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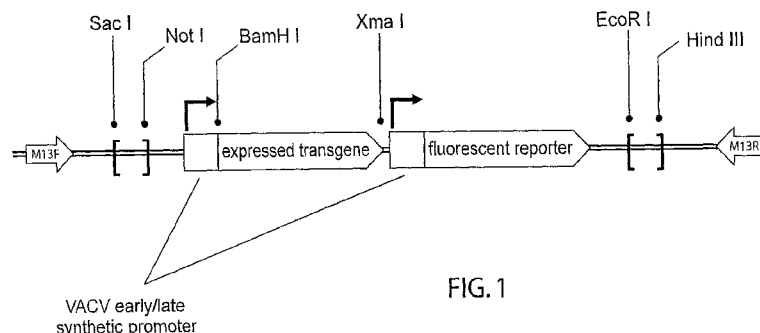


FIG. 1

(57) Abstract: A composition for treating cancerous cells in a subject having an immune system includes a virus in the *Yatapoxvirus* genus having at least one mutation. In one embodiment, the mutation results in suppressed expression of a TNF binding protein by the virus. In another embodiment, the mutation results in suppressed expression of thymidine kinase ("TK") by the virus. In another embodiment, the mutation arms the virus with a transgene to express a bacterial flagellin. The mutations can be present singly or in combination. Additional aspects include a method of treating cancerous cells with a composition as described herein, and a method of delivering at least one gene to cancerous cells in a subject.



COMPOSITION FOR TREATING CANCEROUS CELLS AND A METHOD THEREFOR**FIELD OF THE INVENTION**

[0001] The present disclosure relates to a composition for treating cancerous cells in subjects and a method therefor.

SUMMARY

[0002] One aspect of the present disclosure is a composition for treating cancerous cells in a subject having an immune system. The composition includes a virus in the *Yatapoxvirus* genus having at least one mutation. The at least one mutation results in suppressed expression of a TNF binding protein by the virus.

[0003] Another aspect of the present disclosure is a composition for treating cancerous cells in a subject having an immune system, including a virus in the *Yatapoxvirus* genus, where the virus has at least one mutation, the at least one mutation resulting in suppressed expression of thymidine kinase ("TK").

[0004] In another aspect of the present disclosure, the composition for treating cancerous cells in a subject having an immune system includes a poxvirus which encodes a transgene expressing a bacterial flagellin.

[0005] In yet another aspect of the present disclosure, a method of treating a subject with cancerous cells includes administering a composition to the subject, wherein the composition includes a virus in the *Yatapoxvirus* genus having at least one mutation which results in suppressed expression of a TNF binding protein by the virus.

[0006] According to yet another aspect of the present disclosure, at least one gene is delivered to cancerous cells in a subject by modifying a virus of the *Yatapoxvirus* genus by mutating the virus to suppress expression of a TNF binding protein having a structure capable of binding an MHC-1 light chain. The virus is also modified by encoding at least one gene in the virus, wherein the at least one gene encoded in the virus results in increased apoptosis of the cancerous cells or activates an immune response in the subject. The modified virus is administered to the subject.

[0007] The pharmaceutical compositions for treating cancerous tumors and methods described herein allow for potentially effective treatment of the cancerous cells with limited risk of serious infection or side effects which may be experienced with traditional

treatment methods, and may in some cases be used in combination with traditional treatment methods. The modified poxviruses described herein have exhibited results which indicate increased oncoselectivity and increased oncolethality as compared to unmodified poxviruses, and are expected to maintain preferable OV characteristics such as causing only a mild and self-limiting febrile illness in infected subjects.

[0008] These and other features, advantages, and objects of the present device will be further understood and appreciated by those skilled in the art upon studying the following specification, claims, and appended drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] FIG. 1 is a schematic illustrating one embodiment of a recombinant tanapoxvirus (TPV) as altered by a p2KO method to insert an expressed transgene and a fluorescent reporter;

[0010] FIG. 2A is a graph illustrating average tumor volume in athymic nude mice xenografted with HCT 116 cells and treated with a vehicle-only control solution;

[0011] FIG. 2B is a graph illustrating average tumor volume in athymic nude mice xenografted with HCT 116 cells and treated with one embodiment of recombinant TPV (TPV/*egfp*) as compared to the vehicle-only control solution;

[0012] FIG. 2C is a graph illustrating average tumor volume in athymic nude mice xenografted with HCT 116 cells and treated with one embodiment of recombinant TPV (TPV-p2KO/ Δ 66R/mMCP-1) as compared to the vehicle-only control solution;

[0013] FIG. 2D is a graph illustrating average tumor volume in athymic nude mice xenografted with HCT 116 cells and treated with one embodiment of recombinant TPV (TPV-p2KO/ Δ 66R/mGM-CSF) as compared to the vehicle-only control solution;

[0014] FIG. 2E is a graph illustrating average tumor volume in athymic nude mice xenografted with HCT 116 cells and treated with one embodiment of recombinant TPV (TPV-p2KO/ Δ 66R/fliC) as compared to the vehicle-only control solution;

[0015] FIG. 2F is a graph illustrating average tumor volume in athymic nude mice xenografted with HCT 116 cells and treated with one embodiment of recombinant TPV (TPV-p2KO/ Δ 66R) as compared to the vehicle-only control solution;

- [0016] FIG. 2G is a graph illustrating average tumor volume in athymic nude mice xenografted with HCT 116 cells and treated with one embodiment of recombinant TPV (TPV-p2KO/ Δ 2L) as compared to the vehicle-only control solution;
- [0017] FIG. 2H is a graph illustrating average tumor volume in athymic nude mice xenografted with HCT 116 cells and treated with one embodiment of recombinant TPV (TPV-p2KO/ Δ 2L/ Δ 66R/fliC) as compared to the vehicle-only control solution; and
- [0018] FIG. 3 includes views of one embodiment of a viral plaque at 2 days, 4 days, and 6 days produced by infection of one embodiment of a recombinant TPV which has been altered to express fluorescent reporters.

DETAILED DESCRIPTION

- [0019] For purposes of description herein the terms "upper," "lower," "right," "left," "rear," "front," "vertical," "horizontal," and derivatives thereof shall relate to the composition as oriented in FIG. 1. However, it is to be understood that the composition may assume various alternative orientations and the methods may include various step sequences, except where expressly specified to the contrary. It is also to be understood that the specific devices and processes illustrated in the attached drawings, and described in the following specification are simply exemplary embodiments of the inventive concepts defined in the appended claims. Hence, specific dimensions and other physical characteristics relating to the embodiments disclosed herein are not to be considered as limiting, unless the claims expressly state otherwise.
- [0020] Although some preference for infection of cancerous and/or transformed cells has been shown in oncolytic variants of some viruses such as VACV, wild-type poxviruses such as those in the genus *Yatapoxvirus*, including without limitation tanapoxvirus ("TPV"), are not generally considered to have a high degree of native oncospecificity. In viruses without significant native oncospecificity, genetic engineering has been employed to increase cancer cell selectivity.
- [0021] Although they do not necessarily have a high degree of native oncospecificity, poxviruses, and more specifically poxviruses in the *Yatapoxvirus* genus, have several inherent qualities that make them well-suited for modification for use as OV's. Poxviruses have viral genomes which are able to accommodate a large amount of added genetic material, and come with a built-in array of offensive and defensive capabilities. Poxvirus

genomes encode a variety of immunomodulatory proteins devoted to hiding infection-associated cell-surface epitopes from host immunosurveillance, the inhibition and evasion of some host immune and inflammatory responses, and the disruption of signals from the extracellular environment by means of virally-encoded peptides which mimic host cytokines and cytokine receptors. Poxviruses also produce two distinct types of progeny virions, the mature virion (MV) form and the enveloped virion (EV) form. The MV form of the virus is enclosed in a single lipid bilayer and is released from the host cell only by cytolysis. The EV form is actively exported from the host cell after it has acquired a second, outer envelope, possibly from the host cell trans Golgi network, and is referred to as a wrapped virion (WV) until it is exported from the infected cell, after which time it is referred to as the EV form. The poxvirus EV form is a specialized form of the virus which is responsible for spreading the poxvirus to distant sites within the host by trafficking through the bloodstream and the lymphatic network. The EV form is well-suited for this task, as it has only 6 transmembrane proteins exposed to the extracellular environment vs. approximately 20 for the MV form. Fewer exposed epitopes mean that the EV form is more able to escape neutralizing immunity than the MV form.

[0022] Additionally, TPV, a member of the genus *Yatapoxvirus* and a wild poxvirus, has additional features which are beneficial in developing an OV, making it a preferred member of the *Yatapoxvirus* genus for developing an OV. Humans infected with TPV experience only a mild and self-limiting febrile illness, possibly because TPV infection is normally confined to peripheral areas of the body. Apart from areas in equatorial Africa (where it is endemic) humans are immunologically naïve to TPV. Additionally, the TPV has never been observed to transmit from person to person, a highly desirable safety feature in an OV.

[0023] Genetically engineered specimens of poxviruses, including specimens of the *Yatapoxvirus* genus, are disclosed herein for use as OV, to be incorporated in compositions to treat cancerous cells, and to be used in methods of treating cancerous cells. Several preferred embodiments, including several preferred embodiments incorporating a recombinant TPV, are also described herein.

[0024] In summary, as further described below, one aspect of the present disclosure is a composition for treating cancerous cells in a subject having an immune system. In one aspect, the composition includes a virus in the *Yatapoxvirus* genus having at least one

mutation. The at least one mutation results in suppressed expression of a TNF binding protein by the virus. Another aspect of the present disclosure is a composition for treating cancerous cells in a subject having an immune system, including a virus in the *Yatapoxvirus* genus, where the virus has at least one mutation resulting in suppressed expression of thymidine kinase ("TK"). In another aspect, the composition includes a poxvirus which encodes a transgene expressing a bacterial flagellin. As used herein, the term "subject" includes human and animal subjects, and preferably mammalian subjects.

[0025] Also as described in greater detail below, a method of treating a subject having cancerous cells includes administering a composition to the subject, wherein the composition is as described herein. For example, in one embodiment the composition includes a virus in the *Yatapoxvirus* genus having at least one mutation which results in suppressed expression of a TNF binding protein by the virus. In another embodiment, the composition includes a virus in the *Yatapoxvirus* genus, where the virus has at least one mutation resulting in suppressed expression of TK. In yet another embodiment, the composition includes a poxvirus which encodes a transgene expressing a bacterial flagellin. Any or all of these mutations can be present in the composition, singly or in any combination. Additionally, the composition can be delivered in a targeted manner to a group of cancerous cells, or can be delivered to the subject systemically.

[0026] According to yet another aspect of the present disclosure, as described in greater detail below, at least one gene is delivered to cancerous cells in a subject by modifying a virus of the *Yatapoxvirus* genus by mutating the virus to suppress expression of a TNF binding protein having a structure capable of binding an MHC-1 light chain. The virus is also modified by encoding at least one gene in the virus, wherein the at least one gene encoded in the virus results in increased apoptosis of the cancerous cells or activates an immune response in the subject. The modified virus is administered to the subject.

[0027] In certain embodiments, the poxvirus is genetically modified to suppress expression of a host range factor with TNF-binding activity, also referred to herein as a TNF binding protein. The TNF binding protein which is suppressed is similar in structure to an MHC-1 heavy chain protein, and the encoded TNF binding protein can interact with an MHC-1 light chain. In TPV, the TNF binding protein is encoded in the 2L gene. Recombinant TPV in which the 2L gene has been ablated or otherwise mutated to suppress expression of the TNF binding protein is sometimes referred to herein as "2L-

deleted" or " Δ 2L." In a normal poxvirus infection, secreted TNF binding protein acts to blunt the host inflammatory and antiviral immune response by binding to and effectively reducing the amount of TNF present to interact with the infected cells. While this is a desirable outcome for the poxvirus, when poxvirus is used as an OV it may be advantageous to increase, rather than decrease, the amount of inflammation experienced by the treated tumor. In a human subject whose tumor is treated with an OV based upon the recombinant TPV as the poxvirus, ablation of the 2L gene in the recombinant TPV can result in an effective increase in TNF concentration at the tumor site, (compared to tumors infected with 2L-bearing TPV). Increased levels of TNF can ultimately act to increase tumor clearance. Because the 2L gene has previously been shown to bind to human TNF but not mouse TNF, the ablation of the 2L gene in some of the recombinant TPVs described herein was not expected to be a significant factor in tumor clearance during the mouse experiments, but is expected to be a more significant factor in tumor clearance in primates and humans. Ablation of the 2L gene was undertaken in the specific examples of the recombinant TPV used in xenografted athymic nude mice where the xenograft is made up of human cancer cells, as described herein, because mouse testing is an important step towards further testing of poxvirus-based OVs in more relevant primate models of cancer virotherapy.

