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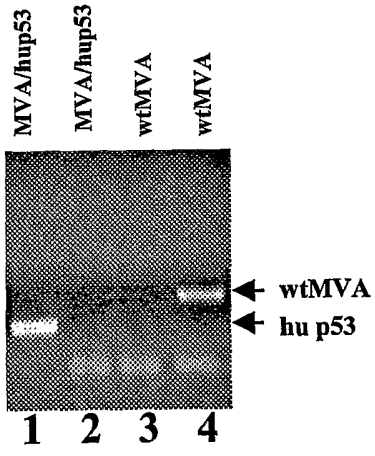
(54) Title: MODIFIED VACCINIA ANKARA EXPRESSING P53 IN CANCER IMMUNOTHERAPY



(57) Abstract: Mutations to the tumor suppressor protein p53 have been observed in 40-60% of all human cancers. These mutations are often associated with high nuclear and cytoplasmic concentrations of p53. Since many tumors exhibit highly elevated p53 levels, the protein is an attractive target for cancer immunotherapy. Unfortunately, p53 is an autoantigen that is likely to be tolerated as a self-protein by the immune system. The present invention is based on the discovery that this self-tolerance can be overcome by administration of recombinant modified vaccinia Ankara (MVA) containing a nucleic acid that encodes p53 (rMVAp53). The invention discloses a method of generating a p53-specific CTL response to tumor cells expressing mutated p53 by administering a composition comprising rMVAp53. Administration of rMVAp53 decreases tumor development, tumor growth, and mortality in a variety of malignant cell types. These effects are enhanced by administration of CTLA-4 blocker and/or CpG oligodeoxynucleotide immunomodulators.

MODIFIED VACCINIA ANKARA EXPRESSING P53 IN CANCER IMMUNOTHERAPY

BACKGROUND OF INVENTION

The present utility application claims priority to provisional patent application U.S. Serial No. 06/436,268 (Ellenhorn and Diamond), filed December 23, 2002, and U.S. Serial No. 06/466,607 (Ellenhorn and Diamond), filed April 30, 2003, the disclosures of which are incorporated by reference in their entirety herein.

GOVERNMENT INTEREST

This invention was made with government support in part by grants from the NIH, Division of AIDS (RO1-AI43267 and R21-AI44313) and NCI: RO1-CA77544, PO1-CA30206, R29-CA70819, and CA33572. The government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to the fields of virology, molecular biology, and tumor immunology. Specifically, this invention relates to compositions and methods for eliciting immune responses effective against malignancies expressing p53.

BACKGROUND

p53 is a tumor suppressor protein that regulates the expression of certain genes required for cell cycle arrest or apoptosis. The tumor suppressor gene encoding p53 is activated by DNA damage, cell stress, or the aberrant expression of certain oncogenes (Levine 1997). Once activated, wild type p53 (wt p53) serves to temporarily arrest the cell cycle, allowing time for DNA repair and preventing cells with damaged DNA from proliferating uncontrollably (Levine 1997). p53 is also

involved in inducing apoptosis in cells with certain types of physiologic damage (Levine 1997).

Mutations in p53 that functionally inactivate its growth suppressing ability have been observed in 40-60% of all human cancers, and are associated with the malignant phenotype (Hainaut 2000). Mutations to p53 occur as early events in tumorigenesis (Millikan 1995; Querzoli 1998; Allred 1993), abrogating the ability of the protein to suppress cell division (Finlay 1989; Eliyahu 1989). The regulation of p53 expression in cells can occur at the level of p53 mRNA abundance or at the level of p53 protein abundance. Mutations of p53 are often associated with high nuclear and cytoplasmic concentrations of the p53 protein, due to the prolonged half-life of the mutated protein. Many tumors are characterized by highly elevated intracellular p53 levels compared to nonmalignant cells. Other tumors synthesize large amount of mutated p53, but contain low or below normal steady-state levels of intracellular p53, presumably as a result of accelerated intracellular degradation of the protein. Overexpression of p53 is an independent predictor of more aggressive cancers (Turner 2000; Elkhuizen 2000; Zellars 2000), lymph node metastases (Pratap 1998), failure to respond to standard therapies (Berns 1998; Berns 2000), and mortality (Sirvent 2001; Querzoli 2001).

Missense point mutations are the most frequent p53 mutations in cancer, leaving the majority of the p53 protein in its wild type form (wt p53). Although p53 mutations may represent true tumor specific antigens, most of these mutations occur at sites that do not correspond to immunologic epitopes recognized by T cells (Wiedenfeld 1994). Because of this, any widely applicable p53-directed

immunotherapy must target wt p53. In experimental models, it has been possible to target p53 because the mutated molecule is associated with high nuclear and cytoplasmic concentrations of the p53 protein (Finlay 1988). p53 is an attractive target for adaptive immune response because the intracellular concentration of nonmutated p53 in healthy cells is very low (Zambetti 1993; Reich 1984). This means that healthy cells expressing non-mutant p53 will most likely escape an enhanced immune response to over-expressed mutant p53 (Offringa 2000).

p53, like most tumor associated antigens that are recognizable by the cellular arm of the immune system, is an autoantigen (Rosenberg 2001). The fact that p53 is an autoantigen widely expressed throughout development (Schmid 1991), coupled with the fact that the majority of mutated p53 being expressed in tumors has the same structure as the wild type protein, means that tumor-expressed p53 is likely to be tolerated as a self-protein by the immune system. This tolerance, which has been shown by functional and tetramer studies in mice to exist at the cytotoxic T lymphocyte level (CTL) (Theobald 1997; Erdile 2000), limits the effectiveness of p53-directed immunotherapies. To be successful, an effective immunotherapy must overcome this tolerance without also inducing autoimmunity against normal cells and tissues (Theobald 1997; Erdile 2000; Hernandez 2000). Small numbers of self-reacting T cells escape during the processes involved in the immune tolerance.

Tumors overexpressing p53 have been eliminated in murine models by the systemic administration of epitope specific CTL (Vierboom 2000a; Vierboom 2000b; Vierboom 1997; Hilburger 2001), epitope pulsed dendritic cells (DC) (Mayordomo 1996), or mutant p53 epitope with IL-12 (Noguchi 1995). Each of these strategies has

considerable drawbacks with regards to clinical applicability. CTL infusion and infusion of epitope pulsed dendritic cells are time consuming and expensive, because the isolation, culturing, and reinfusion of cells must be performed individually for each patient. Conversely, in order to produce any effect, the cell-free vaccination strategies previously used required either intratumoral injections or vaccination prior to tumor challenge, neither of which represents a practical approach in the clinical setting. There is thus a need for simplified, efficient, and widely applicable immunotherapeutic strategies in the treatment of cancer.

SUMMARY OF THE INVENTION

The p53 gene product is overexpressed in a majority of cancers, making it an ideal target for cancer immunotherapy. The efficacy of these therapies has been limited, however, by the fact that tumor-expressed p53 is likely to be tolerated as a self-protein by the immune system. The present invention is based on the discovery that this self-tolerance can be overcome by administration of recombinant MVA containing a nucleic acid that encodes p53 (rMVAp53). Administration of p53 is shown to greatly decrease tumor development, tumor growth, and mortality in mice challenged with a variety of malignant cell types. It is also shown that the therapeutic effects of rMVAp53 are enhanced by administration of a CTLA-4 blocker or CpG oligodeoxynucleotide (CpG ODN) immunomodulator. This enhancement is greatest when both immunomodulators are administered. The present invention provides a recombinant MVA composition for use in the treatment of cancer, a method of treating cancer using this composition, and a kit for administration of the composition.

In a first aspect, the present invention provides a composition comprising recombinant MVA that contains a nucleic acid encoding p53. Preferably, the p53 encoded by the recombinant MVA is wt human p53. According to the present invention, the composition may also contain a CTLA-4 blocker and/or a CpG ODN.

In another aspect, the present invention provides a method for treating a subject having a p53-expressing malignancy. This method is based on administration of a recombinant MVA containing a nucleic acid that encodes p53. Preferably, the method also calls for administration of a CTLA-4 blocker and/or CpG ODN as an immunomodulator. In a third aspect, the present invention provides a kit for treating a p53-expressing malignancy. This kit contains a recombinant MVA containing a nucleic acid that encodes p53, and may also contain a CTLA-4 blocker and/or CpG ODN as an immunomodulator. In a final aspect, this invention provides for an MVA recombination plasmid containing a nucleic acid insert that encodes wt human p53.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: PCR analysis of the pLW22-hup53 construct. rMVAhup53 injected (lanes 1, 2) and wtMVA infected (lanes 3, 4) BHK cells were subjected to total DNA extraction and PCR amplification using wtMVA (lanes 2, 4) or hup53 (lanes 1, 3) specific primers. The rMVAhup53 product was shown to have no contaminating wtMVA.

Figure 2: Expression of mup53 by cells infected with rMVAmup53. Cells infected with rMVAmup53 express mup53 at high levels, confirming that MVA is a suitable vaccine vector. Cell lysates were subjected to SDS-PAGE and Western

blotting. The lanes are designated as follows: 1) Meth A, unmanipulated Meth A sarcoma cells, 2) HCMV IE1 exon4-rMVA infected BHK cells, 3-4) rMVAmup53 infected BHK cells (loaded 0.125 ul, 0.25ul cell lysates respectively), 5) rAdp53, and 6) rAdpp65 infected HEK 293 cells. All lanes were loaded with 20 µl sample unless indicated specifically.

Figure 3: Generation of a p53-specific CTL response by rMVAmup53 *in vitro*. A single intraperitoneal (i.p.) vaccination with rMVAmup53 generates p53 specific CTL responses that efficiently kill cells overexpressing p53. **(a)** Splenocytes from mice treated with rMVAmup53 were harvested at 14 days and restimulated *in vitro* for 6 days with rAdp53 infected syngeneic LPS blasts. CTL activity was evaluated in a standard 4-h ^{51}Cr release assay using rVVp53 (solid line) or rVVpp65 (dashed line) infected 10.1 cells. **(b)** Splenocytes from rMVAmup53 (solid line) or rMVApp65 (dashed line) vaccinated mice were harvested at 14 days following vaccination and restimulated *in vitro* for 6 days with rAdp53 infected syngeneic LPS blasts. Cytotoxicity was measured against rVVp53 infected 10.1 cells. **(c)** Splenocytes harvested 14 days after rMVAmup53 (solid line) or rMVApp65 (dashed line) vaccination were restimulated *in vitro* for 6 days using syngeneic LPS blasts infected with rMVAp53. Cytotoxicity was measured against Meth A cells by a standard 4-h ^{51}Cr release assay.

Figure 4: Effect of vaccination with rMVAmup53 on Meth A tumor prevention. Balb/c mice were injected subcutaneously (s.c.) with 5 x 10^5 Meth A cells. On day 5, mice were vaccinated with either 5 x 10^7 pfu of rMVAmup53 (MVAp53) (n = 16), 5 x 10^7 pfu rMVApp65 (MVApp65) (n = 16), or PBS (n = 12). The survival plot shows

the proportion of surviving animals in each group as a function of days post tumor challenge. The improvement of the mice vaccinated with rMVAmup53 over both control groups is statistically significant ($P < 1$) as determined by the log rank test.

Figure 5: Effect of vaccination with rMVAmup53 plus anti-CTLA-4 mAb on established Meth A tumors. Mice were injected s.c. with a rapidly lethal dose of 10^6 Meth A cells. On days 6, 9, and 12 mice were injected i.p. with either anti-CTLA-4 mAb (CTLA4mAb) or control mAb. On day 7, mice were vaccinated with either 5 x 10^7 pfu rMVAp53 (MVAp53) or 5 x 10^7 pfu rMVApp65 (MVAapp65). The survival plot shows the proportion of surviving animals in each group. The survival advantage of mice vaccinated with rMVAp53 plus anti-CTLA-4 mAb (n=14) over control animals receiving rMVApp65 plus CTLA-4 (n=14), rMVAp53 plus control ab (n=14), or rMVApp65 plus control ab (n=6) is statistically significant ($P < 0.001$) as determined by the log rank test.

Figure 6: Effect of vaccination with rMVAmup53 plus anti-CTLA-4 mAb on established 11A-1 tumors. Balb/c mice were injected s.c. with 2 x 10^6 11A-1 cells ($p = 0.00044$, comparing rMVAmup53 plus anti-CTLA-4 mAb to all other groups). Anti-CTLA-4 mAb 9H10 (CTLA4 mAb) or the control hamster isotype matched polyclonal antibody (isotype matched Ab) were injected i.p. on days 4, 7, and 10 at 100, 50, and 50 µg dose, respectively. On day 5, mice were vaccinated i.p. with either 5 x 10^7 pfu of rMVAmup53 (MVAp53), 5 x 10^7 pfu rMVApp65 (MVApp65), or PBS. Each line represents the mean and standard deviation of eight mice.

Figure 7: Effect of vaccination with rMVAmup53 plus anti-CTLA-4 mAb on established MC-38 tumors. C57BL/6 mice were injected s.c. with 1 x 10^6 MC-38

cells (p = 0.0001, comparing rMVAmup53 plus anti-CTLA-4 mAb to all other groups). Anti-CTLA-4 mAb 9H10 (CTLA4 mAb) or the control hamster isotype matched polyclonal antibody (isotype matched Ab) were injected i.p. on days 4, 7, and 10 at 100, 50, and 50 µg dose, respectively. On day 5, mice were vaccinated i.p. with either 5 x 10^7 pfu of rMVAmup53 (MVAp53), 5 x 10^7 pfu rMVApp65 (MVApp65), or PBS. Each line represents the mean and standard deviation of eight mice.

Figure 8: Effect of vaccination with rMVAmup53 plus CpG ODN on established 11A-1 tumors. Balb/c mice were injected s.c. with 2 x 10^6 11A-1 cells (p = 0.00002, comparing rMVAmup53 plus CpG ODN to all other groups). 15 nmoles of CpG ODN (CpG) was injected i.p. on days 4, 9, and 14. On day 5, the mice were immunized i.p. with either 5 x 10^7 pfu of rMVAmup53 (MVAp53), 5 x 10^7 pfu of rMVApp65 (MVApp65), or PBS.

Figure 9: Effect of vaccination with rMVAmup53 plus CpG ODN on established Meth A tumors. Balb/c mice were injected s.c. with 1 x 10^6 Meth A cells (p = 0.0015, comparing rMVAmup53 plus CpG ODN to all other groups). 15 nmoles of CpG ODN (CpG) was injected i.p. on days 4, 9, and 14. On day 5, the mice were immunized i.p. with either 5 x 10^7 pfu of rMVAmup53 (MVAp53), 5 x 10^7 pfu of rMVApp65 (MVApp65), or PBS.

Figure 10: Effect of vaccination with rMVAmup53 plus CpG ODN on established MC-38 tumors. C57BL/6 mice were injected with 1 x 10^6 MC-38 cells (p = 0.0004, comparing rMVAmup53 plus CpG ODN to all other groups). 15 nmoles of CpG ODN (CpG) was injected i.p. on days 4, 9, and 14. On day 5, the mice were

immunized i.p. with either 5 x 10^7 pfu of rMVAmup53 (MVAp53), 5 x 10^7 pfu of rMVApp65 (MVApp65), or PBS.

Figure 11: Effect of vaccination with rMVAmup53 plus anti-CTLA-4 mAb and CpG ODN on established 11A-1 tumors. Balb/c mice (n = 8) were injected s.c. with 2 x 10^6 11A-1 cells. Anti-CTLA-4 mAb (CTLA4 mAb) was injected i.p. on days 14, 17, and 20 at 100, 50, and 50 µg dose, respectively. 15 nmoles of CpG ODN (CpG) was injected i.p. on days 14, 19, and 24. On day 15, mice were vaccinated i.p. with either 5 x 10^7 pfu of rMVAmup53 (MVAp53), 5 x 10^7 pfu rMVApp65, or PBS. The survival plot shows the proportion of surviving animals in each group as a function of days post tumor challenge. p = 0.02 comparing combined CpG ODN and anti-CTLA-4 mAb to CpG ODN alone, and p = 0.01 comparing combined CpG ODN and anti-CTLA-4 mAb to anti-CTLA-4 mAb alone.

Figure 12: Effect of vaccination with rMVAmup53 plus anti-CTLA-4 mAb and CpG ODN on established MC-38 tumors.

C57BL/6 mice (n = 8) were injected s.c. with 1 x 10^6 MC-38 cells. Anti-CTLA-4 mAb was injected i.p. on days 4, 7, and 10 at 100, 50, and 50 µg dose, respectively. 15 nmoles of CpG ODN was injected i.p. on days 4, 9, and 14. On day 5, mice were vaccinated i.p. with either 5 x 10^7 pfu rMVAmup53, 5 x 10^7 pfu MVApp65, or PBS. The survival plot shows the proportion of surviving animals in each group as a function of days post tumor challenge. p = 0.002 comparing combined CpG ODN and anti-CTLA-4 mAb to CpG alone, and p = 0.001 comparing combined CpG ODN and anti-CTLA-4 mAb with anti-CTLA-4 mAb alone.

