

## ORIGINAL ARTICLE

# Virotherapy induces massive infiltration of neutrophils in a subset of tumors defined by a strong endogenous interferon response activity

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Oncolytic virotherapy has shown substantial promises as an alternative therapeutic modality for solid tumors in both preclinical studies and clinical trials. The main therapeutic activity of virotherapy derives from the direct lytic effect associated with virus replication and the induction of host immune responses to the infected tumor cells. In this study, we show that some human and murine tumor cell lines are highly resistant to the lytic effect of a type II herpes simplex virus-derived oncolytic virus, FusOn-H2, which was constructed by deleting the N-terminal region of the *ICP10* gene. However, these tumor cells still respond exceptionally well to FusOn-H2 virotherapy *in vivo*. Histological examination of the treated tumors revealed that, in contrast to tumors supporting FusOn-H2 replication, implants of these highly resistant lines showed massive infiltration of neutrophils after virotherapy. Further analysis indicated a correlation between an intrinsically strong interferon response activity and the recruitment of neutrophils in these tumors. These results suggest that an innate immune response mainly represented by neutrophils in these tumors may be part of the virotherapy-mediated antitumor mechanism.

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### Introduction

A persistent criticism of many emerging cancer treatments is that their beneficial effects extend only to a subset of patients. This limitation tends to be more common with biotherapeutic interventions such as immunotherapy and gene therapy. For example, studies by Morgan *et al.*<sup>1</sup> showed that only 2 of 15 patients receiving infusions of their own modified T-cells responded with clearly objective regressions of metastatic melanoma. With a few notable exceptions, such as the strong link between a mutated epidermal growth factor (*EGFR*) gene and clinical responses to its tyrosine kinase inhibitor Iressa,<sup>2,3</sup> the mechanisms accounting for the heterogeneous responses of tumors to biotherapy remain poorly understood. New insight into these mechanisms could greatly accelerate progress in the development of effective biotherapeutic agents for use in cancer patients.

Virotherapy is a strategy in which a virus that preferentially replicates in tumor cells is applied either locally or systemically to lyse such cells.<sup>4</sup> Unlike typical forms of gene-based cancer therapy, oncolytic viruses are

thought to kill tumor cells directly through selective replication/cytolysis and consequently spread to surrounding tumor tissues. These properties represent a major advantage over the inherent inefficiency of gene delivery and the resultant limited tumor cell killing of conventional gene therapies. Several viruses, including adenovirus,<sup>5</sup> herpes simplex virus (HSV),<sup>6</sup> retrovirus,<sup>7</sup> vaccinia virus,<sup>8</sup> measles virus<sup>9</sup> and vesicular stomatitis virus<sup>10</sup> have been modified for oncolytic purposes. These viruses can be derived either from naturally occurring viruses that preferentially target tumor cells,<sup>10</sup> or from genetically engineered viruses that target cancer cells by a defined molecular mechanism.<sup>6,11–13</sup> Despite only a relatively short history of research and development, several oncolytic viruses are being tested in clinical trials against tumors of different tissue origins; in general, they have shown excellent safety profiles and some have produced indications of efficacy.<sup>14</sup> However, as with many other biotherapeutic approaches, the clinical utility of virotherapy is restricted by the generally small group of patients with favorable responses. In one recent clinical trial, 26 patients were treated with an oncolytic virus derived from a type I HSV (HSV-1), but only three had favorable responses.<sup>15</sup> This and similar outcomes underscore the need to understand why some tumors (but not others) respond well to treatment with oncolytic viruses.

We recently developed an oncolytic virus based on a type II HSV (HSV-2) by deleting the N-terminal region of the *ICP10* gene from the viral genome.<sup>16</sup> Designated

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FusOn-H2, it has multiple antitumor mechanisms and has shown potent oncolytic activity against tumor cells of different tissue origins.<sup>16–18</sup> Yet, several tumor cell lines that we have screened *in vitro* show almost high resistance to the replication of FusOn-H2 and thus its oncolytic effect. This suggests that patients whose tumor cells are non-permissive to FusOn-H2 replication *in vitro* would be unresponsive to FusOn-H2 virotherapy. In this study, we show that an intrinsically strong interferon (IFN) response activity underlies the resistance of murine and human tumors to FusOn-H2 lytic activity, but does not preclude a favorable therapeutic response. To the contrary, treatment of implanted tumors with FusOn-H2 virotherapy led to their massive infiltration and destruction by neutrophils, suggesting a need to re-examine current strategies of virotherapy for cancer patients.

## Materials and methods

### Cell lines and viruses

The EC9706 cell line was kindly provided by Dr Mingrong Wang (Chinese Academy of Medical Sciences). Panc02-H7 and MiaPaCa-2 cells were gifts from Dr Min Li (Baylor College of Medicine). The remainder of the cell lines were purchased from ATCC (Manassas, VA). EC9706 designates a human esophageal carcinoma line,<sup>19</sup> LLC a murine Lewis lung carcinoma line and Panco2-H7 a murine pancreatic cancer line.<sup>20</sup> All of the cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

FusOn-H2 was derived from the wild-type HSV-2 strain 186 (wt186). The details of its construction are described elsewhere.<sup>16</sup> Viral stocks were prepared by infecting Vero cells with 0.01 plaque-forming units (pfu) per cell. Viruses were harvested 2 days later and purified as described.<sup>21</sup> The purified viruses were titrated, divided into aliquots and stored at  $-80^{\circ}\text{C}$  until use.

### Plasmid construction and assays of endogenous IFN response activity

pJ-interferon-stimulated response element (ISRE)-P contained a murine ISG56 promoter with five copies of ISRE (5'-AGTTTCACTTTCCAGTCTCAGTTTCAGTTTCT-3') that were synthesized by DNA 2.0 (Menlo Park, CA). The sequence of the ISG56 promoter with ISREs was derived from the Gene bank (<sup>#</sup>S77714S1). pJ-ISRE-SEAP was constructed by inserting the gene encoding the secreted form of alkaline phosphatase (SEAP) and SV40-polyA signal into the *Hind*III and *Hpa*I site of pJ-ISRE-P.

