The B cell–controlled development of FAE (as opposed to mere reduction of PP numbers) is crucial for retroviral infection, as tumor necrosis factor receptor 1 (TNFR1) KO mice with the same reduction in number and size of PPs (*21, 22*) as B cell–negative mice but with significant numbers of M cells were found to harbor MMTV efficiently (*9*).

The precise mechanisms of the organogenic function of B cells are unknown. B cells in transgenic mIgM mice do not produce any secreted Ig, excluding involvement of soluble Ig or signaling through Fc receptors in FAE development. The members of the TNFR family have been implicated in organogenesis of lymphoid tissue, including GALT (*23*), and are likely to be involved in the B cell–dependent development of FAE, similar to B cell–dependent generation of follicular dendritic cells (FDCs) (*24*).

The organogenic function of B cells in GALT (and possibly in other mucosal barriers, such as respiratory epithelium) is distinct from their immune functions of Ig secretion or antigen presentation. It affects M cell–dependent translocation of pathogens through mucosal barriers, influences an organism's interactions with environmental flora (*25*), and must be taken into account when interpreting studies that implicate B cells as antigen-presenting cells in immunity and autoimmunity (*26, 27*).

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18. Newborn mice were irradiated with 200 rad, inoculated intravenously with 10^5 B6 BM cells the same day, reinoculated intraperitoneally with the same dose of BM for two additional days, simultaneously fostered by infected females, and then transferred to noninfected mothers. Integrated MMTV proviruses were detected in the splenic DNA of 8-week-old BM recipients by semiquantitative polymerase chain reaction (PCR) [T. V. Golovkina *et al.*, *J. Virol.* **71**, 3895 (1997)]. At that time, all KO mice were fully reconstituted with T and B cells (as determined by fluorescence-activated cell sorter analysis) and developed additional normal-size PPs (5.6 per mouse; 5, 5, 5, 5, 9, 5, 5, 6, and 5 in individual mice). Densitometry was done on the Nighthawk gel documentation system (PDI, Huntington Station, NY).
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Use of Chemokine Receptors by Poxviruses

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Chemokine receptors serve as portals of entry for certain intracellular pathogens, most notably human immunodeficiency virus (HIV). Myxoma virus is a member of the poxvirus family that induces a lethal systemic disease in rabbits, but no poxvirus receptor has ever been defined. Rodent fibroblasts (3T3) that cannot be infected with myxoma virus could be made fully permissive for myxoma virus infection by expression of any one of several human chemokine receptors, including CCR1, CCR5, and CXCR4. Conversely, infection of 3T3-CCR5 cells can be inhibited by RANTES, anti-CCR5 polyclonal antibody, or herbimycin A but not by monoclonal antibodies that block HIV-1 infection or by pertussis toxin. These findings suggest that poxviruses, like HIV, are able to use chemokine receptors to infect specific cell subtypes, notably migratory leukocytes, but that their mechanisms of receptor interactions are distinct.

Viruses can use a wide spectrum of cellular receptors for binding and entry to initiate an infectious process (*1, 2*). For example, HIV and simian immunodeficiency virus (SIV) are known to exploit a variety of members belonging to the chemokine receptor superfamily, most notably CXCR4 and CCR5, which, along with CD4, act as coreceptors to govern viral tropism [reviewed in (*3*)]. The identification of cell-surface receptors for poxvirus

infections, on the other hand, has been hampered by the existence of ubiquitous low-affinity cell-surface binding determinants (*4*), nonspecific effects of unrelated cellular receptors (*5*), and evidence that alternative virion forms use different receptors (*6*). Most studies to evaluate the nature of poxvirus receptors have been based on vaccinia virus as the model system (*7*), but laboratory strains of vaccinia virus exhibit an unusually wide host range that includes a broader spectrum of infectible mammalian cells than most poxviruses directly isolated from native animal hosts (*8*). Here we show that myxoma virus, a rabbit-specific poxvirus pathogen that is relatively restricted to cultured rabbit cells in vitro but also able to infect certain primate cell lines (*9*), can use a spectrum of chemokine receptors in a way that is not species-specific to initiate infection in normally nonpermissive cells.