Figure 13: Cellular requirements for anti-CTLA-4 mAb immunomodulator effect on Meth A tumors. Balb/c mice **(a)** or IFN-γ^{KO} Balb/c mice **(b)** were injected s.c. with a rapidly lethal dose of 10^6 Meth A cells. Groups of mice from both populations were injected i.p. with depleting doses of anti-CD4, anti-CD8, anti-NK1.1, or control mAb on days –1, 1, 3, and 10, and weekly thereafter. On days 6, 9, and 12 mice were injected i.p. with either anti-CTLA-4 mAb (CTLA4mAb) or control mAb. On day 7, mice were vaccinated with either 5 x 10^7 pfu rMVAp53 (MVAp53) or 5 x 10^7 pfu rMVApp65 (MVAapp65). **(a)** Mean tumor growth was calculated for each group of Balb/c mice, with error bars illustrating standard deviation. The last datapoint for each line represents the first mortality. **(b)** The proportion of surviving IFN-γ^{KO} Balb/c mice is plotted.

Figure 14: Cellular requirements for CpG ODN immunomodulator effects on 11A-1 tumors. Balb/c mice were injected s.c. with 2 x 10^6 11A-1 cells. 15 nmoles of CpG ODN was injected i.p. on days 4, 9, and 14. On day 5, mice were vaccinated i.p. with 5 x 10^7 pfu of rMVAmup53. Mice were injected i.p. with depleting doses of anti-CD4 (CD4), anti-CD8 (CD8), anti-NK1.1 (NK), or control mAb on days 4, 6, 8, and 15, and every 7 days thereafter. Tumors were measured twice weekly in three dimensions. p = 0.004 by two-sided Wilcoxon test, comparing $CD8^+$ depleted to all other groups. p = 0.007, comparing anti-NK1.1 to anti-CD4 and control mAb.

Figure 15: Cellular requirements for anti-CTLA-4 mAb immunomodulator effects on 11A-1 tumors. Mice were injected s.c. with 2 x 10^6 11A-1 cells. Anti-CTLA-4 mAb was injected i.p. on days 4, 7, and 10 at 100, 50, and 50 µg/dose, respectively. On day 5, the mice were vaccinated i.p. with 5 x 10^7 pfu rMVAmup53.

The mice were depleted of $CD8^+$, $CD4^+$, or NK cells by i.p. injection with the relevant mAb or control mAb on days 4, 6, 8, and 15, and then every 7 days thereafter. Tumors were measured twice weekly in three dimensions with calipers. Each curve represents the mean and standard deviation of 8 mice. $p = 0.004$, comparing $CD8^+$ depleted to all other groups. $p = 0.008$, comparing $CD4^+$ depleted to NK depleted and control groups.

Figure 16: Contribution of TLR 9 to the CpG ODN immunomodulator effect. $TLR9^{-/-}$ ($p = 0.0009$, comparing anti-CTLA-4 mAb to CpG ODN group) mutant C57BL/6 mice were injected s.c. with 1×10^6 MC-38 cells. Mice were treated with anti-CTLA-4 mAb (CTLA4 mAb) on days 4, 7, and 10 at 100, 50, and 50 µg/dose, respectively, or with 15 nmoles of CpG ODN on days 4, 9, and 14. On day 5, all mice were vaccinated i.p. with 5×10^7 pfu of rMVAmup53. Tumors were measured twice weekly in three dimensions with calipers. Each curve represents the mean and standard deviation of 8 mice.

Figure 17: Contribution of IL-6 to the CpG ODN immunomodulator effect. $IL\text{-}6^{-/-}$ ($p = 0.02$, comparing anti-CTLA-4 mAb to CpG ODN group by Wilcoxon 2-sided RankSum Test) mutant C57BL/6 mice were injected s.c. with 1×10^6 MC-38 cells. Mice were treated with anti-CTLA-4 mAb (CTLA4 mAb) on days 4, 7, and 10 at 100, 50, and 50 µg/dose, respectively, or with 15 nmoles of CpG ODN on days 4, 9, and 14. On day 5, all mice were vaccinated i.p. with 5×10^7 pfu of rMVAmup53. Tumors were measured twice weekly in three dimensions with calipers. Each curve represents the mean and standard deviation of 8 mice.

Figure 18: Expression of hup53 by cells infected with rMVAhup53. BHK cells were injected with purified rMVAhup53 (MVA/p53). Expression of hup53 was measured at 24 and 48 hours. Cell lysates were subjected to SDS-PAGE and Western blotting. Lane 1: BHK cells injected with control MVA; Lane 2: BHK cells infected with rMVAhup53 for 24 hours; Lane 3: BHK cells infected with rMVAhup53 for 48 hours. All lanes were loaded with 20 µl of sample.

Figure 19: Effect of vaccination with rMVAhup53 plus anti-CTLA-4 mAb and CpG ODN on established 4T1/hup53 tumors. Mice were injected s.c. with 5×10^4 4T1/hup53, then vaccinated i.p. with 10^7 pfu rMVAhup53 or PBS control on day 6. On day 16, mice received an rMVAhup53 or PBS booster injection, along with 15 nmole of CpG ODN and 50 µg of anti-CTLA-4 mAb. rMVAhup53 vaccinated mice displayed a significant improvement in survival ($p < 0.05$, two sided T-test) compared to PBS controls.

DETAILED DESCRIPTION

The present invention is based on the discovery that self-tolerance to a protein expressed in both normal and cancerous cells can be overcome, and that a strong anti-tumor immune response can be generated without the requirement for intratumoral administration and without the production of systemic toxicity or auto-immunity. The invention provides novel cell-free compositions and methods for the generation of effective immune responses against a wide variety of human malignancies, independent of the subject's haplotype or genotype. The examples discussed below demonstrate that vaccination with a modified vaccinia Ankara vector engineered to express either wild type murine or wild type human p53

(rMVAmup53 or rMVAhup53) stimulates a vigorous p53-specific CTL response. This response can be enhanced by the co-administration of an immunomodulator consisting of a CTLA-4 blocker and/or CpG ODN.

MVA virus (GenBank Accession Number U94848) is a variant of the Ankara strain of vaccinia virus that was derived by over 570 serial passages on primary chicken embryo fibroblast. Several properties of MVA as an attenuated poxvirus make it ideal for the generation of a therapeutic response to tumors expressing p53. One advantage of MVA is that it is able to efficiently replicate its DNA in mammalian cells, yet it is avirulent and does not propagate. This trait is the result of losing two important host range genes among at least 25 additional mutations and deletions that occurred during its passages through chicken embryo fibroblasts (Meyer 1991; Antoine 1998). In contrast to NYVAC (attenuated Copenhagen strain) and ALVAC (host range restricted avipox), both early and late transcription in MVA are unimpaired, allowing for continuous gene expression throughout the viral life cycle (Carroll 1997a; Carroll 1997b; Blanchard 1998; Sutter 1992). MVA has been found to be more immunogenic than the Western Reserve (WR) strain, and can be used in conditions of pre-existing poxvirus immunity (Ramirez 2000a; Ramirez 2000b). The favorable clinical profile of MVA as a recombinant vaccine delivery vehicle is buttressed by its benign safety profile as a smallpox vaccine in Europe in the late 1970's (Mayr 1999; Mayr 1978). MVA was administered to over 120,000 high-risk individuals, including the aged and very young, without serious side effects (Mayr 1978). More recently, MVA has also been administered to immunocompromised non-human primates without adverse outcome (Stittelaar 2001). This is in stark contrast to

other vectors, such as retroviruses and adenoviruses, which pose documented risks to the human host. Immunotoxicity of the vector, adjuvant, or immunomodulator used is a particular point of concern in the immunotherapy of cancer, as most cancer patients are severely immunocompromised due to chemotherapy, radiation, or the immunosuppressive effects of the cancer itself. MVA was first developed into a vaccine vehicle in the early 1990's, after it became clear that non-attenuated poxviruses such as the WR strain could not be safely administered to immunocompromised individuals (Redfield 1987; Collier 1991). In summary, the potency of MVA as an expression vector combined with its safety profile in primates and humans make it highly attractive as a delivery system for cancer genes.

Construction of rMVAmup53 and rMVAhup53 is achieved by recombinant DNA techniques that are well known in the art (Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory, 2001; Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, 1986 and 2000). The coding sequence of wild type p53 can be conveniently obtained by RT-PCR using p53-specific primers. These primers hybridize to DNA and serve as initiation sites for DNA synthesis. Nucleotide primers are designed to bind at separate sites on opposing duplex strains, thereby defining the intervening sequence as the portion to be amplified. Nucleic acid molecules to be employed as primers will generally include at least a 10 base pair sequence complementary to the DNA segment to be amplified. Primer selection is well known to those of skill in the art. Primers for the amplification of wt mup53 or wt hup53 can be designed to contain appropriate restriction sites for subcloning into a suitable MVA recombination plasmid, such as pMCO3, pLW22, pLW51, pUCII LZ or other

MVA transfer vectors well known in the art. The recombination plasmid contains sequences necessary for expression of the foreign gene insert, as well as the flanking sequences necessary for homologous recombination into a chosen site of deletion in the MVA genome. To generate recombinant MVA virus, cells are infected with MVA virus and transfected with the recombination plasmid containing the foreign gene insert. After homologous recombination between virus and plasmid is allowed to occur, recombinant MVA expressing the inserted gene is isolated.

Cellular expression of p53 protein following infection with rMVAmup53 or rMVAhup53 was analyzed to determine the fidelity and extent of its expression from recombinant virus. Meth A cells, which overexpress mutated p53, were used as a positive control, and HCMV IE1 exon4 rMVA infected BHK cells were used as a negative control. Western blot analysis revealed abundant p53 expression by cells infected with rMVAmup53 or rMVAhup53, as well as by Meth A cells. No detectable expression of p53 by HCMV IE1 exon 4-rMVA infected BHK cells was observed. High levels of p53 expression by rMVAp53 infected BHK cells was also observed by fluorescence microscopy. The high level of p53 expression exhibited by rMVAmup53 and rMVAhup53 compared to other viral and cellular forms demonstrates its usefulness in vaccination protocols.

In animal experimental models, MVA based vaccines stimulate tumor specific CTL activity (Espenschied 2003; Drexler 1999) and effect regression of established tumors (Espenschied 2003; Carroll 1997b; Mulryan 2002; Rosales 2000). There are numerous advantages to immunization with whole protein expressed in MVA. In contrast to peptide immunization, multiple epitopes can be expressed, and a

polyclonal host response can be stimulated. Antigen-specific cognate help, which is essential to the propagation of a CTL response, can be achieved through expression of a protein in MVA. In addition, expression of whole protein can result in the stimulation of responses to otherwise cryptic epitopes. Immunization with recombinant viruses may also avoid the need for a complex and expensive approach involving the expansion and adoptive transfer of antigen-specific cells, or the need to generate an individualized vaccine for a particular cancer patient. This advantage of a recombinant vaccine approach may encourage more widespread clinical use to prevent recurrence in patients with earlier stages of disease.

In vitro experiments were run to determine whether vaccination with rMVAmup53 could break p53 tolerance, resulting in the generation of p53-specific CTL. Splenocytes were harvested from mice following a single intraperitoneal (i.p.) vaccination with rMVAmup53, and restimulated *in vitro* with p53 over-expressing cells. The splenocytes recognized and lysed wt p53 over-expressing targets. In contrast, splenocytes from mice vaccinated with rMVApp65, which stimulates vigorous pp65 specific CTL responses, did not recognize the p53 over-expressing targets, demonstrating the specificity of the lymphocyte response. rMVAmup53 vaccination can also stimulate CTL recognition of Meth A cells, which express mutated p53. Restimulated splenocytes from mice vaccinated with rMVAmup53 recognized mutant p53 over-expressing Meth A, whereas control mice vaccinated with rMVApp65 did not.

Since a single vaccination with rMVAmup53 resulted in enhanced CTL response, there was sufficient justification to examine the effect of rMVAmup53

vaccination on the growth of Meth A tumor cells *in vivo*. Administration of rMVAmup53 was shown to inhibit the outgrowth of murine sarcoma Meth A, an immunogenic tumor cells line that overexpresses mutant p53. Mice inoculated with a lethal dose of Meth A tumor cells and vaccinated with rMVAmup53 by i.p. injection three days later exhibited slower tumor growth and higher survival rates than control animals. A majority of the vaccinated mice failed to develop tumors entirely, and these mice were resistant to rechallenge with Meth A after 52 days (Espenschied 2003).

The above results demonstrate the efficacy of a novel rMVAmup53 cell-free vaccine at targeting p53 expressed by a malignant tumor. Additional experiments were performed to determine whether this effect could be enhanced by addition of a CTLA-4 blocker or CpG ODN immunomodulator. Immunization with vaccinia viral constructs results in the uptake and presentation of viral proteins by DC (Norbury 2002). In draining lymph nodes, the DC present antigen to naïve $CD8^+$ T cells, resulting in T cell activation and the subsequent propagation of an immune response (Norbury 2002). Immunomodulator experiments were designed to determine the feasibility of augmenting the response to rMVAp53 by addressing both the initiation of the response and its propagation.

One potent strategy for optimizing tumor vaccines involves manipulating negative regulation of T cell responsiveness by using a molecule that blocks CTLA-4 engagement with ligand, a phenomenon referred to as "CTLA-4 blockade." CTLA-4 is a cell surface receptor found on T cells. Activation of CTLA-4 leads to inhibition of T cell responses. CTLA-4 plays a significant role in regulating peripheral T-cell

tolerance by interfering with T-cell activation through both passive and active mechanisms (Egen 2002). Application of a CTLA-4 blocker in combination with cancer vaccines expressing tumor associated autoantigens can, in some cases, result in tumor rejection along with breaking of tolerance, albeit with the concomitant induction of autoimmunity (Espenschied 2003; Hurwitz 2000; van Elsas 1999). *In vitro*, CTLA-4 blockade lowers the T-cell activation threshold and removes the attenuating effects of CTLA-4. CTLA-4 blockade also inhibits Treg cell activity *in vivo* (Read 2000). When combined with GM-CSF producing tumor cell vaccines, CTLA-4 blockade results in rejection of established poorly immunogenic melanoma, mammary carcinoma, and prostate carcinoma grafts (Hurwitz 1998; Hurwitz 2000; van Elsas 1999). This occurs through a process, which involves breaking tolerance to tumor associated antigens. CTLA-4 blocking agents are molecules that specifically bind to the CTLA-4 receptor and interfere with the binding of CTLA-4 to its counter-receptors. A CTLA-4 blocking agent can be a monoclonal or polyclonal antibody, a fragment of an antibody, a peptide, a small organic molecule, a peptidomimetic, a nucleic acid such as interfering RNA (iRNA) or antisense molecule, an aptamer, or any domains from CTLA-4 ligands, including members of the B7 family of CTLA-4 ligands, wherein said ligands can be preferably synthesized as recombinant soluble proteins capable of binding CTLA-4 present on immune cells and blocking CTLA-4 function. Anti-CTLA-4 antibodies may be generated by immunizing a host animal with CTLA-4 protein or with cells expressing CTLA-4. Monoclonal antibodies to CTLA-4 (anti-CTLA-4 mAb) can be produced by conventional techniques, namely fusing a hybridoma cell with a mammalian immune

cell that produces anti-CTLA-4 antibody. Mammalian cells used to generated anti-CTLA-4 mAb may include rat, mouse, hamster, sheep, or human cells. Anti-CTLA-4 mAbs may be purified from hybridoma cell supernatants or from ascites fluid. Anti-CTLA-4 antibodies may be human antibodies generated using transgenic animals (Bruggemann 1991; Mendez 1997) or human immunoglobulin phage display libraries (Winter 1994). Anti-CTLA-4 antibodies also encompasses chimeric and humanized (or "reshaped") antibodies. Chimeric antibodies to CTLA-4 may be generated through recombinant methods to contain the CTLA-4 binding domain of a non-human antibody and the constant domain of a human antibody. Humanized antibodies to CTLA-4 may be generated by recombinant methods to contain only the CDR regions of non-human anti-CTLA-4 antibodies placed on a human antibody structural framework (Jones 1986; Low 1986). Individual residues within the non-human region may be substituted with residues from the human antibody framework. Conversely, individual residues within the human antibody framework may be substituted with residues from the non-human antibody (Foote 1992). Such substitutions may be used to increase the binding capabilities of the humanized antibody or to decrease the immune response against the antibody. Humanized antibodies to CTLA-4 can be the product of an animal having transgenic human immunoglobulin constant region genes. They can also be engineered by recombinant DNA techniques to substitute the C_H1, C_H2, C_H3, hinge domains, or other domains with the corresponding human sequence, by methods known in the art.

