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Attenuated, Oncolytic, but Not Wild-Type Measles Virus Infection Has Pleiotropic Effects on Human Neutrophil Function

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We previously showed that neutrophils play a role in regression of human tumor xenografts in immunodeficient mice following oncolytic vaccine measles virus (MV-Vac) treatment. In this study, we sought, using normal human neutrophils, to identify potential neutrophil-mediated mechanisms for the attenuated MV-Vac induced effects seen in vivo, by comparison with those consequent on wild-type (WT-MV) infection. Both MV-Vac and WT-MV infected and replicated within neutrophils, despite lack of SLAM expression. In both cases, neutrophils survived longer ex vivo postinfection. Furthermore, MV-Vac (but not WT-MV) infection activated neutrophils and stimulated secretion of several specific antitumor cytokines (IL-8, TNF-α, MCP-1, and IFN-α) via induction of de novo RNA and protein synthesis. In addition, MV-Vac (but not WT-MV) infection caused TRAIL secretion in the absence of de novo synthesis by triggering release of prefabricated TRAIL, via a direct effect upon degranulation. The differences between the outcome of infection by MV-Vac and WT-MV were not entirely explained by differential infection and replication of the viruses within neutrophils. To our knowledge, this is the first demonstration of potential mechanisms of oncolytic activity of an attenuated MV as compared with its WT parent. Furthermore, our study suggests that neutrophils have an important role to play in the antitumor effects of oncolytic MV. The Journal of Immunology, 2012, 188: 1002–1010.

The ability of numerous viruses to replicate within tumor cells and exert selective inhibitory effects on tumor growth has long been recognized (1). Measles virus (MV), a negative sense, ssRNA virus has well documented lytic activity in several in vivo models of human cancer (2–5) and is already in early-phase clinical trials (6). Mechanisms of oncolytic activity, particularly in vivo, are unclear.

Although the immune system—in particular an anamnestic Ab response—has rightly been considered as posing a significant barrier to the efficacy of oncolytic therapy, it is also a realistic contention that viral cytolysis and tumor cell elimination could be favored by cellular cytotoxicity directed toward virally infected tumor cells. In this context, cells of the innate immune system are increasingly recognized as players (7). Evidence from both our own work and the studies of others has led us to consider the role of neutrophils in MV-mediated oncolysis. We previously demonstrated MV-induced regression of human tumor xenografts in SCID mice. Both MV-infected and noninfected cells were lysed, suggesting that local antitumor response was enhanced by MV infection of tumor cells. This hypothesis is supported by our finding that tumor regression was associated with an intense neutrophil infiltration. The density of the neutrophil infiltrate and the antitumor response was augmented when MV expressing GM-CSF—a neutrophil survival factor—was used (5). Other groups have also implicated microorganisms in stimulating an antitumor response by neutrophils. In a clinical study of the antitumor activity of Mycobacterium bovis bacillus Calmette-Guérin (BCG), in patients with bladder cancer, neutrophils were shown to be involved in the BCG-induced antitumor response (8). This response correlated with increased levels of TRAIL in urine (9). In a murine bladder cancer model, neutrophil depletion eliminated the antitumor effect of the BCG treatment (10). Studies of replicating oncolytic virus therapy have also shown that neutrophils are involved in the in vivo tumor killing activity mediated by such viruses (11, 12). Both vesicular stomatitis virus (VSV) and vaccinia virus-mediated oncolysis has been attributed to indirect killing of uninfected tumor cells by neutrophils (11). In a B16 melanoma model in immunocompetent mice, VSV-induced IL-28 secretion by neutrophils contributed to VSV-mediated regression of the tumors (11). Work from the Ottawa group using VSV to treat syngeneic CT-26 colon cancer also underscored the relevance of a virus-induced neutrophil response in tumor regression. Recruitment of neutrophils to the infected tumor beds was proposed to as the mechanism for the observation of blood flow shut down within the tumor following VSV therapy (12). Further investigation showed that VSV replication in the tumor neovasculature initiated an inflammatory reaction, which included neutrophil-dependent initiation of microclots within tumor blood vessels (13).