To measure ISRE activity, we seeded tumor cells in 24-well plates in duplicate and transfected them with 1  $\mu\text{g}$  of pJ-ISRE-SEAP or control plasmid (pcDNA-green fluorescent protein). In some experiments, 1000 units of IFN- $\alpha$ ,  $\beta$  or  $\gamma$  were added to the medium immediately after the plasmid transfection. Supernatants were collected daily from day 1 to day 13 according to experimental design. SEAP in the supernatants was quantified with the

Great EscAPE SEAP Chemiluminescence Detection Kit from Clontech Laboratories (Mountain View, CA).

### Characterization of viral growth

Cells were seeded in triplicate into 24-well plates at 50% density. On the next day, they were infected with the test viruses at 0.1 pfu per cell for 1 h and washed once with phosphate-buffered saline (PBS) to remove unabsorbed and uninternalized viruses before fresh medium was added. At 48 h post-infection, the cells were harvested and viruses were released by repeated freezing and thawing and sonication. Virus titers were determined on Vero cells by a plaque assay.

### Real-time PCR (RT-PCR) assays

RNA was extracted from tumor cells with Trizol Reagent from Invitrogen (Carlsbad, CA). Human IFN- $\beta$  and ISG56 transcripts were detected by two-step RT-PCR, using the TaqMan Gene Expression Assay kit (Applied Biosystems, Carlsbad, CA). ISG56 primer sequences were: ISG56 forward: 5'-GGGAGTTATCCATTGATGACGATGA-3'; ISG56 reverse: 5'-GGTGTC TAGGAATTC AATCTGATCCAA-3'; FAM (6'-carboxyfluorescein)-labeled ISG56 probe: 5'-ATGCCTGATTTAGAAAACA-3'. RT-PCR was performed according to the TaqMan Gene Expression Assay protocol with the ABI Prism 7700 Sequence Detection System (Foster City, CA). The reaction was run with the following cycling conditions. After being held at  $50^{\circ}\text{C}$  for 2 min and at  $95^{\circ}\text{C}$  for 10 min, the samples were run for 40 cycles including  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 1 min. RT-PCR data were analyzed with the ABI Prism 7700 Sequence Detection System.

### Experimental animals

All animal experiments and procedures were approved by Animal Care and Use Committees of Baylor College of Medicine and University of Houston. Female Hsd athymic (nu/nu) mice (obtained from Harlan, Indianapolis, IN) were kept under specific pathogen-free conditions and used in experiments when they attained the age of 5–6 weeks. EC9706 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.05% EDTA. After trypsinization was stopped with medium containing 10% fetal bovine serum, the cells were washed once in a serum-free medium and resuspended in PBS. On day 0,  $5 \times 10^6$  EC9706 cells were inoculated into the right flank of nude mice. After 2 weeks of tumor cell implantation, when the tumors reached approximately 3–5 mm in diameter, mice received a single intratumor injection of either  $3 \times 10^6$  or  $6 \times 10^4$  pfu of FusOn-H2 in a volume of 100  $\mu\text{l}$ , or the same volume of PBS. The tumors were measured weekly and their volumes were determined by the formula: tumor volume ( $\text{mm}^3$ ) = (length (mm))  $\times$  (width (mm))<sup>2</sup>  $\times$  0.52. For histological examination, mice were euthanized by CO<sub>2</sub> exposure at days 1, 2, 3 and 5 after receiving intratumoral injection of FusOn-H2. Tumor tissues were explanted and sectioned for H&E staining. For immunohistochemical staining for neutrophils, tumor sections were initially incubated with a rat antimouse neutrophil monoclonal antibody (NIMP-R14)

purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). The second antibody was goat-antirat IgG (Invitrogen) and the third antibody was horseradish peroxidase-labeled donkey-antigoat IgG (Vector Laboratories, Burlingame, CA). The slides were then stained with Vectastain ABC kit (Vector Laboratories).

For isolation of neutrophils from tumors, excised tumor tissues were minced in 1 ml of Hanks' balanced saline solution containing 0.05% collagenase and 0.002% DNase I, and filtered through a 70- $\mu$ m cell strainer (BD Falcon, Bedford, MA). After centrifugation ( $400 \times g$ , 10 min and at  $4^\circ\text{C}$ ), the pellets were resuspended in Hanks' balanced saline solution with 15 mM EDTA and 1% bovine serum albumin. The cell suspensions were then loaded onto a three-layer Percoll gradient of 78, 69 and 52%, respectively (Amersham Pharmacia Biotech, Uppsala, Sweden), and centrifuged at  $1500 \times g$  for 30 min at room temperature without braking. Neutrophils were retrieved from the 69/78% interface and transferred into 1% bovine serum albumin-coated tubes. The collected neutrophils were washed twice with Hanks' balanced saline solution-EDTA-bovine serum albumin buffer and resuspended in 1640 medium. To isolate neutrophils from the peritoneal cavity, we injected mice intraperitoneally with EC9706 cells infected with FusOn-H2 or mock-infected. After 24 h, the peritoneal cavity was washed with 1640 medium and the cells collected by centrifugation ( $400 \times g$ , 10 min and at  $4^\circ\text{C}$ ), resuspended in Hanks' balanced saline solution with 15 mM EDTA and 1% bovine serum albumin and loaded onto a Percoll gradient for separation and collection.

For measurement of neutrophil cytotoxicity against tumor cells, EC9706 cells (at  $20 \times 10^6$  cells per ml) were initially labeled with  $10 \mu\text{M}$  CFSE (Molecular Probes Europe, Leiden, The Netherlands) for 10 min at  $37^\circ\text{C}$ . The labeling was stopped by addition of an equal volume of fetal calf serum. After two washes with PBS, the labeled tumor cells were resuspended in 1640 medium. Then,  $3 \times 10^5$  tumor cells were mixed with the purified neutrophils at different ratios in a total of 200  $\mu$ l volume per well in 96-well plates and incubated at  $37^\circ\text{C}$  for 24 h. The cells were harvested and further stained with propidium iodide (1  $\mu\text{g}$  per ml) before they were analyzed with BD FACSAria flow cytometer (BD Biosciences, San Jose, CA) to quantify dead (double staining for propidium iodide and CFSE) and viable (single staining for CFSE) EC9706 cells. The percentage of surviving cells was calculated by dividing the number of input viable CFSE cells by the number of CFSE-stained viable cells 24 h after incubation with neutrophils.