While investigating the inhibitory properties of a recently discovered class of secreted chemokine binding proteins expressed by myxoma virus (*10*), we unexpectedly observed that pretreatment of permissive baby Green monkey kidney (BGMK) cells with one human chemokine, RANTES (regulated-upon-activation, normal T-cell expressed and secreted), could substantially reduce cellular infection by myxoma virus. This suppressive effect of RANTES on infection of BGMK cells by vMyxlac (a recombinant muxoma virus that expresses β-galactosidase under the control of a late viral promoter that drives a lacZ transgene reporter) (*11*), was not exhibited by two other human CC chemokines (macrophage inflammatory proteins MIP-1α and MIP-1β) that have been shown also to block HIV-1 infection via CCR5 (Fig. 1A) (*12*). The inhibitory effect of RANTES was dose-dependent (Fig. 1B), required pretreatment of the cell monolayer before virus adsorption (Fig. 1C), and was still demonstrated by RANTES variants containing point mutations (P2A, Y3A, D6A, T7A) that inhibit receptor signaling for one or more of the known RANTES receptors (CCR1, CCR3, or CCR5) (*13*). However, NH_2-terminal RANTES deletions (6–68; 10–68) that compromise receptor binding specificities (*14*) were less effective at blocking myxoma virus infection (Fig. 1D).

Studies have shown that a related African Green monkey primate fibroblast cell line expresses CCR5 which can function as a coreceptor for SIV entry (*15*). To investigate the possibility that human RANTES was able to bind and down-regulate the simian version of one or more chemokine receptors that function as a myxoma virus receptor, other cell lines that stably express RANTES-specific chemokine receptors or HIV coreceptors were tested for susceptibility to myxoma virus infection. Myxoma virus cannot infect murine cells, and therefore a series of 3T3 murine fibroblasts that stably express CD4 plus one of a variety of human chemokine receptors (*16*) were tested for the induction of β-galactosidase expression after infection with vMyxlac. The control 3T3 cells expressing only human CD4 did not support substantive levels of viral gene expression as monitored by the synthesis of β-galactosidase (Fig. 2A). However, the coexpression of any one of the human chemokine receptors, CCR1, CCR5, or CXCR4, conferred substantial induction of the viral transgene (Fig. 2A). In each case, surface expression levels of the human chemokine receptors on the 3T3 transfectants were monitored by flow cytometry (Fig. 2B).

The ability of normally nonpermissive cells to become infected as a result of ectopic expression of a cell-surface receptor molecule is considered substantive proof that the molecule functions as a binding or entry receptor (*1*). To assess whether the 3T3 transfectants were fully permissive for multiple and successive rounds of myxoma virus replication, low multiplicity infections by vMyxlac were allowed to proceed for 3 days before detection of β-galactosidase to assess the development of classic myxoma foci. The control 3T3 cells that express CD4 alone were resistant to viral replication, whereas the 3T3 cells expressing CCR1, CCR5, or CXCR4 allowed establishment of classic myxoma foci that were morphologically indistinguishable from parallel infections with fully permissive rabbit kidney fibroblasts (not shown) or BGMK cells (Fig. 3).