[0028] In certain embodiments, the oncoselectivity of the poxvirus is increased by modifying the poxvirus to suppress expression of thymidine kinase (TK). In TPV, the TK encoding gene is known as 66R. Recombinant TPV in which the 66R gene has been ablated or otherwise mutated to suppress expression of TK is sometimes referred to herein as "66R-deleted" or " Δ 66R." The TK activity in neoplastic cells is constitutively high, due to the action of the cellular TK1 in cancerous cells. This is in contrast to normal cells, where TK activity levels peak during the S phase of the cell cycle and are nearly undetectable at other times. Cellular TK1 catalyses a step in nucleotide synthesis, the conversion of thymidine to thymidine monophosphate. For this reason, cancerous cells express TK1 throughout the cell cycle, and as a result tend to have large cytoplasmic pools of thymidine monophosphate available at all stages of the cell cycle. By suppressing the TK encoding gene, particularly in poxviruses in the *Yatapoxvirus* genus, the poxvirus has greater cancer cell selectivity than if the TK encoding gene was left intact. Ablation of the 66R gene was undertaken in some of the specific examples of the recombinant TPV

used in xenografted athymic nude mice described herein even though mice are not normally animal hosts for TPV. Ablation of the 66R gene in this environment demonstrates that the ablation of the 66R gene did not result in non-replicative TPV in permissive cells (such as the human cancerous tumor cells).

[0029] Additionally, the oncolethality of the poxviruses can be increased by encoding the poxvirus with transgenes to increase apoptosis of the cancer cells or to activate the immune system of the subject. Examples of transgenes which can be used to encode the poxvirus include without limitation genes to express cytokines, chemokines, antigen-presenting polypeptides, or bacterial antigens. As used herein, cytokine refers to a protein or a polypeptide having immune cell or system modulating effects, such as stimulating immune cells, promoting growth of immune cells, or directing immune cells to a particular site. In certain preferred embodiments, the poxvirus used is recombinant TPV, armed with a granulocyte-monocyte colony stimulating factor (GM-CSF), macrophage chemotactic protein 1 (CCL2, also referred to as MCP-1 and MCF-1), or bacterial flagellin (FliC, the product of the *fliC* gene in *Salmonella enterica*). When experimenting with recombinant TPV for use with mice, the mouse (m) version of these transgenes was used where relevant in the recombinant TPV, *i.e.*, mGM-CSF, mCCL2, mMCP1, mMCF-1. It is preferred to use the appropriate or effective versions of these transgenes for the subject upon which testing or treatment will be carried out.

[0030] Polymerized flagellin is the main component of the bacterial flagellum for use according to the present disclosure. The flagellin used for the specific experiments described herein was the product of the *Salmonella enterica serovar typhimurium* gene, *fliC*. FliC and other bacterial flagellins are cognate ligands of the toll-like receptor 5 (TLR5), and are strong activators of the innate immune response in mammalian cells *via* MyD88-dependent intracellular signaling and, ultimately, the activation of transcription factor NFκB. The flagellins are potent and pleiotropic virulence factors which have other important roles in bacterial pathogenesis.

[0031] In addition to the modification of the genome in the poxvirus embodiments described above, a fluorescent reporter transgene is optionally inserted into the genome of the poxvirus. Visualization of viral infection in cultured cells is greatly facilitated by the inclusion of the fluorescent reporter transgene, thereby facilitating research using the poxvirus variants described herein. Preferred fluorescent reporter transgenes include the

reporters mCherry (excitation/emission 587 nm/610 nm) and enhanced green fluorescent protein (GFP, excitation/emission 475 nm/509 nm).

[0032] Preferred embodiments of the poxvirus include viruses, preferably of the *Yatapoxvirus* genus, having any or all of the mutations or insertions described above, and the mutations and insertions are preferably carried out using a p2KO vector method. In one preferred embodiment, recombinant TPV, a member of the *Yatapoxvirus* genus, is altered by the p2KO method as used herein, a schematic of which is shown in FIG. 1. As illustrated, two vaccinia virus (VACV)-derived early/late synthetic promoters are used to drive the expression of the desired expressed transgene to encode the virus (e.g., mGM-CSF, mCCL2, mMCP1, mMCF-1, fliC) and the optional fluorescent reporter transgene. The embodiment of the p2KO vector method depicted in FIG. 1 includes transferring a p2KO expression cassette (including left and right flanks, at least one intervening open reading frame (ORF) including the expressed transgene or the fluorescent reporter transgene, and at least one promoter) to the viral genome of the TPV through a homologous recombination double-crossover event during a transfection/infection procedure as described in greater detail below. In this way, the p2KO expression cassette is guided to a specific point in the viral genome of the TPV by the use of viral genomic flanking sequences, resulting in a targeted ablation of the desired gene(s) with the simultaneous expression of the desired expressed transgene and optional fluorescent reporter transgene in the recombinant TPV.

[0033] In the embodiment of the TPV p2KO expression cassette as illustrated in FIG. 1, a plurality of poxvirus early/late synthetic promoters allowed for the expression of multiple transgenes. The p2KO expression cassette guided insertion to a specific point in the viral genome by use of viral genomic flanking sequences derived from a target gene, resulting in the targeted ablation of the desired gene(s) with the simultaneous expression of the fluorescent reporter transgene and the expressed transgene to arm the virus. In the embodiment shown in FIG. 1, both a fluorescent reporter transgene and expressed transgene are shown. In alternate embodiments, either of the fluorescent reporter transgene or the expressed transgene could be present in the p2KO expression cassette for insertion into the viral genome. The left and right flanks are bounded by pairs of unique restriction sites. The flanking regions are ligated into a p2KO vector between a Sac I restriction site and a Not I restriction site on a 5'- (left) flank, and between a EcoR I

restriction site and a Hind III restriction site on the 3'- (right) flank. The gene(s) to be expressed (the fluorescent reporter and/or the expressed transgene) are bounded by a unique 5'-BamH I restriction site and a 3'-Xma I restriction site. These allow for the simple and directional ligation of PCR amplicons bounded by the appropriate restriction sites.

[0034] Relevant primers used in the p2KO method for the examples described herein are shown in Table 1, below. In each case, an inserted restriction endonuclease site is indicated by underlining. Where applicable, in a forward primer the start codon is indicated in bold with grey shading and in a reverse primer a stop codon is indicated in bold with grey shading. Left and right flank primers used in the examples described herein did not include start or stop codons.

Table 1: Primers used to prepare the p2KO ablation/insertion vector

Primer name	sequence
left flank	
66R L SacI (f)	5'-AATGGATCACATAAAGGAGCTCTTAACG-3'
66R L NotI (r)	5'- CAGAAAACATGCGGCCGCATATAATCT-3'
right flank	
66R R EcoRI	5'-GGAGATGAACAAGAAATAGAATTCATAGG-3'
66R R HindIII	5'- CTGTTCTTTATCACAAAGCTTCTATCGGGTG-3'
mGM-CSF	
hmGMCSF BamHI (f)	5'-TAGGCCTGGGATCCGATCCACCGGTCGCCACC ATG TGGCTGCAGA-3'
mGMCSF XmaI (r)	5'-CTCATCAATGTATCTTATCATCCCGGGCTAGCT-3'
mCCL2/MCP-1	
mMCP-1 BamHI (f)	5'-TAGGCCTGGGATCCGATCCACCGGTCGCCACC ATG CAGGTCCCTG-3'
mMCP-1 XmaI (r)	5'-CGGCGATCCCGGGAGATA CTA GTTCAC-3'
fliC (<i>S. typhimurium</i>)	
FliC BamHI (f)	5'-ACCCGGGGATCCTCTAGAAATAATTTTG-3'
FliC XmaI (r)	5'-GGAGCTCGAACCCGGGTCC TTA AC-3'
M13 (f)	5'-
M13 (r)	3'-

[0035] The ORFs used in various embodiments of the p2KO method for an expressed transgene insertion site include the mCCL2 transgene, the mGM-CSF transgene, and the fliC transgene. The mCCL2 transgene used in the examples cited below was produced

using a mCCL2 cDNA clone ORF purchased as an ORF-bearing plasmid (available from Sino Biological, Incorporated). The mGM-CSF transgene used in the examples cited below was produced using a cDNA clone ORF of mGM-CSF provided by Dr. Grant McFadden. The mCCL2, mGM-CSF and *fliC* ORFs were amplified from their vectors by PCR and given a BamHI restriction sequence and an XmaI restriction sequences on the 5'- and 3'- termini of the product amplicons, respectively. The mCCL2, mGM-CSF, and *fliC* ORFs were ligated into the p2KO ablation/insertion vector.

[0036] The following abbreviations, as shown in Table 2 below, are used herein to describe various embodiments of the recombinant TPV which were produced using the p2KO method. Although the p2KO ablation/insertion method is described herein, it is understood that any method known for ablating genes from the genome or inserting transgenes into the genome can be used to form the recombinant TPV described herein.

Table 2: Recombinant TPV abbreviations

TPV recombinant	gene ablated	gene added	reporter(s)
TPV/ <i>egfp</i>	---	---	EGFP
TPV-p2KO/ Δ 66R	66R	---	mCherry
TPV-p2KO/ Δ 66R/mGM-CSF	66R	mGM-CSF	mCherry
TPV-p2KO/ Δ 66R/mMCP-1	66R	mMCP-1	mCherry
TPV-p2KO/ Δ 66R/ <i>fliC</i>	66R	<i>fliC</i>	mCherry
TPV-p2KO/ Δ 2L	2L	---	mCherry
TPV-p2KO/2L/ Δ 66R/ <i>fliC</i>	66R, 2L	<i>fliC</i>	EGFP, mCherry

[0037] To choose an appropriate cell line for testing the recombinant TPV in mouse hosts, the minimally-altered recombinant TPV/*egfp* was tested against a panel of human colorectal cancer cell lines to select the cell line which allowed the best viral replication, thereby maximizing the effect of direct viral tumor cell lysis. The hCRC cell lines tested for TPV/*egfp* replication included HCT 116, COLO205, SW1463 and WiDr. Viral lysis of tumor cells is of importance to tumor clearance in some cases, but viral cytolysis is only one of many factors impinging upon tumor survival and clearance, and immune cell recruitment can also play a role. Although HCT 116 produced fewer progeny virions than the control cell line OMK, HCT 116 was the most productive of the hCRC cell lines tested.

Additionally, many OV's have been characterized in tumors induced with HCT 116. For these reasons HCT 116 was used for experiments to further characterize the oncolytic potential of the recombinant TPV *in vivo*.

[0038] To evaluate various embodiments of recombinant TPV, tumors were induced in athymic nude mice with the HCT 116 cell line by subcutaneous injection of 5×10^6 HCT 116 cells onto a dorsal surface of the athymic nude mice. The mice were randomly segregated into control or experimental groups when tumor size reached 75 mm^3 , with 5 mice in each group. A single injection containing 100 μL of vehicle only (group a) or recombinant TPV (groups b-h) was administered at day 0 (after reaching the tumor volume of 75 mm^3) and tumor volume was measured at three-day intervals thereafter. The average tumor volume was calculated using the formula:

$$\text{Average tumor volume} = (\text{length}) \times (\text{width}) \times (\text{height}) \times \pi/6 \quad (1)$$

[0039] The HCT 116-induced tumor xenografts did not increase in volume to the expected level during treatment with the recombinant TPV. However, multiple secondary tumors developed in mice undergoing treatment with the recombinant TPV. Additionally, in some *in vitro* studies, including an HCT 116 orthotopic xenotransplant model, HCT 116 cells have been reported to be highly motile and invasive.