For *in vitro* neutrophil migration assay, neutrophils were freshly purified from healthy C57BL6 mouse blood as described by Boxio *et al.*<sup>22</sup> The viability of neutrophil population was confirmed as being >95% by trypan blue exclusion staining. The purified neutrophils were used immediately in the matrigel invasion assay with the BD BioCoat Matrigel Invasion Chambers (BD Biosciences, cat. no. 354480) as described elsewhere.<sup>23</sup> Briefly, tumor cells (with or without FusOn-H2 infection) were seeded on the plates at a density of  $5 \times 10^5$ . The plates were

agitated for 30 min in a shaker to allow adsorption. After that, neutrophils at a density of  $25 \times 10^4$  were seeded into the invasion chamber in serum-free medium. After 22 h of incubation at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere, the non-migrating cells were removed from the upper surface of the inserts with a cotton swab. The cells clinging on the lower side of the insert were fixed with ice-cold methanol and stained with Hoechst 33258 (300 nM) (Sigma-Aldrich, St Louis, MO, cat. no. 861405). Six images covering the membrane were randomly taken under microscope, and the mean number of migrating cells was determined. Neutrophils were identified by staining and recognizing the nucleus's characteristic multilobulated shape with Türk's solution (Cat # 109277, Merck, Whitehouse Station, NJ).

#### Statistical analysis

Quantitative results are reported as means and standard errors. Statistical analyses were performed by the two-way *t*-test. *P* values of less than 0.05 were considered statistically significant.

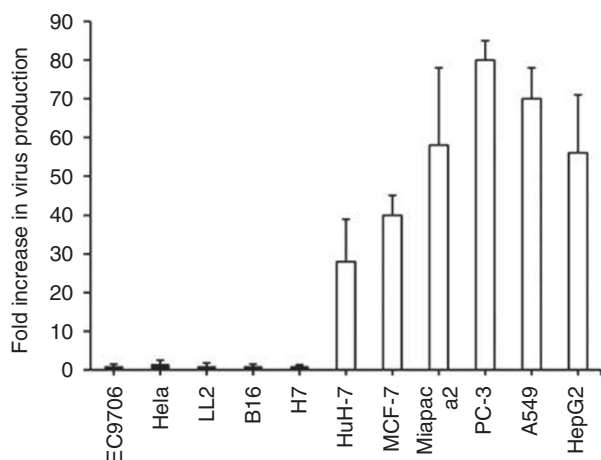
## Results

### *Tumor cells are permissive to FusOn-H2 replication*

As the construction of FusOn-H2, we have characterized this oncolytic virus in more than two dozen tumor cell lines derived from different tissues of both humans and mice. FusOn-H2 efficiently lysed most of the tumor cells that we screened, and effectively shrank tumors established from these cells when injected either locally or systemically.<sup>16-18</sup> However, approximately 20% of the tumor cell lines were highly resistant to FusOn-H2 replication. In contrast to the fully permissive tumor cells, in which the input virus replicated as much as 100-fold within 48 h after infection (Figure 1), the yield of FusOn-H2 in each of the five tumor cell lines representing esophageal carcinoma, cervical cancer, lung carcinoma, melanoma and pancreatic cancer, barely increased over the same time period (Figure 1). In most cases, the oncolytic virus can infect the tumor cells, as indicated by the expression of green fluorescent protein (*GFP*) gene inserted into the viral genome during its construction.<sup>16</sup> The blockage of virus propagation in these tumor cells mainly occurred during the virus replication. Because the therapeutic effect of an oncolytic virus is believed to depend mainly on its ability to replicate and spread, these results indicate that FusOn-H2 would be largely ineffective against tumors established from these cell lines.

### *FusOn-H2 is highly effective against implanted tumors established from some of the cancer cell lines resistant to viral replication*

As tumor cell resistance to viral replication generally predicts a poor response to virotherapy, we initially excluded tumors established from such resistant cells from *in vivo* evaluation of the antitumor effects of FusOn-H2. Recently, however, we chose to include several highly resistant tumor cell lines in our *in vivo* experiments,

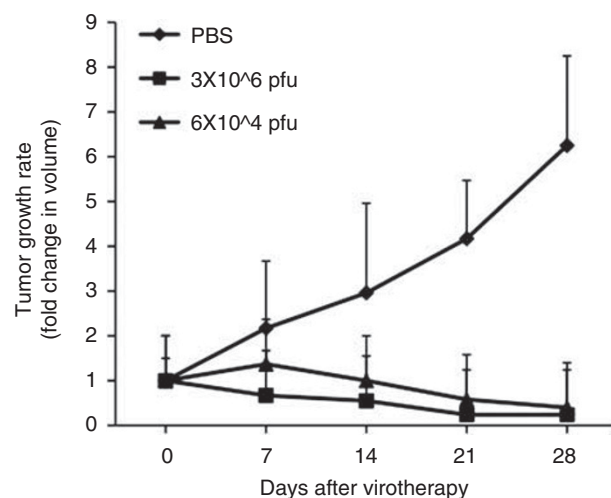


**Figure 1** FusOn-H2 replication in tumor cells of different tissue origins. Cells were seeded in 24-well plates in duplicate and infected with FusOn-H2 at 0.1 pfu per cell for 1 h. Cells were washed and harvested with or without 24-h incubation. The fold increase in viral replication was calculated by dividing the virus titer at 24 h after infection by the values of titer for the same cells harvested immediately after washing without incubation. The data are reported as means of triplicate experiments.

primarily as negative controls. Surprisingly, a single injection of FusOn-H2 at  $3 \times 10^6$  pfu produced a dramatic antitumor effect, nearly eradicating tumors established from implants of EC9706 cells, which are resistant to FusOn-H2 replication (Figure 2). This effect was essentially duplicated when the virus dose was reduced 50-fold, to as low as  $6 \times 10^4$  pfu. Other than tumor disappearance, the animals showed no sign of toxicity during the virotherapy. Together, these observations suggest that FusOn-H2 destroyed the highly resistant tumor cells *in vivo* through mechanisms other than direct oncolysis.

