h intervals, and analyzed for M-T1 expression throughout the virus replication cycle. Processed M-T1 was observed to be continuously secreted from infected cells at comparable rates ($\sim 1 \times 10^6$ molecules/cell/h) at both early and late times of infection with maximal expression (6.67×10^6 molecules/cell) occurring between 4 and 8 h p.i. (Fig. 1B and Table 1). In addition, soluble M-T1 detected at both early and late times were equally efficacious at binding CC-chemokines (data not shown). Thus biologically active and significant amounts of soluble M-T1 appear to be continuously secreted through the course of myxoma virus infection and accumulate in the extracellular environment as a highly stable 40- to 45-kDa species.

Construction and analysis of a recombinant M-T1-deletion mutant virus

To determine the biological role of M-T1 in viral virulence, we constructed a recombinant M-T1-deletion mutant myxoma virus, vMyxlacT1$^-$, that failed to express M-T1. Because the genomic location of the M-T1 ORF is within the terminal inverted repeats, it is present as duplicate copies in the myxoma virus genome. Both copies of the M-T1 ORF were deleted and replaced with a dominant selectable marker gene, *Escherichia coli* guanosine phosphoribosyl transferase (Ecogpt), using a similar homologous recombination procedure between the parental virus (vMyxlac) and an engineered transfer vector as outlined elsewhere (Barry *et al.,* 1997; Macen *et al.,* 1993; Mossman *et al.,* 1996b). A construct bearing flanking sequences of M-T1 separated by Ecogpt, pKS-T1KO (Fig. 2 and Materials and Methods), was transfected into vMyxlac-infected cells, and individual foci surviving three rounds of passage in mycophenolic acid selection were harvested. Plaque-purified viral isolates were propagated further and analyzed by PCR and West-

TABLE 1

Quantification of Secreted Myxoma Virus M-T1 Glycoprotein

	Total M-T1 Secreted		
Time (h p.i.)	μg/10^6 cells	Molecules/cell	Molecules/cell/h
Cumulative			
4	0.19	2.70×10^6	0.66×10^6
8	0.65	9.12×10^6	1.14×10^6
12	1.00	1.39×10^7	1.16×10^6
18	1.06	1.48×10^7	0.82×10^6
24	1.40	1.97×10^7	0.82×10^6
Pulse-chase			
0–4	0.27	3.84×10^6	0.96×10^6
4–8	0.48	6.67×10^6	1.67×10^6
8–12	0.24	3.43×10^6	0.86×10^6
12–18	0.28	3.93×10^6	0.65×10^6
18–24	0.30	4.19×10^6	0.70×10^6

Note. Tabulation of M-T1 levels from infected BGMK cells at various times p.i. was determined according to the procedures outlined in Materials and Methods.