Oligodeoxynucleotides containing unmethylated CpG (cytosine-phosphate-guanine) motifs are potent immunostimulatory agents that can enhance vaccine potency (Krieg 2002). Immune activation by CpG ODN initiates with specific binding to the Toll-like Receptor-9 (TLR9) in B cells and plasmacytoid dendritic cells (Krieg 2002). TLR9 ligation in DC results in secondary activation of lymphocyte, macrophage, monocyte, natural killer (NK), and T-cell populations through the elaboration of cytokines generating a T_H1 cytokine milieu (Krieg 2003). This results in increased NK activity, improved antigen presentation, and T cell help that can augment both humoral and cell-mediated immune responses. In addition, TLR9 ligation results in the production of IL-6 by DCs, which helps overcome the suppressive effect of $CD4^+CD25^+$ Treg cells (Pasare 2003). Administration of CpG ODN alone has been shown to exert modest anti-tumor effects in a number of murine tumor models (Carpentier 1999; Kawarada 2001; Ballas 2001; Baines 2003; Sharma 2003). CpG ODN has been shown to be an effective adjuvant for a variety of experimental tumor vaccines in mice. It is at least as effective as Freund's adjuvant, but with higher T_H1 activity and less toxicity (Chu 1997; Weiner 1997). CpG ODN can enhance the effect of peptide (Davila 2000; Stern 2002), protein (Kim 2002), DC (Heckelsmiller 2002), idiotype (Baral 2003), and GM-CSF secreting tumor cell vaccines (Sandler 2003). The ability of CpG ODN to prime for T_H1 responses and stimulation of NK cells probably accounts for the immunomodulator activity in these vaccine approaches and in those described below.

To determine whether administration of a CTLA-4 blocking agent in conjunction with rMVAmup53 vaccination would be beneficial or would induce

autoimmune disease, a monoclonal antibody specific to CTLA-4 (anti-CTLA-4 mAb) was used. Vaccination with rMVAmup53 and anti-CTLA-4 mAb was shown to effect the rejection of established, palpable Meth A tumors. Mice injected with a high dose of Meth A and vaccinated with rMVAmup53 and anti-CTLA-4 mAb (9H10) only after formation of a palpable tumor nodule exhibited complete tumor regression and lasting tumor immunity. *In vivo* antibody depletion studies confirmed that this antitumor effect was primarily $CD8^+$, and to a lesser extent $CD4^+$, dependent.

To establish that the above results were not tumor specific, vaccination with rMVAmup53 and a CTLA-4 blocker immunomodulator was performed on mice injected with 11A-1 or MC-38 tumor cells. 11A-1 is a rapidly growing malignant cell line that is poorly immunogenic. MC-38 is a colon carcinoma cell line. Mice injected with 11A-1 or MC-38 tumor cells and vaccinated 4 days later with rMVAmup53 and anti-CTLA-4 mAb rejected their tumors. Similar results were seen when the anti-CTLA-4 mAb was replaced with CpG ODN. The majority of mice treated with rMVAmup53 and CpG ODN did not develop palpable tumors and developed lasting tumor immunity, rejecting a rechallenge at 60 days.

The potential additive effect of the anti-CTLA-4 mAb and CpG ODN immunomodulators was examined by administering both immunomodulators in conjunction with rMVAmup53 to 11A-1injected mice with palpable tumors. Tumor rejection and prolonged survival were observed in the majority of mice receiving both immunomodulators in conjunction with rMVAmup53. Mice that received only one immunomodulator in conjunction with rMVA, on the other hand, all eventually succumbed to tumor growth. Not only did the combination of both

immunomodulators provide a greater benefit than either immunomodulator acting alone, but their combined benefit was greater than the simple addition of the effects of the immunomodulators. Similar results were seen in mice bearing MC 38 tumors.

To determine the efficacy of a recombinant MVA containing a human p53 sequence, rMVAhup53 was administered to hupki mice injected with 4T1(H-2^d) cells that had been transfected with human p53. 4T1(H-2^d) is a murine breast carcinoma cell line. Mice were vaccinated with rMVAhup53 6 days after injection with 4T1 cells, and vaccinated again ten days later. During the second vaccination, CpG ODN and anti-CTLA-4 mAb were administered as well. Mice treated with vaccine and both immunomodulators exhibited a statistically significant improvement in survival.

The above results demonstrate the efficacy of a novel rMVAmup53 or rMVAhup53 cell-free vaccine at eliciting an immune response targeting p53 in a variety of malignant tumor types, as well as the efficacy of anti-CTLA-4 mAb and CpG ODN as immunomodulators to this vaccine. Accordingly, the present invention provides a composition comprising a recombinant MVA virus engineered to express p53 (rMVAp53). The present invention further provides an immunotherapeutic method for eliciting an immune response against a wide range of p53-expressing malignancies by administering rMVAp53

Introduction of rMVAp53 into a subject can be performed by any procedure known to those skilled in the art, and is not dependent on the location of tumor nodules for efficacy or safety. Thus, rMVAp53 can be administered by intravascular, subcutaneous, peritoneal, intramuscular, intradermal or transdermal injection, to name a few possible modes of delivery. rMVAp53 can be prepared as a formulation

at an effective dose in pharmaceutically acceptable media, such as normal saline, vegetable oil, mineral oil, PBS, etc. Therapeutic preparations may include physiologically tolerable liquids, gel or solid carriers, diluents, adjuvants and excipients. Additives may include bactericidal agents, additives that maintain isotonicity (e.g., NaCl, mannitol), additives that maintain chemical stability (e.g., buffers, preservatives) and other ingredients. For parenteral administration, the rMVAp53 may be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Liposomes or non-aqueous vehicles, such as fixed oils, may also be used. The formulation may be sterilized by techniques known in the art.

The rMVAp53 formulation can be further enhanced with a costimulator, such as a cytokine, tumor antigen, an antigen derived from a pathogen, or any immunomodulator. The costimulator can be any agent that directly or indirectly stimulates an immune response in combination with the rMVAp53, and may be selected for its ability to modulate APC or T-cell function. For example, MVA can be engineered to express GM-CSF, IL-12, or other stimulatory cytokines to produce a costimulator, and the combination of rMVAp53 and costimulator (here: MVA expressing the stimulatory cytokine) can be introduced into the subject. The treatment may be performed in combination with administration of cytokines that stimulate antigen presenting cells, such as granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), interleukin 3 (IL-3), interleukin 12 (IL-12), and others well known in the art. Other costimulators include cytokine-

transduced tumor cells, such as tumor cells transduced with GM-CSF, as well as tumor cells that have been irradiated and/or treated with a chemotherapeutic agent *ex vivo* or *in vivo*. Chemotherapeutic or radiotherapeutic agents are further examples of costimulators. Thus, rMVAp53 can be administered in conjunction with a variety of costimulators known to those of skill in the art.

The formulation is administered at a dose effective to increase the response of T cells to antigenic stimulation. The determination of the T cell response will vary with the condition that is being treated. Useful measures of T cell activity are proliferation, the release of cytokines, including, IL-2, IFNγ, TNFα, etc; T cell expression of markers such as CD25 and CD69; and other measures of T cell activity as known in the art. The dosage of the therapeutic formulation will vary widely, depending upon the stage of the cancer, the frequency of administration, the manner or purpose of the administration, the clearance of rMVAp53 from the subject, and other considerations. The dosage administered will vary depending on known factors, such as the pharmacodynamic characteristics of the particular agent, mode and route of administration, age, health and weight of the recipient, nature and extent of symptoms, concurrent treatments, frequency of treatment, and effect desired. The dose may be administered as infrequently as weekly or biweekly, or fractionated into smaller doses and administered daily, semi-weekly, etc., to maintain an effective dosage level.

Generally, a daily dosage of active ingredient can be about 10^6-10^{11} IU (infectious units)/kg of body weight. Dosage forms suitable for internal administration generally contain from about 10^6 to 10^{12} IU of active ingredient per

unit. The active ingredient may vary from 0.5 to 95% by weight based on the total weight of the composition. In some cases it may be desirable to limit the period of treatment due to excessive T cell proliferation. The limitations will be empirically determined, depending on the response of the patient to therapy, the number of T cells in the patient, etc. The number of T cells may be monitored in a patient by methods known in the art, including staining with T cell specific antibodies and flow cytometry.

In a preferred embodiment of the present invention, rMVAp53 is administered in conjunction with an immunomodulator, specifically a CTLA-4 blocking agent or a CpG ODN. The combined administration of rMVAp53 and the CTLA-4 blocking agent anti-CTLA-4 mAb is unexpectedly potent in producing regression of advanced tumors that are rapidly lethal when left untreated. The same is true of the combined administration of rMVAp53 and CpG ODN. Potency is even greater when both immunomodulators are administered in conjunction with rMVAp53. In addition, the anti-CTLA-4 mAb CpG ODN immunomodulators are nontoxic to the subject, and capable of generating long lasting immunity to lethal challenges with tumor cells when administered in conjunction with rMVAp53.. As is the case with rMVAp53 alone, introduction of rMVAp53 plus anti-CTLA-4 mAb and/or CpG ODN into a subject can be performed by any procedure known to those skilled in the art, and is not dependent on the location of tumor nodules for efficacy or safety. Thus, rMVAp53, anti-CTLA-4 mAb, and CpG ODN can be administered by intravascular, subcutaneous, peritoneal, intramuscular, intradermal or transdermal injection, to name a few possible modes of delivery. rMVAp53, anti-CTLA-4 mAb, and CpG ODN can be administered together,

separately, or sequentially, in any order, by the same route of administration or by different routes. rMVAp53 plus anti-CTLA-4 mAb and/or CpG ODN can be prepared as formulations at an effective dose in pharmaceutically acceptable media, for example normal saline, vegetable oil, mineral oil, PBS, etc. Therapeutic preparations may include physiologically tolerable liquids, gel or solid carriers, diluents, adjuvants and excipients. Additives may include bactericidal agents, additives that maintain isotonicity, e.g. NaCl, mannitol; and chemical stability, e.g. buffers and preservatives and other ingredients. rMVAmup53 plus anti-CTLA-4 mAb and/or CpG ODN may be administered as a cocktail or as single agents. For parenteral administration, anti-CTLA-4 mAb and CpG ODN may be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Liposomes or non-aqueous vehicles, such as fixed oils, may also be used. The formulation may be sterilized by techniques as known in the art.

The rMVAp53 plus anti-CTLA-4 mAb and/or CpG ODN combination can be further enhanced with a costimulator such as a cytokine, tumor antigen, or antigen derived from a pathogen. A costimulator can be any agent that directly or indirectly stimulates an immune response in combination with rMVAp53 or in combination with rMVAp53 plus anti-CTLA-4 mAb and/or CpG ODN. For example, MVA can be engineered to express GM-CSF, IL-12, or other stimulatory cytokine to produce a costimulator, and the combination of rMVAp53 and costimulator (here: MVA expressing the stimulatory cytokine), or rMVAp53 plus anti-CTLA-4 mAb and/or CpG ODN and costimulator can be introduced into the subject. The treatment may be

performed in combination with administration of cytokines that stimulate antigen presenting cells, such as granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), interleukin 3 (IL-3), interleukin 12 (IL-12) and others well known in the art. Other costimulators include cytokine-transduced tumor cells such as tumor cells transduced with GM-CSF, or tumor cells that have been irradiated and/or treated with a chemotherapeutic agent *ex vivo* or *in vivo*. Chemotherapeutic or radiotherapeutic agents are further examples of costimulators. Thus, rMVAp53 either alone or in combination with anti-CTLA-4 mAb and/or CpG ODN can be administered in conjunction with a variety of costimulators known to those of skill in the art.

The dosage of the therapeutic formulation will vary widely, depending upon the stage of the cancer, the frequency of administration, the manner or purpose of the administration, and the clearance of rMVAp53, anti-CTLA-4 mAb, and CpG ODN from the subject, among other considerations. The dosage administered will vary depending on known factors, such as the pharmacodynamic characteristics of the particular agent, mode and route of administration, age, health and weight of the recipient, nature and extent of symptoms, concurrent treatments, frequency of treatment and effect desired. The dose may be administered as infrequently as weekly or biweekly, or fractionated into smaller doses and administered daily, semi-weekly, etc. to maintain an effective dosage level.

Generally, a daily dosage of active ingredient (antibody) can be about 0.1 to 100 mg/kg of body weight. Dosage forms suitable for internal administration

generally contain from about 0.1 mg to 500 mgs of active ingredient per unit. The active ingredient may vary from 0.5 to 95% by weight based on the total weight of the composition. In some cases it may be desirable to limit the period of treatment due to excessive T cell proliferation. The limitations will be empirically determined, depending on the response of the patient to therapy, the number of T cells in the patient, etc. The number of T cells may be monitored in a patient by methods known in the art, including staining with T cell specific antibodies and flow cytometry. The formulation is administered at a dose effective to increase the response of T cells to antigenic stimulation. The determination of the T cell response will vary with the condition that is being treated. Useful measures of T cell activity are proliferation, the release of cytokines, including. IL-2, IFNγ, TNFα, etc; T cell expression of markers such as CD25 and CD69; and other measures of T cell activity as known in the art.

The present invention further provides a kit that will allow the artisan to prepare an immunotherapeutic regimen for eliciting an immune response against a p53-expressing malignancy. An example of a kit comprises rMVAp53, a CTLA-4 blocking agent and/or a CpG ODN, and instructions for using these compounds to elicit an immune response against a p53-expressing malignancy in a subject. The kit may further comprise one or more pharmaceutically acceptable carriers. When administered, the compositions of the kit are administered in pharmaceutically acceptable preparations. The terms administration, administering, and introducing refer to providing the compositions of the invention as a medicament to an individual in need of treatment or prevention of a p53-expressing malignancy. This

medicament, which contains compositions of the present invention as the principal or active ingredients, can be administered in a wide variety of therapeutic dosage forms in the conventional vehicles for topical, oral, systemic, local, and parenteral administration. Thus, the kits of the invention provide compositions for parenteral administration that comprise a solution of the compositions dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, including sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and many others. Actual methods for preparing compounds for parenteral administration will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington: The Science and Practice of Pharmacy ("Remington's Pharmaceutical Sciences") Gennaro AR ed. 20th edition, 2000: Williams & Wilkins PA, USA, which is incorporated herein by reference.

Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents. All the preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, stimulates the desired response. In the case of treating cancer, the desired response is inhibiting

the initiation or progression of the cancer, or producing regression of the cancer. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. These desired responses can be monitored by routine methods or can be monitored according to diagnostic methods of the invention discussed herein. It is believed that doses of immunogens ranging from 10^4 IU/kilogram to 10^{11} IU/kilogram, depending upon the mode of administration, would be effective. The preferred range is believed to be between 10^6 IU and 10^9 IU per kilogram. The absolute amount will depend upon a variety of factors, including the combination selected for administration, whether the administration is in single or multiple doses, and individual patient parameters including age, physical condition, size, weight, and the stage of the disease. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation.

The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (2001), Ausubel et al. (Eds.) Current Protocols in Molecular Biology, John Wiley & Sons (1986, 2000) are used. One skilled in the art may develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

It will be understood that many variations can be made in the procedures herein described while still remaining within the bounds of the present invention. Likewise, it is understood that, due to the degeneracy of the genetic code, nucleic acid sequences with codons equivalent to those disclosed will encode functionally equivalent or identical proteins as disclosed herein. It is the intention of the inventors that such variations are included within the scope of the invention.

EXAMPLES

Materials and Methods

Animals

Female 6-8 week old Balb/c, C57BL/6, B6.129S2-*IL6*^{tm1Kopf} (IL-6$^{-/-}$, and IFN-γ knock out (IFN-γ^{KO}) mice on the Balb/c background were obtained from The Jackson Laboratory (Bar Harbor, Maine). TLR9$^{-/-}$ mice were a kind gift from Dr. Shizuo Akira (Osaka University, Osaka, Japan). Mice were maintained in a specific pathogen-free environment. All studies were approved by the Research Animal Care Committee of the City of Hope National Medical Center, and performed under the AAALAC guidelines.