#### *FusOn-H2 induces massive infiltration of neutrophils into resistant tumors*

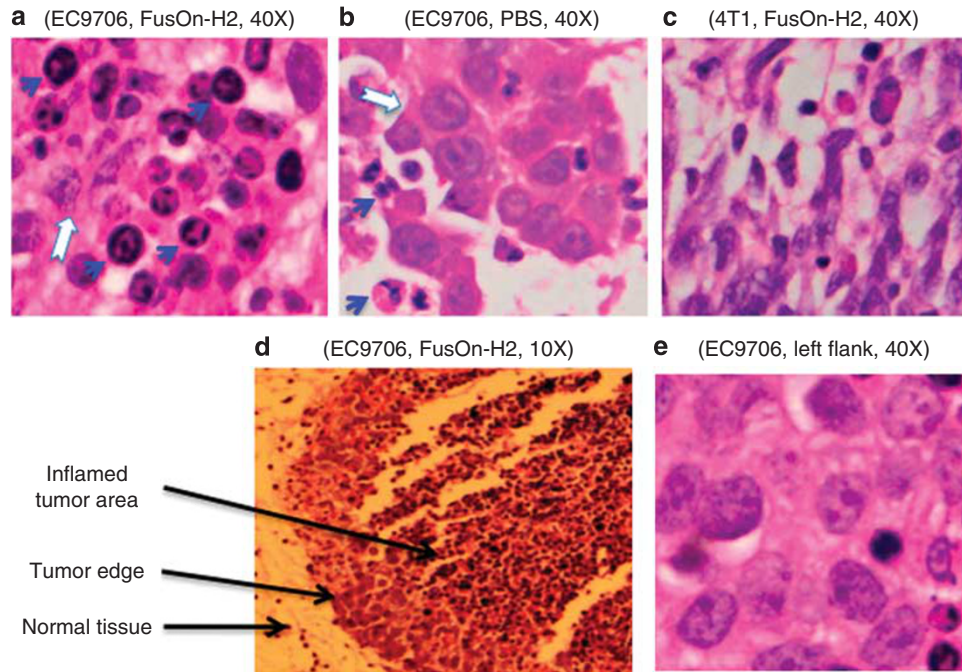
To account for the unexpected antitumor effects of FusOn-H2 virotherapy, we initially established tumors from EC9706 or 4T1 cells (a murine mammary tumor line that is significantly more permissive than EC9706 to FusOn-H2 replication but otherwise is similar to EC9706 in that they both form tumors aggressively once implanted into mice). After their injection with FusOn-H2, the tumors were harvested at days 1, 2, 3 and 5 for histological examination. The results revealed a massive infiltration of neutrophils in EC9706 tumors treated with FusOn-H2 (Figure 3, blue arrows). The inner areas of the tumors were almost entirely filled with neutrophils; the few remaining tumor cells did not appear healthy (white arrow in Figure 3a). Tumor cells near the periphery seemed viable and formed a ring surrounding the inflamed interior (Figure 3d). Infiltrating neutrophils were much less common in EC9706 tumors treated with PBS (Figure 3b) and were virtually undetectable in 4T1 tumors, which showed obvious oncolytic effects due to



**Figure 2** Therapeutic effect of FusOn-H2 against established EC9706 tumors. Tumors were initially established by implanting  $5 \times 10^6$  EC9706 cells in the right flank of nude mice. Once tumors reached the approximate size of 5 mm in diameter, they were injected with FusOn-H2 at a dose of  $3 \times 10^6$  or  $6 \times 10^4$  pfu. Tumors were measured weekly post-treatment, and the tumor growth rate was determined by dividing the tumor volume before treatment by the tumor volume after treatment.

robust FusOn-H2 replication (Figure 3c). In a subsequent experiment, we inoculated EC9706 tumor cells into both flanks and treated tumors on the right flank with FusOn-H2. The treated tumors shrank, but tumor growth on the opposite flank was not affected (data not shown). Histological examination of the untreated tumors did not reveal any increases in neutrophil infiltration (Figure 3e), indicating that the massive infiltration of neutrophils was a regional effect that was directly associated with virus infection.

To further characterize the infiltrating neutrophils, we conducted another *in vivo* experiment. Established EC9706 tumors were injected with either  $3 \times 10^6$  pfu of FusOn-H2 or the same amount of virus that had been inactivated by UV radiation, or PBS. Tumors were explanted 2 days later and divided into halves. One half was frozen to visualize virus infection by examining GFP expression under a fluorescent microscope. As FusOn-H2 contains the *GFP* gene, the virus infectivity could be conveniently determined by this way. The other half was used for preparation of paraffin sections for immunohistochemical staining of neutrophils. The results are shown in Figure 4. The micrographs, taken at a low magnitude ( $\times 10$ ) from sections immunohistochemically stained for neutrophils, showed that there was a widespread neutrophil infiltration in tumors treated with FusOn-H2 (Figure 4a). However, the extent of neutrophil infiltration was drastically reduced in tumors treated with the inactivated virus (Figure 4b and f), indicating that virus infectivity was probably necessary for the induction of neutrophil infiltration. Neutrophils were not readily visible in untreated tumors, suggesting that these tumors were not intrinsically associated with neutrophil infiltration. Similar neutrophil infiltration was also detected in



**Figure 3** Massive infiltration of resistant tumors by neutrophils after treatment with FusOn-H2. Highly resistant EC9706 (**a**, **b**, **d** and **e**) or permissive 4T1 (**c**) tumor cells were implanted on the right flank (**a**, **b**, **c** and **d**) or on both flanks (**e**) of female nu/nu mice. Once tumors reached the approximate size of 5 mm in diameter, FusOn-H2 or PBS was injected into the tumors on the right flank, as indicated. Tumors were explanted on days 1, 2, 3 and 5 and sectioned for H&E staining. The sections shown in this study represent day 2 after virus or PBS administration. Blue arrows indicate infiltration; the white arrow marks degenerating tumor cells.

tumors established from another highly resistant tumor cells, B16 murine melanoma, after FusOn-H2-virotherapy (Supplementary data).