In the current study, we have extended our own observations of the relevance of neutrophils in the measles-mediated antitumor response to investigate specific mechanisms by which infection by the attenuated, oncolytic MV might directly stimulate potential anticancer properties of human neutrophils. To distinguish the generalized responses to a viral infection per se from those which might specifically result from infection by a so-called “oncolytic strain,” we compared the response to oncolytic MV to that seen...
when a wild-type (WT) strain of MV was used. We demonstrated several novel mechanisms whereby infection of an oncolytic MV can specifically stimulate a neutrophil response that favors the elimination of transformed cells. In addition, we have demonstrated multiple differential effects between the oncolytic vaccine strain and the WT strain.

Materials and Methods

Cell culture

Vero (African green monkey kidney) cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Invitrogen, Paisley, U.K.) supplemented with 5% FBS, 100 U/ml streptomycin, 100 μg/ml penicillin, and 2 mM l-glutamine (Invitrogen). The marmoset lymphoblastoid cell line B95a (American Type Culture Collection) was maintained in DMEM (Invitrogen), supplemented with 10% FBS, 100 U/ml streptomycin, 100 μg/ml penicillin, and 2 mM l-glutamine. The human pre-B leukemia cell line Nalm-6 was cultured in RPMI 1640 medium (Invitrogen), supplemented with 10% FBS, 100 U/ml streptomycin, 100 μg/ml penicillin, and 2 mM l-glutamine.

Neutrophil isolation

Heparinized peripheral blood was obtained from healthy volunteers after they gave informed consent. Buffy coats were purchased from the North London Blood Transfusion Centre (Colindale, U.K.). Neutrophils were isolated as described previously (14). Briefly, RBCs were sedimented with 5% dextran (Fisher Scientific, Leicestershire, U.K.) at room temperature for 40 min. Leukocyte-rich upper fraction was carefully layered on LymphoPrep (Axis-Shield, Oslo, Norway) and subject to density gradient centrifugation at 400 g for 30 min without brake. Contaminating RBCs were removed by hypotonic lysis. Giemsa-stained cytopsin slides were prepared with isolated cells, and neutrophils were recognized by their lobular or segmented nuclei. Total number of cells and number of neutrophils were counted within three random fields of each slide. The average purity of isolated neutrophils was always >95%. Isolated human neutrophils were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% FBS, 100 U/ml streptomycin, 100 μg/ml penicillin, and 2 mM l-glutamine.

Virus propagation and infection

The attenuated, oncolytic strain of MV, denoted MV-Vac was derived from the currently available Moraten vaccine strain (15), a gift from Dr. R. Cattaneo (Mayo Clinic, Rochester, MN.). WT MV-Dublin denoted WT-MV was a gift from Dr. P. Duprex (Queen’s University, Belfast, U.K.) (16). Vaccine strain MV was propagated on Vero cells. WT MV-Dublin denoted WT-MV was propagated on Vero cells by infection at a multiplicity of infection (MOI) of 0.01, followed by incubation at 37°C for 2 h. The viral inoculum was removed and replaced with medium. Cells were scraped into small volumes of Opti-MEM (Invitrogen) when the maximum cytopathic effect was observed. Viral particles were released by two freeze-thaw cycles and titrated at 50% tissue culture infectious dose on Vero cells. WT measles virus was propagated using the same method but using B95a cells. All viral stocks were frozen in aliquots at −80°C. For MV infection, freshly isolated neutrophils were washed once with 1× PBS before resuspended in Opti-MEM (Invitrogen) with viral inoculation at a MOI of 1.0. After 2 h of adsorption, the virus was removed, and neutrophils were maintained in RPMI 1640 complete medium.

Antibodies

FITC-conjugated anti-CD44 mAb, PE-conjugated anti-CD150 (anti-SLAM) mAb, FITC-conjugated anti-CD47, Annexin V–allophycocyanin, propidium iodide (PI), and Annexin V binding buffer were all purchased from BD Biosciences (Oxford, U.K.). PE-conjugated anti–nectin-4, anti-human–L-selectin mAb, and goat F(ab)2 anti-mouse IgG–allophycocyanin were purchased from R&D Systems (Abingdon, U.K.). For neutrophil degranulation, purified anti-CD63 (clone H5C6), anti-CD35 (clone E11), anti-CD66b (clone G10F5), and mouse IgG1 isotype control were purchased from R&D Systems (Abingdon, U.K.). PE-conjugated anti–nectin-4, FITC-conjugated anti-CD147, Annexin V–allophycocyanin were purchased from R&D Systems (Abingdon, U.K.). For neutrophil degranulation, purified anti-CD63 (clone H5C6), anti-CD35 (clone E11), anti-CD66b (clone G10F5), and mouse IgG1 isotype control were purchased from BD Biosciences. Anti-MV H-PioC was a gift from Dr. R. Cattaneo.