[0040] FIG. 2A illustrates the observed average tumor development over a span of 36 days (beginning at the time when the tumor mass exceeded 75 mm^3) in athymic nude mice xenografted with 5×10^6 HCT 116 cells and subsequently treated with a vehicle only control solution. The average tumor volume, shown in an open circle, increased until approximately 15 days, at which point its volume stabilized at approximately 100 mm^3 for the remainder of the time span. The standard error of the mean is shown with bars ($\pm 1 \text{ SEM}$). The stabilization of the volume of the tumor is in contrast to some previous studies which have shown that untreated HCT 116 tumors in nude mice gradually increase in volume over the same interval, when using the same or similar numbers of HCT 116 cells in the initial xenograft. For example, it has been reported that HCT 116-induced tumors have a doubling time of approximately 8 days. Also, a recent study which examined the VACV as an OV therapeutic against HCT 116 xenografts in nude mice showed HCT 116 tumor growth up to 4000 mm^3 in a time interval similar to the time span illustrated in FIG. 2A.

- [0041] In the embodiment depicted in FIG. 2B, the black filled squares illustrate the average tumor volume of mice in group B, which were treated with TPV/*egfp* and the average tumor volume of mice in control group A. The bars illustrate the standard error of the mean ($\pm$ 1 SEM) for the tumor volume in control group A and the tumor volume in group B.
- [0042] In the embodiment depicted in FIG. 2C, the black filled squares illustrate the average tumor volume of mice in group C which were treated with TPV-p2KO/ Δ 66R/mMCP-1 and the average tumor volume of mice in control group Aa. The bars illustrate the standard error of the mean ($\pm$ 1 SEM) for the tumor volume in control group A and the tumor volume in group C.
- [0043] In the embodiment depicted in FIG. 2D, the black filled squares illustrate the average tumor volume of mice in group D, which were treated with TPV-p2KO/ Δ 66R/mGM-CSF and the average tumor volume of mice in control group A. The bars illustrate the standard error of the mean ($\pm$ 1 SEM) for the tumor volume in control group A and the tumor volume in group D.
- [0044] In the embodiment depicted in FIG. 2E, the black filled squares illustrate the average tumor volume of mice in group E, which were treated with TPV-p2KO/ Δ 66R/fliC and the average tumor volume of mice in control group A. The bars illustrate the standard error of the mean ($\pm$ 1 SEM) for the tumor volume in control group A and the tumor volume in group E.
- [0045] In the embodiment depicted in FIG. 2F, the black filled squares illustrate the average tumor volume of mice in group F, which were treated with TPV-p2KO/ Δ 66R and the average tumor volume of mice in control group A. The bars illustrate the standard error of the mean ($\pm$ 1 SEM) for the tumor volume in control group A and the tumor volume in group F.
- [0046] In the embodiment depicted in FIG. 2G, the black filled squares illustrate the average tumor volume of mice in group G, which were treated with TPV-p2KO/ Δ 2L and the average tumor volume of mice in control group A. The bars illustrate the standard error of the mean ($\pm$ 1 SEM) for the tumor volume in control group A and the tumor volume in group G.
- [0047] In the embodiment depicted in FIG. 2H, the black filled squares illustrate the average tumor volume of mice in group H, which were treated with TPV-

p2KO/ Δ 2L/ Δ 66R/fliC and the average tumor volume of mice in control group H. The bars illustrate the standard error of the mean ($\pm$ 1 SEM) for the tumor volume in control group A and the tumor volume in group H.

[0048] One particularly preferred embodiment of the recombinant TPV for use herein is a recombinant TPV with the *fliC* transgene added to a double-knockout background (Δ 66R and Δ 2L). Our results demonstrate that the recombinant TPV deleted for both 2L and 66R which expressed the fliC transgene produced a robust and durable therapeutic effect upon HCT 116 tumor xenografts. Another preferred embodiment of the recombinant TPV for use herein is a recombinant TPV with the *fliC* transgene added to a single-knockout virus (Δ 66R). Both of the single knockout recombinant TPV embodiments (TPV-p2KO/ Δ 66R and TPV-p2KO/ Δ 2L) showed statistically significant reductions in tumor volume at at least two time points, and in each case the observed significant reduction in tumor volume was temporally distant from the point of virotherapeutic inoculation. Both single knockout recombinant TPVs appeared to trend towards an effect at these later time points. Indeed, with the exception of the TPV/*egfp* virus, all embodiments of the recombinant TPVs tested appeared to produce some degree of tumor ablation, with the recombinant TPVs mentioned above being preferred. Since the T cell-dependent adaptive immune response is severely impaired in nude mice, the examples described herein demonstrate that the innate immune response is potentially capable of reducing the tumor burden in subjects and therefore recombinant TPV armed with an innate immune response activator is expected to be useful in subjects with immunodeficiency syndromes. We therefore conclude that OV armed with activators of the innate immune response will also be useful in individuals with immunodeficiency syndromes.

[0049] As a highly-conserved pathogen-associated molecular pattern (PAMP), flagellins are targets for detector molecules involved in cytosolic immunosurveillance. For example, detection of flagellin by the Nod-like receptor NCLR4 (also known as Ipaf) triggers activation of the Ipaf inflammasome which in turn activates caspase-1 and maturation of the cytokine interleukin 1 β (IL-1 β) in macrophages. Although the expected amount of FliC produced in the course of an OV infection is expected to be small, in mice even minute amounts of bacterial flagellin ($\leq$ 5 μ g/animal) administered by tail vein injection cause global (*i.e.*, in both organs and plasma) elevations of the cytokines TNF, IL-1 β , IL-6, and the chemokine MIP-2 (IL-8), as well as changes in the MEK intracellular

signaling pathway. The action of FliC in mammalian cells is therefore partially independent of the TLR-based PAMP detectors, and has not yet been fully elucidated. However, based on the results described herein, the activation of the innate immune response by recombinant TPV expressing FliC appears to contribute to the reduction of tumor mass in nude mice, which have an intact innate immune response.

List of Non-limiting Embodiments

- [0050] Embodiment A is a composition for treating cancerous cells in a subject having an immune system, comprising: a virus in the *Yatapoxvirus* genus having at least one mutation, wherein the at least one mutation results in suppressed expression of a TNF binding protein by the virus.
- [0051] The composition of Embodiment A wherein the virus to suppress expression of the TNF binding protein has a structure capable of binding to an MHC-1 light chain.
- [0052] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein the virus is a yatapoxvirus which encodes a transgene expressing a bacterial flaggellin protein.
- [0053] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein a polymerized flaggellin protein is the main component of the bacterial flagellin.
- [0054] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein the transgene is a product of the *Salmonella enteric serovar typhimurium* gene ("fliC").
- [0055] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein the virus is a tanapoxvirus (TPV), and wherein the at least one mutation suppresses expression of the TNF binding protein encoded by a 2L gene of the TPV.
- [0056] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein the virus has a second mutation, and wherein a second mutation results in suppressed expression of thymidine kinase by the virus.
- [0057] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein the virus is a tanapoxvirus (TPV), and wherein a second mutation suppresses expression of thymidine kinase encoded by a 66R gene of the TPV.

- [0058] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein the virus further encodes a transgene to increase apoptosis of the cancerous cells.
- [0059] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein the virus further encodes a transgene to activate the immune system of the subject.
- [0060] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein the virus further encodes a transgene to introduce a mCherry fluorescent reporter.
- [0061] The composition of Embodiment A or Embodiment A with one or more of the intervening features wherein the virus further encodes a transgene to introduce a green fluorescent protein fluorescent reporter.
- [0062] Embodiment B is a composition for treating cancerous cells in a subject having an immune system, comprising: a virus in the *Yatapoxvirus* genus having at least one mutation, wherein the at least one mutation results in suppressed expression of thymidine kinase (TK).
- [0063] The composition of Embodiment B wherein the virus is a poxvirus which encodes a transgene expressing a bacterial flaggellin protein.
- [0064] The composition of Embodiment B or Embodiment B with one or more of the intervening features wherein the transgene is a product of the *Salmonella enteric serovar typhimurium* gene ("fliC").
- [0065] The composition of Embodiment B or Embodiment B with one or more of the intervening features wherein the virus is a tanapoxvirus (TPV), and wherein the at least one mutation suppresses expression of the thymidine kinase encoded by a 66R gene of the TPV.
- [0066] The composition of Embodiment B or Embodiment B with one or more of the intervening features wherein the virus has a second mutation, and wherein a second mutation results in suppressed expression of a TNF binding protein by the virus.
- [0067] The composition of Embodiment B or Embodiment B with one or more of the intervening features wherein the virus is a tanapoxvirus (TPV), and wherein a second mutation suppresses expression of the TNF binding protein encoded by a 2L gene of the TPV.

- [0068] The composition of Embodiment B or Embodiment B with one or more of the intervening features wherein the virus to suppress expression of the TNF binding protein has a structure capable of binding to an MHC-1 light chain.
- [0069] The composition of Embodiment B or Embodiment B with one or more of the intervening features wherein the virus further encodes a transgene to increase apoptosis of the cancerous cells.
- [0070] The composition of Embodiment B or Embodiment B with one or more of the intervening features wherein the virus further encodes a transgene to activate the immune system of the subject.
- [0071] Embodiment C is a method of delivering at least one gene to cancerous cells in a subject, comprising: modifying a virus of the *Yatapoxvirus* genus by mutating the virus to suppress expression of a TNF binding protein having a structure capable of binding an MHC-1 light chain; and administering the modified *Yatapoxvirus* genus virus to the subject systemically.
- [0072] The method of Embodiment C further comprising: modifying the virus by encoding the at least one gene in the virus, wherein the at least one gene results in increased apoptosis of the cancerous cells or activates an immune response in the subject.

Detailed Description of Experiments:

- [0073] OMK (Owl Monkey kidney) cells, HCT 116, COLO 205, SW1463 and WiDr cell lines from the American Type Culture Collection were used (available as American Type Culture Collection product numbers CRL-1556, CCL-247, CCL-222, CCL-234 and CCL-218 respectively). OMK cells were used for the virus amplification and viral titrations described herein. The cell lines were propagated in complete growth medium consisting of DMEM (available from Gibco/Life Technologies) supplemented with 10% (vol/vol) fetal bovine serum (available from Atlanta Biologicals), 2 mM L-glutamine (available from Sigma-Aldrich) and 50 µg/ml gentamicin sulfate (available from AMRESCO). After virus infection, cell monolayers of the cell lines were maintained in maintenance medium, which was identical to growth medium except that the concentration of fetal bovine serum was reduced to 2 %. The cells were incubated at 37 °C in a 5 % CO₂ atmosphere. Cell counting and cell viability assays were done with an Improved Neubauer hemacytometer using 0.2 % (wt/vol) trypan blue.

Experiment 1: Control

[0074] Wild-type TPV (Kenya strain) was provided by Dr. Joseph Esposito (of the Centers for Disease Control, Atlanta, GA, USA). The wild-type TPV was modified as described herein to form a control recombinant TPV which expresses the fluorescent reporter EGFP (with no other genetic modifications). Briefly, two identical vaccinia virus (VACV)-derived early/late synthetic promoters were used to drive the expression of a fluorescent reporter gene in the control recombinant TPV using the p2KO method. A p2KO expression cassette (including left and right flanks, plus the intervening open reading frames (ORF) and the promoters) was transferred to the viral genome of the wild-type TPV through a homologous recombination double-crossover event during a transfection/infection procedure, to form the control recombinant TPV. Flanking regions for recombination were ligated into the p2KO vector between the Sac I restriction site and the Not I restriction site on the 5'- (left) flank, and between the EcoR I restriction site and the Hind III restriction site on the 3'- (right) flank

Experiment 2: Transfection/infection procedure

[0075] A transfection/infection procedure was used to produce the recombinant TPV used in these example embodiments. Briefly, OMK cells were transfected using a jetPRIME transfection reagent (available from PolyPlus Transfection SA) at a concentration of 1 µl transfection reagent per µg of purified p2KO vector according to the manufacturer of the transfection reagent's transfection protocol. At approximately 5 hours post transfection, OMK monolayers were inoculated with 1 plaque-forming unit per cell (pfu/cell) of wild-type TPV-Kenya strain (non-fluorescent). At five days post-inoculation the infected monolayers were scraped with a rubber cell scraper on ice, subjected to three cycles of freezing and thawing at -80 °C, 15 seconds of sonication at 4 °C, serially diluted and plated onto freshly-seeded OMK monolayers at approximately 90 % confluence and overlaid with maintenance medium containing 0.5 % methylcellulose. Fluorescent, well-separated plaques were picked and each pick subjected to at least three rounds of plaque purification to produce a virus preparation which contained no visible wild type (non-fluorescent) plaques. Samples were considered pure only if no wild-type plaques were visible in culture and no wild-type TPV DNA was detectable by PCR.