Next, to examine the effect of the infiltrating neutrophils on EC9706 cells more directly, we performed an additional *in vivo* experiment similar to that illustrated in Figure 3. After harvesting infiltrating neutrophils from established tumors at 2 days post-treatment, we immediately mixed them with EC9706 tumor cells at different ratios and measured cytolysis 24 h later (FusOn-H2 was undetectable in the purified neutrophils). The neutrophils retrieved from the FusOn-H2-treated EC9706 tumors had a significantly higher killing activity against EC9706 tumor cells than did those isolated from untreated tumors (Figure 5a). These results demonstrate a critical difference in cytolytic capacity between neutrophils in FusOn-H2-treated versus untreated EC9706 tumors.

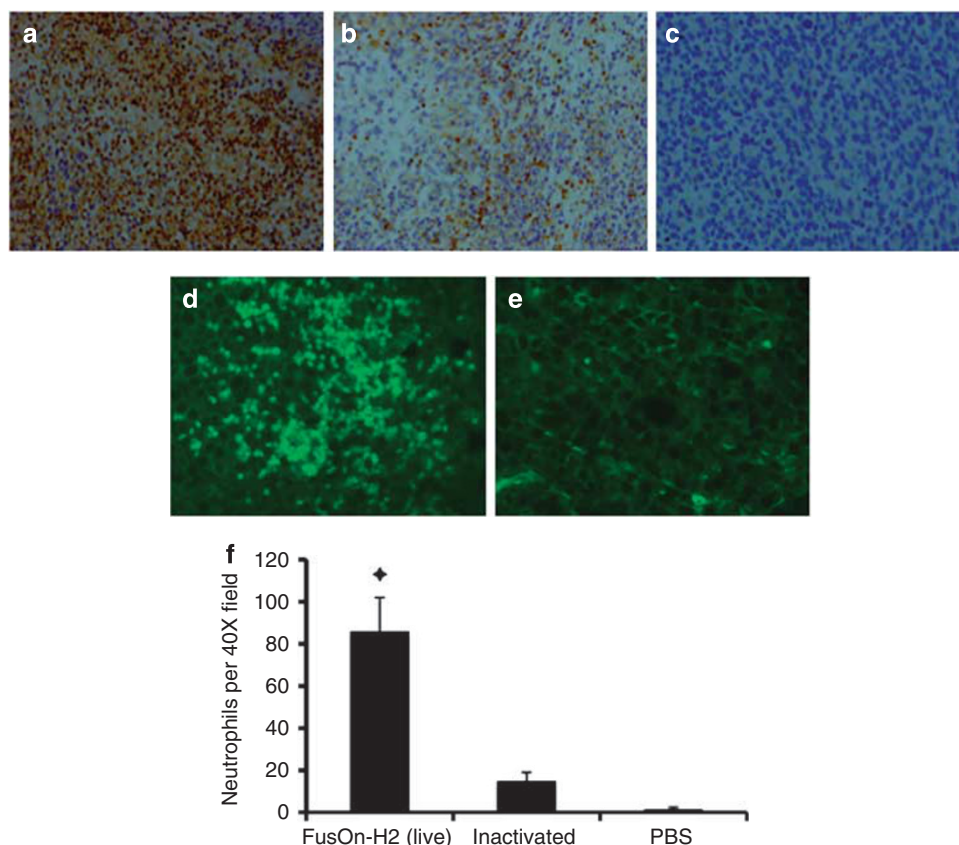
We also measured the effect of FusOn-H2-infected tumor cells on the migration ability of neutrophils in an *in vitro* experiment. Freshly isolated neutrophils and tumor cells of different preparations (mock-infected, infected with 1 pfu per cell of FusOn-H2 or UV-inactivated FusOn-H2) were seeded in matrigel invasion chambers for cell migration assay as described.<sup>23</sup> The results show that significantly more neutrophils were migrating toward the well seeded with FusOn-H2-infected EC9706 cells than to the wells seeded with two permissive cell lines, 4T1 and MD-MBA-435 (Figure 5b). As compared with the mock-infected cells, cells infected with UV-inactivated FusOn-H2 can increase neutrophil migra-

tion. However, to achieve the maximal chemoattractant effect, full infectivity of the virus is required.

*Strong endogenous IFN response activity occurs in tumor cells with resistance to FusOn-H2 replication*

The observation that only FusOn-H2-resistant tumors showed massive neutrophil infiltration after virotherapy indicates an intrinsic biological difference between the resistant and non-resistant tumors. To pursue this notion, we first evaluated the IFN response status of the tumor cells, as this response functions as a critical innate antiviral mechanism and could explain the failure of FusOn-H2 to replicate well in some tumor lines but not others. For this purpose, we constructed a test plasmid, pJ-ISRE-SEAP, in which *SEAP* gene is driven by a minimal promoter linked to three tandem repeats of ISRE, derived from the *ISG56* promoter region. When transfected into tumor cells, this construct enabled us to measure SEAP levels in the culture medium and hence to monitor the cells' IFN response activity.

Figure 6a shows the results of transfecting pJ-ISRE-SEAP into the five lines of tumor cells that showed resistance to FusOn-H2, as well as a panel of tumor cells that are permissive to the virus. Supernatants were collected and the secreted SEAP was quantified at different time points after transfection. All five highly resistant cell lines had much higher levels of SEAP secretion than did the permissive lines (Figure 6a). Among the five resistant lines, EC9706 (human esophageal carcinoma) and B16 (murine melanoma) showed an extremely high level of ISRE activity. We also monitored