Flow cytometry

Cells were incubated on ice with the appropriate primary Abs at 4°C for 40 min, washed twice with BD FACSFlow (BD Biosciences), and then incubated with conjugated secondary Abs in the dark for 30 min. The cells were washed again and resuspended with FACSFlow before data acquisition using FACSCalibur (BD Biosciences). For Annexin V and PI staining, cells were washed twice in cold 1× PBS before being resuspended in 1× Annexin V buffer with Annexin V and PI. The cells were incubated at room temperature for 15 min in the dark before acquisition. All FACS analysis was carried out using FlowJo (Tree Star).

Relative quantitative real-time PCR

Total RNA was extracted from neutrophils using TRizol (Invitrogen, San Diego, CA), and concentration was measured by NanoDrop spectrophotometer (Thermo Scientific, Essex, U.K.). RNA was converted to first-strand cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Paisley, U.K.) and random hexamer (Promega, Southampton, U.K.). Relative quantitative real-time PCR (RQ-PCR) for MV-nucleoprotein (MV-N) was carried out in triplicates of 25 μl-reaction using a customized TaqMan gene expression assay (Applied Biosystems, Warrington, U.K.). MV-N primer sequences were 5′-GTATCTGGCTTGGGACTGGATC-3′ (forward), 5′-GTTCTACAGGACTCAAGTTGGFAR-3′ (reverse) TaqMan probe sequence was 5′-TCAACGCAAATCT-3′. Expression of TRAIL was quantified using an inventoried Taqman gene expression assay. For TNF-α, IL-8, MCP-1, and IFN-α, RQ-PCR was performed using Quantitech primer assays and a Quantitech SYBR green kit (Qiagen, Crawley, U.K.). Dissociation curves were generated for gene expression studies with SYBR green to confirm the amplification products. All PCRs were carried out on ABI 7500 system and used GAPDH as the housekeeping gene. Relative quantity of mRNA was calculated using ΔΔCt method (17) and expressed as fold change relative to the appropriate calibrator sample.

ELISA

Quantification of TNF-α, IL-8, MCP-1, and TRAIL in supernatants of cultured neutrophils was carried out using Quantikine ELISA kits (R&D Systems), according to the manufacturer’s instructions. For IFN-α, IFN-β, and IFN-γ quantification, ELISA was performed using VeriKine kits (PBL IFN Source). All results were normalized to the amount produced by 5 × 10⁶ neutrophils in picograms per milliliter.

Western blotting

Proteins were separated on 12% polyacrylamide gel (Invitrogen) and transferred to nitrocellular membranes as described previously (18). Mouse anti-MV-H primary Ab (HpioC) was used to detect MV protein expression. After staining with HRP-linked secondary Abs (DakoCytomation, Cambridge, U.K.), protein bands were visualized by ECL (GE Healthcare, Little Chalfont, U.K.). All immunoblots were stained with Ponceau S to ensure even protein loading.

Inhibitor experiment

Neutrophils were treated with 20 μg/ml cycloheximide (Sigma-Aldrich) for 1 h before MV infection. Supernatants from MV-infected neutrophils and uninfected control, with or without inhibitor treatment, were collected at 24-h time point and stored at −80°C before use.

Neutrophil degranulation

Neutrophil degranulation was induced by cytochalasin B and FMLF (19). Briefly, freshly isolated neutrophils were incubated with 5 μg/ml cytochalasin B at 37°C for 5 min, followed by 1 μM FMLF at 37°C for 2 min with gentle agitation. Neutrophils were then pelleted and washed once with 1× PBS before staining with Abs for neutrophil granule markers.