Experiment 3: Confirmation of viral transgene expression

[0076] Verification of FliC expression was done by Western blot. Verification of mCCL2 and mGM-CSF expression was done by Luminex multianalyte cytokine detection assay (performed by the University of Maryland cytokine core laboratory). Samples for analysis were prepared by infecting semi-confluent OMK cell monolayers in 60 mm tissue culture dishes (having 22.1 cm² surface area available for cell growth) with TPV-p2KO/ Δ 66R/mCCL2, TPV-p2KO/ Δ 66R/mGM-CSF, and TPV-p2KO/ Δ 2L/ Δ 66R/*fliC* using 10 pfu/cell. Supernatant (3 ml/dish) and cytoplasmic extracts were prepared at the indicated times post-infection. For FliC detection, extracted lysates were analyzed by Western blot. Proteins were transferred to a PVDF membrane (available from Millipore) and probed with an anti-FliC monoclonal antibody (available from BioLegend) at a 1:2000 dilution (vol/vol). Powdered milk 5 % (wt/vol) was used as the blocking agent. The secondary antibody was a monoclonal anti-mouse IgG conjugated to horseradish peroxidase (available from Abcam), used at a 1:2500 dilution. Visualization was by ECL (Thermo Scientific/Pierce). Embodiments of TPV recombinants containing the p2KO vector but without a *fliC*, mGM-CSF or mCCL2 insert served as controls.

Experiment 4: Cell density determinations

[0077] Four human colorectal cancer cell lines, and the OMK cell control were separately inoculated into 12-well plates (3 wells per cell line) such that one day later the cells would achieve 90 % confluency. Each well (having 3.8 cm² surface area available for cell growth) was trypsinized, counted and scored for viability by trypan blue exclusion. This was done to ensure that experiments at a specified number of viral pfu/cell for each cell line would be accurate.

Experiment 5: Virus titration

[0078] To assay the number of viable recombinant TPV virions present in a sample, a plaque assay was used. Briefly, virus samples were sonicated for 15 seconds on ice, serially diluted in maintenance medium and inoculated onto nearly confluent OMK monolayers in 6-well plates (n = 3 for each dilution of sample). Virus was allowed to adsorb at room temperature with gentle rocking for one hour. The inoculum was then removed and each well gently washed two times with 1 ml of pre-warmed maintenance medium. After washing, 2 ml of an overlay medium was added and the infected OMK monolayers incubated for 10 days at 37 °C. The overlay medium was then removed and

monolayers were stained (using 0.1 % crystal violet in 37 % formaldehyde). Plates were washed with distilled water, dried in air, and plaques were counted.

Experiment 6: Animals

- [0079] Male neonatal athymic nude (Nude-*Foxn1*^{nu/nu}) mice (available through Harlan Laboratories) were received at four weeks of age and allowed to acclimate for one week before the beginning of experimentation. Mice were individually housed in clear polycarbonate cages under a 12 hour/12 hour light/dark cycle. Food and water was available *ad libitum*. All animal housing conditions, manipulations and treatments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Western Michigan University (IACUC protocol number 13-07-01).

Experiment 7: Choice of cell line for tumor xenografts in nude mice

- [0080] Before initiating *in vivo* studies in athymic nude mice, we determined which hCRC cell line had the highest viral productivity when infected with TPV/*egfp*. TPV/*egfp* was assayed for its ability to replicate in four hCRC-derived cell lines: HCT 116, WiDr, SW1463 and COLO 205. OMK cells were used as a positive control. Each cell line was seeded into 12-well tissue culture plates (having 3.8 cm² surface area available for cell growth) and 0.1 pfu/cell of TPV/*egfp* was inoculated into each well. Lysates were collected at 4 days post-infection and assayed by plaque assay.

Experiment 8: Tumor induction and measurement

- [0081] Tumors were produced in athymic nude mice by subcutaneous injection of 5 x 10⁶ HCT 116 cells on the dorsal surface, approximately above the first lumbar vertebra. Each injection was followed by an assessment of viability by trypan blue exclusion to ensure that the cells were viable at and after the time of injection. Once visible, tumors were measured using a digital caliper (Pittsburgh model 6ZBTMCO) along a major axis (length), a minor axis (width) and a z dimension (height). The volume of the tumors was then estimated using formula 1 presented herein. When the estimation of tumor size reached or surpassed 75 mm³ each animal was randomly segregated into the control group (group a) or one of the seven experimental groups (groups b-h).

Experiment 9: Virotherapy of HCT 116 xenografts in nude mice

- [0082] Each treatment group was composed of five or six tumor-bearing athymic nude mice. A single virotherapeutic injection was administered to each tumor-bearing mouse once tumor volume reached or exceeded 75 mm³. Virotherapeutic injections were given

intratumorally as a single injection of 5×10^6 pfu suspended in 100 μ l of crude OMK cell lysate diluted in normal saline. Each mouse's weight and tumor volume was measured and recorded at three-day intervals thereafter. Data was collected for 13 time points over a total of 39 days. To control for unanticipated inflammation or other injection effects produced by the administration of the recombinant TPV injection, a vehicle control group was used, denoted as group a herein. This control group consisted animals which received the HCT 116 cells but experienced only a mock recombinant TPV injection (100 μ l of vehicle only). This group is referred to as the "mock virotherapy" group or group A. All experimental groups were compared to group A to assess the therapeutic efficacy of the recombinant TPV.

[0083] To assess treatment effects each experimental group was compared to the control group a using the Mann-Whitney *U* test (sometimes referred to as the Wilcoxon rank-sum test). Treatment with the recombinant TPV was judged to have produced a significant therapeutic effect if the average tumor volume within a group was significantly reduced when compared to the mock-injected control. A significance level of $p < 0.05$ was used throughout the study.

Results: The p2KO poxvirus ablation/insertion vector

[0084] The p2KO poxvirus ablation/insertion vector was designed and constructed to provide a rapid and reliable way to simultaneously ablate any desired TPV gene(s) and replace the ablated gene(s) with the desired expressed fluorescent reporter and/or the desired expressed transgene. Visualization of viral infection in cultured cells was greatly facilitated by the inclusion of the fluorescent reporters, mCherry and EGFP. The use of two fluorescent reporters made it possible to identify and isolate the double-deleted recombinant TPV with the *fliC* insertion (TPV-p2KO/ Δ 2L/ Δ 66R/*fliC*). A viral plaque produced by infection with the TPV-p2KO/ Δ 2L/ Δ 66R/*fliC* virus on an OMK cell monolayer is shown in FIG. 3, and demonstrates the simultaneous expression of the brilliant orange-red and green color associated with mCherry and EGFP, respectively.

[0085] The overall sequence of a base vector (*i.e.*, with the fluorescent reporter but without the optional transgene to be expressed) was verified by DNA sequencing of an amplicon produced by PCR amplification of the region between the M13 forward and reverse primer binding sequences. The insertion of ORFs encoding mCCL2, mGM-CSF, or *fliC* was verified by DNA sequencing of the p2KO vector to ensure correct placement and

orientation before they were used in the transfection/infection procedure. The recombinant TPVs were verified to be knockouts for either *2L*, *66R*, or both by agarose gel analysis of PCR products using recombinant viral DNA as a template.

Results: Transfection/infection

- [0086] By 4-5 days post-inoculation, expression of the fluorescent reporter was evident in the OMK cell monolayer, indicating gene expression from the p2KO vector in the cytosolic compartment of wild-type TPV-infected cells. In control cultures which were transfected with the p2KO vector but not subsequently inoculated with wild type TPV, no fluorescence was observed. Purity of the viral sample was then verified by PCR using the recombinant viral genomic DNA as the template before further use. All viral DNA samples were probed for the presence of the ampicillin resistance gene, which was not detected in any recombinant TPV. This indicates that all of the recombinant TPVs resulted from a double-crossover event rather than a single-crossover event.

Results: Confirmation of viral transgene expression

- [0087] To demonstrate that the inserted ORFs were expressed in cells infected with the recombinant TPVs (including TPV-p2KO/ Δ 66R/*mCCL2*, p2KO/ Δ 66R/*mGM-CSF*, and TPV-p2KO/ Δ 2L/ Δ 66R/*fliC*) OMK monolayers in 60 mm dishes (having 20 cm² growth area) were inoculated with the relevant recombinant TPV and assayed for transgene expression in cell lysates and culture supernatants as described previously. A Western blot of lysates from OMK cells inoculated TPV-p2KO/ Δ 2L/ Δ 66R/*fliC* was probed with a monoclonal anti-FliC antibody. A single band with an apparent molecular mass of 50 kDa was observed, identical to the FliC positive control, as expected. The intensity of this band gradually increased between day three and day six post-infection. The FliC transgene was not detected in mock infected cells. Infected cell lysates and their culture supernatants were assayed to determine the presence of mCCL2 and mGM-CSF. Both transgenes were highly expressed, and present in large amounts in infected cell supernatants (4.9 ng/ml mCCL2, and greater than 10.0 ng/ml mGM-CSF). Both mGM-CSF and mCCL2 were only weakly detectable or undetectable in cytoplasmic extracts of infected cells or control uninfected cells, and in cells infected with TPVs not expressing these transgenes. This data indicates that both mCCL2 and mGM-CSF were secreted from infected cells, as anticipated, while FliC accumulated within the cytoplasm of infected cells, again as anticipated.

Results: Cell density determinations

[0088] Each cell line used in this study had its confluent density (cells/cm²) determined at near confluence. The OMK control cell line had a confluent density of approximately 1.0×10^5 cells/cm². The densities determined for the colorectal cancer cell lines were as follows: HCT 116 had a confluent density of approximately 1.4×10^5 cells/cm²; COLO205 had a confluent density of approximately 6.9×10^5 cells/cm²; SW1463 had a confluent density of approximately 4.5×10^5 cells/cm²; WiDr had a confluent density of approximately 2.5×10^5 cells/cm². These values were used to calculate the number of pfu to use when inoculating these cell lines.

Results: Virotherapy HCT 116 xenografts in nude mice

[0089] Before initiating *in vivo* studies in athymic nude mice, OMK cells were found to be the hCRC cell line with the highest viral productivity when infected with TPV/*egfp*, as described above. OMK cells were the best host cells, allowing the production of approximately 3×10^6 progeny pfu/well. Of the hCRC cell lines tested, HCT 116 produced the most progeny virions with an average yield (n = 3) of approximately 7×10^5 progeny pfu/well. We therefore chose HCT 116 for the *in vivo* phase of this study.

[0090] In order to evaluate the oncolytic potential of the recombinant TPV, tumors were induced in athymic nude mice (Nude-*Foxn1*^{nu/nu}). Viability counts of the HCT 116 cells demonstrated that > 99% were viable at the time of injection. Tumors generally reached 75 mm³ within one to three weeks after injection of the HCT 116. Treatment with TPV/ $\Delta 66R$ (group F), TPV/ $\Delta 2L$ (group G), and TPV/ $\Delta 2L/\Delta 66R/fliC$ (group H) all produced significant reductions in tumor size at 2 or more time points when compared to the mock injected controls. TPV/ $\Delta 2L$ -treated tumors (group G) were significantly smaller than mock-injected tumors at two time points, 33 days (a 47.6% reduction) and 36 days (a 65.2% reduction) post-treatment. TPV/ $\Delta 66R$ -treated tumors (group F) were significantly smaller than mock-injected tumors at two time points, 27 days (a 34.9% reduction) and 36 days (a 52% reduction) post-treatment. TPV/ $\Delta 2L/\Delta 66R/fliC$ -treated tumors (group H) showed a robust and durable therapeutic effect, and were significantly reduced in volume when compared to mock-injected tumors at six time points, 15 days (a 56.1% reduction), 21 days (a 62.0% reduction), 24 days (a 63.8% reduction), 27 days (a 59.5% reduction), 33 days (a 55.3% reduction) and 36 days (a 69.6% reduction) post-treatment.