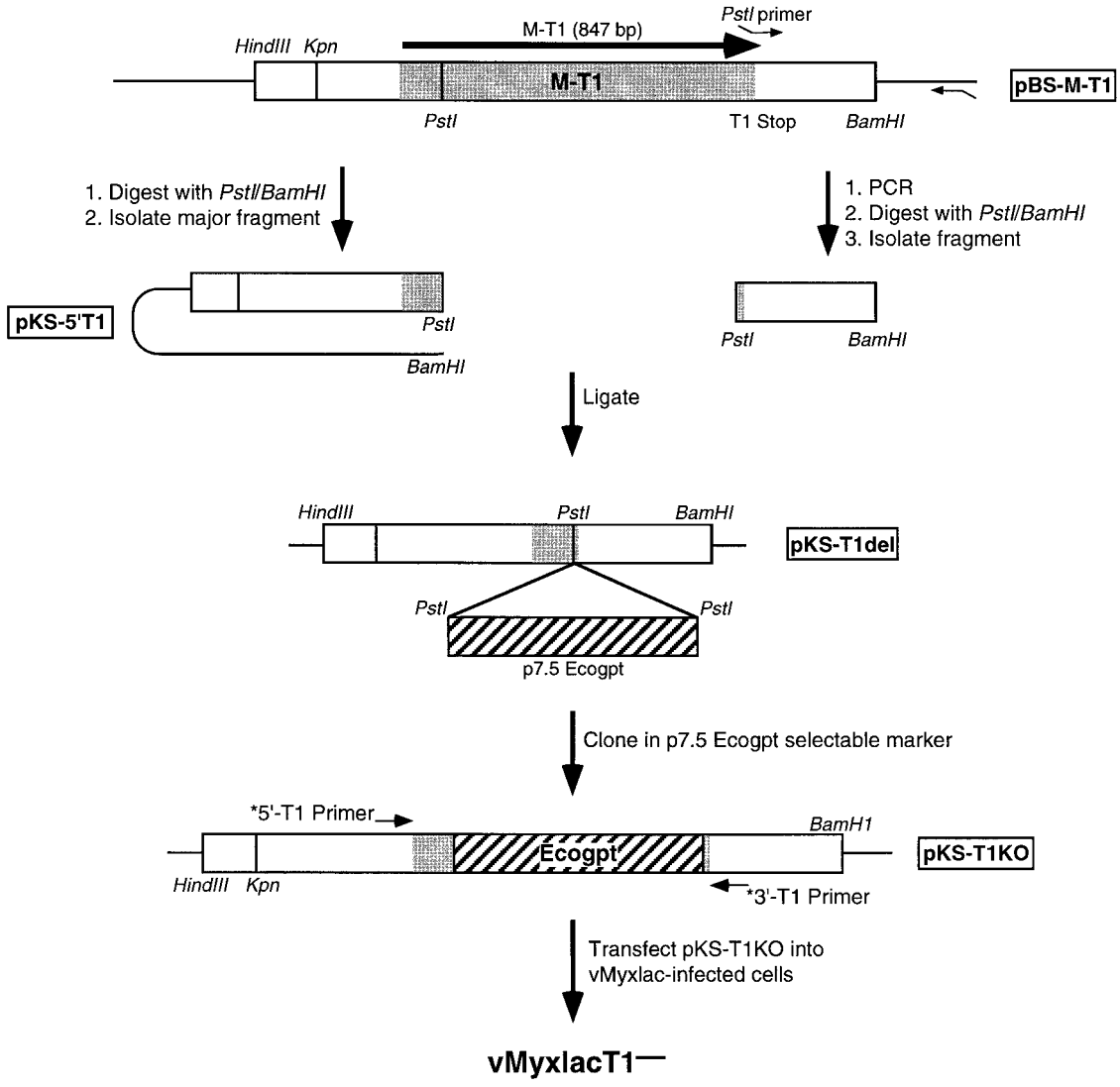

FIG. 2. Construction of a recombinant mutant myxoma virus containing a M-T1 disruption. A construct containing a 64-bp 5′ coding sequence of M-T1, pKS-5′T1, was generated by restriction digestion of pBS-M-T1 with *Pst*I and *Bam*HI. A 3′ terminal fragment of M-T1 was generated by PCR amplification using a primer containing an engineered *Pst*I site and cloned into a pT7Blue T-vector. The 3′ M-T1 fragment was isolated after restriction digestion with *Pst*I and *Bam*HI and cloned into the complementary sites of pKS-5′T1 to generate the plasmid pKS-T1del. Digestion with *Pst*I and insertion of a *Pst*I p7.5Ecogpt cassette was used to form the M-T1 disruption plasmid pBS-T1KO. vMyxlac-infected cells were subsequently transfected with pBS-T1KO DNA, and recombinant viruses (vMyxlacT1$^-$) resistant to Ecogpt selection were harvested and plaque purified. Primers directed toward the 5′ and 3′ flanking sequences of M-T1 (*) were used in PCR diagnostic analysis to verify the disruption of M-T1 and insertion of Ecogpt.

ern blotting. As a control, a recombinant M-T1 revertant virus, vMyxT1R, in which the intact M-T1 ORF was restored fully into its original loci, was constructed using a similar genetic approach.

To verify the deletion of M-T1, viral DNA harvested from plaque-purified isolates was subjected to PCR analysis using 5′ and 3′ primers that hybridize to specific M-T1 flanking sequences. Deletion/disruption of M-T1 by Ecogpt insertion can be easily visualized by size discrimination of the PCR products under agarose gel electrophoresis; the presence of any contaminating isolates bearing wild-type M-T1 sequences can also be readily identified. As shown in Fig. 3A, although the wild-type M-T1 sequence produced an amplified product of ~890 bp (lanes 1 and 3), the integration of Ecogpt within the disrupted M-T1 sequence yielded a PCR product of ~1000 bp (lane 2). In addition to an isolate containing a mixed pool of both wild-type and vMyxlacT1$^-$ recombinants (Fig. 3A, lane 4), one isolate composed of a pure population of vMyxlacT1$^-$ recombinants (Fig. 3A, lane 5) was identified and chosen for further characterization. PCR analysis from a vMyxT1R viral recombinant demonstrated only the reverted wild-type M-T1 sequence (Fig. 3A, lane 6).

To verify whether the vMyxlacT1$^-$ recombinant virus was defective in M-T1 expression, secreted proteins from virus-infected cells were analyzed by Western blotting using specific anti-M-T1 antisera. Although the 43-

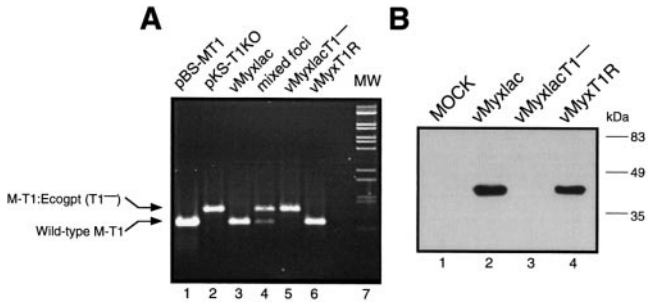

FIG. 3. Characterization of M-T1 from vMyxlac (wild-type), recombinant vMyxlacT1$^-$, and vMyxT1R viruses. (A) Agarose gel electrophoresis of PCR products amplified by flanking M-T1 5′ and 3′ primers (see Fig. 2). Migration of wild-type M-T1 (lane 1) and M-T1:Ecogpt (lane 2) DNA amplified from pBS-M-T1 and pKS-T1KO plasmids, respectively. PCR analysis of viral DNA harvested from vMyxlac-infected cells (lane 3). PCR analysis of viral DNA after homologous recombination and Ecogpt dominant selection identified a mixed foci (lane 4) composed of both M-T1 and M-T1:Ecogpt and a pure recombinant M-T1-deletion virus (lane 5) containing only M-T1:Ecogpt. Viral DNA harvested from cells infected with vMyxT1R demonstrates the presence of wild-type M-T1. Molecular size markers corresponding to *Bst*EII-cut λ standards are shown in lane 7. (B) Western blotting analysis of supernatants from mock-infected (lane 1), vMyxlac-infected (lane 2), vMyxlacT1$^-$-infected (lane 3), and vMyxT1R-infected (lane 4) cells using specific anti-M-T1 antisera. SDS–PAGE low-molecular-weight markers are indicated to the right.

kDa secreted M-T1 glycoprotein was readily detected in the supernatants of cells infected with the parental virus (Fig. 3B, lane 2) or vMyxT1R recombinant (Fig. 3B, lane 4), M-T1 was completely absent from the medium of vMyxlacT1$^-$-infected (Fig. 3B, lane 3) or mock-infected (Fig. 3B, lane 1) cultures. Similarly, lysates from vMyxlacT1$^-$-infected cells were devoid of intracellular M-T1 (data not shown). Furthermore, several other distinct myxoma soluble glycoproteins, namely SERP-1 and M-T7, were observed to be expressed from vMyxlacT1$^-$ and vMyxlac in comparable amounts, indicating that the deletion of M-T1 did not alter the expression of additional myxoma-soluble proteins (data not shown). Taken together, this data confirm that vMyxlacT1$^-$ is a pure recombinant myxoma M-T1 deletion mutant virus that is incapable of M-T1 expression.