Statistical analysis

Graphs were plotted using Prism 5.0 (GraphPad Software). The Mann–Whitney U test was used to assess statistical significance between two groups. p < 0.05 was considered statistically significant.

Results

Impact of MV infection on neutrophil activation status and survival

Expression of the MV receptors CD46 and CD150/SLAM was quantified on neutrophils. All donor neutrophils expressed high levels of CD46 (Fig. 1A); mean expression from seven donors was 61.4 ± 5.22 (the mean of mean fluorescence intensity [MFI] ± SEM) versus isotype control 4.1 ± 0.21. By contrast, no donor
neutrophils expressed SLAM, and mean expression on eight donors was 5.1 ± 0.88 versus isotype control 4.2 ± 0.44.

To determine the efficiency with which the viruses could infect and replicate within neutrophils, we evaluated genome replication, via quantitation of MV-N mRNA using RQ-PCR and sought evidence for subsequent viral protein expression. By 1 h postinfection, MV-N RNA was already expressed with both MV-Vac and WT-MV (Fig. 1A). MV-N copy number increased steadily in the hours following MV-Vac infection, reaching a peak 80-fold higher than UV-MV at 6 h. Following WT-MV infection, MV-N mRNA was ∼10-fold greater than following UV-MV but increased only marginally over 24 h, suggesting that genome replication did occur following WT-MV infection, but was less efficient than that seen postinfection with MV-Vac. MV-H glycoprotein was expressed on the surface of MV-Vac–infected neutrophils as demonstrated by flow cytometry at 24 h postinfection (Fig. 1C), but cell surface expression of MV-H following WT-MV infection could not be demonstrated.

To ascertain whether lack of cell surface expression of MV-H on WT-MV–infected neutrophils was due to a complete failure of viral protein production or otherwise, Western blotting was carried out on lysed neutrophils following infection. MV-H glycoprotein was detected in the cell lysate of WT-MV–infected neutrophils but not in UV-MV–infected or noninfected controls (Fig. 1D), indicating that MV-H is translated and processed following WT-MV infection of human neutrophils, although not transported to the cell surface in sufficient quantity to be detected by FACS. Hence, MV-Vac was able to infect neutrophils and replicate efficiently. WT-MV also infected neutrophils and replicated with modest efficiency, despite the lack of SLAM expression.

Recently, two additional cellular membrane proteins, CD147 (20) and nectin-4 (21), were identified as additional entry receptors for WT-MV. We examined the expression of CD147 and nectin-4 on human neutrophils by FACS (Fig. 1E). Abundant CD147 but no nectin-4 was found. This offers a potential explanation for the low-level infection by WT-MV we observed in this study.

Next, we determined the effect of MV infection on neutrophil viability and activation. L-Selectin shedding, a marker of neutrophil activation, was assessed by flow cytometry (22, 23). Freshly isolated neutrophils expressed high level of surface L-selectin, indicating that the cells were not already activated by the isolation procedures. When neutrophils were cultured in medium alone, L-selectin was only slightly downregulated at 4 h postisolation (Fig. 2B), which is consistent with previously reported shedding of L-selectin over time, in culture medium alone (24). MV-Vac infection resulted in L-selectin shedding in an identical manner to the positive control, LPS, at the same time point. By contrast, WT-MV infection induced no such activation at 4 h postinfection; L-selectin expression on WT-MV–infected neutrophils was similar to that of the uninfected control at 4 h.

Next, we determined the effect of MV infection on neutrophil survival using flow cytometry to quantify the percentage of Annexin V−PI− population. There was a statistically significant enhancement of viability following both MV-Vac and WT-MV infection, as compared with no infection (Fig. 3A). Thus, MV-Vac infection both activated neutrophils and enhanced their survival ex vivo. WT-MV infection also enhanced survival ex vivo, but no evidence of activation was found.