[0091] Although initial testing was performed using human colorectal cancer cell lines, the pharmaceutical virotherapy described herein is intended for use in treating a broad array of cancers. A non-limiting list of cancers to be treated with a pharmaceutical composition as described herein includes: basal cell carcinoma, carcinoma, choriocarcinoma, glioma tumor, intra-epithelial neoplasm, leukemia, lymphoma, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, melanoma, myeloma, neuroblastoma, retinoblastoma, rhabdomyosarcoma, sarcoma, and cancers of the biliary tract, bladder, bone, brain, breast, CNS, cervix, colon and rectum, connective tissue, digestive system, endometrial cells, esophagus, eye, stomach, head and neck, kidney, larynx, liver, lung, pancreas, prostate, oral cavity, ovaries, respiratory system, skin, stomach, testicles, thyroid, uterus, and urinary system.

[0092] The pharmaceutical composition as described herein is to be administered at a therapeutically effective dose. The term "therapeutically effective dose" as used herein refers to an amount of the pharmaceutical which, after administration, is effective to achieve the desired therapeutic result. A therapeutically effective dose can vary from patient to patient according to factors such as the disease state, age, sex, and weight of the individual, form of the pharmaceutical, and the ability of the dosage form to elicit the desired response in the individual. The therapeutically effective dose may be determined by starting with a low, safe dose and escalating to higher doses, while monitoring for therapeutic effects (e.g. a reduction in cancer cell growth) along with the presence of any deleterious side effects. The pharmaceutical composition may include the poxvirus, viral nucleic acids, or expression vectors to produce the desired virotherapeutic effect.

[0093] The pharmaceutical composition can be administered via any suitable dosage forms or routes known in the art, including without limitation, parenteral, oral, enteral, buccal, nasal, topical, rectal, vaginal, transmucosal, epidermal, transdermal, dermal, ophthalmic, pulmonary, and subcutaneous administration routes to provide a systemic or localized, therapeutically effective dose. The pharmaceutical will be administered to a subject in formulations or preparations suitable for the particular administration route. Formulations suitable for administration of the pharmaceutical dosage form may include, without limitation; aerosols, dispersions, emulsions, implants, liposome based formulations, nose drops, patches, powders, solutions, sprays, suppositories and suspensions. The formulations may be presented in unit dosage form and may be

prepared by any methods known in the art. Methods of preparing these formulations or dosage forms include the step of combining the poxvirus or nucleic acid of the present disclosure with one or more pharmaceutically acceptable carriers and may further comprise additives, such as without limitation: stabilizers, preservatives, and transfection facilitating agents which assist in the cellular uptake of the medicines. Suitable stabilizers may include, without limitation: albumin, EDTA, glycine and monosodium glutamate. Suitable preservatives may include, without limitation: antibiotics, methyl hydroxybenzoate, phenols, 2-phenoxyethanol, potassium sorbate, sodium benzoate, and thimerosal.

[0094] The pharmaceutical composition can be delivered locally into the target tissue or organ at a tumor site of a subject in need of treatment. An effective dose of the composition is directly injected to the tumor site through the subject's skin or in an exposed surgical field using a syringe. In certain embodiments, the pharmaceutical composition can be injected using an implantable dosing device.

[0095] It is also important to note that the construction and arrangement of the elements of the composition as shown and described in the exemplary embodiments is illustrative only. Although only a few embodiments of the present innovations have been described in detail in this disclosure, those skilled in the art who review this disclosure will readily appreciate that many modifications are possible (e.g., variations in sizes, dimensions, structures, shapes and proportions of the various elements, values of parameters, mounting arrangements, use of materials, colors, orientations, etc.) without materially departing from the novel teachings and advantages of the subject matter recited. For example, elements shown as integrally formed may be constructed of multiple parts or elements shown as multiple parts may be integrally formed, the operation of the interfaces may be reversed or otherwise varied, the length or width of the structures and/or members or connector or other elements of the system may be varied, the nature or number of adjustment positions provided between the elements may be varied. It should be noted that the elements and/or assemblies of the system may be constructed from any of a wide variety of materials that provide sufficient strength or durability, in any of a wide variety of colors, textures, and combinations. Accordingly, all such modifications are intended to be included within the scope of the present innovations. Other substitutions, modifications, changes, and omissions may be

made in the design, operating conditions, and arrangement of the desired and other exemplary embodiments without departing from the spirit of the present innovations.

[0096] It will be understood that any described processes or steps within described processes may be combined with other disclosed processes or steps to form structures within the scope of the present device. The exemplary structures and processes disclosed herein are for illustrative purposes and are not to be construed as limiting.

[0097] It is also to be understood that variations and modifications can be made on the aforementioned structures and methods without departing from the concepts of the present device, and further it is to be understood that such concepts are intended to be covered by the following claims unless these claims by their language expressly state otherwise.

[0098] The above description is considered that of the illustrated embodiments only. Modifications of the device will occur to those skilled in the art and to those who make or use the device. Therefore, it is understood that the embodiments shown in the drawings and described above is merely for illustrative purposes and not intended to limit the scope of the device, which is defined by the following claims as interpreted according to the principles of patent law, including the Doctrine of Equivalents.

CLAIMS

What is claimed is:

1. A composition for treating cancerous cells in a subject having an immune system, comprising:
a virus in the *Yatapoxvirus* genus having at least one mutation, wherein the at least one mutation results in suppressed expression of a TNF binding protein by the virus.
2. The composition of claim 1, wherein the virus to suppress expression of the TNF binding protein has a structure capable of binding to an MHC-1 light chain.
3. The composition of any one of claims 1 and 2, wherein the virus is a yatapoxvirus which encodes a transgene expressing a bacterial flaggellin protein.
4. The composition of claim 3, wherein a polymerized flaggellin protein is the main component of the bacterial flaggellin.
5. The composition of any one of claims 3 and 4, wherein the transgene is a product of the *Salmonella enteric serovar typhimurium* gene ("fliC").
6. The composition of any one of claims 1-5, wherein the virus is a tanapoxvirus (TPV).
7. The composition of any one of claims 1-6, wherein the at least one mutation suppresses expression of the TNF binding protein encoded by a 2L gene of the TPV.
8. The composition of any one of claims 1-7, wherein the virus has a second mutation, and wherein the second mutation results in suppressed expression of thymidine kinase by the virus.

9. The composition of any one of claims 1-5 and 7-8, wherein the virus is a tanapoxvirus (TPV), and wherein a second mutation suppresses expression of thymidine kinase encoded by a 66R gene of the TPV.
10. The composition of any one of claims 1-9, cells wherein the virus further encodes a transgene to activate the immune system of the subject.
11. The composition of any one of claims 1-10, wherein the virus further encodes a transgene to increase apoptosis of the cancerous cells.
12. The composition of any one of claims 1-11, wherein the virus further encodes a transgene to introduce a mCherry fluorescent reporter.
13. The composition of any one of claims 1-12, wherein the virus further encodes a transgene to introduce a green fluorescent protein fluorescent reporter.
14. A method of delivering at least one gene to cancerous cells in a subject, comprising:
 - modifying a virus of the *Yatapoxvirus* genus by mutating the virus to suppress expression of a TNF binding protein having a structure capable of binding an MHC-1 light chain; and
 - administering the modified *Yatapoxvirus* genus virus to the subject systemically.
15. The method of claim 14 further comprising:
 - modifying the virus by encoding the at least one gene in the virus, wherein the at least one gene results in increased apoptosis of the cancerous cells or activates an immune response in the subject.
16. A composition for treating cancerous cells in a subject having an immune system, comprising:

a virus in the *Yatapoxvirus* genus having at least one mutation, wherein the at least one mutation results in suppressed expression of a TNF binding protein by the virus.

17. The composition of claim 16, wherein the virus to suppress expression of the TNF binding protein has a structure capable of binding to an MHC-1 light chain.

18. The composition of claim 16, wherein the virus is a yatapoxvirus which encodes a transgene expressing a bacterial flaggellin.

19. The composition of claim 18, wherein the transgene is a product of the *Salmonella enteric seroval typhimurium* gene ("fliC").

20. The composition of claim 16, wherein the virus is a tanapoxvirus (TPV), and wherein the at least one mutation suppresses expression of the TNF binding protein encoded by a 2L gene of the TPV.

21. The composition of claim 16, wherein the virus has a second mutation, and wherein a second mutation results in suppressed expression of thymidine kinase by the virus.

22. The composition of claim 16, wherein the virus is a tanapoxvirus (TPV), and wherein a second mutation suppresses expression of thymidine kinase encoded by a 66R gene of the TPV.

23. The composition of claim 16, wherein the virus further encodes a transgene to activate the immune system of the subject.

24. The composition of claim 16, wherein the virus further encodes a transgene to increase apoptosis of the cancerous cells.

25. A composition for treating cancerous cells in a subject having an immune system, comprising:

a virus in the *Yatapoxvirus* genus having at least one mutation, wherein the at least one mutation results in suppressed expression of thymidine kinase (TK).

26. The composition of claim 25, wherein the virus is a yatapoxvirus which encodes a transgene expressing a bacterial flaggellin protein.

27. The composition of claim 26, wherein the transgene is a product of the *Salmonella enteric serovar typhimurium* gene ("fliC").

28. The composition of claim 25, wherein the virus is a tanapoxvirus (TPV), and wherein the at least one mutation suppresses expression of the thymidine kinase encoded by a 66R gene of the TPV.

29. The composition of claim 25, wherein the virus has a second mutation, and wherein a second mutation results in suppressed expression of a TNF binding protein by the virus.

30. The composition of claim 25, wherein the virus is a tanapoxvirus (TPV), and wherein a second mutation suppresses expression of the TNF binding protein encoded by a 2L gene of the TPV.

31. The composition of claim 30, wherein the virus to suppress expression of the TNF binding protein has a structure capable of binding to an MHC-1 light chain.

32. The composition of claim 25, wherein the virus further encodes a transgene to increase apoptosis of the cancerous cells.

33. The composition of claim 25, wherein the virus further encodes a transgene to activate the immune system of the subject.

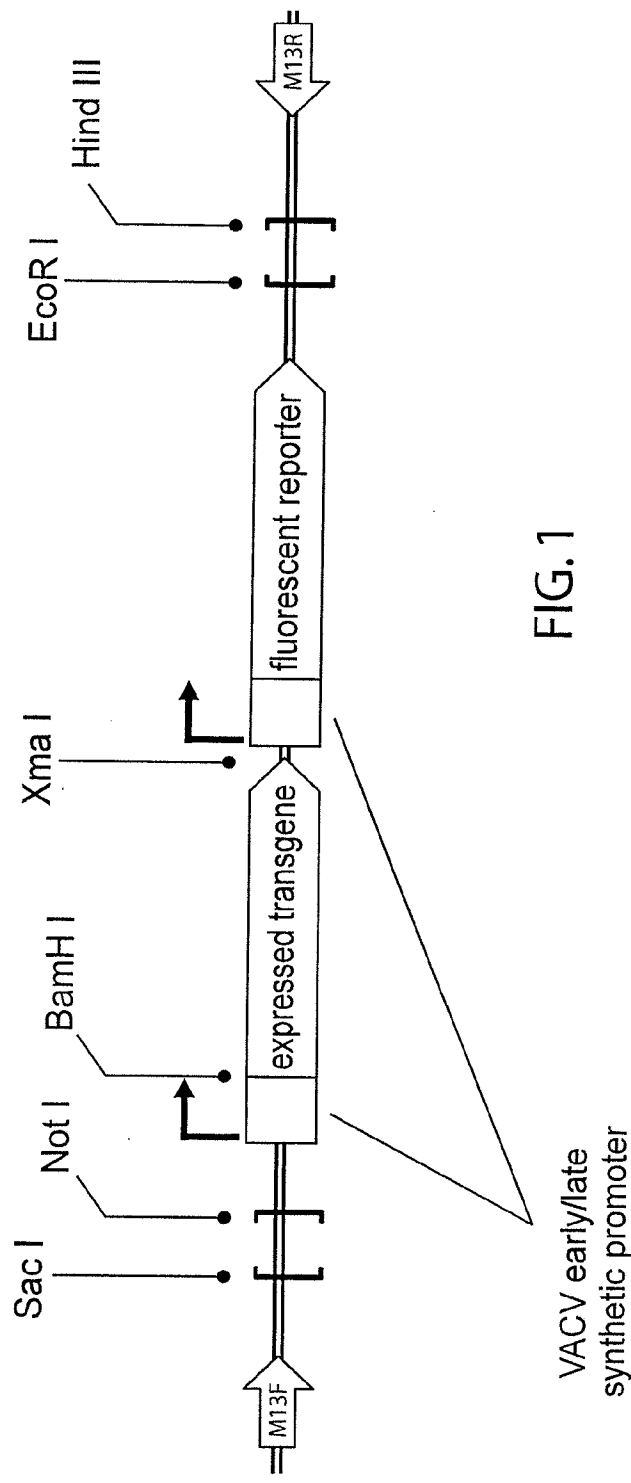
34. A method of delivering at least one gene to cancerous cells in a subject, comprising:

modifying a virus of the *Yatapoxvirus* genus by mutating the virus to suppress expression of a TNF binding protein having a structure capable of binding an MHC-1 light chain; and

administering the modified *Yatapoxvirus* genus virus to the subject systemically.