Cell lines

CV-1 (Kit 1965), TK^{-} (Berson 1996), and Baby Hamster Kidney cells (BHK-21) (Macpherson 1962) were purchased from American Type Culture Collection (ATCC) (Manassas, VA), and grown in MEM supplemented with non-essential amino acids, L-glutamine, and 10% FCS. 11A-1 (Selvanayagam 1995) was a kind gift from Dr. R.L. Ullrich (University of Texas Medical Branch, Galveston, TX). Hek 293 cells and

p53null 10.1 cells were kind gifts from Dr. K.K. Wong and Dr. Susan Kane (City of Hope National Medical Center, Duarte, CA). MC-38 (Tan 1976) was a kind gift from Dr. S.A. Rosenberg (National Cancer Institute, Bethesda, MD. Meth A sarcoma cells (Meth A) (DeLeo 1977) were a kind gift from Dr. L. J. Old (Memorial Sloan-Kettering Cancer Center, New York, NY). Meth A was passaged as an ascitic tumor. Cells were harvested, counted and washed with PBS prior to use. The characteristics of the Meth A, 11A-1, and MC-38 tumor cell lines are summarized in the following table:

Cell line	Tumor	MHC Background	P53 mutation position(s)
Meth A	Fibrosarcoma	H-2^d	132, 168, 234
11A-1	Mammary cell carcinoma	H-2^d	173
MC-38	Colon carcinoma	H-2^b	242

Antibodies

Anti-CD4 (GK1.5) (Dialynas 1983) and anti-NK1.1 (PK136) (Koo 1984) were purchased from ATCC. Anti-CD8 (H35) (Miconnet 2001) and anti-CTLA-4 mAb (9H10) (Krummel 1995) were kind gifts from James P. Allison (University of California, Berkeley, CA). Antibodies were produced using a CELLine Device (BD Biosciences, Bedford, MA). IgG antibodies were purified by absorbance over protein G-Sepharose (Amersham, Uppsala, Sweden) followed by elution with 0.1M Glycine-HCl, pH 2.7. The product was then dialyzed against phosphate-buffered normal saline (PBS) and concentrated using a Centriplus centrifugal filter device (Millipore, Bedford, MA). Control Syrian Hamster IgG was obtained from Jackson Immuno Research (West Grove, PA).

Viral Constructs

rMVA expressing murine p53 (rMVAmup53):

Wild type MVA (wtMVA) was obtained from Dr. Bernard Moss and Dr. Linda Wyatt (National Institutes of Health Bethesda, MD). wtMVA stocks for the generation of recombinant MVA (rMVA) containing mup53 are propagated on specific pathogen free chicken embryo fibroblasts (SPF/CEF). The wtMVA stock is titrated by immunostaining, aliquoted, and stored at -80°C.

Murine p53 (mup53) is analogous to human p53, with 80% sequence homology (Halevy 1991; Sukumar 1995). The mRNA coding sequence for full-length wild type mup53 is shown in SEQ ID NO: 1. The level of homology between murine and human p53 makes the murine system an excellent preclinical model for evaluating immunologic approaches for overcoming tolerance to p53. rMVA expressing murine p53 was generated by homologous recombination of wtMVA and a pMCO3 insertion vector containing a murine p53 insert, as described in Espenschied 2003. The entire cDNA of murine wild type p53 was amplified by PCR of mRNA obtained from murine splenocytes. The murine p53 PCR product was ligated into the cloning site of the MVA expression vector pMCO3 (also obtained from Dr. Moss and Dr. Wyatt). This vector contains sequences that insert into deletion III of the MVA genome, and also contains the *gus* (*E. coli* B-glucuronidase) operon for screening purposes (Ourmanov 2000). Generation of recombinant MVA was achieved on monolayers of BHK-21 cells (Espenschied 2003). Briefly, BHK-21 cells were transfected with 20 μg of plasmid DNA using Lipofectin (Invitrogen, Carlsbad, CA) and infected with wtMVA at an moi of 0.01. The infected cells were

incubated for 48 hours, then harvested, pelleted, and subjected to 3 cycles of freeze/thaw and sonication to lyse the cells. rMVA virus expressing murine p53 (rMVAmup53) was screened for *gus* expression by adding X-GlcA (5-Bromo-4-Chloro-3-Indolyl B-D-Glucuronide, Sigma-Aldrich, St Louis, MO). After 10 rounds of purification, the rMVAmup53 was expanded on BHK-21 monolayers. The rMVAmup53 titer was determined by immunostaining infected cultures using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA).

rMVA expressing human p53 (rMVAhup53):

Two different constructs of rMVA expressing human p53 (rMVAhup53) were made. The mRNA sequence encoding full-length wild type hup53 is shown in SEQ ID NO: 2. The first was made using the pLW51 insertion plasmid, while the second was made using the pLW22 insertion plasmid. wtMVA used to make the first construct was propagated on SPF/CEF. wtMVA used to make the second construct was propagated on BHK-21 (BHK) cells. wtMVA stock was titrated by immunostaining, aliquoted, and stored at -80°C.

pLW51 was used as the insertion plasmid for generating the first rMVAhup53 construct. pLW51 has four important features. First, it contains MVA flanking regions of deletion III that allow it to insert into the deletion III region of MVA via homologous recombination. Second, it contains a color screening marker gene, β-glucoronidase (*gus*), under control of a vaccinia promoter called P_{11}. Third, it contains two direct repeats composed of MVA sequence (designated as DR1 and DR2) flanking the *gus* screening marker gene to allow the *gus* gene to be removed from recombinant MVA. Finally, it contains two vaccinia promoters (P_{SYN} and $P_{7.5}$)

and two multiple cloning sites (MCS), permitting the insertion of two separate foreign genes under the control of the P_{SYN} and $P_{7.5}$ promoters. The first MCS is behind an early/late P_{SYN} promoter, while the second MCS uses an early/late P_{mH5} promoter. This enables the elimination of the *gus* marker gene through recombination via a set of direct repeats, which flank it. The generation of the initial rMVA stock is done on CEF utilizing methods that were previously described for BHK cells, with modifications to account for good laboratory practice (GLP) conditions. About 40-50 foci are pulled from the first rounds of screening to ensure that a correct recombinant will be found, after which 5-10 are pulled in each subsequent round. After each round of selection, either immunostaining or immunofluorescence is performed on each plug to make sure that the plug is expressing the hup53 gene. To achieve a virus that will be deleted of the bacterial gene marker, purified rMVA expressing hup53 is plated at low dilution in 24 well plates. Wells that do not have a color reaction demonstrating the *gus* gene are further analyzed for the presence of the hup53 gene product. This is accomplished by antibody staining using conditions that allow recovery of the virus from the cells. Those wells that exhibit hup53 immunostaining in the absence of a color reaction are further propagated and confirmed to be the correct phenotype. A portion of the viral plug pulled from the final round of screening absent the *gus* marker is expanded in a 100 mm tissue culture dish of CEF. This is followed by DNA extraction and PCR analysis (discussed below).

pLW22 was used as the insertion plasmid for generating the second rMVAhup53 construct. pLW22 has MVA flanking regions that allow it to insert into

MVA via homologous recombination. It also has a color screening marker gene, β-galactosidase. To obtain DNA encoding wt hup53, pHp53B plasmid in *E. coli* was obtained from the ATCC (#57254). Hup53 was amplified from the pHp53B plasmid using the forward primer of SEQ ID NO: 3 and the reverse primer of SEQ ID NO: 4. Amplified wt hup53 DNA was inserted into the pLW22 vector between restriction sites Pme-1 and Asc-1, generating pLW22-hup53. The plasmid sequence of pLW22-hup53 is shown in SEQ ID NO: 5.

Generation of rMVA was achieved on monolayers of BHK cells. BHK cells were transfected with 20 μg of plasmid DNA using Lipofectin (Invitrogen, Carlsbad, CA), and infected with wtMVA at an moi of 0.01. The infected cells were incubated for 48 hours, then harvested, pelleted, and subjected to three cycles of freeze/thaw and sonication to lyse the cells. rMVA expressing hup53 was screened for β-gal expression by adding presence of Bluo-gal™ substrate (Sigma-Aldrich, St Louis, MO) (Chakrabarti 1985). After 10 rounds of purification, the rMVAhup53 was expanded on BHK monolayers. The rMVA titer was determined by immunostaining infected cultures using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

For both constructs, a standard DNA extraction is performed. Ethanol precipitation of 50 μL of the cell lysate resulted in enough DNA to run a PCR reaction to assure the absence of contaminating wtMVA. One set of PCR primers are designed outside the flanking regions of the recombination site for which the gene has been inserted. The presence of unmodified wtMVA sequence will generate a 500 bp PCR product, whereas the insertion of the sequence containing

hup53 has a much larger fragment (>6 kb), which is usually difficult to amplify under standard PCR conditions. A second set of PCR primers are designed to amplify a sequence within the hup53 insert. The presence of the hup53 insert will generate a 300 bp PCR product. The PCR samples are run on a 1% agarose gel and analyzed to determine if additional screenings are necessary to remove any remaining wtMVA. Examples of purified MVA containing human p53 have been shown to be absolutely homogenous (Figure 1).

rMVA expressing pp65 (rMVApp65):

rMVA expressing pp65 (rMVApp65), a CMV tegument protein, was constructed using techniques similar to those used to construct rMVAmup53 (Gibson).

rVV expressing murine p53 or pp65:

Recombinant Western Reserve strain Vaccinia Virus expressing murine wild type p53 or pp65 (rVVp53, rVVpp65) was constructed using published techniques (Diamond 1997).

rAd expressing murine p53:

Recombinant adenovirus expressing wild type murine p53 (rAd-mup53) was constructed using the pAd Easy system (He 1998). Both pAd Track-CMV and pAd Easy-1 plasmids were kindly provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). Wild type murine p53 cDNA was cloned into the Bgl II and Xba I site of a pAd Track-CMV shuttle vector containing green fluorescent

protein (GFP) with a CMV promoter (p53-pAd Track-CMV). The p53-pAd Track-CMV was cotransformed into BJ5183 cells with the pAd Easy-1 plasmid to generate the p53 recombinant adenoviral construct by homologous recombination. The presence of the p53 gene in the recombinants was confirmed by DNA sequencing. The p53 recombinant adenoviral construct was cleaved with Pac I and transfected into HEK-293 cells. rAd-mup53 was harvested 5 days after transfection and p53 protein expression was confirmed by western blot. The adenovirus was expanded on HEK-293 cells and purified by cesium chloride gradient. The purified virus was dialyzed in PBS, titered on HEK-293 cells, and stored at –80°C in 20% glycerol.

Oligodeoxynucleotides (ODN)

Synthetic ODN 1826 with CpG motifs (SEQ ID NO: 6) and non-CpG ODN 1982 (SEQ ID NO: 7) (Moldoveanu 1998) were synthesized with nuclease-resistant phosphorothioate backbones by Trilink (San Diego, CA). The Na^+ salts of the ODNs were resuspended at 5 mg ml^{-1} in 10 mM Tris (pH 7.0) 1 mM EDTA and stored as 50 µl aliquots at -20°C before dilution in aqueous 0.9% sodium chloride solution prior to injection.

Example 1: Expression of murine p53 protein by rMVAmup53:

Expression of murine p53 protein following infection with rMVAmup53 was analyzed to determine the fidelity and extent of its expression from recombinant virus. Lysates were prepared from BHK or HEK 293 cells infected with rMVAmup53 and subjected to SDS-PAGE and Western blotting. Standard Western Blotting techniques were performed using an ECL Western Blot Kit (Amersham Pharmacia Biotech, England). The samples were incubated with a purified mouse anti-p53

monoclonal antibody, PAb 122 (Gurney 1980), followed by incubation with a peroxidase labeled goat anti-mouse secondary antibody provided in the ECL Western Blot kit. Western blot analysis of BHK cells infected with rMVAmup53 demonstrates abundant p53 expression (Figure 2). The remarkable level of expression exhibited by rMVAmup53 compared to other viral and cellular forms demonstrates its usefulness in vaccination protocols. As shown in Figure 1, the volume on the rMVAmup53 lane is between 80-160 fold less than what was applied to the gel in the other lanes, yet the intensity of the band is several fold higher. This demonstrates a very high level of p53 expression by rMVAmup53. Meth A cells were used as a positive control and BHK cells infected with HCMV IE1 exon 4 rMVA were used as negative controls. Meth A is a Balb/c derived, tumorigenic 3-methylcholanthrene-induced sarcoma that over-expresses mutated p53. A 53 kilodalton band was observed in both the p53 overexpressing Meth A sarcoma and the rMVAmup53 infected BHK cells (Figure 1). This contrasts with the absence of detectable p53 expression in the HCMV IE1 exon 4-rMVA infected BHK cells. Strong p53 expression was also observed by fluorescence microscopy in BHK cells infected with rMVAmup53 (data not shown).

Example 2: *In vitro* generation of a p53-specific CTL response by rMVAmup53:

Vaccination of mice with rMVA expressing viral and tumor associated antigens results in enhanced antigen specific CTL responses. One goal of this example was to determine if vaccination with rMVAmup53 could break p53 tolerance, resulting in the generation of p53-specific CTL. Mice were vaccinated i.p. with $5x10^7$ pfu of either rMVAmup53 or rMVApp65. After two weeks, spleens were

harvested and disassociated, and splenocytes were washed and counted. Splenocytes were restimulated *in vitro* for 6 days with syngeneic LPS blasts infected with rAd-mup53 or rMVAmup53. Na-$^{51}CrO_4$-labeled target cells that overexpress wt p53 were added to 96 well plates with the effectors, in triplicate, at various effector to target ratios, in 200 µl of complete medium. The plates were incubated for 4 hours at 37°C, and the supernatant was harvested and analyzed. Percent specific lysis was calculated using the formula: percent specific release = (experimental release – spontaneous release) / (total release - spontaneous release) X 100. Splenocytes vaccinated with rMVAmup53 recognized and lysed target cells that overexpressed wt p53 (Figure 3). In contrast, splenocytes from mice vaccinated with rMVApp65, which stimulates a vigorous pp65 specific CTL response, did not recognize the p53 over-expressing targets (Figure 3B), demonstrating the specificity of the lymphocyte response. rMVAmup53 vaccination can also stimulate CTL recognition of a cell line bearing mutated p53, Meth A. Restimulated splenocytes vaccinated with rMVAmup53 recognized mutant p53 over-expressing Meth A cells, but splenocytes vaccinated with rMVAmup53 did not (Figure 3c).

Example 3: *In vivo* rMVAmup53 tumor challenge experiments:

Since a single vaccination with rMVAmup53 resulted in enhanced CTL responses, there was sufficient justification to examine the effect of rMVAmup53 vaccination on the growth of tumor cells *in vivo*.

Statistical methods

For experiments where the growth rate of some tumors necessitated early sacrifice, growth curves were compared by the time to a fixed size using a logrank

test. Contrasts of single groups to all others were conducted after a single omnibus test. For cell depletion experiments, all mice were followed for a fixed amount of time, and final tumor size was compared by the Wilcoxon rank-sum test, after a significant Kruskal-Wallis test if there were more than two groups. For survival experiments, a logrank test was used.

rMVAmup53 vs. Meth A cells

Six-week-old female Balb/c mice were injected by subcutaneous (s.c.) route in the left flank with $5x10^5$ Meth A cells. Mice injected s.c. with Meth A cells develop a rapidly growing fibrosarcoma that kills the majority of mice within 21 days (Figure 3). On day 3, the mice were vaccinated with $5x10^7$ pfu of rMVAmup53 by intraperitoneal (i.p.) injection. Negative control mice were injected with $5x10^7$ rMVApp65 or PBS. The s.c. tumors were measured twice weekly in three dimensions with calipers. Tumors in rMVAmup53 treated animals grew at a much slower rate than those in control animals. At 14 days, the mean s.c. tumor volume for the rMVAmup53 treated group (n=16) was dramatically lower than both the rMVApp65 (n=16) and PBS (n=12) controls ($22mm^3$ versus $348\ mm^3$, $p<0.001$ and $22mm^3$ versus $252mm^3$, $p<0.001$ by Student's t-test). Survival of rMVAmup53 treated animals was also significantly prolonged compared to either control group (Figure 4). 12 of the 16 rMVAmup53 immunized mice failed to develop tumors entirely. The 12 tumor free rMVAmup53 treated animals were re-challenged at day 52 with $5x10^5$ Meth A tumor cells. All animals remained tumor free for the duration of a 30 day observation period (data not shown).

rMVAmup53 plus anti-CTLA-4 mAb vs. Meth A cells

One potent strategy for optimizing tumor vaccines involves manipulating negative regulation of T cell responsiveness using an antibody that blocks CTLA-4 engagement with ligand. This phenomenon has been referred to as CTLA-4 blockade. Application of anti-CTLA-4 mAb in combination with cancer vaccines expressing tumor associated autoantigens, in some cases, results in tumor rejection along with breaking of tolerance and induction of autoimmunity. Therefore, mAb specific to CTLA-4 was added to rMVAmup53 vaccination to determine whether it would synergize and augment the anti-tumor activity against Meth A *in vivo*. A more rigorous tumor model was designed in order to overcome the potent antitumor effect of CTLA-4 blockade alone. Six-week-old Balb/c mice were injected s.c. in the left flank with 10^6 Meth A cells rather than $5x10^5$ Meth A cells, and treatment was postponed until a palpable tumor nodule was identified (Day 6). This more rigorous model overcame the effect of the CTLA-4 blockade, producing a rapidly lethal tumor in the majority of mice despite anti-CTLA-4 mAb treatment (Figure 5). On day 7, mice were injected i.p. with $5x10^7$ pfu of rMVAmup53. Controls were the same as above. Anti-CTLA-4 mAb antibody or control hamster Ab were injected i.p. on days 6, 9, and 12 at 100, 50 and 50 μg dose, respectively. 11 of the 14 mice immunized with rMVAmup53 plus anti-CTLA-4 mAb rejected tumors, resulting in tumor free survival for the duration of the 60 day observation period (Figure 5). By contrast, mice treated with rMVApp65 and control antibody died rapidly of progressive tumor (Figure 5) as did PBS treated controls (data not shown). The 11 tumor-free rMVAmup53 plus anti-CTLA-4 mAb treated mice also rejected a re-challenge with