Vaccine MV but not WT-MV provokes specific de novo antitumor cytokine secretion by neutrophils

Next, we quantified the effect of MV-Vac and WT-MV infection of neutrophils on the expression of a variety of antitumor prion-
Inflammation genes by RQ-PCR and their corresponding proteins by ELISA. We chose to examine IL-8 (a potent chemoattractant and activator of neutrophils), TNF-α (a pleiotropic cytokine that can cause destruction of tumor-associated vasculature), TRAIL (a cytokine with tumor-specific cytotoxic activity) and MCP-1 (a chemoattractant for monocytes and T lymphocytes), IFN-α/β, IFN-γ, and Fas ligand. For TNF-α, IL-8, MCP-1, and IFN-α mRNA, a similar and striking pattern was observed—infection with MV-Vac resulted in a 10- to 100-fold upregulation of the mRNA, which was statistically significant compared with mock infection or WT-MV infection (Fig. 4). There was a corresponding increase in protein secretion of these cytokines (Fig. 5) with similar pattern seen for TNF-α, IL-8, MCP-1, and IFN-α mRNA data. We did not detect any IFN-β, IFN-γ, or Fas ligand secretion by neutrophils in any conditions (data not shown).

De novo TRAIL synthesis is not required for MV-mediated secretion

The TRAIL response to MV-infection of neutrophils was very different from the antitumor cytokine response. By contrast to the upregulation of various cytokine mRNAs, which were shown in Fig. 4, infection with both MV-Vac and WT-MV resulted in a relative decrease in TRAIL mRNA production, as shown in Fig. 6A. Despite this, TRAIL protein was secreted into the supernatant in response to MV-Vac infection (Fig. 6B). Neither WT-MV nor mock infection resulted in any change in TRAIL secretion. This finding...
led us to speculate that the oncolytic MV-Vac may be stimulating release of preformed TRAIL from storage in neutrophil granules. To investigate further, we first confirmed that de novo protein synthesis was not required for MV-Vac–mediated TRAIL release by repeating the experiment in the presence of a protein synthesis inhibitor, cycloheximide. This inhibitor suppressed TRAIL secretion resulting from the positive control IFN-α treatment but had no effect on TRAIL secretion in response to MV-Vac infection (Fig. 6C), confirming that protein synthesis is not required for the MV-Vac–stimulated TRAIL release from neutrophils.

**MV-Vac infection directly stimulates neutrophil degranulation**

To determine whether MV-Vac infection was directly affecting neutrophil granule release, we quantified granule exocytosis by flow cytometry, taking advantage of the fact that increases in cell surface expression of CD66b (specific granule marker), CD35 (secretory granule marker), and CD63 (azurophil granule marker) are consequent on the relevant granule exocytosis (25, 26). Fig. 7A shows that expression of all three markers (CD63, CD66b, and CD35) on the cell surface was upregulated after cytochalasin B and fMLF-positive control (27) treatment compared with the

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**FIGURE 4.** MV-Vac infection of neutrophils upregulates mRNA expression of various proinflammatory antitumor genes. RQ-PCR expression of TNF-α (n = 8) (A), IL-8 (n = 8) (B), MCP-1 (n = 7) (C), and IFN-α (n = 5) (D) in neutrophils at 5 h following MV-Vac infection, WT-MV infection, or no infection. The y-axis represents the fold change in mRNA, which was normalized against the calibrator sample of freshly isolated neutrophils (baseline). GAPDH was used as the housekeeping gene. The x-axis shows the mean ± SEM for each condition **p < 0.01, *p < 0.05.

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**FIGURE 5.** Neutrophils secrete proinflammatory cytokines when infected by MV-Vac. Freshly isolated neutrophils were infected by MV-Vac or WT-MV at MOI = 1.0. Supernatants were analyzed for level of TNF-α (A), IL-8 (B), MCP-1 (C), and IFN-α (D) by ELISA at 24 h postinfection. Uninfected neutrophils at the same time point were used as the negative control. The y-axis represents the amount of cytokine released by 5 × 10^6 neutrophils (pg/ml). Each symbol represents one individual neutrophil donor. The bars on the x-axis show the mean ± SEM. **p < 0.01, *p < 0.05.
Infection can directly stimulate neutrophil degranulation. Together, these data strongly suggest that MV-Vac but not WT-MV increased over baseline following MV-Vac infection. Taken together, these findings. First, we demonstrated that the oncolytic strain of MV is very efficient at infecting and replicating within freshly isolated primary human neutrophils and that viral proteins are expressed, even by these terminally differentiated cells cultured ex vivo. That MV-Vac infection did not kill the neutrophils despite efficient infection is consistent with numerous reports of the selectivity of the oncolytic effect (2, 4). However, extension of the ex-vivo lifespan of these infected primary cells, was unexpected. This may relate to the induction of an activated state, which we demonstrated by both L-selectin shedding and IL-8 secretion. It is known that once activated, neutrophils have reduced susceptibility to apoptosis and an increased longevity (33, 34).