35. The method of claim 34 further comprising:

modifying the virus by encoding the at least one gene in the virus, wherein the at least one gene results in increased apoptosis of the cancerous cells or activates an immune response in the subject.



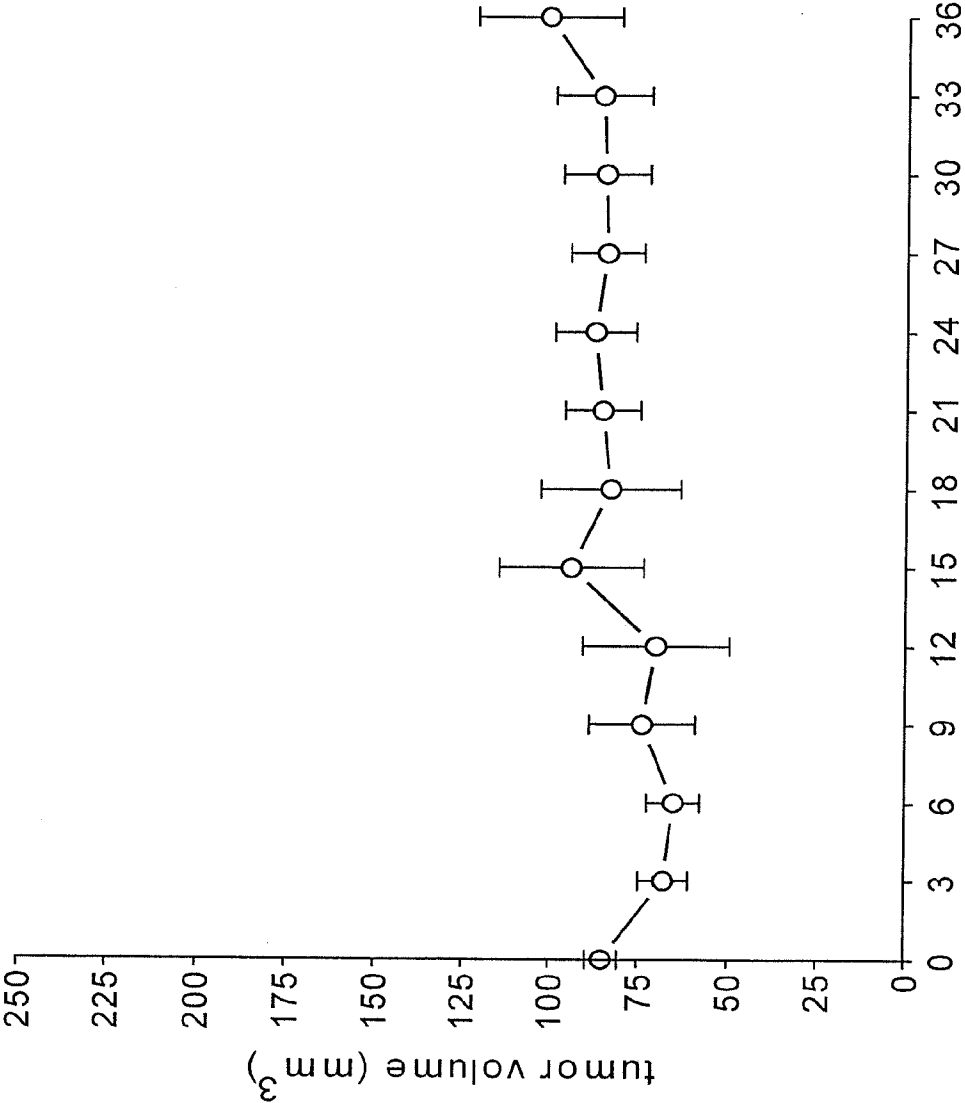
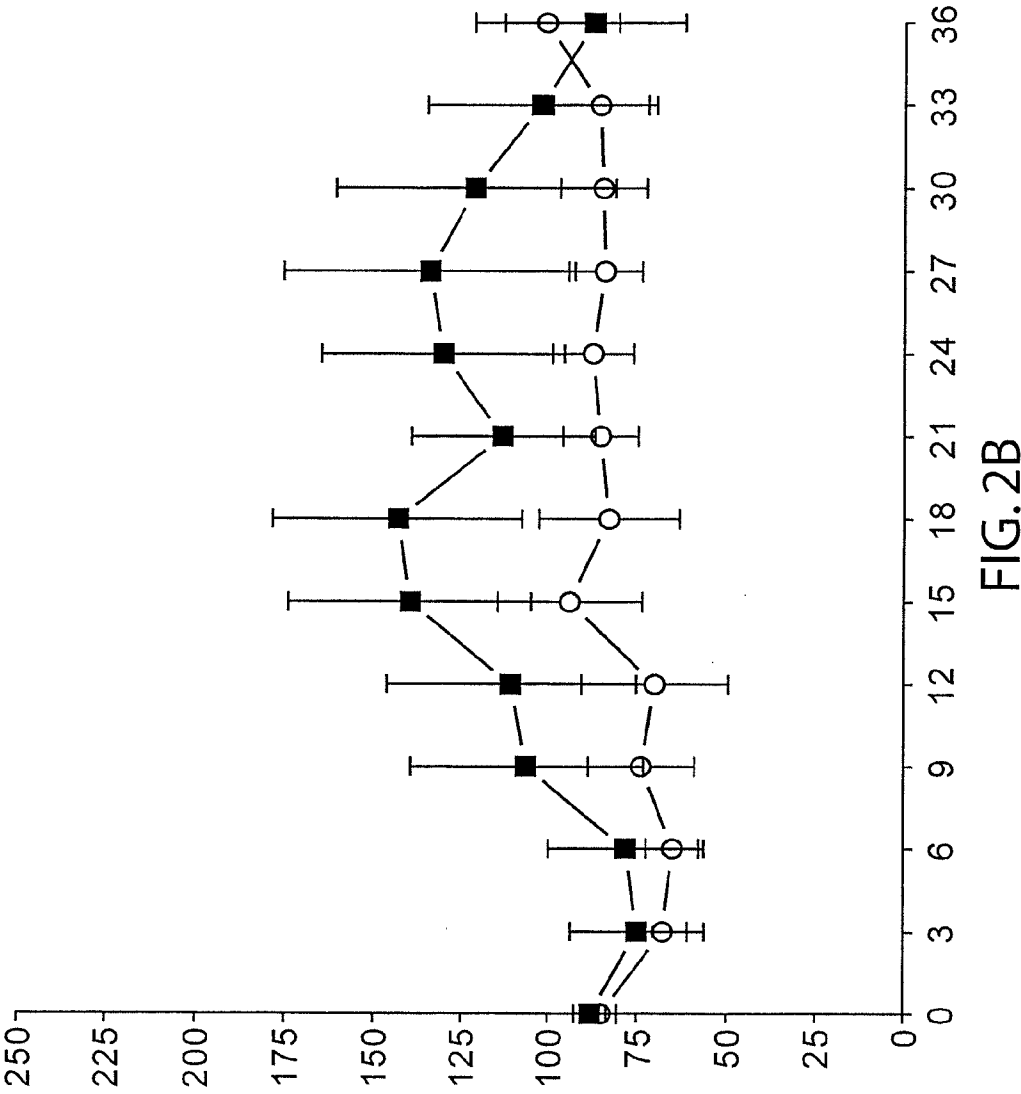
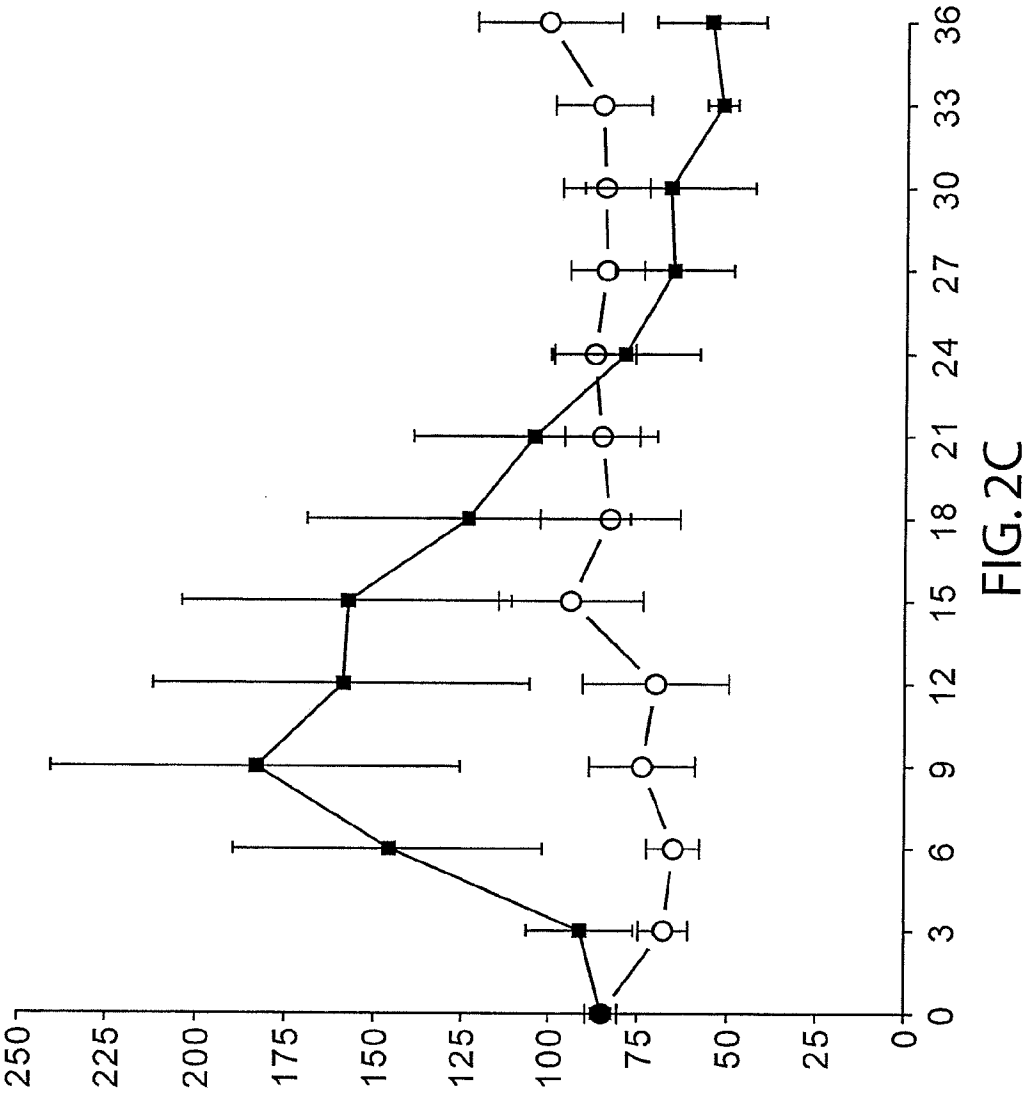
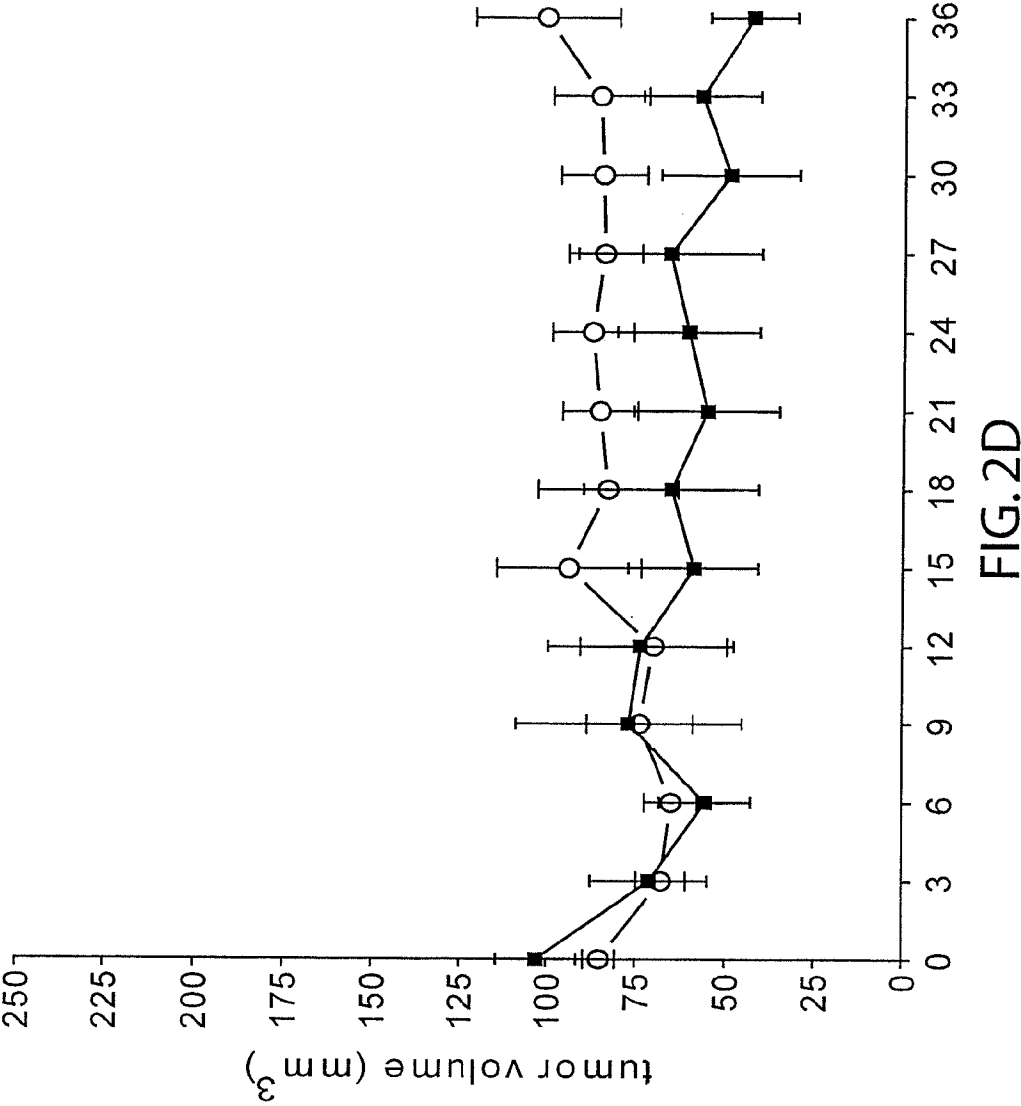
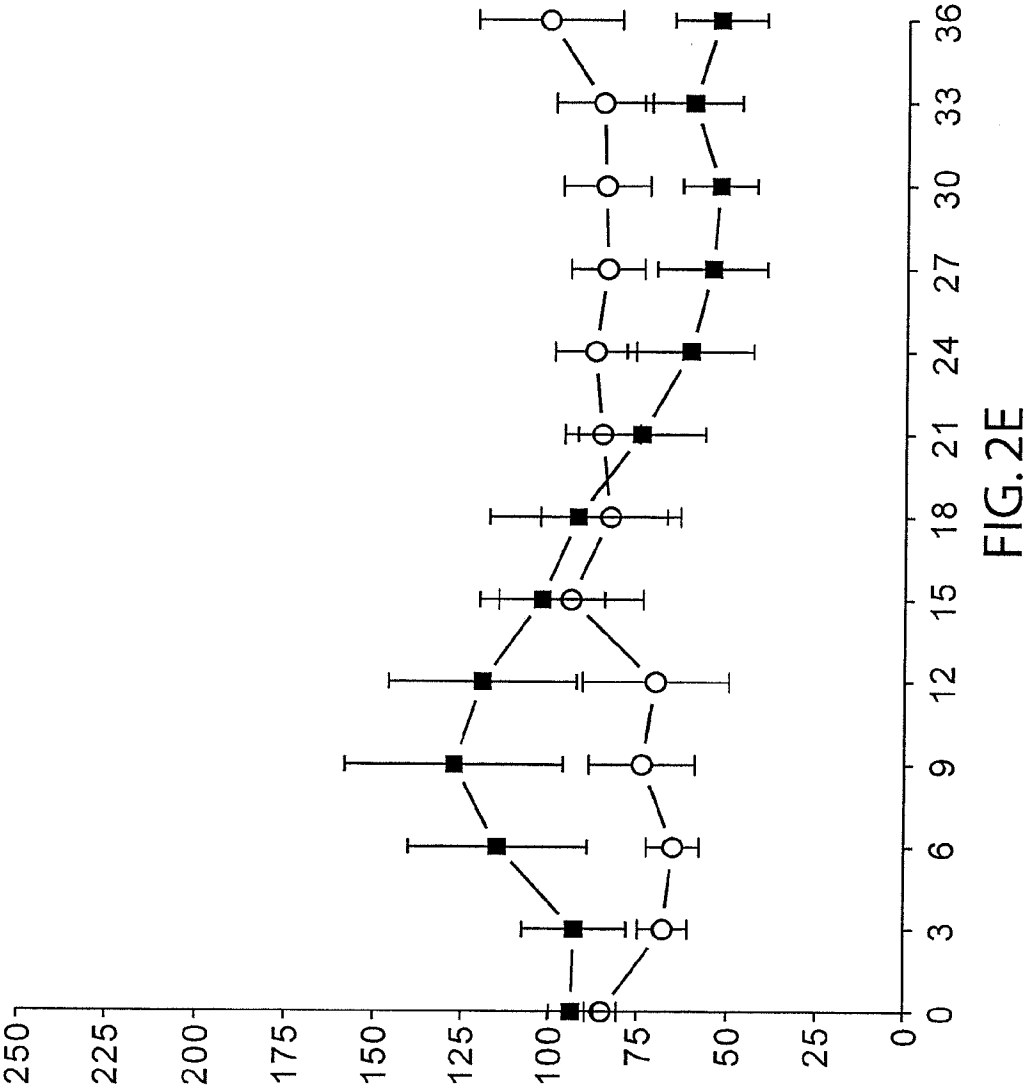