10^6 Meth A tumor cells at 60 days, and remained tumor free for the duration of a 30 day observation period (data not shown).

rMVAmup53 plus anti-CTLA-4 mAb vs. 11A-1 cells

Six-week-old Balb/c mice were injected s.c. in the left flank with 2 x 10^6 11A-1 cells. 11A-1 is a rapidly growing malignant cell line that is poorly immunogenic. Mice vaccinated with 10^6 irradiated 11A-1 tumor cells failed to reject a subsequent challenge with 11A-1 (data not shown). Anti-CTLA-4 mAb or the control hamster antibody was injected i.p. on days 4, 7, and 10 at 100, 50, and 50 µg/dose, respectively. On day 5, mice were vaccinated i.p. with either 5 x 10^7 pfu of rMVAmup53, 5 x 10^7 MVApp65, or PBS. s.c. tumors were measured twice weekly in three dimensions with calipers. Mice vaccinated with rMVAmup53 plus anti-CTLA-4 mAb rejected their tumors (Figure 6). Animals treated with anti-CTLA-4 mAb alone or with a control MVA vaccine developed rapidly progressing lethal tumors ($p = 0.00044$, comparing rMVAmup53 with anti-CTLA-4 mAb blockade to control groups).

rMVAmup53 plus anti-CTLA-4 mAb vs. MC-38 cells

Six-week-old C57BL/6 mice, $TLR9^{-/-}$, or $IL\text{-}6^{-/-}$ mice were injected s.c. in the left flank with 1 x 10^6 MC-38 cells. Anti-CTLA-4 mAb or the control hamster antibody was injected i.p. on days 4, 7, and 10 at 100, 50, and 50 µg/dose, respectively. On day 5, mice were vaccinated i.p. with either 5 x 10^7 pfu of rMVAmup53, 5 x 10^7 rMVApp65, or PBS. s.c. tumors were measured twice weekly in three dimensions with calipers. Mice vaccinated with rMVAmup53 plus anti-CTLA-4 mAb rejected their tumors, while those treated with anti-CTLA-4 mAb alone or with a control MVA

vaccine developed rapidly progressing tumors (p = 0.0001, comparing rMVAmup53 with anti-CTLA-4 mAb to control groups) (Figure 7).

rMVAmup53 plus CpG ODN vs. 11A-1 cells

CpG ODN treatment has been shown to be an effective immunomodulator in a number of experimental tumor vaccine models (Krieg 2002). Mice were challenged with 11A-1 tumor as above. 15 nmoles of CpG ODN or the non-CpG ODN control were injected i.p. on days 4, 9, and 14. On day 5, the mice were vaccinated i.p. with either 5 x 10^7 pfu of rMVAmup53, 5 x 10^7 rMVApp65, or PBS. The s.c. tumors were measured twice weekly in three dimensions with calipers. While rMVAmup53 and CpG ODN each separately resulted in minimal attenuation of tumor growth, all animals developed progressively lethal tumors. The combination of CpG ODN and rMVAmup53 vaccination resulted in significantly diminished tumor outgrowth (p = 0.00002) (Figure 8). 6 of the 8 animals treated with rMVAmup53 plus CpG ODN did not develop palpable tumors and developed lasting tumor immunity, rejecting a rechallenge with 11A-1 at 60 days (data not shown).

rMVAmup53 plus CpG ODN vs. Meth A cells

A pattern of tumor rejection similar to that for 11A-1 was seen following treatment of early established Meth A tumors in Balb/c mice (p = 0.0015) (Figure 9).

rMVAmup53 plus CpG ODN vs. MC-38 cells

To demonstrate that the immunomodulator effect of CpG ODN on rMVAmup53 vaccination is not strain specific, the vaccination strategy was repeated in C57BL/6 mice bearing early established MC38 colon cancers. Vaccination with

rMVAmup53 plus CpG ODN resulted in significant suppression of tumor growth (p = 0.0004) (Figure 10).

rMVAmup53 plus anti-CTLA-4 mAb plus CpG ODN vs. 11A-1 cells

A more rigorous tumor model was designed to evaluate the potential additive effects of CpG ODN and anti-CTLA-4 mAb on rMVAmup53 vaccination. Six-week-old Balb/c mice were injected s.c. in the left flank with 2 x 10^6 11A-1 cells and followed for two weeks until palpable tumors were present. Anti-CTLA-4 mAb or the control hamster antibody was injected i.p. on days 14, 17, and 20, at 100, 50, and 50 µg/dose, respectively. 15 nmoles of CpG ODN was injected i.p. on days 14, 19, and 24. On day 15, the mice were vaccinated i.p. with either 5 x 10^7 pfu of rMVAmup53, 5 x 10^7 MVApp65, or PBS.

rMVAmup53 vaccination combined with either anti-CTLA-4 mAb or CpG ODN immunomodulators resulted in prolonged survival, but all animals eventually succumbed to progressive tumor growth. The combination of anti-CTLA-4 mAb and CpG ODN administration with rMVAmup53 vaccination resulted in tumor rejection and prolonged survival in the majority of treated animals (Figure 11). The combination of anti-CTLA-4 mAb and CpG ODN provides better immunomodulator activity than either CpG ODN alone (p = 0.02) or anti-CTLA-4 mAb alone (p = 0.01). The effect of combined anti-CTLA-4 mAb and CpG ODN administration provides a greater benefit in terms of survival at 60 days than the simple addition of the effects of both immunomodulators separately.

rMVAmup53 plus anti-CTLA-4 mAb plus CpG ODN vs. MC-38 cells

A similar pattern was seen in C57BL/6 mice bearing MC 38 tumors (Figure 12). C57BL/6 mice bearing MC-38 tumors were treated with rMVAmup53 plus a combination of anti-CTLA-4 mAb and CpG ODN as described above for 11A-1. In this tumor model, the combination of anti-CTLA-4 mAb and CpG ODN also provided better immunomodulator activity than either CpG ODN alone ($p = 0.002$) or anti-CTLA-4 mAb alone ($p = 0.001$). The combined effect in both tumor models is not simply a dose additive effect, as the CpG ODN and anti-CTLA-4 mAb were both already administered at doses of maximal efficacy. The striking increases in activity found when both immunomodulators are used together in at least two different tumors suggests that further investigation of the combined modality is warranted in humans.

Example 4: Cellular requirements for anti-CTLA-4 mAb and CpG ODN immunomodulator effect:

To determine the cellular requirements for the immunomodulator effect of anti-CTLA-4 mAb and CpG ODN, Balb/c mice were depleted of $CD4^+$, $CD8^+$, or NK cells prior to vaccination. Depletion was accomplished by i.p. injection of 200 µg of $CD4^+$, $CD8^+$, or NK1.1 cell specific mAbs, or a control mAb. Injections were given on days -1, 1, 3, 4, 6, 8, and 15, with a maintenance dose every 7 days until the termination of the animals. This regimen was shown to deplete (>95%) Balb/c mice of $CD4^+$, $CD8^+$, or NK 1.1 cells based on flow cytometry of peripheral blood from treated animals (data not shown).

The cellular requirements for the immunomodulator effect of CTLA-4 blockade on rMVAmup53 vaccination were evaluated using the Meth A tumor model in Balb/c mice. Mice depleted of $CD8^+$ T cells or $CD4^+$ and $CD8^+$ T cells simultaneously develop rapidly lethal tumors. These tumors are resistant to vaccination with rMVAmup53 and anti-CTLA-4 mAb. In contrast, $CD4^+$ T cell depletion resulted in only a partial abrogation of response to the vaccine. NK1.1 cell depletion had little effect on the ability of vaccinated mice to reject Meth A (Figure 13a). Results were the same when the depleting mAbs were administered after vaccine and anti-CTLA-4 mAb treatment (data now shown). Similar results were also obtained when the 11A-1 tumor model was used rather than the Meth A tumor model. The therapeutic effect of rMVAmup53 and anti-CTLA-4 mAb could be eliminated by administering depleting doses of anti-$CD8^+$ mAb ($p = 0.004$) (Figure 15). The antitumor effect was partially blocked by the administration of depleting anti-$CD4^+$ mAb ($p = 0.008$), and unaffected by the administration of an NK depleting mAb. These results show that the immunomodulator effect of anti-CTLA-4 mAb is entirely dependent on $CD8^+$ cells, partially dependent on $CD4^+$ cells, and not dependent at all on NK cells (Espenschied 2003).

The cellular requirements for the immunomodulator effect of CpG ODN on rMVAmup53 vaccination were evaluated using Balb/c mice with four-day established 11A-1 tumors. As with anti-CTLA-4 mAb, the immunomodulator effect of CpG ODN on MVAmup53 vaccination could be completely abrogated by the administration of depleting $CD8^+$ mAb ($p = 0.004$) (Figure 14). However, unlike anti-CTLA-4 mAb, the immunomodulator effect of CpG ODN was unaffected by $CD4^+$ depletion, while

depletion of NK cells partially abrogated the vaccine effect ($p = 0.007$, comparing NK to $CD4^+$ and control antibody depletions). The difference in cellular requirements for $CD4^+$ and NK between anti-CTLA-4 mAb and CpG ODN is striking, because both immunomodulators cause equivalent levels of rejection. These results suggest that the two immunomodulators act through differing immunologic mechanisms. This information, combined with the data regarding the effects of combined anti-CTLA-4 mAb /CpG ODN administration on rMVAmup53, suggest a synergistic effect by the two immunomodulators on tumor growth.

Contribution of IFN-γ

The contribution of IFN-γ secretion to the effect of CTLA-4 blockade and rMVAmup53 vaccination was evaluated in IFN-γ^{KO} mice. Both unvaccinated mice and mice vaccinated with rMVApp65 and anti-CTLA-4 mAb developed lethal tumors at a rate similar to that seen in normal Balb/c mice (Figure 13b). 3 of the 5 IFN-γ^{KO} mice that were vaccinated with rMVAmup53 and anti-CTLA-4 mAb developed lethal tumor growth, confirming a contribution of IFN-γ to the vaccine/CTLA-4 blockade effect.

Contribution of TLR 9

The cell subset depletion studies suggest that the mechanism of immunomodulator activity of CTLA-4 blockade and CpG ODN is different. CpG ODN activity results from the stimulation of B-cells and plasmacytoid dendritic cells through an interaction with the TLR9 receptor (Chu 1997). CpG treatment causes a bias towards the TH1 cytokine milieu and stimulation of NK cell proliferation, which may account for the partial effect on tumor rejection. To further delineate the

divergent pathways involved in the CpG ODN and CTLA-4 blockade immunomodulator effects, MC-38 tumor challenge experiments were conducted in $TLR9^{-/-}$ mice. $TLR9^{-/-}$ mice fail to immunologically respond to CpG ODN administration (Hemmi 2000). As expected, $TLR9^{-/-}$ mice bearing early established MC-38 tumors failed to immunologically respond to CpG ODN and rMVAmup53 vaccination (Figure 16). In contrast, inclusion of anti-CTLA-4 mAb with rMVAmup53 vaccination resulted in tumor rejection in $TLR9^{-/-}$ mice ($p = 0.0009$) that was similar to that seen in wt C57BL/6 mice (Figure 16, Figure 7).

Contribution of IL-6

Both CpG ODN and CTLA-4 blockade inhibit $CD25^{+}$ $CD4^{+}$ suppressor or regulatory T cells (Treg), and this effect may contribute to their immunomodulator activity in the described tumor models. Blocking CTLA-4 is thought to have a direct inhibitory affect on Tregs, most of which constitutively express CTLA-4 (Read 2000). In contrast, CpG ODN inhibits Treg activity through the secretion of IL-6 by DC (Pasare 2003). To evaluate the role of IL-6 on the CpG ODN and anti-CTLA-4 mAb immunomodulator effects, tumor challenge experiments were conducted in $IL\text{-}6^{-/-}$ mice. $IL\text{-}6^{-/-}$ mice bearing early established MC-38 tumors failed to immunologically respond to rMVAmup53 vaccination with CpG ODN by rejecting tumor (Figure 17). This suggests that CpG ODN could be mediating its immunomodulator effects, at least in part, through the IL-6 dependent pathway of Treg cell inhibition. In contrast, anti-CTLA-4 mAB inclusion with rMVAmup53 vaccination resulted in tumor rejection in $IL\text{-}6^{-/-}$ mice ($p = 0.02$) to an extent similar to that seen in wt C57BL/6 mice (Figure 17, Figure 7).

Example 5: Expression of human p53 by rMVAhup53:

BHK cells were infected with purified rMVAhup53. Expression of hup53 was measured at 24 and 48 hours, and analyzed by Western blot and immunohistochemistry. The infected rMVAhup53 cells demonstrated vigorous expression of hup53 at both time periods (Figure 18).

Example 6: *In vivo* rMVAhu53 tumor challenge experiments:

Hupki mice, a novel murine knock-in model expressing human p53, were obtained from Dr. Monica Hollstein (DKFZ, Heidelberg, Germany) in the 129/Sv genetic background. The mice were backcrossed for 4 generations onto the Balb/c(H-2^d) background in order to take advantage of the knock-in transgene in a murine background where tumors and other reagents are readily available. The hupki mice on the Balb/c background were backcrossed to homozygosity as confirmed by PCR analysis, using a mating procedure that minimized inbreeding effects (data not shown). The 4T1(H-2^d) murine breast carcinoma cell line was stably transfected with human p53, and hupki mice were s.c. injected with 5 x 10^4 4T1/hup53 in the flank. Mice injected with 4T1/hup53 grow progressive tumors, and the majority succumb to these tumors by day 35. To test the efficacy of rMVAhup53, mice were vaccinated with 10^7 pfu rMVAhup53 by i.p. injection on day 6 after 4T1/hup53 injection. Ten days later, the mice received an rMVAhup53 booster injection, along with CpG-ODN (15 nmole of ODN 1826) and anti-CTLA-4 mAb (50 µg/mouse). rMVAhup53 vaccination resulted in a statistically significant

improvement in survival ($p < 0.05$, two sided T-test) compared to PBS controls (Figure 19).

As stated above, the foregoing are merely intended to illustrate the various embodiments of the present invention. As such, the specific modifications discussed above are not to be construed as limitations on the scope of the invention. It will be apparent to one skilled in the art that various equivalents, changes, and modifications may be made without departing from the scope of the invention, and it is understood that such equivalent embodiments are to be included herein. All references cited herein are incorporated by reference as if fully set forth herein.

Abbreviations used herein: GFP, green fluorescent protein; DC, dendritic cells; IFN-γ^{KO}, IFN-γ knock out; MVA, modified vaccinia virus Ankara; rMVA, recombinant modified vaccinia virus Ankara; rAd-mup53, recombinant Adenovirus expressing murine wild type p53; hup53, wild type human p53; mup53, wild type murine p53; rMVAp53, recombinant MVA expressing p53; rMVAmup53, recombinant MVA expressing wild type murine p53; rMVAhup53, recombinant MVA expressing wild type human p53; rMVApp65, recombinant MVA expressing pp65; rVVmup53, recombinant vaccinia virus expressing murine wild type p53; rVVpp65, recombinant vaccinia virus expressing pp65; wtMVA, wild type MVA; WR, Western Reserve; i.p., intraperitoneal; s.c., subcutaneous; mAb, monoclonal antibody.

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What is claimed is:

1. A composition comprising a recombinant MVA virus (rMVA) containing a nucleic acid sequence encoding p53.

2. The composition of claim 1, wherein said p53 is wild type murine p53.

3. The composition of claim 2, wherein said nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO: 1.

4. The composition of claim 1, wherein said p53 is wild type human p53.

5. The composition of claim 4, wherein said nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO: 2.

6. The composition of claim 1, further comprising an immunomodulator comprising a CTLA-4 blocking agent, a CpG oligodeoxynucleotide, or both a CTLA-4 blocking agent and a CpG oligodeoxynucleotide.

7. The composition of claim 6, wherein said CTLA-4 blocking agent is an antibody.

8. The composition of claim 7, wherein said antibody is a monoclonal antibody.

9. A method of treating a subject having a p53-expressing malignancy, comprising introducing into said subject a composition comprising recombinant MVA virus containing a nucleic acid sequence encoding p53.

10. The method of claim 9, wherein said subject is human.

11. The method of claim 9, wherein introduction of said composition elicits an immune response effective against said p53-expressing malignancy.

12. The method of claim 9, wherein said p53 is wild type human p53.

13. The method of claim 12, wherein said nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO: 2.

14. The method of claim 9, wherein introduction of said composition is repeated one or more times.

15. The method of claim 9, further comprising introducing into said subject an immunomodulator comprising a CTLA-4 blocking agent, a CpG oligodeoxynucleotide, or both a CTLA-4 blocking agent and a CpG oligodeoxynucleotide.