M-T1 is nonessential for replication *in vitro*. Previous studies have demonstrated that virulence factors from poxviruses are typically nonessential for growth in tissue culture. We characterized the growth characteristics of vMyxlacT1$^-$ *in vitro* in several susceptible rabbit and primate cell lines. Single-step growth curve analysis demonstrated no defects in the ability of vMyxlacT1$^-$ to replicate in cultured primate baby green monkey kidney (BGMK) or rabbit RK-13 fibroblast cells compared with the parental vMyxlac virus (data not shown). The productive infection of rabbit lymphocytes is thought to be crucial for efficient dissemination of the virus via the host afferent lymphatic channels *in vivo*. vMyxlacT1$^-$ was observed to productively infect a rabbit T lymphocyte cell line, RL-5, with no observable defects in replication or in causing the premature cell death of infected cells (data not shown). Thus vMyxlacT1$^-$ appears to be dispensable for virus replication in cultured cells *in vitro*.

Pathogenesis of vMyxlacT1$^-$-infected rabbits

To examine the biological role of M-T1 in virus virulence, European rabbits were infected with vMyxlacT1$^-$ and monitored for any changes in the clinical manifestations of myxomatosis. As summarized in Table 2, rabbits infected with either the parental vMyxlac or recombinant vMyxT1R viruses were indistinguishable and developed the classic symptoms of myxomatosis, including the development of fulminating lesions at the primary site of inoculation, multiple secondary lesions, blepharoconjunctivitis, and supervening bacterial infections. By days 10–14, all vMyxlac- and vMyxT1R-infected rabbits were sacrificed due to the increased severity of the disease. Surprisingly, vMyxlacT1$^-$-infected rabbits also experienced a similar pathogenic profile, including the development of primary and secondary lesions, oppor-

TABLE 2

Pathogenicity of vMyxlac, vMyxT1R, and vMyxlacT1$^-$ Infected European Rabbits

Day	vMyxlac (wild-type) and vMyxT1R	vMyxlacT1$^-$
0	Six NZW rabbits inoculated with 1000 PFU intradermally	Six NZW rabbits inoculated with 1000 PFU intradermally
2–3	Small (0.5–1 cm), slightly inflamed primary lesions	Larger (1–2 cm) very inflamed primary lesions; edema of face and eyes; mild conjunctivitis
4–7	Large (2–2.5 cm) protuberant demarcated primary lesions with necrotic centers; few secondary lesions; mild edema, conjunctivitis, and rhinitis	Large (2.5 cm) protuberant demarcated primary lesions with necrotic centers; multiple secondary lesions; moderate edema, conjunctivitis, and rhinitis
7–12	Multiple secondary lesions; severe supervening bacterial infections (mucopurulent discharge of eyes/nares); extreme conjunctivitis, edema, and rhinitis; acute dyspnea	Multiple secondary lesions; severe supervening bacterial infections (mucopurulent discharge of eyes/nares); extreme conjunctivitis, edema, and rhinitis; acute dyspnea; one rabbit with resolving infection
10–14	All rabbits sacrificed due to disease severity	Five of six rabbits sacrificed due to disease severity
14–21		Complete recovery of remaining rabbit

tunistic bacterial infections, and five of six (>80%) rabbits became moribund and required euthanasia (Table 2). However, one striking phenotypic difference was an augmented inflammation of the primary lesions from the vMyxlacT1$^-$-infected rabbits during the early stages of the disease. By 2–3 days p.i., the primary lesions at the initial site of vMyxlac inoculation were barely discernible (Table 2). In contrast, the primary lesions of rabbits infected with the deletion-mutant virus were significantly larger, displaying heightened inflammation (reddening and swelling) and a notable surrounding edematous zone (Table 2). Although the elevated size and inflammation of the vMyxlacT1$^-$ lesions were morphologically distinct from the vMyxlac lesions between 1 and 3 days p.i., the primary nodules became indistinguishable by 7 days after infection. In general, the vMyxlacT1$^-$-infected rabbits also experienced more edema, conjunctivitis, and a slightly accelerated development of bacterial infections, but given the variability of symptomology between rabbits, we were not able to assess these differences as significant. Thus the absence of the M-T1 viral chemokine binding protein resulted in an increased localized inflammatory response at the primary inoculation site during the early phases of the infection but had no major significant effects in attenuating the progression of disease or on the mortality rate of infected European rabbits.

Histological analysis of vMyxlacT1$^-$-infected tissues

Gross pathological examinations showed that the primary vMyxlacT1$^-$ tissue lesions exhibited greater inflammation and were considerably larger than their wild-type infected counterparts during the first 3 days of infection. Given these notable differences in the early inflammatory response at the primary site, tissue samples were resected at 2, 3, and 7 days p.i. for complete histological analysis. Microscopically, the epidermis of the vMyxlacT1$^-$ primary sites showed more reactivity and hyperplasia than vMyxlac tissues, indicative of an intense inflammatory response occurring at 2–3 days p.i. (data not shown). Stained tissue sections revealed striking differences in cellular infiltrates within the deep dermis of the primary lesions between the two sets of rabbits (Fig. 4). By 2 and 3 days p.i., a mild focal inflammatory response with a sparse number of infiltrating leukocytes was observed in the dermal tissues of vMyxlac primary sites (Figs. 4, A and C). In contrast, a intense widespread inflammatory reaction with a marked increase in heterophil and mononuclear rabbit leukocytes was observed in the deep dermal layer of vMyxlacT1$^-$ primary tissues (Figs. 4, B and D). To determine whether M-T1 was modulating the influx of leukocytes within infected tissues, rabbit leukocytes permeating the dermal tissues were enumerated. In the absence of M-T1 expression, we observed an approximate threefold to fourfold increase in the number of leukocytes migrating deeply into the primary tissues (Fig. 5A), suggesting that M-T1 alters the trafficking of inflammatory cells *in vivo* during the early phases of myxoma virus infection.