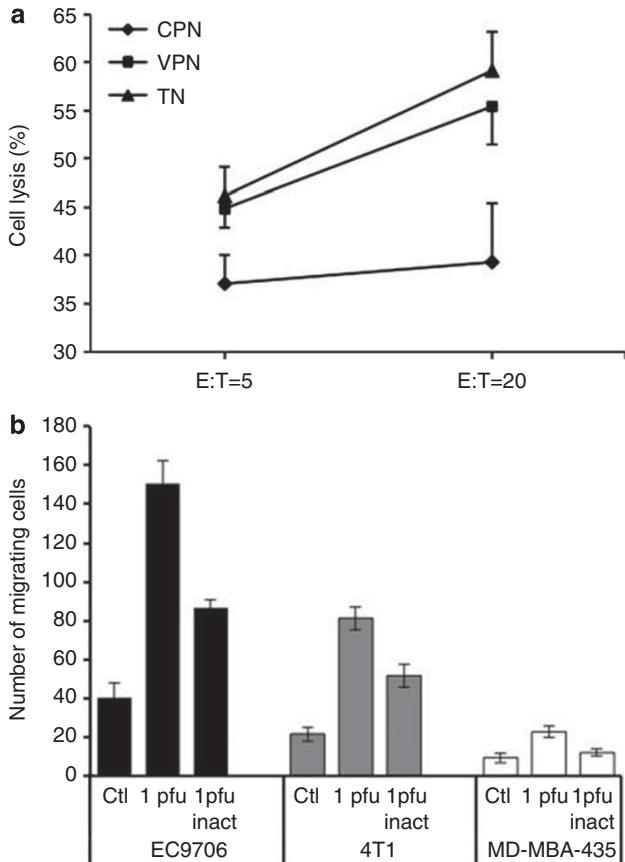


**Figure 4** Further characterization of the infiltrating neutrophils. EC9706 tumors were established on the right flank of nude mice and injected with  $3 \times 10^6$  pfu of FusOn-H2 (**a, d**) or the same of FusOn-H2 that had been inactivated by UV radiation (**b, e**) or PBS (**c**). Tumors were explanted 2 days later and divided into halves; one half for preparation of frozen sections for examining green fluorescent protein expression under a fluorescent microscope (**d, e**) and the other half for immunohistochemical staining of neutrophils (**a-c**). The infiltrating neutrophils from **a-c** were quantitated by counting 10 microscopic fields ( $\times 40$ ) and the average numbers are plotted in (**f**).  $\star P < 0.01$  versus inactivated FusOn-H2.

SEAP secretion by EC9706 cells for an extended time, demonstrating that it peaked on day 5 after transfection. Thereafter, it declined slightly but remained at a relatively high level for up to 2 weeks, the longest time span that we monitored (Figure 6b).

Because *SEAP* is an exogenous marker gene that was introduced into tumor cells by transfection, we thought it important to measure the expression of endogenous genes that are normally regulated via ISRE. For this purpose, we chose to measure *ISG56* expression, again by RT-PCR. We also asked if FusOn-H2 infection would further regulate ISRE activity. Thus, total RNA was extracted from the highly resistant EC9706 cell line and from two permissive lines (MCF-7 and HuH-7) with or without the FusOn-H2 infection. When quantified by RT-PCR, *ISG56* transcripts were significantly more abundant in EC9706 cells than in the other two lines (Figure 7a). FusOn-H2 infection further increased the level of *ISG56* transcripts in EC9706 cells, in contrast to results in the MCF-7 and HuH-7 lines, where FusOn-H2 infection led to reductions in the levels of these transcripts. These results further confirm the high-level intrinsic IFN response activity in these highly resistant tumor lines.

We next asked if the strong endogenous IFN response activity in the highly resistant tumor cells is interferon-dependent, by comparing IFN- $\alpha$  and IFN- $\beta$  secretions in resistant versus permissive tumor cells with or without FusOn-H2 infection. Using an enzyme-linked immunosorbent assay, we were unable to detect IFN- $\alpha$  in most tumor cells, whether or not they were infected with FusOn-H2 (data not shown). Although IFN- $\beta$  transcripts could be detected with RT-PCR, which is thought to be more sensitive than enzyme-linked immunosorbent assay, their amounts did not differ significantly between the highly resistant (EC9706) and permissive (MCF-7 and HuH-7) cells (Figure 7b). FusOn-H2 infection increased the amount of IFN- $\beta$  transcripts but only marginally. We then determined if addition of interferons to the culture medium would affect *SEAP* expression from pJ-ISRE-SEAP. After transfecting a panel of tumor cells with this construct and adding different types of IFNs to the medium, we collected the supernatants 24 h later for quantification of SEAP. Addition of both type I and type II IFNs led to varied but consistently significant increases of *SEAP* expression in permissive tumor cells (Figure 7c). In the Huh-7 line, for example, *SEAP* expression increased more than 100-fold after addition

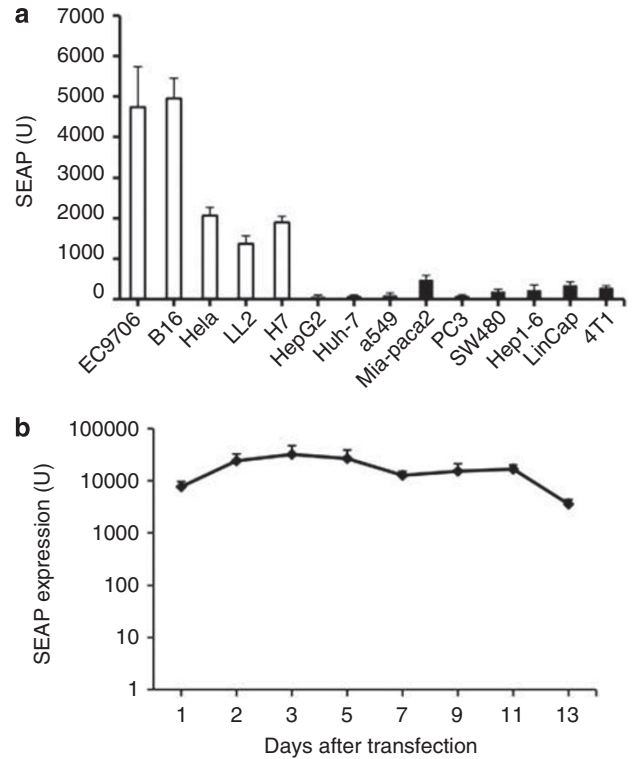


**Figure 5** *In vitro* assay for killing and migration activity of neutrophils. (a) Killing activity of neutrophils isolated from tumor tissues or from peritoneal cavity. Neutrophils were isolated from either established EC9706 tumors that had been treated with FusOn-H2 (TN) or from peritoneal cavity that had been injected with EC9706 cells infected with FusOn-H2 (VPN) or mock-infected (CPN). The purified neutrophils were then mixed with EC9706 cells at the ratio of either 5 or 20, and cytolysis was determined 24 h later. (b) *In vitro* neutrophil migration assay using the BD BioCoat Matrigel Invasion Chambers. Tumor cells (with or without infection with FusOn-H2) were seeded on the lower wells and neutrophils were seeded into the invasion chambers. After 22 h of incubation, the non-migrating cells were removed and the cells clinging on the lower side of the insert were fixed and quantified.

of type I IFNs. By contrast, the presence of exogenous IFNs had little effect on *SEAP* expression in highly resistant tumor cells (EC9706 and HeLa lines). Together, these results suggest that the high level of endogenous ISRE activity in these highly resistant cell lines is IFN-independent and that other, co-existing mechanisms likely contributed to this intrinsic activity.