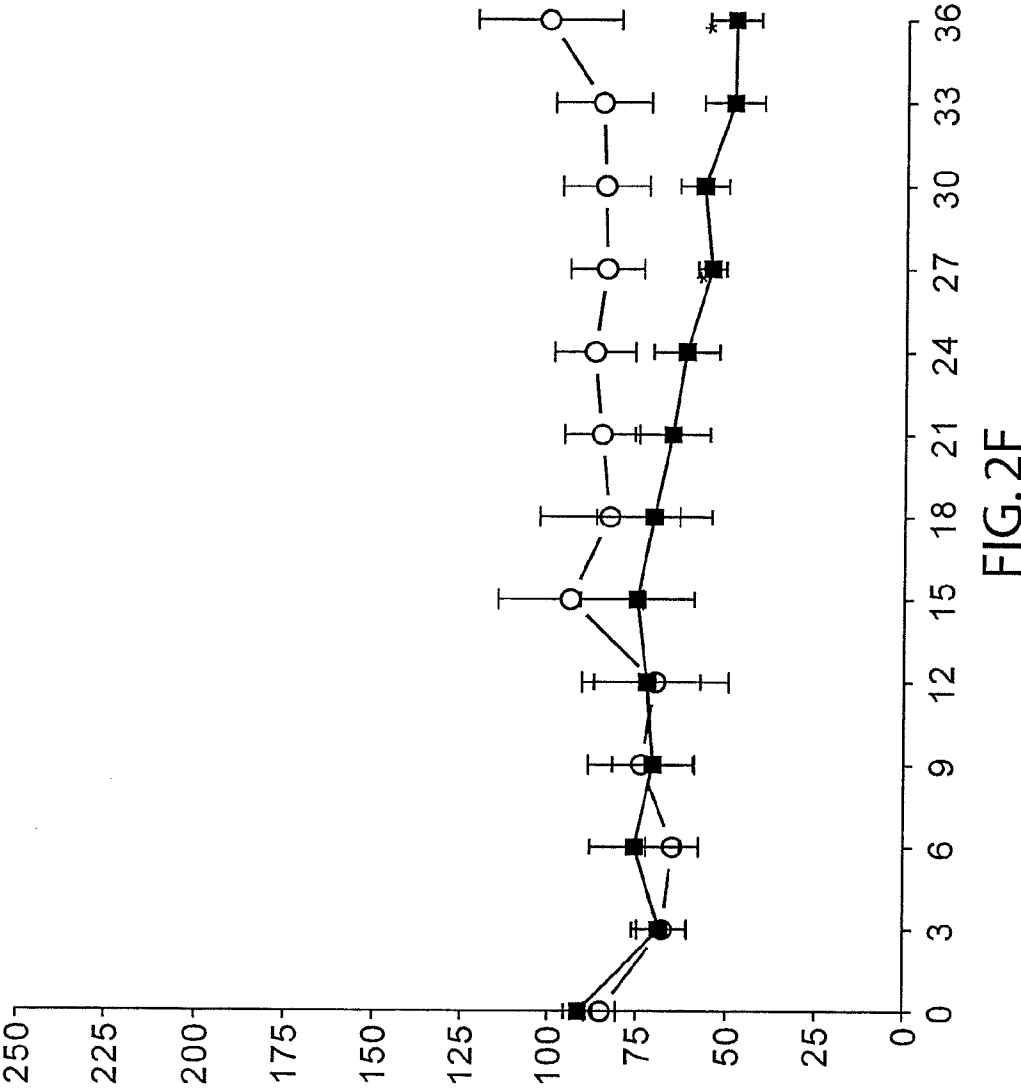
FIG. 2A

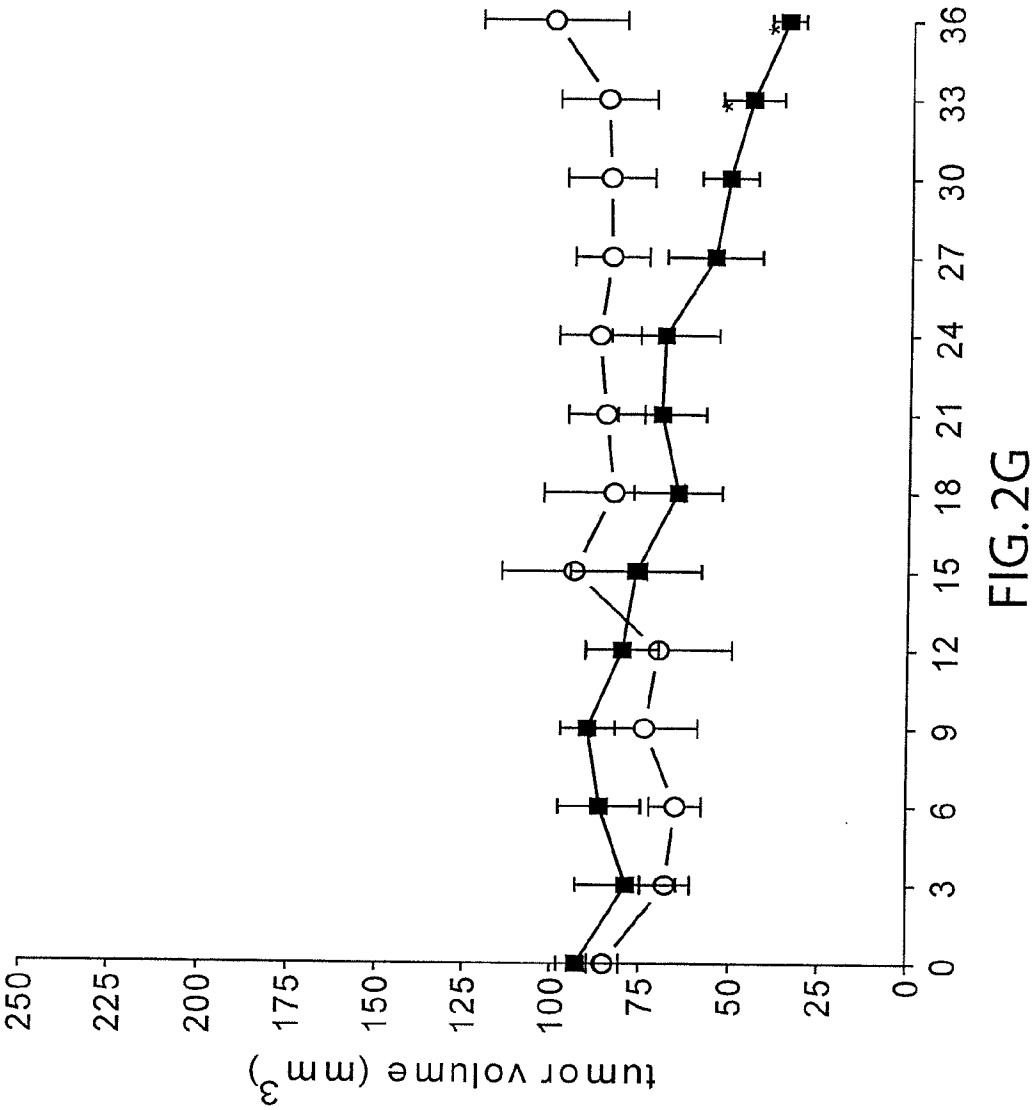




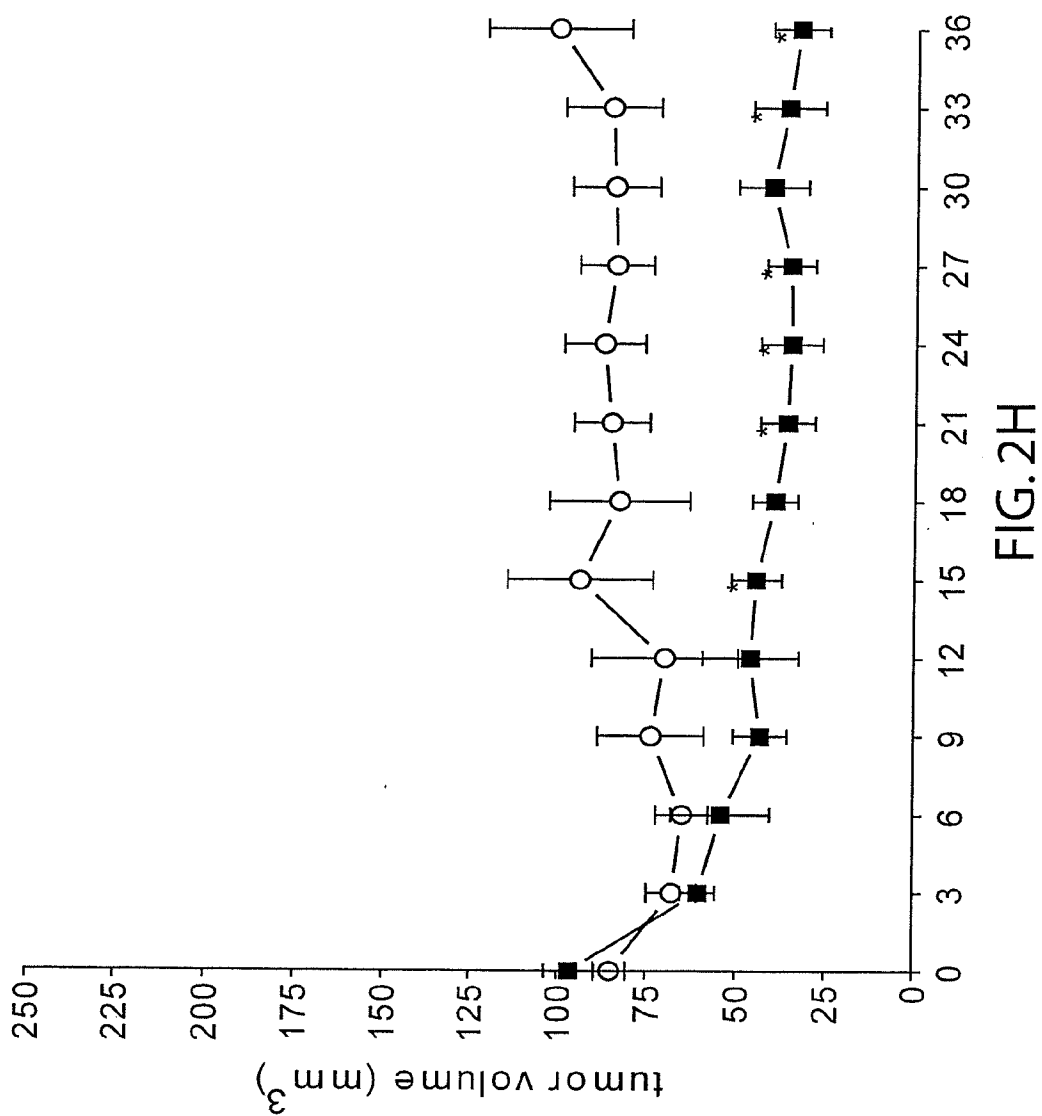








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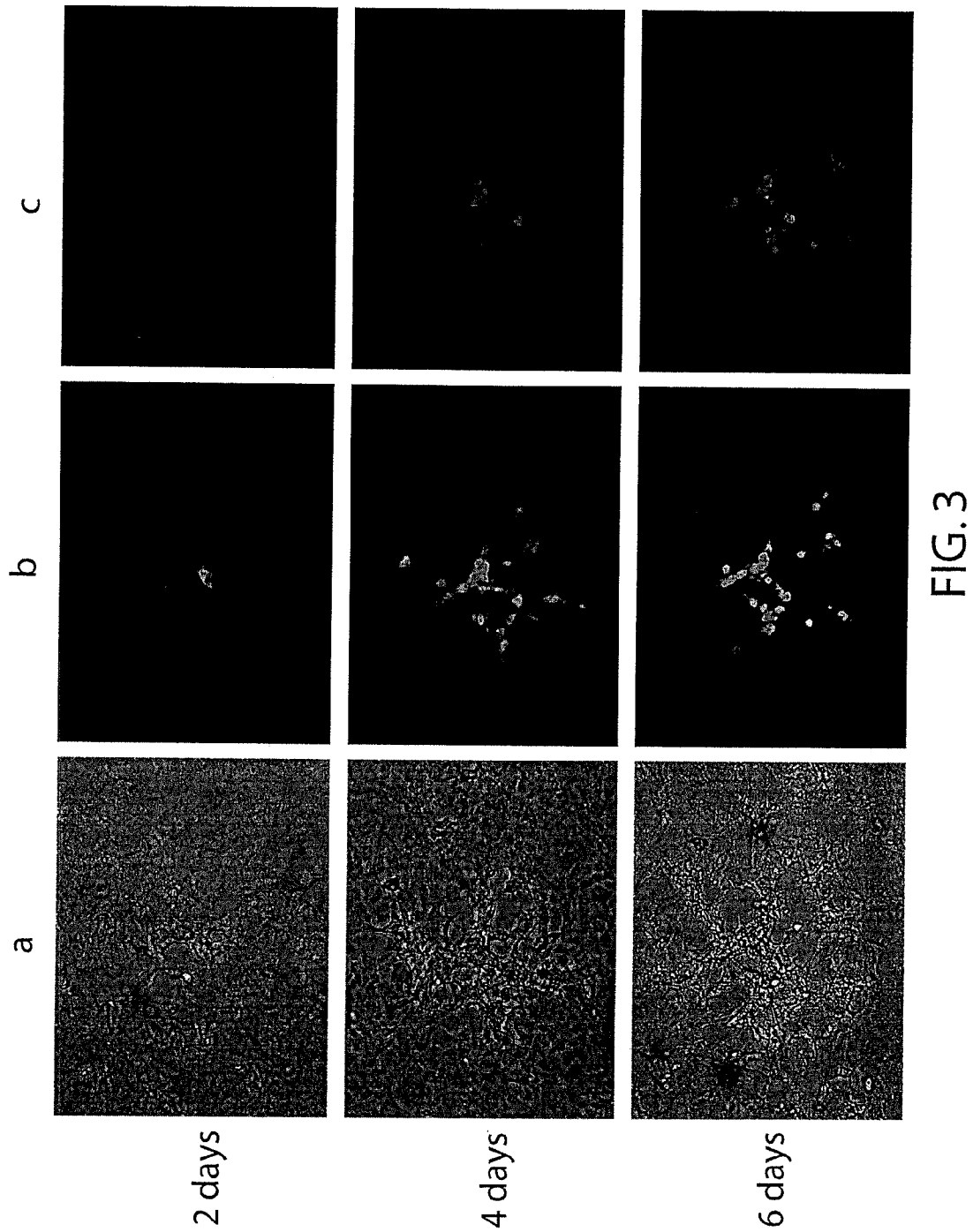


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2015/040881

A. CLASSIFICATION OF SUBJECT MATTER <i>A61K 35/768 (2015.01)</i> <i>C12N 15/39 (2006.01)</i> <i>A61P 35/00 (2006.01)</i> According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K 35/768, C12N 15/39, A61P 35/00 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) ESPACENET, PATENTSCOPE, JPO, PubMed, USPTO, EBI, DWPI, ELIBRARY, CIPO, KIPO, SIPO, Patsearch (RUPTO interanal)														
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category*</th> <th style="width: 70%;">Citation of document, with indication, where appropriate, of the relevant passages</th> <th style="width: 20%;">Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td style="text-align: center;">Y A</td> <td>WO 2004/014314 A2 (KIRN DAVID) 19.02.2004, p.8, last paragraph , p.13, paragraph 4, p.20, paragraph 1, pp.24-25, p.29, last paragraph - p.30, line 8, p.31, paragraph 2, p.65, paragraphs 1, 3, claims 50, 51, 99, 101</td> <td>1-2, 14-17, 20, 23-24, 34-35 3-4, 18-19, 21-22</td> </tr> <tr> <td style="text-align: center;">Y</td> <td>RAHMAN M.M. et al. "Interaction of human TNF and β2-microglobulin with Tanapox virus-encoded TNF inhibitor, TPV-2L", Virology, 2009, Vol. 386, pp. 462-468</td> <td>1-2, 14-17, 20, 23-24, 34-35</td> </tr> <tr> <td style="text-align: center;">X A</td> <td>US 7208313 B2 (MCCART et al.) 24.04.2007, col.1, lines 16-26, col.3, lines 13-36, col.4, lines 36-50, example 4, claims</td> <td>25, 32-33 26-31</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	Y A	WO 2004/014314 A2 (KIRN DAVID) 19.02.2004, p.8, last paragraph , p.13, paragraph 4, p.20, paragraph 1, pp.24-25, p.29, last paragraph - p.30, line 8, p.31, paragraph 2, p.65, paragraphs 1, 3, claims 50, 51, 99, 101	1-2, 14-17, 20, 23-24, 34-35 3-4, 18-19, 21-22	Y	RAHMAN M.M. et al. "Interaction of human TNF and β 2-microglobulin with Tanapox virus-encoded TNF inhibitor, TPV-2L", Virology, 2009, Vol. 386, pp. 462-468	1-2, 14-17, 20, 23-24, 34-35	X A	US 7208313 B2 (MCCART et al.) 24.04.2007, col.1, lines 16-26, col.3, lines 13-36, col.4, lines 36-50, example 4, claims	25, 32-33 26-31
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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.														
<table border="0" style="width: 100%;"> <tr> <td style="width: 50%; vertical-align: top;"> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; vertical-align: top;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family </td> </tr> </table>			* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family										
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family													
Date of the actual completion of the international search 13 October 2015 (13.10.2015)		Date of mailing of the international search report 29 October 2015 (29.10.2015)												
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezkhovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37		Authorized officer E.Redo Telephone No. 495 531 65 15												

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2015/040881

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-13
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.