M-T1 blocks monocyte/macrophage influx *in vivo*

We previously demonstrated that M-T1 potently inhibits its CC-chemokines from inducing the chemotactic migration of primary monocytes *in vitro* (Lalani *et al.,* 1998). We predicted, therefore, that M-T1 may also abrogate CC-chemokine-mediated migration of inflammatory effectors belonging to the monocyte/macrophage cell lineage during virus infection *in vivo*. The discrimination of rabbit leukocyte subclasses by surface marker expression is poorly defined, and few reagents currently exist for optimal immunohistochemical analysis of rabbit tissues. To characterize the effects of M-T1 on monocytic influx during myxoma virus infection, tissue sections were immunostained with RAM11, a murine monoclonal antibody that is specific to rabbit monocytes/macrophages (Lucas *et al.,* 1996; Tsukada *et al.,* 1986). As shown in Fig. 5B, there was a significant elevation in the percentage of infiltrating rabbit monocytes/macrophages into the dermal tissues early (2–3 days) during vMyxlacT1$^-$ infection. We were unable to accurately assess the differences in RAM11-positive leukocytes in the day-7 tissues due to widespread tissue necrosis or determine which additional leukocyte subpopulations may have altered migration patterns by vMyxlacT1$^-$. Nevertheless, our data suggest that the expression of the M-T1 glycoprotein has profound effects on influencing the effective migration of rabbit inflammatory leukocytes, such as monocytes/macrophages, during viral infection *in vivo*, consistent with the biochemical property of M-T1 as a potent CC-chemokine inhibitor *in vitro*.

Viral burden in infected lesions

Macrophages play a central role in the early response to virus infection by facilitating intrinsic resistance mechanisms and generating secreted antiviral cytokines (Nathanson, 1997). Because myxoma virus expresses a variety of proteins that target host inflammatory cells, we examined tissues from primary lesions to determine whether the increased level of macrophages observed during vMyxlacT1$^-$ infection had any effects on virus clearance. Because the parental vMyxlac and mutant vMyxlacT1$^-$ viruses both express an *E. coli lacZ* transgene, localization of either virus within infected tissues can be readily identified by staining sections with an antibody against β-galactosidase. No significant qualitative differences in the level of β-galactosidase staining, as a marker for viral gene expression, were observed in the primary or secondary dermal tissues from vMyxlac- or vMyxlacT1$^-$-infected rabbits at 3 days (Fig. 6) or 7 days (data not shown) p.i. Thus although the increased