## Discussion

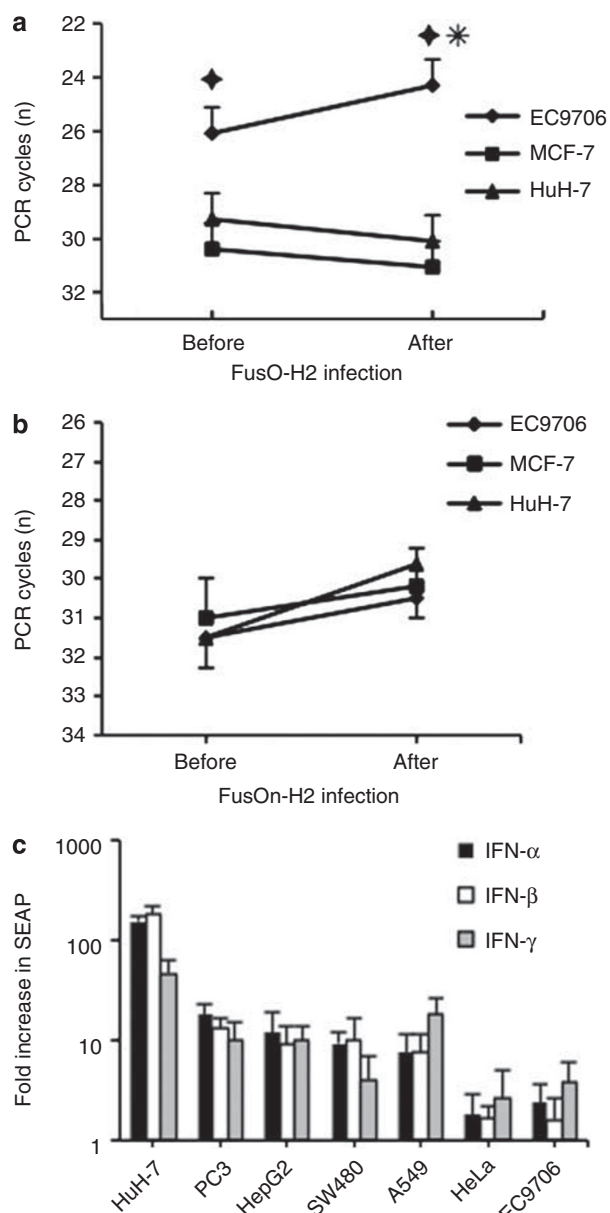
The majority of studies evaluating the therapeutic effects of virotherapy are conducted with implanted tumor cells selected *in vitro* for their permissiveness to a particular oncolytic virus, as exemplified by our previous



**Figure 6** Endogenous IFN response activity. (a) Both resistant and non-resistant tumor cells were transfected with 1  $\mu$ g of pJ-ISRE-SEAP, which contains the *SEAP* gene driven by a minimal promoter linked to four copies of ISRE. After 24 h of the transfection, supernatants were collected and the SEAP concentration was quantified. (b) The duration of SEAP expression from pJ-ISRE-SEAP was further characterized in a separate experiment. Again EC9706 cells were transfected with 1  $\mu$ g of pJ-ISRE-SEAP. Supernatants were collected at the indicated times for quantification of SEAP. The near twofold difference in SEAP level between 6a and 6b is probably due to the variations in either cell density or transfection efficiency between these two experiments.

work with the HSV-2-derived oncolytic virus FusOn-H2.<sup>16–18,24</sup> In our experience, approximately 20% of all tumor lines show resistance to FusOn-H2 *in vitro* and therefore would not be expected to have appreciable responses to virotherapy *in vivo*. In this study, we report the surprising finding that some of the tumor cells unable to support FusOn-H2 replication can nonetheless respond well to therapy with the virus. Indeed, even an intermediate dose of FusOn-H2 virtually eradicated tumor implants that had shown resistance to this virus *in vitro*; a similar result was obtained when the virus dose was further reduced by as much as 50-fold. Our data therefore indicate that the outcome of FusOn-H2 virotherapy against these highly resistant tumors depends on mechanisms other than direct oncolysis.

The most straightforward explanation for these favorable responses to virotherapy despite intrinsic resistance to FusOn-H2 lies in the massive infiltration of neutrophils that could be seen throughout the entire tumor tissue. Indeed, subsequent studies of neutrophils extracted from the treated tumors revealed that they could lyse tumor



**Figure 7** Endogenous *ISG56* and IFN expression in tumor cells with or without FusOn-H2 infection and the effect of externally added IFNs on ISRE activity. **(a)** and **(b)**. Endogenous *ISG56* and IFN expression in tumor cells with or without FusOn-H2 infection. Tumor cells were seeded into six-well plates in duplicate and incubated overnight at 37 °C. One set of cells was harvested and the others were infected with 1 pfu per cell of FusOn-H2 for 24 h before harvesting. The total RNA was prepared for RT-PCR quantification of *ISG56* transcripts **(a)** or *IFN- $\beta$*  transcripts **(b)** as described in Materials and methods. \* $P < 0.01$  versus MCF-7 or HuH-7, \* $P < 0.05$  versus before infection. **(c)** Effect of externally added IFNs on ISRE activity. Tumor cells were initially transfected with pJ-ISRE-SEAP. Then, different types of IFNs were added to the medium followed by 24 h of incubation before the collection of supernatants for SEAP assay. The fold of increase in SEAP activity was calculated by dividing the amount of SEAP released into the medium before addition of IFNs by that measured 24 h after IFN incubation. The Huh-7, PC3, HepG2, SW480 and A549 tumor cells are all permissive to FusOn-H2 replication.