16. The method of claim 15, wherein said CTLA-4 blocking agent is an antibody.

17. The method of claim 16, wherein said antibody is a monoclonal antibody.

18. The method of claim 15, wherein introduction of said immunomodulator occurs prior to, simultaneous with, or after introduction of the composition comprising recombinant MVA virus.

19. The method of claim 15, wherein introduction of said immunomodulator is repeated one or more times.

20. The method of claims 9 or 15, wherein the method of said introduction is selected from the group consisting of subcutaneous, percutaneous, intradermal, intraperitoneal, intramuscular, intratumoral and intravenous injection.

21. A kit for treating a subject having a p53-expressing malignancy comprising a composition comprising recombinant MVA virus containing a nucleic acid sequence encoding p53 and instructions for administration of said composition,

wherein said administration elicits an immune response effective against said p53-expressing malignancy.

22. The kit of claim 21, wherein said p53 is human p53.

23. The kit of claim 22, wherein said nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO: 2.

24. The kit of claim 21, further comprising an immunomodulator comprising a CTLA-4 blocking agent, a CpG oligodeoxynucleotide, or both a CTLA-4 blocking agent and a CpG oligodeoxynucleotide.

25. The kit of claim 24, wherein said CTLA-4 blocking agent is an antibody.

26. The kit of claim 25, wherein said antibody is a monoclonal antibody.

27. The kit of claim 21, wherein said subject is human.

28. A vector comprising an MVA recombination plasmid containing a nucleic acid insert encoding p53.

29. The vector of claim 28, wherein said MVA recombination plasmid is pLW22.

30. The vector of claim 28, wherein said nucleic acid insert encodes human wild type p53.

31. The vector of claim 30, wherein said vector comprises the sequence of SEQ ID NO: 5.

32. A method of generating a p53 specific cytotoxic T lymphocyte (CTL) response against cells overexpressing mutant p53 comprising administering a composition comprising recombinant MVA virus containing a nucleic acid sequence encoding p53.

33. The method of claim 32, wherein said nucleic acid sequence encoding p53 encodes murine wild type p53.

34. The method of claim 33, wherein said nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO: 1.

35. The method of claim 32, wherein said nucleic acid sequence encoding p53 encodes human wild type p53.

36. The method of claim 35, wherein said nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO: 2.

37. The method of claim 32, further comprising administering an immunomodulator comprising a CTLA-4 blocking agent, a CpG oligodeoxynucleotide, or both a CTLA-4 blocking agent and a CpG oligodeoxynucleotide.

38. The method of claim 37, wherein said CTLA-4 blocking agent is an antibody.

39. The method of claim 38, wherein said antibody is a monoclonal antibody.

40. The method of claim 37, wherein administration of said immunomodulator occurs prior to, simultaneous with, or after introduction of the composition comprising recombinant MVA virus.

41. The method of claim 37, wherein introduction of said immunomodulator is repeated one or more times.

Fig. 1

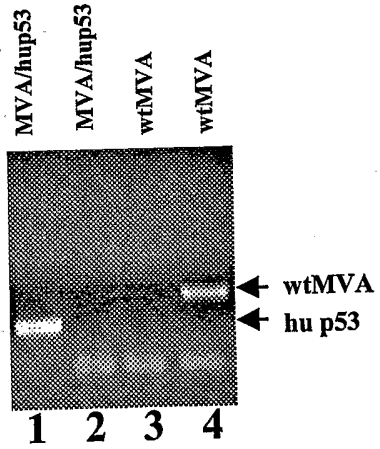
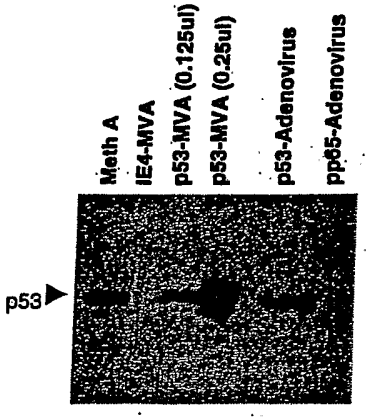


Fig. 2


Meth A
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p53-MVA (0.125ul)
p53-MVA (0.25ul)
p53-Adenovirus
pp65-Adenovirus
p53

Fig. 3

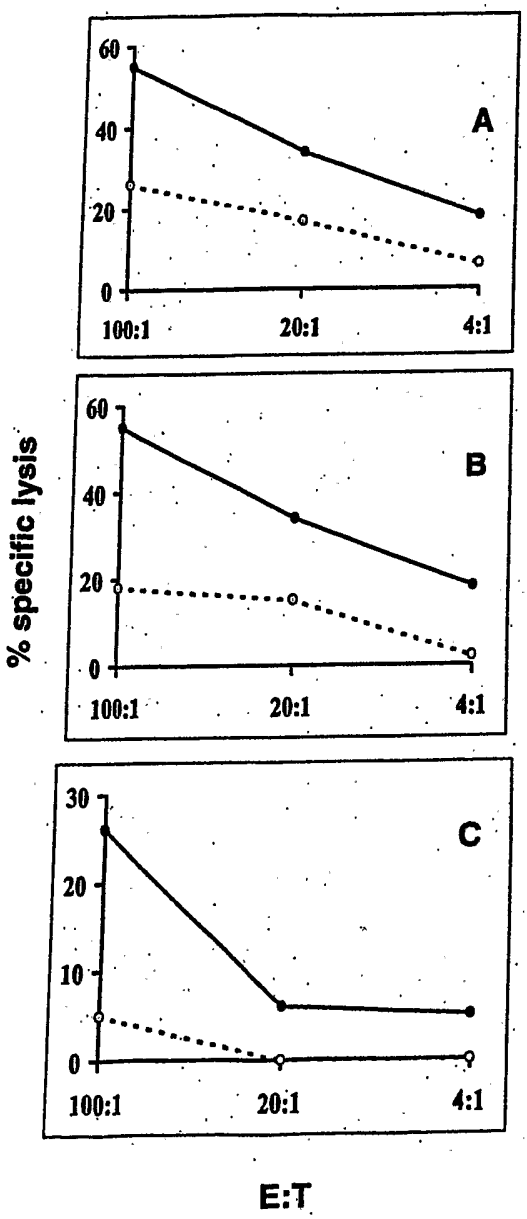


Fig. 4

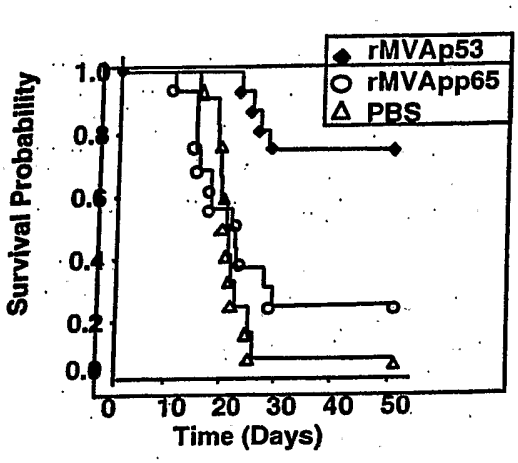

rMVAp53
rMVApp65
PBS
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Time (Days)

Fig. 5

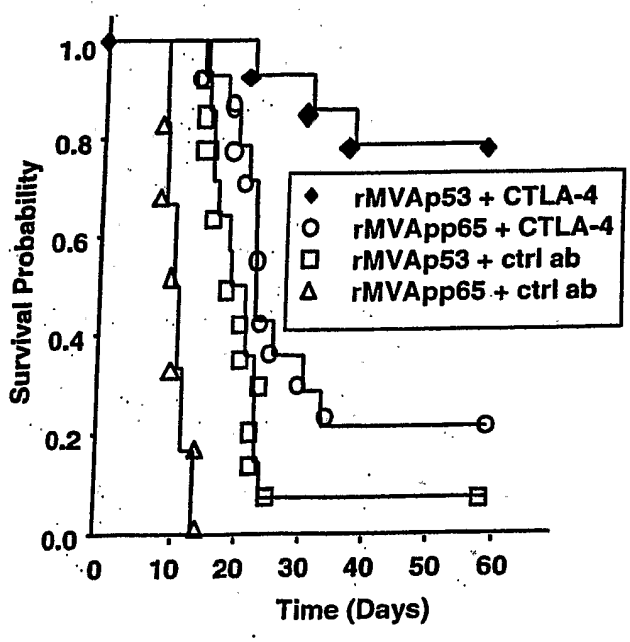
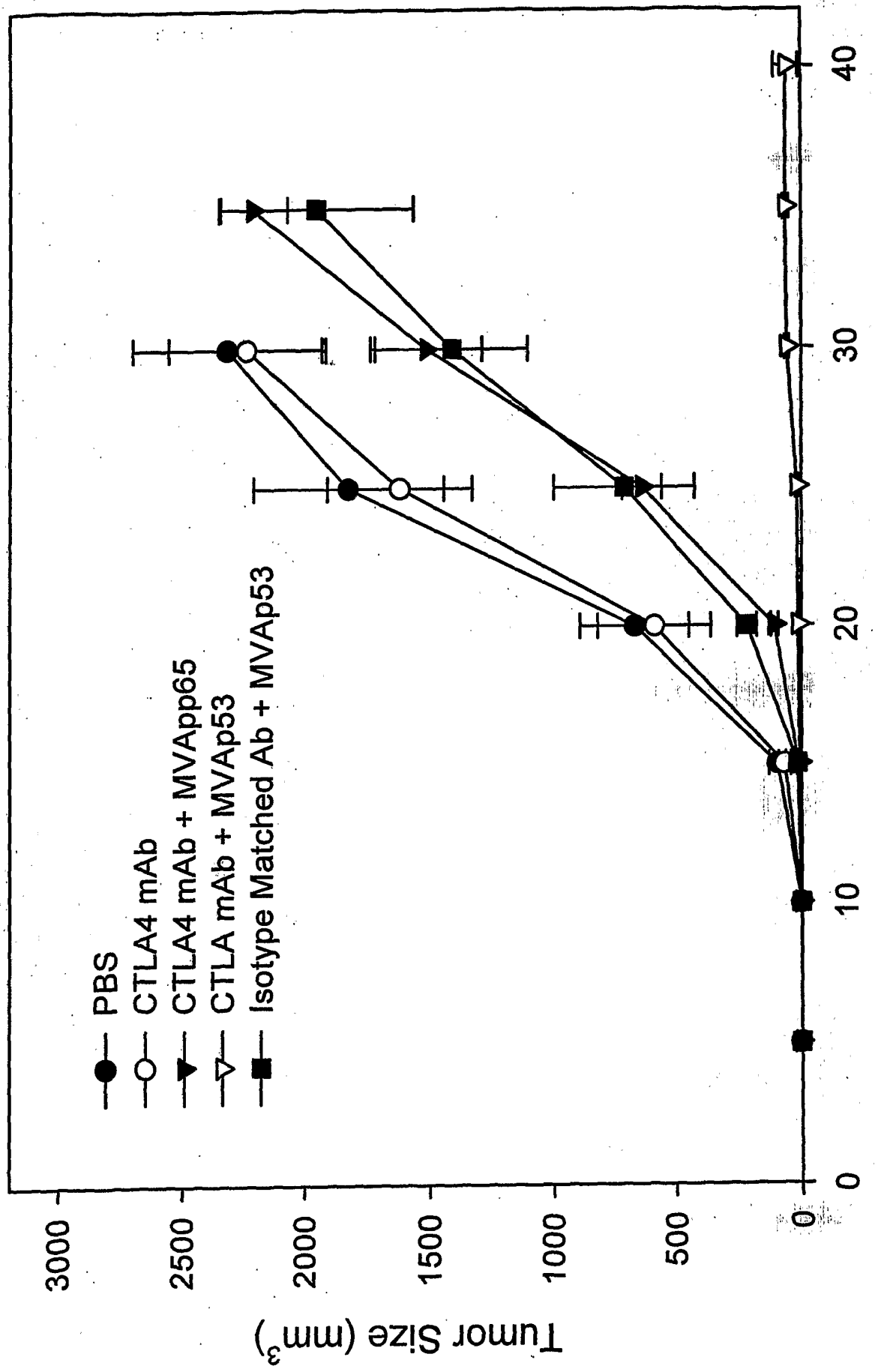

1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
0
10
20
30
40
50
60
Time (Days)
rMVAp53 + CTLA-4
rMVApp65 + CTLA-4
rMVAp53 + ctrl ab
rMVApp65 + ctrl ab

Fig. 6


Tumor Size (mm³)
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
PBS
CTLA4 mAb
CTLA4 mAb + MVApp65
CTLA mAb + MVAp53
Isotype Matched Ab + MVAp53

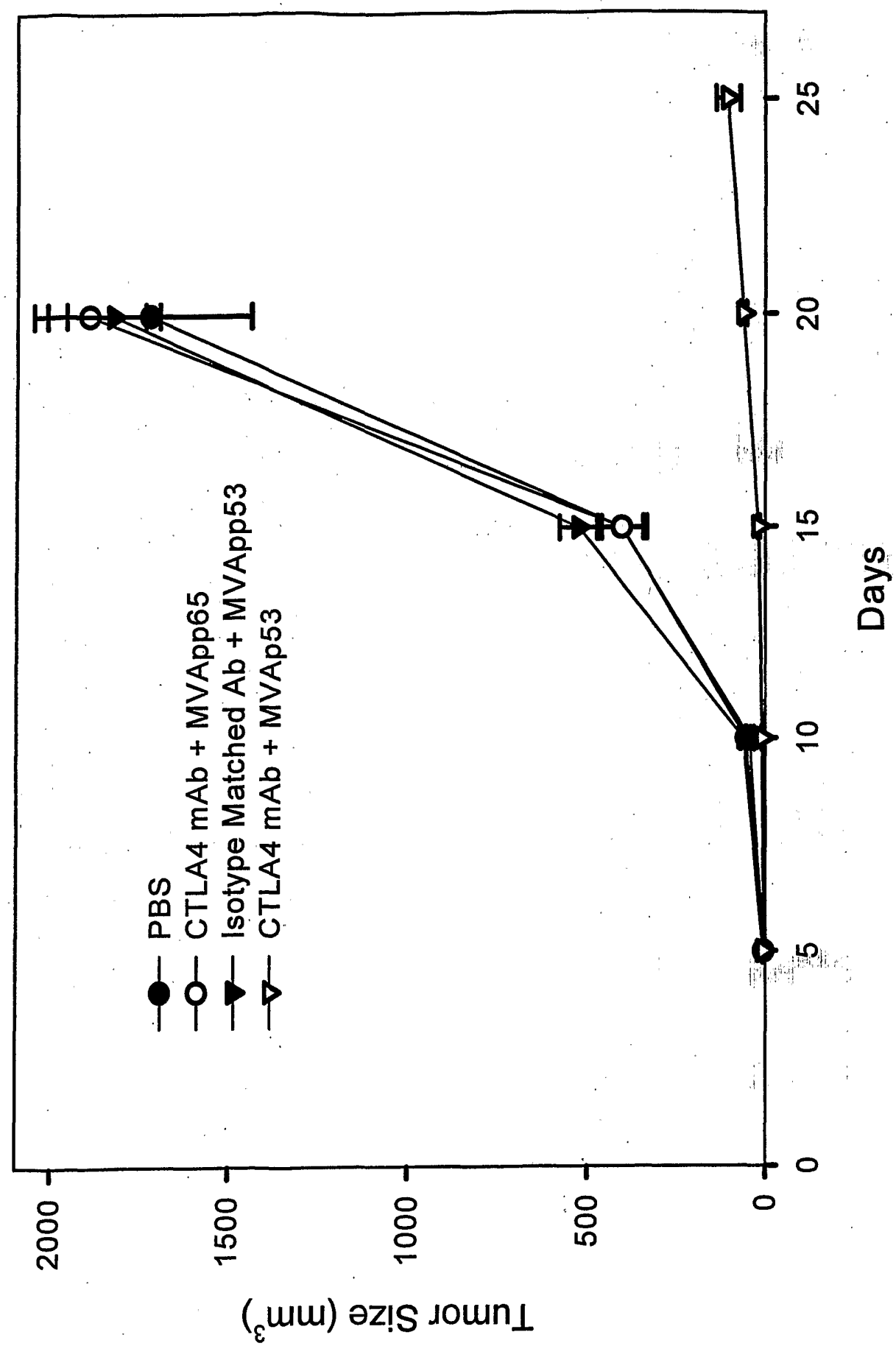

Tumor Size (mm³)
2000
1500
1000
500
0
0
5
10
15
20
25
Days
PBS
CTLA4 mAb + MVApp65
Isotype Matched Ab + MVApp53
CTLA4 mAb + MVAp53