Contrary to expectation—because we demonstrated that SLAM is absent on human neutrophils—WT-MV also infected and replicated within human neutrophils, albeit with less efficiency than the vaccine strain. This may relate to low-level infection mediated by the recently reported MV receptor CD147 (20). MV infection occurring via CD147 is independent of binding to MV H and occurs via and virion-associated cyclophilin B. This less efficient infection is consistent with numerous reports of the selectivity of the oncolytic effect (2, 4). However, extension of the ex-vivo lifespan of these infected primary cells, was unexpected. This may relate to the induction of an activated state, which we demonstrated by both L-selectin shedding and IL-8 secretion. It is known that once activated, neutrophils have reduced susceptibility to apoptosis and an increased longevity (33, 34).

DMSO vehicle-treated negative controls, indicating effective degranulation of azurophil, specific granules and secretory vesicles by the positive control. Fig. 7B shows the percentage of cells expressing CD63, CD66b and CD35, as well as the mean fluorescence intensity (MFI) on freshly isolated neutrophils (baseline) and at 24 h following infection with MV-Vac, WT-MV, or mock infection. For each of the markers, representative examples are shown first. Quantification of the mean (±SEM) results from between 6 and 10 individual donors is then shown. CD35 (28) was expressed on ~60% of freshly isolated neutrophils. The percentage of cells expressing CD35 decreased (mean = 14.8%) following 24 h in culture alone. Infection with MV-Vac resulted in a significant increase in the percentage of cells expressing CD35 (mean = 34.6%) but not with WT-MV infection (mean = 6.5%). There was also a highly significant difference in MFI of CD35 expression between MV-Vac–infected neutrophils (mean = 9.6) and uninfected controls (mean = 4.3) or WT-MV infection (mean = 2.9), although none of the experimental conditions resulted in higher CD35 MFI than baseline. Over 90% of neutrophils expressed surface CD66b at baseline, and the percentage of cells expressing CD66b did not change significantly with MV infection. However, the MFI increased significantly after MV-Vac infection (mean = 160.2) compared with baseline (mean = 81.3), mock (mean = 44.0), or WT-MV infection (mean = 40.6). The azurophil granule marker CD63 was expressed on only a small percentage of neutrophils at baseline (mean = 1.3%). Both the percentage of cells expressing CD63 (mean = 9.7%) and the MFI significantly increased over baseline following MV-Vac infection. Taken together, these data strongly suggest that MV-Vac but not WT-MV infection can directly stimulate neutrophil degranulation.

Discussion
In this study, prompted by our own and others’ observations of microbial organism-stimulated, neutrophil-mediated tumor lysis (5, 10, 12, 29–31), we have investigated mechanistic aspects of how an oncolytic virus, measles, might stimulate the functional anticancer properties of neutrophils, which have been previously demonstrated observationally, in murine models. In our experiments, we used freshly isolated human neutrophils from numerous healthy donors. To isolate which aspects of MV infection that are particularly pertinent to “oncolytic” activity, we used a WT-MV as a control, allowing us to assess which, if any, virally mediated effects on neutrophils are specific to an attenuated oncolytic strain of MV.

Numerous antitumor properties of neutrophils have been proposed; among these are direct-cell killing by H2O2 or superoxide (30) and calprotectin (32). In our previous work, we were not able to demonstrate that MV had any effect on those functions of neutrophils (5); hence, our current study focused on other potential anticancer mechanisms. In particular, we focused on the activation status of neutrophils and the secretion of cytokines with known antitumor properties. Our studies generated some surprising findings. First, we demonstrated that the oncolytic strain of MV is very efficient at infecting and replicating within freshly isolated human neutrophils and that viral proteins are expressed, even by these terminally differentiated cells cultured ex vivo. That MV-Vac infection did not kill the neutrophils despite efficient infection is consistent with numerous reports of the selectivity of the oncolytic effect (2, 4). However, extension of the ex-vivo lifespan of these infected primary cells, was unexpected. This may relate to the induction of an activated state, which we demonstrated by both L-selectin shedding and IL-8 secretion. It is known that once activated, neutrophils have reduced susceptibility to apoptosis and an increased longevity (33, 34).