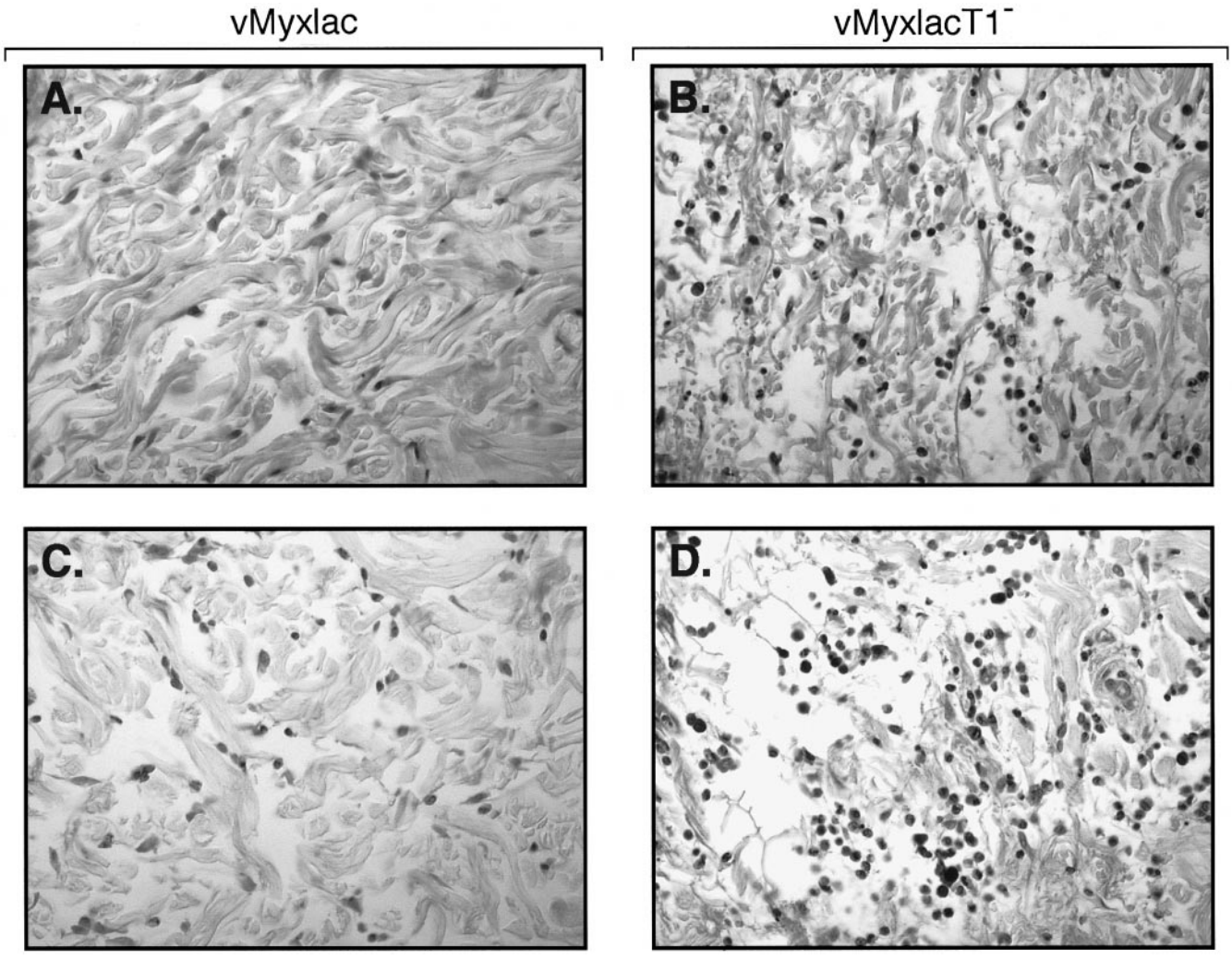

FIG. 4. Histological analysis of primary lesion tissues. Microscopic analyses (magnification 400×) of the deep dermal layer of rabbit tissues from the primary site of inoculation with vMyxlac (A and C) or vMyxlacT1$^-$ (B and D) harvested at 2 days (A and B) and 3 days (C and D) p.i.

number of infiltrating phagocytes were present in vMyxlacT1$^-$-infected primary tissues at 3 days p.i., the viral burdens of both the mutant and the wild-type myxoma viruses remain at comparable levels. We conclude that despite the increased levels of infiltrating macrophages in lesions infected with vMyxlacT1$^-$ virus, the effective antiviral activities of these responding phagocytes were likely compromised by the summated activities of other anti-inflammatory myxoma viral proteins.

DISCUSSION

A major strategy used by poxviruses in thwarting the host-inflammatory response to virus infection involves the sequestration of crucial host biological response modifiers such as key cytokines critical for orchestrating the antiviral responses (Alcamí *et al.,* 1998b; Barry and McFadden, 1998b; Spriggs, 1996). A growing number of secreted poxvirus glycoproteins, including vIFN-α/β receptors, vIFN-γ receptors, vIL-1β receptors, and vTNF receptors, have been identified with activities aimed at disrupting host cytokine networks (McFadden *et al.,* 1995). In addition to being capable of effectively neutralizing the antiviral activities of their respective cytokines *in vitro*, many of these viroceptors have been shown to function as anti-inflammatory virulence factors because their deletion alters the pathogenesis of their infected hosts *in vivo* (McFadden *et al.,* 1998).

The coordinated recruitment of immune cells to sites of virus infection is a key feature of the early host inflammatory response to pathogenic insult (Nathanson, 1997). Chemokines are believed to be critical in this process by selectively activating and mobilizing monocytes, T lymphocytes, dendritic cells, natural killer cells, and granulocytes to sites of infection and damage (Luster, 1998). As a consequence of the important roles that chemokines play in orchestrating inflammatory processes, certain viruses have devised a number of countermeasures for neutralizing the activities of chemokines or their re-

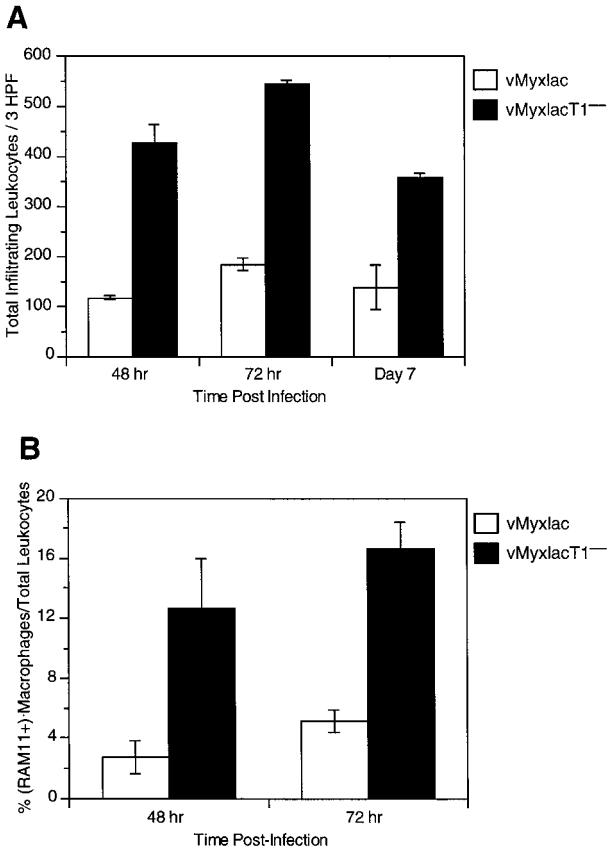

FIG. 5. M-T1 blocks leukocyte infiltration in virus-infected tissues. (A) Infiltrating rabbit leukocytes within the deep dermal layer of tissues from vMyxlac (open bars) and vMyxlacT1$^-$ (filled bars) primary lesions were enumerated in three high-power fields from hematoxylin and eosin-sectioned tissues as outlined in Materials and Methods. (B) Primary tissues from vMyxlac (open bars) and vMyxlacT1$^-$ (filled bars)-infected rabbits were harvested at 2 and 3 days p.i. and stained with RAM11 antibody. Peroxidase-stained RAM11+ monocytes/macrophages in the deep dermis of infected tissues were counted in three random high-power fields, and the results are expressed as the percentage of infiltrating RAM11+ macrophages/total infiltrating rabbit leukocytes within virus-infected tissues.

ceptors (Lalani and McFadden, 1997; Murphy and Pease, 1998; Nelson and Krensky, 1998; Wells and Schwartz, 1998). There are three strategies of chemokine subversion known to be used by viruses: (1) virus-encoded chemokine ligand homologues that function as antagonists, (2) virus-encoded cell-surface chemokine receptor homologues, and (3) virus-encoded secreted chemokine binding proteins such as the myxoma virus M-T1 glycoprotein. However, the significance of many of these viral proteins in contributing to viral pathogenesis or in abrogating the activities of chemokines *in vivo* has not been fully addressed.