These observations suggest that myxoma

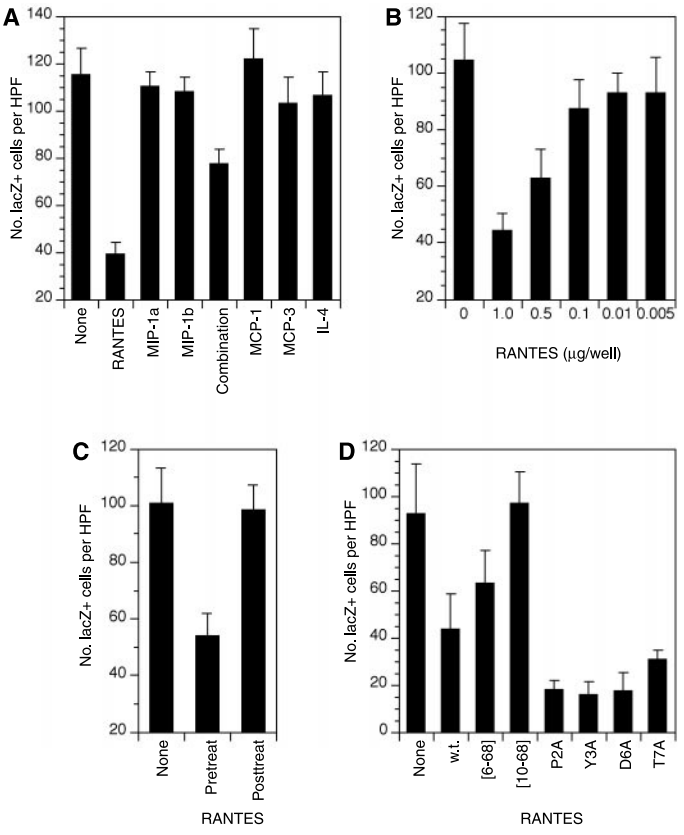

Fig. 1. Pretreatment with the CC chemokine RANTES inhibits myxoma virus infection of BGMK cells. (**A**) Wells containing 4×10^4 BGMK (Green monkey kidney fibroblast) cells were left untreated (none) or pretreated (10 μg/ml) with the following human cytokines: RANTES, MIP- 1α, MIP-1β, or a combination (3.3 μg/ml each RANTES, MIP-1α, MIP-1β), monocyte chemoattractant proteins MCP-1 and MCP-3, or interleukin 4 (IL-4) for 1 hour, washed with phosphate-buffered saline and then infected with vMyxlac (a recombinant myxoma virus that expresses β-galactosidase) at a multiplicity of infection (m.o.i.) of 1. After 16 hours, infected-monolayers were fixed and stained with X-gal, and the number of $lacZ^+$ (vMyxlac-infected) cells were enumerated per high-power field (HPF) by light microscopy. (**B**) BGMK cells were pretreated with indicated concentrations of RANTES per well (0.1 ml) for 1 hour and infected with vMyxlac, and $lacZ^+$ cells were enumerated as indicated above. (**C**) BGMK cells were not treated (none), treated with RANTES as in (A) before vMyxlac infection (pretreat), or treated with RANTES for 16 hours after virus adsorption (posttreat) and analyzed as above. (**D**) BGMK cells were not treated (none) or were pretreated (10 μg/ml) with wild-type (w.t.) RANTES or the indicated RANTES mutants defective for either receptor binding (6–68; 10–68) or signaling (P2A, Y3A, D6A, T7A) for 1 hour. They were washed and then infected with vMyxlac for 16 hours as indicated above.

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virus can use diverse chemokine receptor family members for binding, internalization, or possibly a downstream event that requires receptor signal transduction. To determine whether chemokine receptor signaling itself might be necessary to confer the permissive phenotype, viral infection of 3T3-CCR5 cells was carried out in the presence of the G-protein inhibitor, pertussis toxin; the tyrosine kinase inhibitor, herbimycin A; or the generalized phosphatase inhibitor, PP2 {4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyrimidine}. Only herbimycin A could block myxoma infection, suggesting that, unlike the case for HIV, downstream tyrosine kinases, but not G protein–coupled signaling events, are critical for productive myxoma infection (Fig. 4A). To verify that viral replication in 3T3-CCR5 cells required surface CCR5 expression, preincubation of 3T3-CCR5 cells with either RANTES or a nonaggregating variant (E26A) of RANTES effectively blocked myxoma infection with an apparent median inhibitory concentration (IC_{50}) of 475 nM (Fig. 4B). This result is also distinct from the inhibition of HIV infection by RANTES, which becomes stimulatory upon aggregation at higher concentrations (*17*). To further demonstrate receptor specificity, polyclonal antibody to the NH_2-terminus of CCR5 (*18*) effectively reduced