Contrary to expectation—because we demonstrated that SLAM is absent on human neutrophils—WT-MV also infected and replicated within human neutrophils, albeit with less efficiency than the vaccine strain. This may relate to low-level infection mediated by the recently reported MV receptor CD147 (20). MV infection occurring via CD147 is independent of binding to MV H and occurs via and virion-associated cyclophilin B. This less efficient mechanism of entry has been suggested as a possible receptor for SLAM-independent entry. Hence, the high level of CD147 expression on neutrophils provides an explanation for our observations. Nectin-4 (another recently reported MV receptor) (21) was absent on neutrophils in our study, ruling this out as a possible mechanism of entry for WT-MV.

Following infection by WT-MV, low-level MV-H glycoprotein expression was demonstrated by Western blotting, although cell surface expression could not be detected by FACS, indicating...
a possible failure of protein transport to the cell surface. Again, this is consistent with an H-independent mechanism of WT-MV entry and replication within neutrophils, because expression of MV proteins at the cell surface generally facilitates cell-to-cell viral spread. Interestingly, neutrophils infected by WT-MV had a similar increase in longevity ex vivo as did MV-Vac–infected cells, although activation by WT-MV could not be demonstrated by L-selectin downregulation. However, IL-8 secretion did occur after WT-MV infection, albeit a much more muted IL-8 response was seen than following MV-Vac infection, suggesting that the activation of neutrophils by MV may relate at least in part to the efficiency of viral replication.

MV-Vac infection consistently induced human neutrophils to secrete considerable quantities of cancer-relevant soluble cytokines. On the basis of our previous observations (5) and the reports of others of the association of neutrophil infiltration with a better anticancer response in murine models, we suggest that our findings are relevant to therapeutic approaches involving MV and other oncolytic viruses.

The IFN pathway has been a common starting point for the investigation of “oncolytic” properties. First described in a pivotal study regarding VSV (35), several studies have since demonstrated that one mechanism of the selective tumor cytotoxicity of oncolytic viruses relates to deficiencies in the IFN-mediated antiviral responses of tumor rendering viral replication and killing more potent in tumor cells than in normal cells with an intact pathway. There is some evidence that an activated RAS/Raf1/MEK/ERK pathway plays a critical role in the defects, at least as far as VSV-mediated lysis is concerned (36). Other viruses include Newcastle Disease Virus (37) and adenovirus (38). This is
consistent with our clear demonstration that the oncolytic strain of MV stimulated a very specific, brisk IFN response from the neutrophils, which did not occur at all, following mock or WT infection.

We also investigated other, specifically chosen cytokines, which we hypothesized might be of particular relevance to the in vivo observations, which have already been reported (5, 12). Neutrophils are known to produce TNF-α; local production of high levels of TNF-α can specifically kill tumor vessel endothelial cells and lead to hyperpermeability of tumor vessels and destruction of tumor vasculature (39). As such, virally directed secretion of TNF-α by neutrophils could explain the neutrophil-mediated blood flow shutdown, which was observed in VSV-induced tumor regression (12). This TNF-α induced hyperpermeability of tumor-associated vasculature also potentially facilitates accumulation of systemically administered oncolytic virus or chemotherapy drugs within tumor (39, 40). There was some low level secretion of TNF-α in response to WT-MV but a very significant difference between vaccine and WT-MV.

We quantified considerable IL-8 secretion by neutrophils upon infection by both vaccine and WT-MV strains, although we documented ∼2 orders of magnitude difference in the vaccine-induced response as compared with the WT response. Our interest in quantifying the IL-8 response stemmed from the fact that IL-8 secretion is a surrogate marker for neutrophil activation, and we were interested to confirm the L-selectin activation data using another method. In addition, the IL-8 response, which is an early neutrophil response, can amplify local antitumor responses via recruitment of additional activated neutrophils to the tumor site (41). In our previous in vivo studies, we observed considerable accumulation of neutrophils at the tumor site, and the magnitude of the response correlated with VM-mediated tumor regression.