We have previously shown that the myxoma virus M-T1 glycoprotein binds to a broad spectrum of CC-chemokines with high affinity and effectively sequesters soluble chemokine ligands from engaging their cell-surface receptors (Graham *et al.,* 1997; Lalani *et al.,* 1998). In addition, using *in vitro* chemotaxis assays, M-T1 was shown to be capable of blocking MIP-1α and MCP-1 from stimulating the unidirectional migration of primary human monocytes, suggesting that M-T1 may play a fundamental biological role in myxoma virus pathogenesis by functionally retarding the proper trafficking of rabbit phagocytes *in vivo* (Lalani *et al.,* 1998). Here we demonstrate that the expression of M-T1 during myxoma virus infection *in vivo* results in marked perturbations of the extent and amount of leukocyte infiltration into virus-infected tissues, although these additional phagocytes exert only minimal effects on virus-induced mortality rates.

Our present study demonstrates that the myxoma M-T1 glycoprotein is secreted continuously during early and late times of virus infection and is nonessential for virus replication *in vitro*. Given the potent CC-chemokine inhibitory properties of M-T1 previously displayed *in vitro*, we were surprised to discover that the deletion of M-T1 from myxoma virus does not significantly reduce the lethality of the myxomatosis disease progression in infected European rabbits. Rather, M-T1 was shown to have demonstrable alterations in localized inflammation surrounding the primary sites of infection, but the increased numbers of infiltrating phagocytes were still relatively ineffective at virus clearance. Rabbits infected with an M-T1-null mutant experienced a notable elevation of inflammation at the primary lesions in comparison to an infection with the parental virus during the early phases of infection. The heightened inflammatory response observed during gross examinations of vMyxlacT1$^-$-infected rabbits was also confirmed by histological analysis. The deletion of M-T1 resulted in an increase in the extravasation of rabbit monocytes/macrophages, and perhaps additional leukocytes, into infected dermal tissues. Thus M-T1 appears to play a significant role in altering the directional migratory signals that are required for influx of inflammatory cells during the acute-phase response to myxoma virus infection as predicted by the *in vitro* activities of all the T1/35kDa family members as potent CC-chemokine inhibitors (Alcamí *et al.,* 1998a; Lalani *et al.,* 1998; Smith *et al.,* 1997).

Our results are in agreement with prior investigations of a distinct M-T1 family member, namely RPV-35kDa, in a rabbitpox virus model of pathogenesis (Martinez-Pomares *et al.,* 1995). The deletion of 35-kDa ORF from RPV resulted in a modest acceleration of illness in infected Balb/c mice and no overall difference in the pathogenesis of infected European rabbits (Martinez-Pomares *et al.,* 1995). However, histological analysis in subsequent studies revealed dramatic alterations of lymphocyte chemotaxis in primary rabbit tissues during early phases of infection (Graham *et al.,* 1997). Another example of the difficulty of using morbidity as an end-point measurement for poxvirus virulence is the case of deleting the