Fig. 7

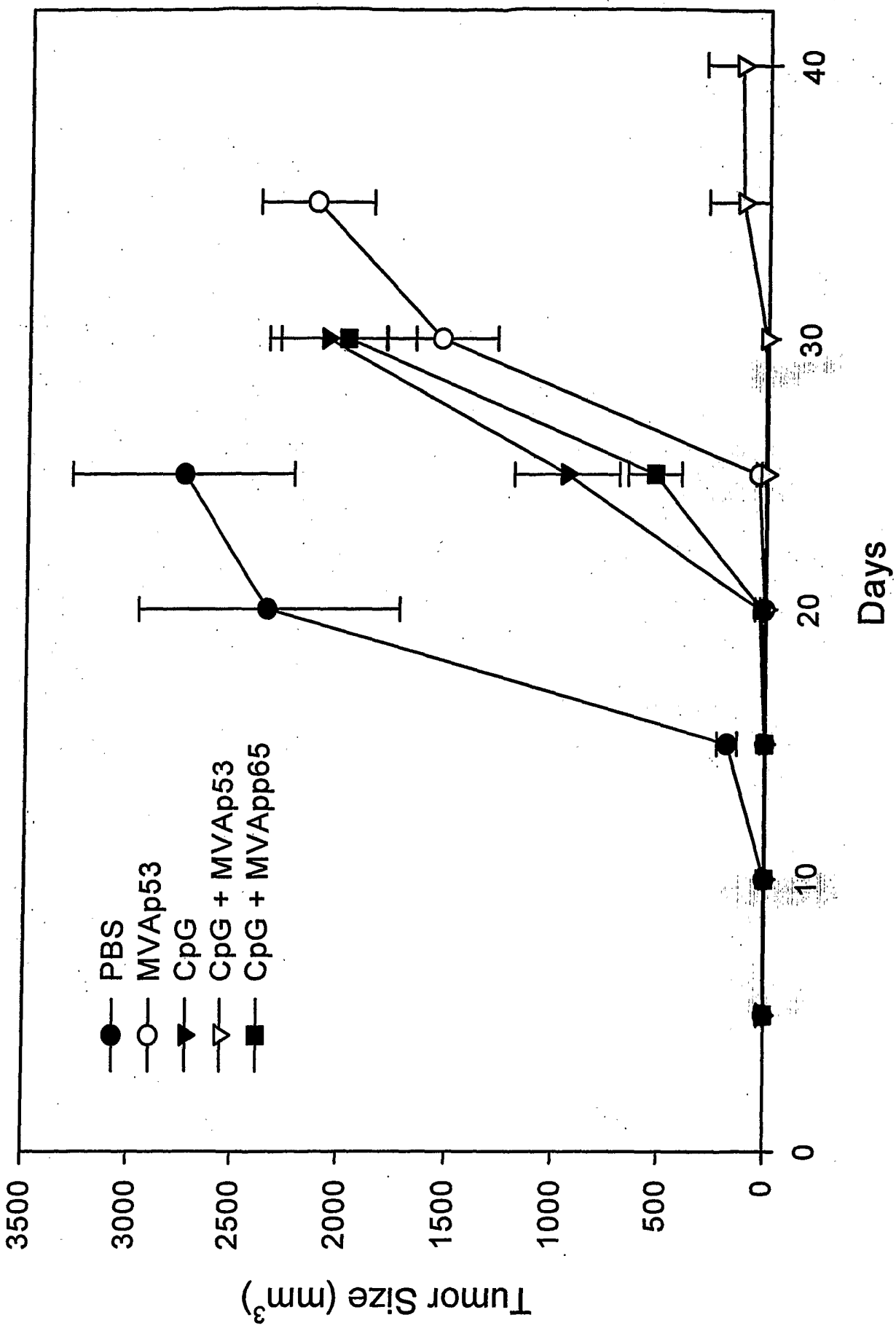
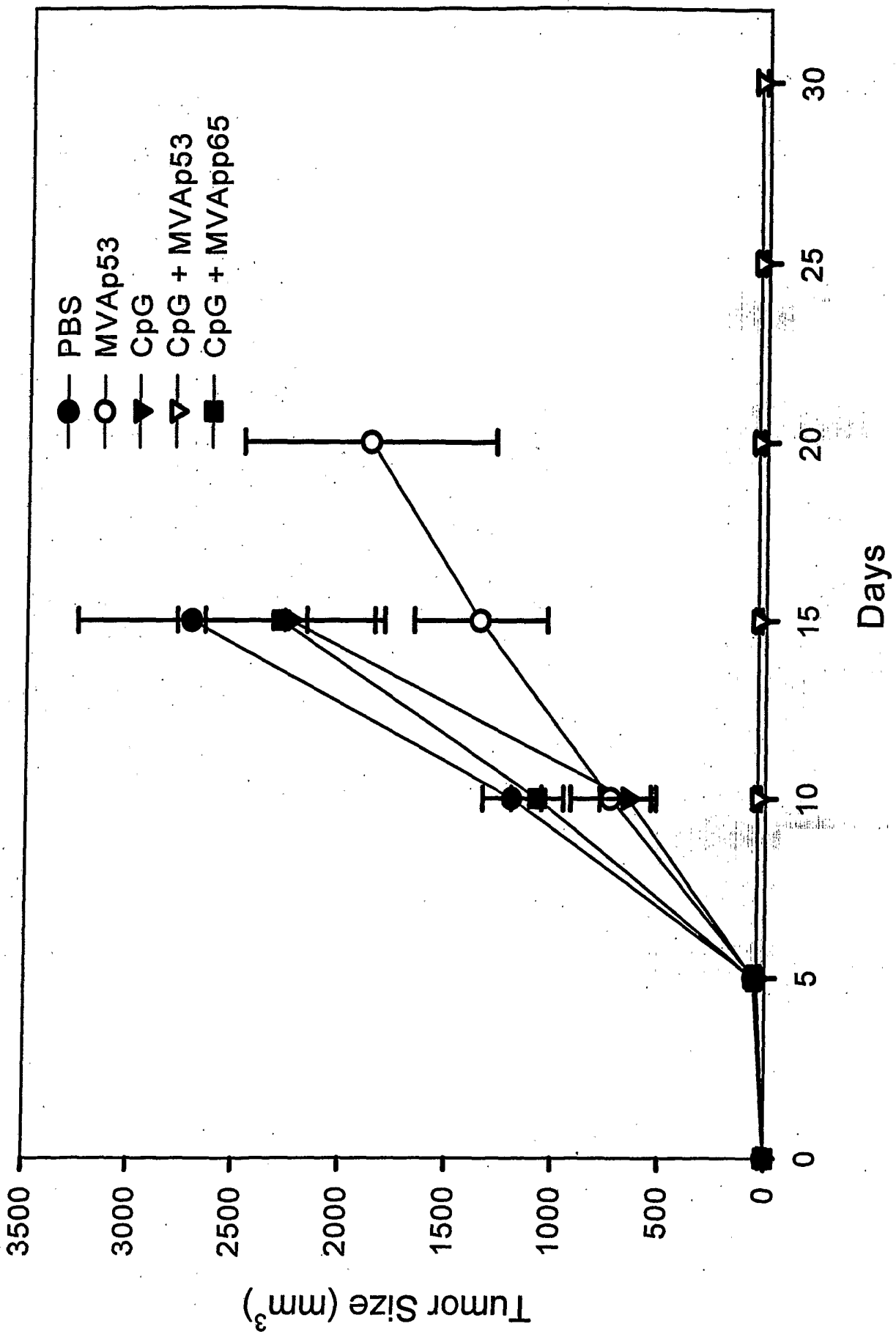

Tumor Size (mm³)
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
Days
PBS
MVAp53
CpG
CpG + MVAp53
CpG + MVApp65

Fig. 8


PBS
MVAp53
CpG
CpG + MVAp53
CpG + MVApp65
Tumor Size (mm³)
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3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Days

Fig. 9

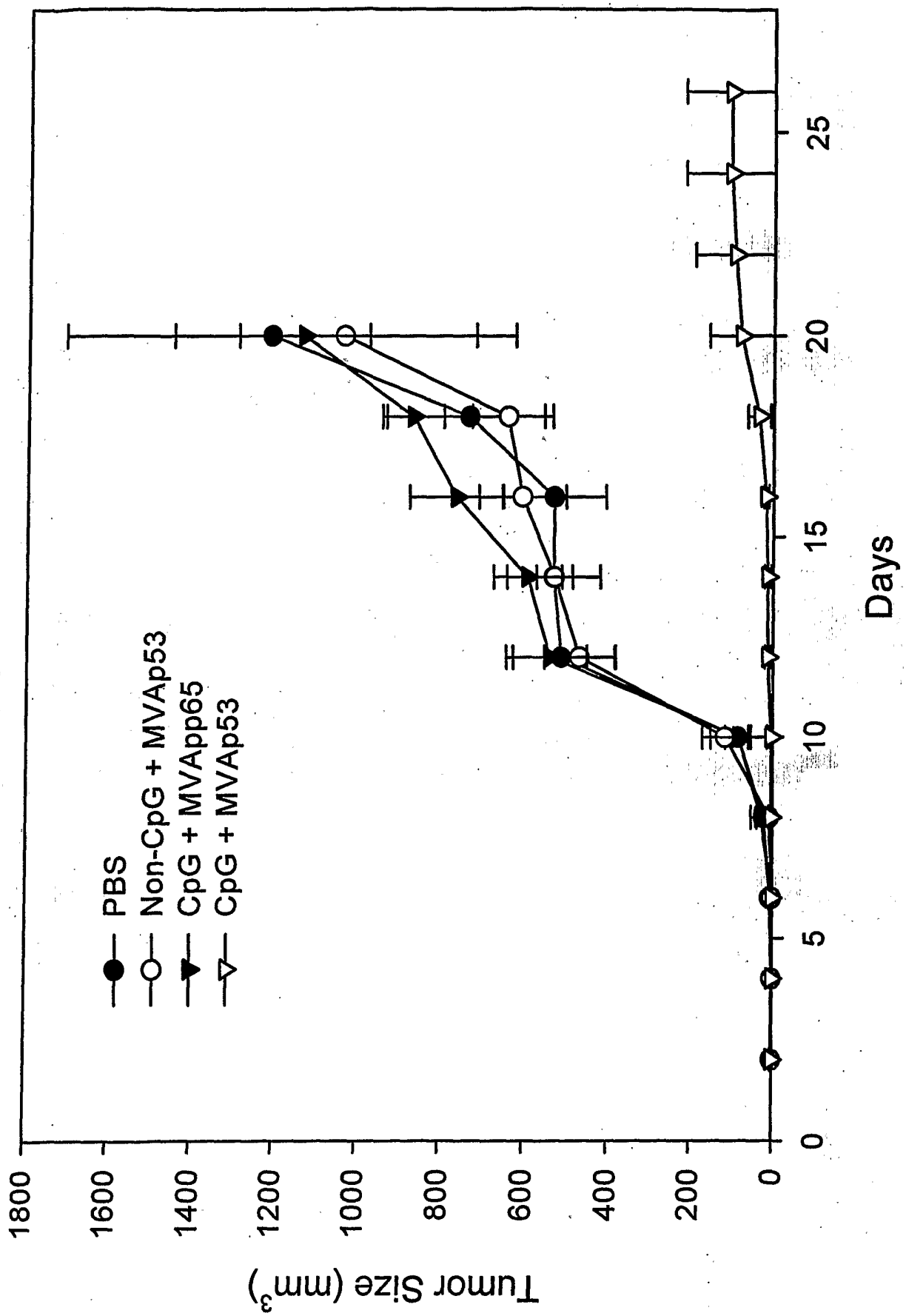

Tumor Size (mm³)
1800
1600
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
Days
PBS
Non-CpG + MVAp53
CpG + MVApp65
CpG + MVAp53

Fig. 10

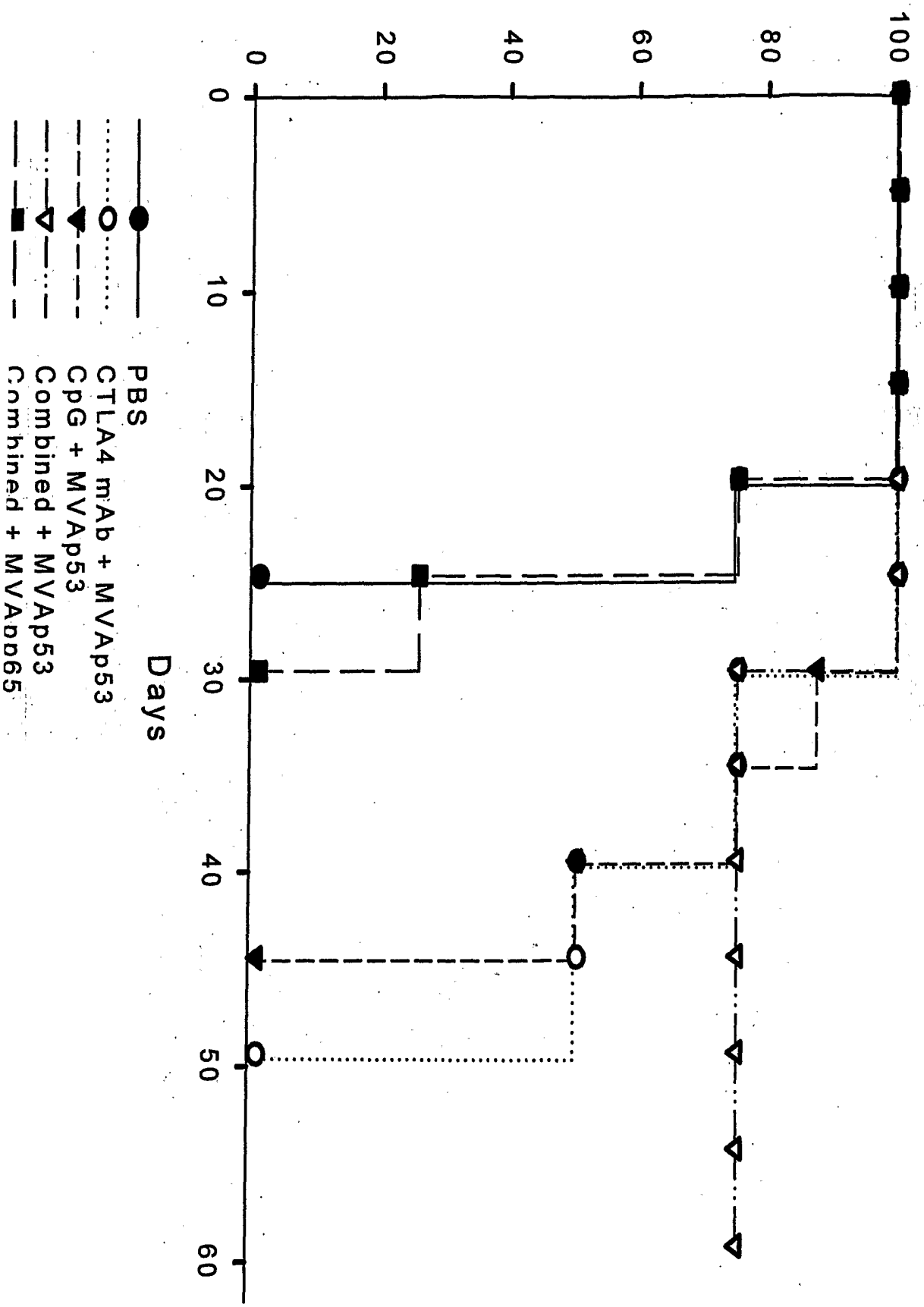

0
20
40
60
80
100
0
10
20
30
40
50
60
Days
PBS
CTLA4 mAb + MVAp53
CpG + MVAp53
Combined + MVAp53
Combined + MVAp65