We investigated the production of MCP-1, because we wanted to understand whether the VM-mediated neutrophil response could be documented as potentially linking with antitumor responses of other immune cells. MCP-1 has a potent in vivo monocyte recruitment activity (42) and can also attract T lymphocytes (43). VM-induced production of MCP-1 suggests that neutrophil may also play an immunoregulatory role by recruiting other immune cells with antitumor properties to the tumor site. MCP-1 protein itself is also cytostatic against several types of tumor cells (44). In the case of all of the cytokines noted above, there was complete congruence between RNA and protein upregulation and statistically significant difference between the vaccine and WT-MV-induced effects.

The secretion of the cytotoxic molecule TRAIL was different and intriguing mechanistically, because the secretion of soluble TRAIL did not depend on mRNA up-regulation and was subsequently demonstrated to be independent of de novo protein synthesis. Thus, it appeared to be secreted directly as a result of neutrophil degranulation. Undoubtedly, the presence of IFN in a local microenvironment can stimulate production of cytotoxic molecule TRAIL by neutrophils and monocytes (45, 46). Indeed, our data confirm that IFN can stimulate neutrophils to produce TRAIL. However, it is sensitive to protein synthesis inhibition in a manner in which the VM-mediated TRAIL production is not suggesting that the TRAIL response we observed is not a result of IFN stimulation. The TRAIL response is highly relevant to antitumor activity; this is the best-studied candidate molecule to date as a mediator of the cytotoxic properties of neutrophils upon microbial infection. TRAIL is typically selectively tumor toxic while sparing normal cells (47–49). Neutrophils are known to produce TRAIL in response to M. bovis BCG but have not previously been shown to respond in this way following viral infection. In a clinical study of the antitumor activity of in patients with bladder cancer, neutrophils were implicated in the BCG-induced antitumor response (8). This response correlated with increased levels of TRAIL in urine (9). Analysis of neutrophils from patients undergoing BCG treatment showed that they are a rich source of TRAIL. Indeed, depletion of neutrophils in a murine bladder cancer model eliminated the antitumor effect of BCG treatment (10). In our study, it is also of interest that the neutrophils themselves were not sensitive to the secreted TRAIL. This may be related, not only to the state of activation that we demonstrated but also to the low level of TRAIL death receptors coupled to the high level of TRAIL decoy receptors we observed on neutrophils (data not shown).

Within neutrophils, TRAIL protein is reported to be detectable in all types of granules, but it is especially abundant in azurophil granules and secretory vesicles (8, 50). We demonstrated clearly that MV-Vac infection per se was associated with granule release from neutrophils. This supports the contention that the TRAIL response was a result of degranulation, rather than de novo upregulation on TRAIL. We are not aware that any viruses have yet been clearly reported as directly causing neutrophil degranulation. Respiratory syncytial virus was reported as causing neutrophils to produce cytokines such as IL-8 and myeloperoxidase. The production of myeloperoxidase was taken as evidence of direct release from granules. However, the chemokine release was transcription dependent as it was inhibited by actinomycin D (51). Our data are of particular interest in that there was a very strong differential between vaccine MV and WT-MV in their ability to stimulate granule release—WT-MV did not have this property at all.

In summary, our observations show that neutrophils are readily infectable by an oncolytic vaccine strain of MV and, upon infection, become activated, live longer, and develop potential tumoricidal properties. Combined with previous in vivo observations of a striking relationship between MV-induced neutrophil infiltration into tumors and antitumor efficacy of MV, our present study suggests that neutrophils may have an important role to play in the antitumor efficacy of oncolytic MV, both directly and indirectly. These observations go some way toward providing a mechanistic explanation of observations that have already been made in vivo and hence are likely to have considerable biological relevance. There were important differences between the outcome of infection by vaccine versus WT infection, and these differences are not entirely explained by a failure of WT-MV to infect and replicate within neutrophils.

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Disclosures
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References