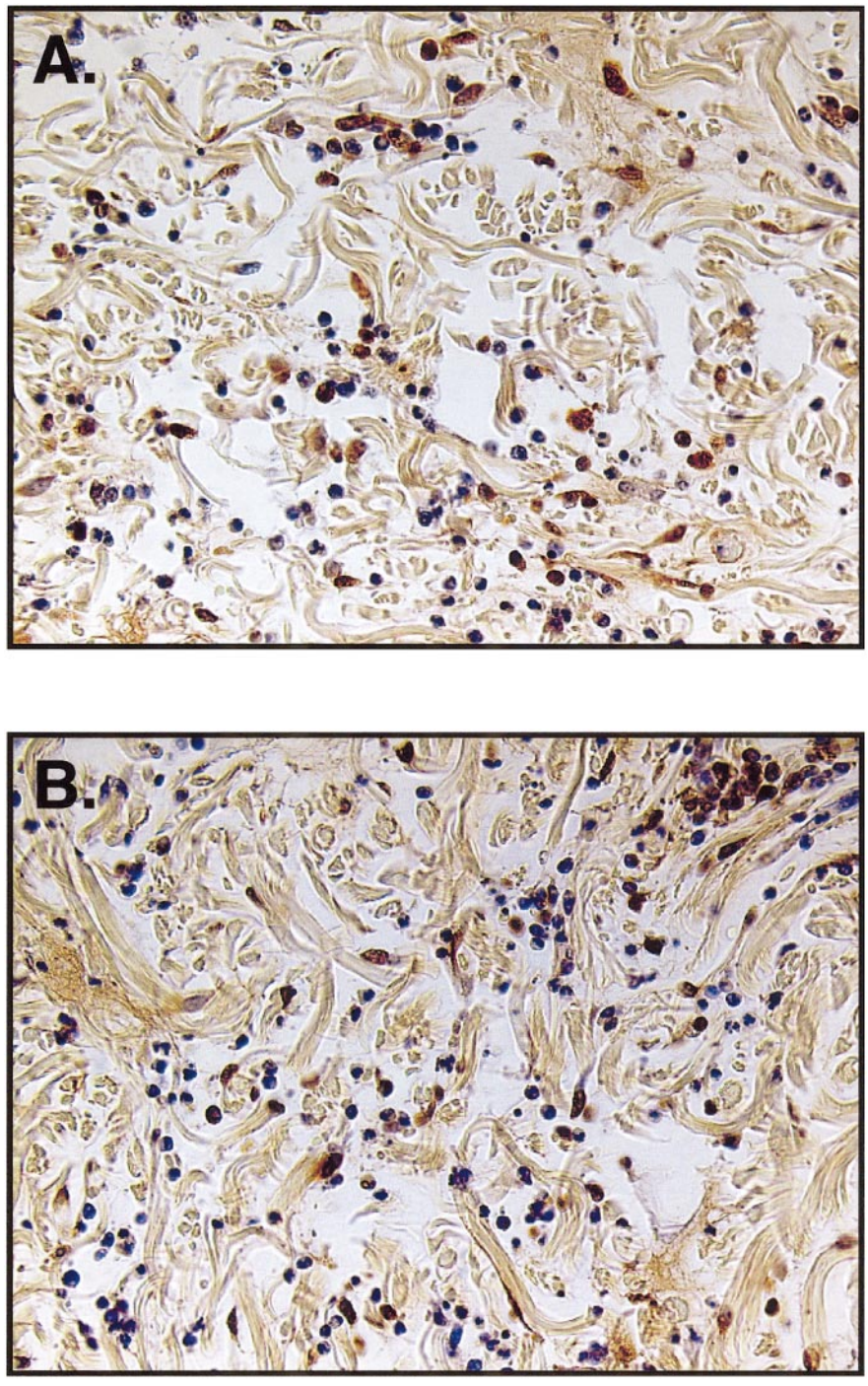

FIG. 6. Viral load in infected primary dermal tissues. Primary tissue sections from vMyxlac (A) and vMyxlacT1$^-$ (B)-infected rabbits were harvested at 3 days p.i. and subjected to immunoperoxidase staining with an anti-β-galactosidase antibody as outlined in Materials and Methods. Peroxidase staining of β-galactosidase correlates with the presence of vMyxlac or vMyxlacT1$^-$ within infected tissues because both viruses encode a *lacZ* gene marker.

vIL-1β receptor of vaccinia virus, which resulted in an accelerated pathogenicity and morbidity after intranasal inoculation but attentuation after intracerebral injection (Alcamí and Smith, 1992; Spriggs *et al.,* 1992). Thus it has been proposed that certain viroceptors may play a role in protecting the host from the detrimental effects caused by the excessive production of certain cytokines in response to infection (Alcamí and Smith, 1995). In this respect, it is entirely plausible that M-T1 may function in moderating the level of the host acute-phase response to facilitate viral replication and spread during the early phases of myxoma virus infection.

The elevated levels of infiltrating phagocytes observed in vMyxlacT1$^-$ tissues did not appear to be sufficient to

resolve virus infection because the detectable viral burden was essentially identical between vMyxlac- and vMyxlacT1$^-$-infected tissues. It is entirely plausible that the infiltrating phagocytes during vMyxlacT1$^-$ infection remain poorly activated and unable to clear virus infection and that at least one of the other known secreted myxoma virulence factors, SERP-1, functions by directly inhibiting inflammatory cells (Lucas *et al.,* 1996; Macen *et al.,* 1993). Thus although M-T1 may effectively suppress the chemoattraction of inflammatory effector cells early during the course of infection, the consolidated activities of additional myxoma virus factors likely contribute to preventing these increased numbers of phagocytes from blocking the development of full-scale myxomatosis pathogenesis.

In addition to M-T1, myxoma virus expresses a second distinct soluble glycoprotein, M-T7, which has been demonstrated to bind chemokines *in vitro* and influence leukocyte influx during myxoma virus infection (Lalani *et al.,* 1997; Mossman *et al.,* 1996b). However, unlike M-T1, which prevents chemokine–receptor interactions, M-T7 has been postulated to function in a distinct manner by disrupting the establishment of solid-phase chemokine gradients along the extracellular matrix (Lalani *et al.,* 1997; Lalani and McFadden, 1997). In addition, M-T1 modulates cellular influx significantly earlier than the inhibition of infiltration observed previously with M-T7 (Mossman *et al.,* 1996b), suggesting that these proteins are functionally nonredundant during myxoma virus infection.

MATERIALS AND METHODS

Cells and viruses

vMyxlac, a myxoma virus (strain Lausanne) derivative bearing a β-galactosidase marker cassette in an intergenic location, was used as the parental wild-type strain in this study and has been described previously (Opgenorth *et al.,* 1992a). The constructions of both recombinant myxoma M-T1-deletion mutant (vMyxlacT1$^-$) and revertant (vMyxT1R) viruses are described below. All viruses were propagated in African BGMK cells (American Type Culture Collection). BGMK cells were passaged in DMEM supplemented with 10% newborn calf serum (GIBCO BRL), 100 units/ml penicillin (BioWhittaker Inc.), 100 μg/ml streptomycin (BioWhittaker Inc.), and 2 mM glutamine (BioWhittaker Inc.). RK-13 cells, a rabbit kidney cell line (American Type Culture Collection), was maintained under similar conditions.

Construction of recombinant vMyxlacT1$^-$ and vMyxT1R viruses

To create the myxoma M-T1-deletion mutant virus, vMyxlacT1$^-$, both copies of the M-T1 ORF were deleted/disrupted by insertion with a dominant selectable marker as shown by the schematic in Fig. 2. A pBluescript vector containing the entire M-T1 gene, pBS-M-T1 (Graham *et al.,* 1997), was restriction digested with *Pst*I and *Bam*HI to remove a majority of the M-T1 coding sequence creating the vector pKS-5′T1. A fragment containing a small 3′ flanking portion of M-T1 was amplified from pBS-M-T1 by PCR using a 3′ M-T1 primer containing an engineered *Pst*I site, T1–3′Pst (CTACCCTGCAGGCGTGTAAC), and a forward primer corresponding to a downstream vector sequence. The amplified products were cloned into a pT7Blue T-vector (Novagen), and a 192-bp *Pst*I–*Bam*HI fragment was excised and ligated into pKS-5′T1, creating the plasmid pKS-T1del. In addition to flanking sequences, the construct contained the 5′ 64-bp and 3′ 20-bp coding sequences of M-T1 separated by an internal *Pst*I site. A *Pst*I cassette containing the Ecogpt gene, whose expression is driven by the vaccinia 7.5K promoter (Falkner and Moss, 1988), was inserted into pKS-T1del, creating pKS-T1KO.