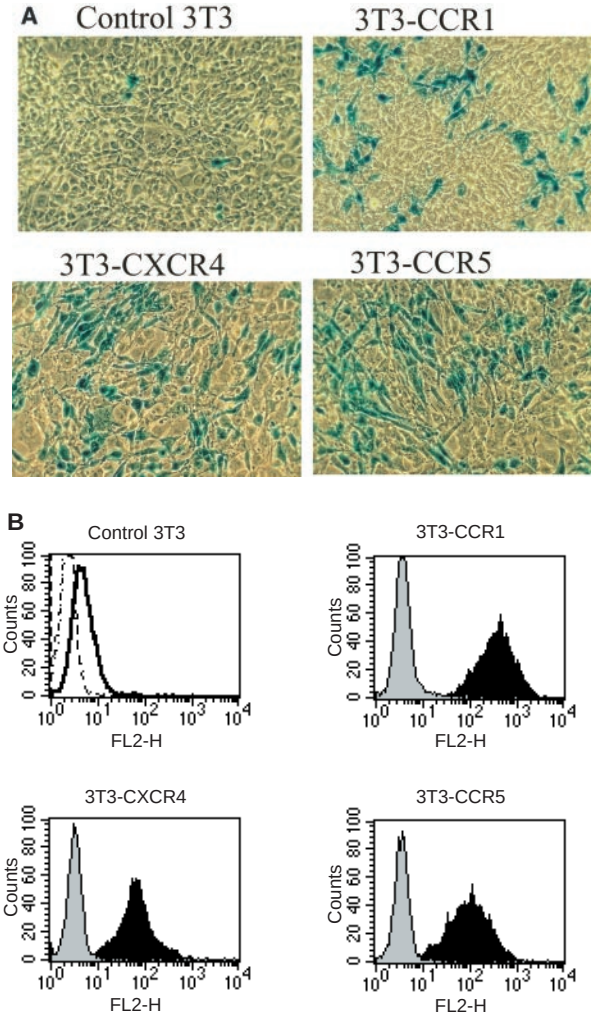

Fig. 2. Ectopic expression of human CC or CXC chemokine receptors permits myxoma virus infection of murine cells. (**A**) 3T3.T4. pMX (control 3T3) cells or various 3T3.T4-derived chemokine receptor expressing stable transfectant cell lines (3T3-CCR1, -CCR5, -CXCR4) were infected with vMyxlac at a multiplicity of 1. After a 16-hour infection, monolayers were fixed, stained with X-gal to detect vMyxlac-infected cells, and photographed using a Zeiss Axiovert light microscope. Original magnification, ×100. (**B**) Analysis of human chemokine receptor expression levels of 3T3 transfectants by flow cytometry. (upper left panel) CCR1 (solid line), CCR5 (dashed line), or CXCR4 (dotted line) expression in parental 3T3.T4 cells. (Other three panels) Expression of CCR1, CCR5, CXCR4 (black curves) versus isotype-matched control antibody (gray curves) on 3T3.T4 stable transfectant sublines expressing human CCR1, CCR5, or CXCR4. All samples were analyzed on a FACScan instrument (Becton-Dickinson) by using CellQuest software after staining with anti-CCR1 (R&D Systems), anti-CCR5PE (PharMingen), anti-CXCR4PE (R&D Systems), anti-mouse IgGPE (Caltag), or the isotype control mouse IgG2aPE (Caltag).

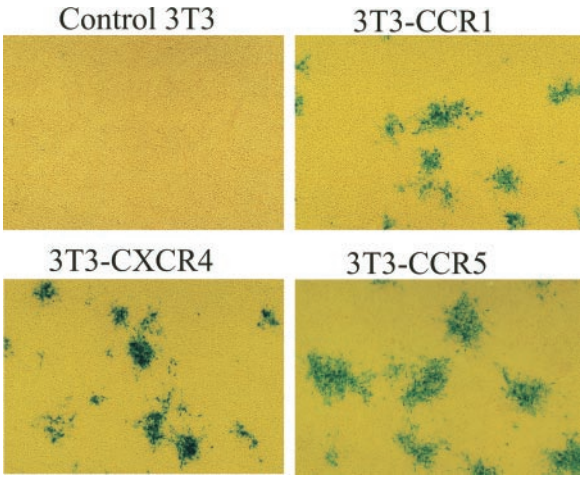

Fig. 3. Human CC and CXC chemokine receptors permit fully productive myxoma virus infection of murine 3T3 cells. 3T3.T4. pMX (control 3T3) cells or stable transfectant 3T3 cell lines that express human chemokine receptors (3T3-CCR1, -CCR5, -CXCR4) were infected with vMyxlac at a multiplicity of <0.001. Three days after the initial infection, monolayers were fixed, stained with X-gal to detect the formation of individual vMyxlac foci, and photographed by using a Zeiss Axiovert light microscope. Original magnification, ×50.