cells *ex vivo*. Thus, FusOn-H2 virotherapy for certain highly resistant tumor cells appears to induce an innate antitumor immune response dominated by neutrophil infiltration. This echoes recent studies suggesting that under certain conditions, neutrophils can function as a key antitumor mediator.<sup>25,26</sup> For example, it has been reported that transforming growth factor- $\beta$  blockade increases neutrophil-attracting chemokines, resulting in an influx of CD11b + /Ly6G + tumor-associated neutrophils that are cytotoxic to tumor cells.<sup>27</sup> Studies by Breitbach *et al.*<sup>28</sup> suggest that infiltrating neutrophils contributed to a reduced blood flow to tumor tissues during virotherapy with vesicular stomatitis virus and vaccinia virus-derived oncolytic viruses. Another studies by Grote *et al.*<sup>29</sup> show that an oncolytic measles virus engineered to express granulocyte-monocyte colony stimulating factor could enhance the antitumor effect by recruiting neutrophils to the tumor site. Our studies suggest that there are two types of tumor cells that respond to FusOn-H2 virotherapy by different ways. The majority of tumor cells are permissive to virus replication and these tumor cells are mainly destroyed by direct cytolysis of the virus. However, there are approximately 20% of tumor cells that are highly resistant to virus replication. Some of these resistant tumor cells are still effectively destroyed by the virotherapy, partly through induction of innate antitumor immunity including massive neutrophil infiltration.

An innate immune response dominated by neutrophils would offer some distinct and unique advantages over adaptive immunity. First, infiltration of tumors is uniformly massive, as demonstrated in Figure 3. By contrast, during adaptive immune responses, T effector cells are usually found at low frequencies in tumor tissues, which may have limited their antitumor efficacy. Indeed, for adaptive antitumor immunity to be successful, substantial expansion of the initially generated tumor-specific T cells is crucial.<sup>30</sup> In most cases, however, T effector cell proliferation has proved extremely inefficient within the tumor microenvironment, probably accounting, at least in part, for the disappointing overall results from an array of clinical trials of T cell-based immunotherapy. Another major advantage of neutrophils over T cells in tumor destruction is that the former has the ability to liquefy the entire tumor tissues, which include tumor cells and tumor stromas such as collagen fibrils, stromal cells, lymphatics and capillaries.<sup>31</sup> By contrast, T cells can only lyse tumor cells and their effects are frequently limited or actively inhibited by the remaining tumor stroma. Thus, given the relative ease with which large numbers of tumor-killing neutrophils were recruited to tumor sites in this study, we suggest that FusOn-H2 virotherapy may represent a unique strategy for enhancing the impact of immunotherapy against certain subgroups of tumors.

Analyses of the tumor cells highly resistant to FusOn-H2 replication revealed that they have strong intrinsic IFN response activity, yet the release of IFN from the tumor cells did not appear to be increased, even after FusOn-H2 infection. This is probably because many tumor cells have a defective IFN pathway;<sup>32</sup> however,



external addition of interferons led to greater ISRE activity in permissive tumor cells but not in the highly resistant cells, indicating that the high levels of endogenous ISRE activity we describe are almost entirely independent of type I interferons themselves. Although the mechanism underlying this enhanced activity is unclear, it was recently reported that overexpression of an intracellular cytoplasmic protein, termed MITA (or STING), can activate IFN regulatory factor 3,<sup>33–35</sup> which then forms a complex with CREB (cAMP response element-binding) protein.<sup>36</sup> This complex subsequently translocates to the nucleus to activate interferon-induced genes through stimulation of ISRE.<sup>37</sup> Thus, we suggest that one possibility for the elevated endogenous ISRE activity in these highly resistant tumor cells is probably due to aberrant overexpression of this protein. Another possibility may be due to more efficient sensitization by toll-like receptors, such as TLR-9, to FusOn-H2 in these tumor cells. These possibilities are under investigation.

How the intrinsically high ISRE activity in these tumor cells contribute to the induction of massive neutrophil infiltration during FusOn-H2 virotherapy is not clear. We suggest that neutrophil recruitment results from the combinatorial effect of the endogenously inflamed tumor cells due to high ISRE activity and the subsequent infection of the cells by FusOn-H2, as neither effect alone was able to elicit this response. It is also possible that cytokines/chemokines other than interferons may be needed to trigger this event. One attractive candidate is interleukin 8, a proinflammatory cytokine released at sites of tissue damage by various cell types. An important function of interleukin 8 is to recruit neutrophils into sites of inflammation and to activate their biological activity by enhancing superoxide anion production.<sup>38</sup> It has been reported that HSV infection of epithelial cells induces interleukin 8 (*IL-8*) gene expression when there is an abundance of inflammatory cytokines in the local tissue.<sup>39</sup> Another cytokine candidate is RANTES, which is upregulated via ISRE and has been reported to preferentially recruit neutrophils.<sup>40</sup> Indeed, if RANTES and interleukin 8 were overexpressed in the same cell, their protein products might act synergistically to recruit neutrophils to tumor sites. Our attempt to detect release of RANTES from FusOn-H2-infected tumor cells in an *in vitro* experiment turned out to be negative (data not shown). Thus, these cytokines might have been released from non-tumor cells in the *in vivo* tumor environment after animals receiving FusOn-H2 virotherapy. Additionally, HSV-2 encodes a secreted form of glycoprotein G, which has recently been described to have proinflammatory properties and can function as a chemoattractant for both monocytes and neutrophils in a dose-dependent fashion.<sup>41</sup> As FusOn-H2 is derived from HSV-2, the secreted glycoprotein G from the virus may have contributed partly to the induction of neutrophil infiltration in these resistant tumors.

Identification of tumor cells, such as the EC9706 line, that respond favorably to FusOn-H2 virotherapy despite their apparent semi-permissiveness suggests that a

screening procedure could be established to identify this subset of tumors, enabling the selection of a low FusOn-H2 dose and/or for a maximal therapeutic effect. The finding of a strong endogenous IFN response activity in these tumor cells may have other clinical implications. For example, it may be possible to devise a 'molecular probe' to specifically identify circulating tumor cells with a high endogenous IFN response, thus accelerating the diagnosis of cancer and providing an estimate of prognosis.

### Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (<http://www.nature.com/cgt>)