vMyxlacT1$^-$ virus was generated by homologous recombination with the pKS-T1KO plasmid with a vMyxlac infection/transfection procedure using mycophenolic acid selection (Falkner and Moss, 1988) as previously described (Mossman *et al.,* 1996a, 1996b). To verify the disruption of M-T1 and insertion of the Ecogpt cassette, primers flanking the 5′ (5′T1-ATAGAAGGATCCTATCATGAAAC) and 3′ (3′T1-CGACATGGATCCTAGATAGGATTA) sequences of M-T1 were generated, and PCR analysis was performed. vMyxlacT1$^-$ was subsequently used along with the plasmid pBS-M-T1 to create a myxoma M-T1 revertant virus, vMyxT1R, in which the intact M-T1 gene was restored fully into its original loci by a similar procedure described elsewhere (Macen *et al.,* 1996; Mossman *et al.,* 1996a).

Immunoblotting analysis

Soluble M-T1 was detected from virus-infected cellular supernatants using Western blotting analysis as described previously (Lalani *et al.,* 1998). Briefly, BGMK cells were infected with vMyxlac, vMyxlacT1$^-$, or vMyxT1R at an m.o.i. of 10. After 1 h, cells were washed thoroughly with PBS to remove unabsorbed virus and cultured overnight (or for various times as indicated) with fresh serum-free media. Supernatants were then harvested, clarified of virus and cellular debris, and concentrated 10-fold using spin microconcentrators (Millipore). Concentrated supernatants were resolved by 12% SDS–PAGE and transferred to HyBond-C supported nitrocellulose (Amersham Inc.) using a semidry transfer apparatus (Tyler Corp.) for 1 h at 50 mA. Membranes were blocked overnight in Tris-buffered saline (TBS) containing 5% skim milk powder and 0.2% Tween 20 (ICN Biomedicals Inc.), and then probed with (1:5000 dilution) polyclonal anti-M-T1 peptide antisera (Lalani *et al.,* 1998) for 1 h. Blots were washed with TBS-0.2% Tween 20, reprobed

with (1:500 dilution) horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Jackson Laboratories), and subsequently visualized by enhanced chemiluminescence (NEN Life Sciences).

Quantification of secreted M-T1 from infected cells

Supernatants containing myxoma virus secreted proteins were harvested at various times p.i. and immunoblotted according to a similar procedure as that described. To calculate the amount of soluble M-T1 produced during myxoma virus infection, chemiluminescent M-T1 bands were detected using an FX Molecular Imaging System (Bio-Rad) and quantified using purified M-T1 protein standards.

Single-step growth curve analysis

Six-well multidishes containing 5×10^5 BGMK or RK-13 cells were infected with vMyxlac or vMyxlacT1$^-$ at an m.o.i. of 5 for 1 h at 37°C. After viral absorption, the cells were washed three times with PBS and incubated with DMEM-containing serum. Infected cultures were harvested at 0, 4, 8, 24, and 48 h p.i., and viral titers were determined on BGMK cells as described previously (Mossman *et al.,* 1996a). All infections were performed in triplicate, and results are expressed as the average viral titers (log PFU/10^5 cells) ± S.D.

Pathogenesis of virus-infected rabbits

Adult female New Zealand White rabbits (*Oryctolagus cuniculus*) were purchased from a local supplier and housed within a biohazard level 3 containment facility in compliance with guidelines of the Canadian Council on Animal Care. Rabbits were injected intradermally with 1000 PFU of virus below each thigh. Virus titers were verified before experimentation to ensure the accuracy of the dose inoculum. For gross pathological studies, six rabbits were inoculated with vMyxlacT1$^-$ (M-T1-deletion mutant virus); three rabbits with vMyxlac (parental wild-type strain); and three rabbits with vMyxT1R (recombinant M-T1 revertant virus). The infected rabbits were observed daily for clinical symptoms of myxomatosis, and any rabbits that appeared moribund were promptly euthanized with an intravenous overdose of pentobarbital. For histological studies, six rabbits were each inoculated with vMyxlac or vMyxlacT1$^-$ as described above. Two rabbits from each group were sacrificed at 48 h, 72 h, and 7 days p.i. and subjected to a complete postmortem examination. Tissue sections were harvested after necropsy and stored in neutral-buffered 10% formalin before histological analysis (discussed below).

Immunohistological analysis

Infected rabbit tissue samples were paraffin embedded, and 5-μm sections were stained with hematoxylin and eosin for microscopic analysis. To determine specific infiltrating leukocyte subpopulations within infected lesions, sections were further analyzed by immunohistochemical staining as described elsewhere (Mossman *et al.,* 1996b). To detect rabbit monocyte/macrophages infiltrates, samples were stained with a rabbit macrophage-specific monoclonal antibody, RAM11 (a kind gift from Dr. E. Raines, University of Washington), at a dilution of 1:500 (Tsukada *et al.,* 1986). Positive-stained (RAM11) rabbit monocytes/macrophages were enumerated from two independent sets of blinded rabbit sections per time point by microscopy using three random high-power fields, and the results are expressed as the percentage of infiltrating RAM11+ macrophages/total number of leukocytes/field in the deep dermal tissue. To evaluate the presence of the virus within infected tissues, samples were immunostained with (1:500 dilution) anti-β-galactosidase antibody (Promega) to detect the presence of the *E. coli* lacZ gene product expressed from vMyxlac and vMyxlacT1$^-$ (Mossman *et al.,* 1996b).

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