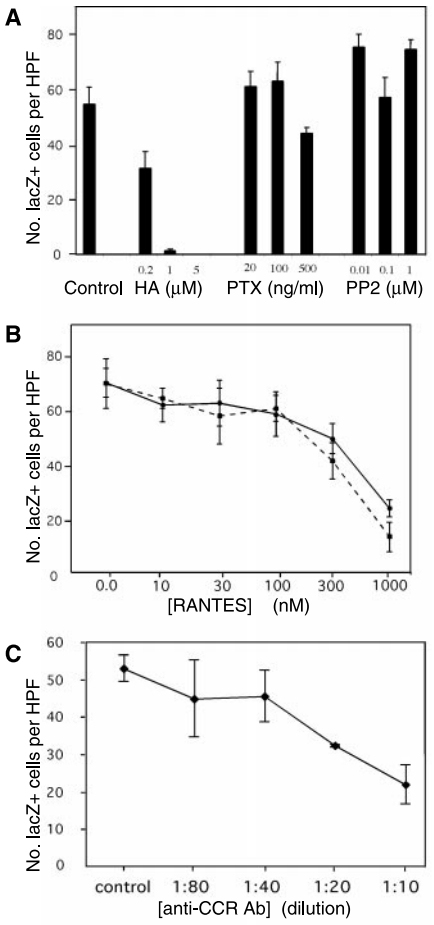

Fig. 4. Inhibition of myxoma virus infection of 3T3-CCR5 cells. Wells containing 4×10^4 3T3-CCR5 cells were infected with vMyxlac as in Fig. 2. (**A**) 3T3-CCR5 cells were left untreated (control), or treated at the indicated concentrations with herbimycin A (HA), pertussis toxin (PTX), or PP2 (all from Calbiochem) for 1 hour before virus adsorption, and left throughout the infection period. (**B**) 3T3-CCR5 cells were pretreated for 1 hour with the indicated concentrations of RANTES (circles, solid line), or a nonaggregating mutant (E26A) of RANTES (squares, dashed line) before vMyxlac infection as indicated earlier. (**C**) 3T3-CCR5 cells were pretreated for 1 hour with the indicated dilutions of a polyclonal antibody against the NH_2-terminus of human CCR5 (*18*) before vMyxlac infection and analyzed as described above. Isotype control antisera exhibited no inhibition at these dilutions (not shown).

virus infection of 3T3-CCR5 cells in a dose-dependent manner (Fig. 4C). On the other hand, no inhibition of myxoma virus infectivity was observed after pretreatment of 3T3-CCR5 with a monoclonal CCR5 antibody (anti–CCR5-2D7) that blocks HIV-1 infection of $CD4^+$ and $CCR5^+$ cells (*19*), indicating that the CCR5 receptor epitopes used by myxoma virus are distinct from those recognized by the HIV-1 envelope glycoprotein, gp120.

A variety of human cell lines that have been used to express one or more chemokine receptors were also screened for infectibility. One human cell line, GHOST (human osteocarcinoma fibroblasts) (*20*) was found to be fully permissive for myxoma infection, which was blocked by stromal-derived growth factor–1 (SDF-1), the only known ligand for CXCR4 (*21*). We conclude that the ability of chemokine receptors to facilitate infection by myxoma virus is not species-specific.

The exploitation of seven-transmembrane spanning receptors by microbial pathogens has been documented for HIV (*3, 22*), *Plasmodium vivax* (*23*), and *Streptococcus pneumoniae* (*24*). In the case of HIV-1 usage of CCR5 or CXCR4, for example, the virus does not utilize chemokine receptor down-regulation to mediate entry (*25*), but the data reported here do not distinguish whether myxoma virus uses chemokine receptors for cell-surface interactions or for events after binding that lead to the initiation of late gene expression. Human AIDS and rabbit myxomatosis share some notable disease similarities, in that myxoma virus and HIV each induce a systemic cellular immune dysfunction associated with extensive virus dissemination via infected migratory leukocytes (*9, 26*), but the viruses are otherwise quite distinct.

Despite these caveats, there is relevance to the issue of potential chemokine receptor usage by poxviruses that can infect humans. CCR5 mutations that down-regulate surface expression, such as the Δ32 alleles, are associated with increased resistance to AIDS, but the original infectious agent that led to the selection of such mutant alleles must have preceded HIV entry into the human population by many centuries (*3, 27*). The exploitation of chemokine receptors by myxoma virus provides a basis to speculate that the selective expansion of polymorphic CCR5 alleles in the human population originally provided increased resistance to variola (smallpox) virus, and only later to HIV.

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