Fig. 11

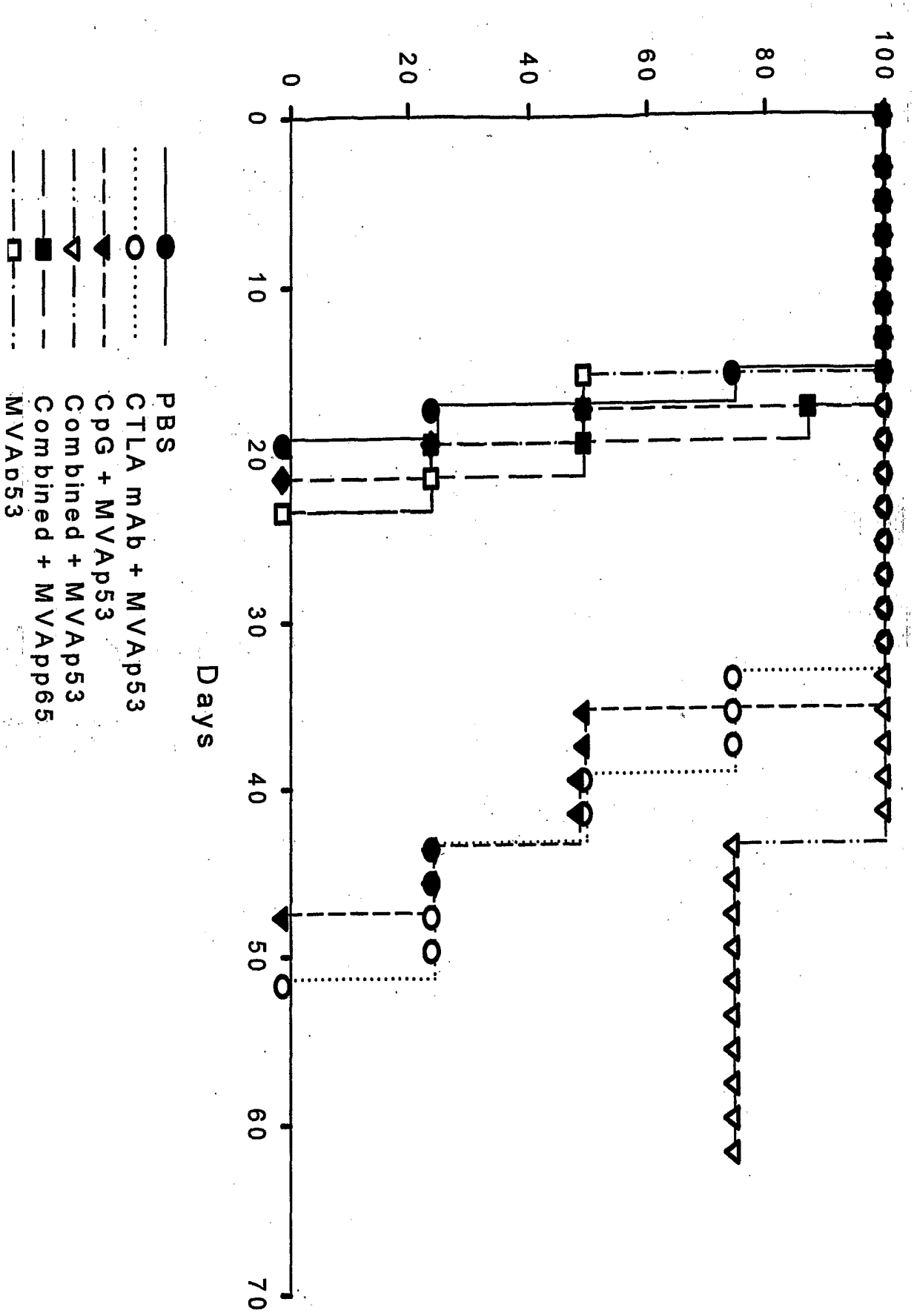
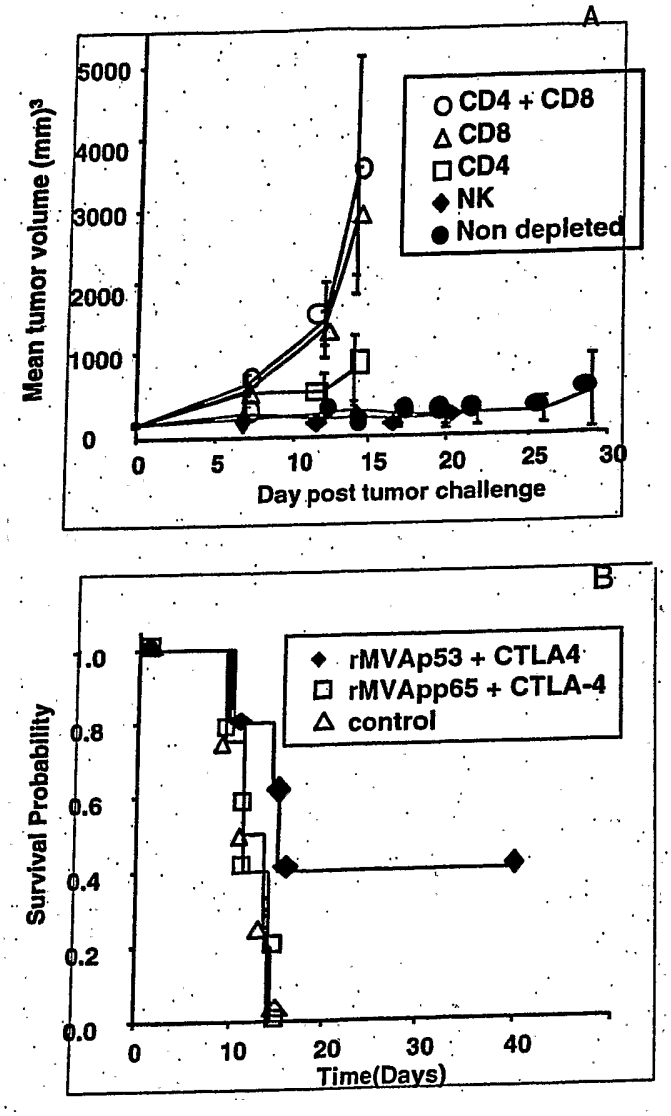
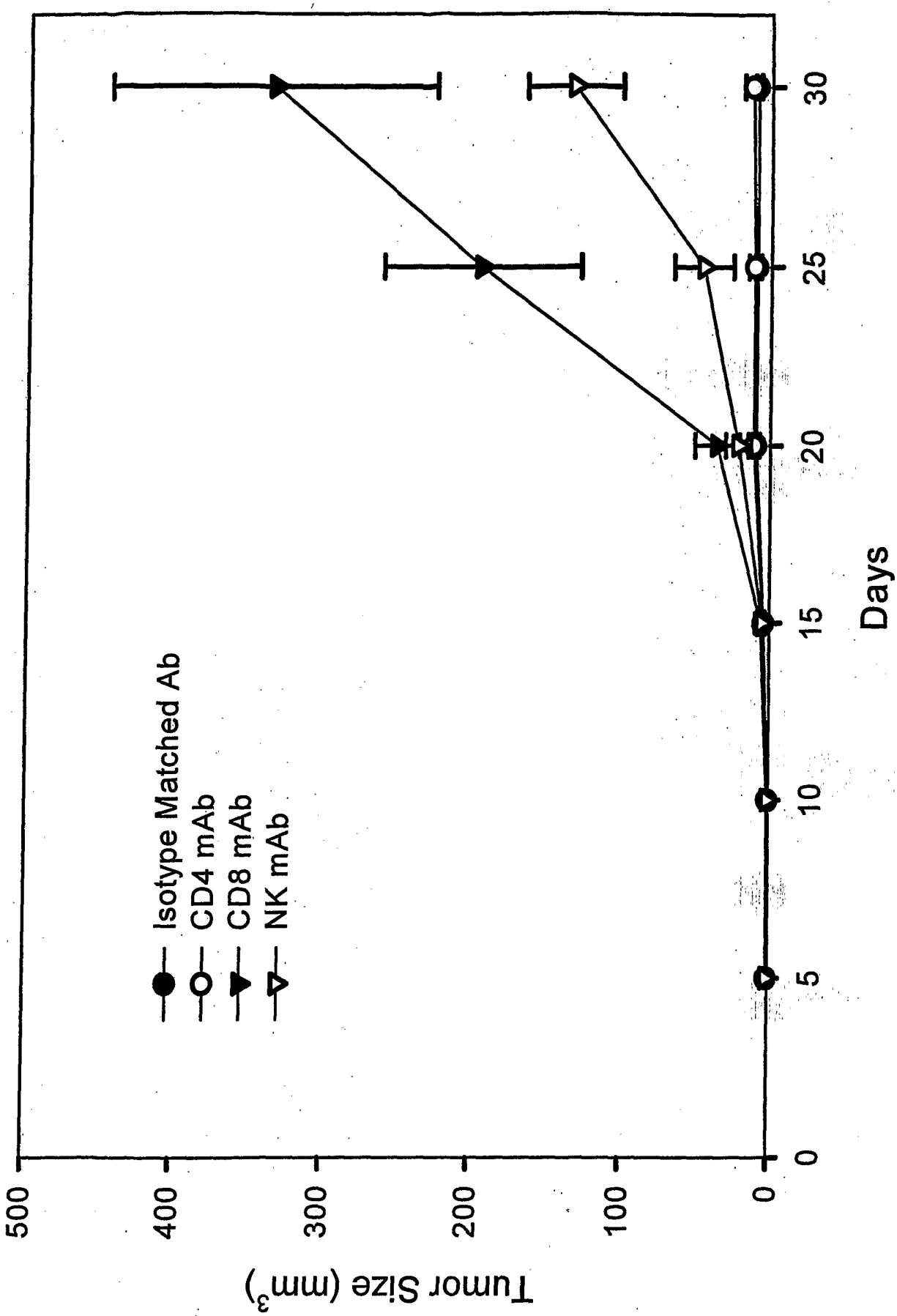

0
20
40
60
80
100
0
10
20
30
40
50
60
70
Days
PBS
CTLA mAb + MVAp53
CpG + MVAp53
Combined + MVAp53
Combined + MVApp65
MVAp53

Fig. 12

Fig. 13


A
Mean tumor volume (mm)3
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
Day post tumor challenge
CD4 + CD8
CD8
CD4
NK
Non depleted
B
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Time(Days)
rMVAp53 + CTLA4
rMVApp65 + CTLA-4
control


Tumor Size (mm³)
500
400
300
200
100
0
0
5
10
15
20
25
30
Days
Isotype Matched Ab
CD4 mAb
CD8 mAb
NK mAb

ig. 14

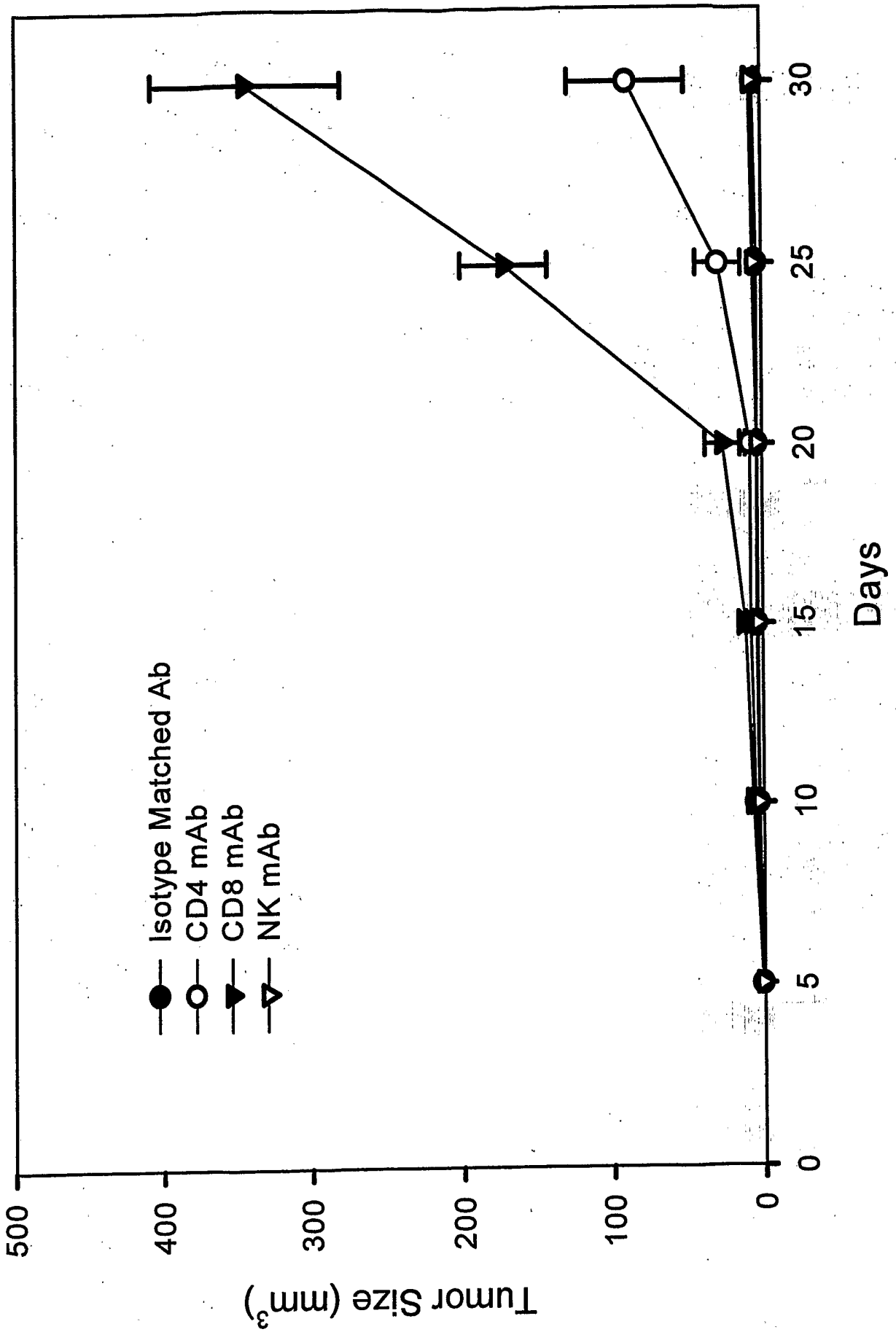

Tumor Size (mm³)
500
400
300
200
100
0
0
5
10
15
20
25
30
Days
Isotype Matched Ab
CD4 mAb
CD8 mAb
NK mAb

Fig. 15

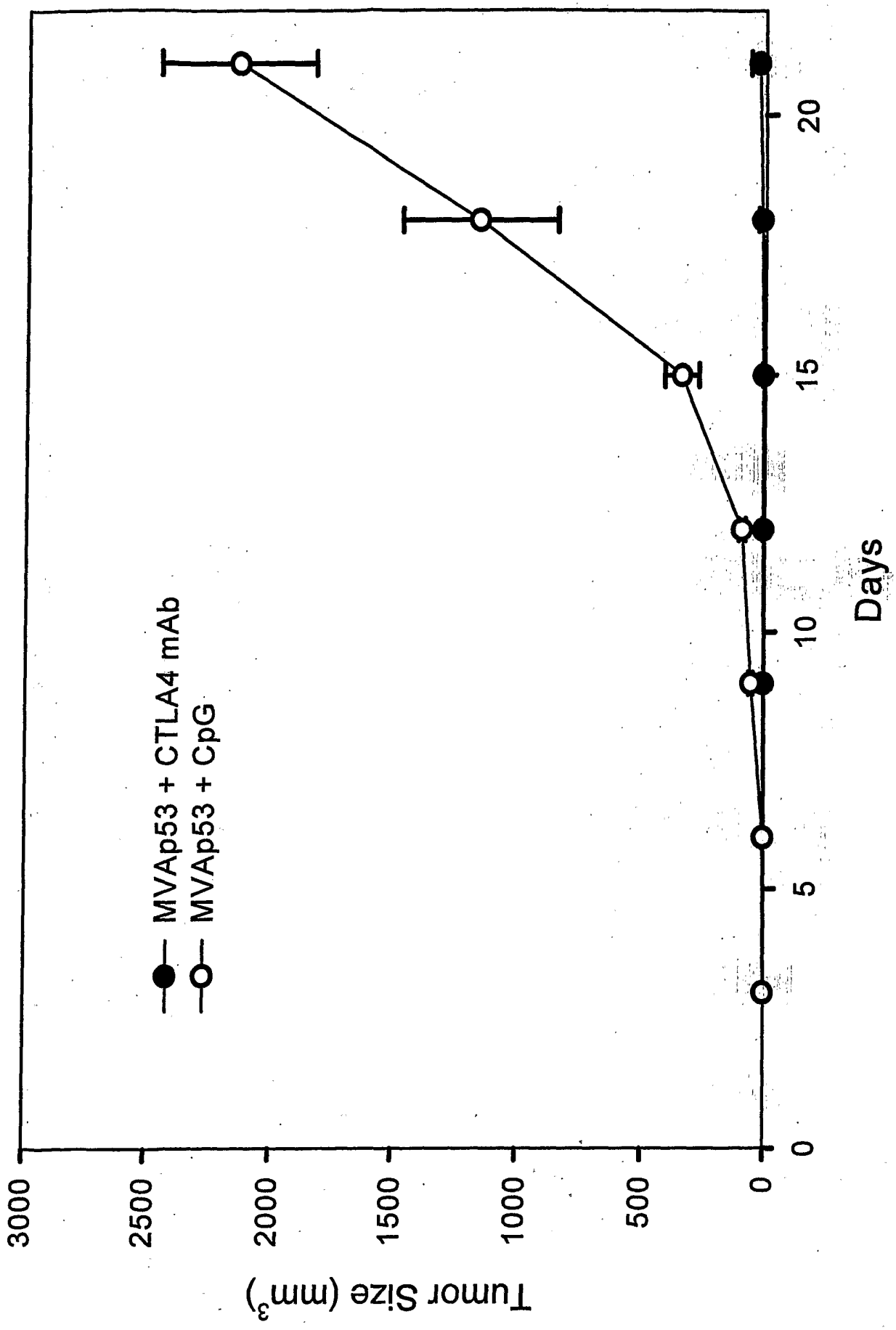

Tumor Size (mm³)
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
Days
MVAp53 + CTLA4 mAb
MVAp53 + CpG

Fig. 16

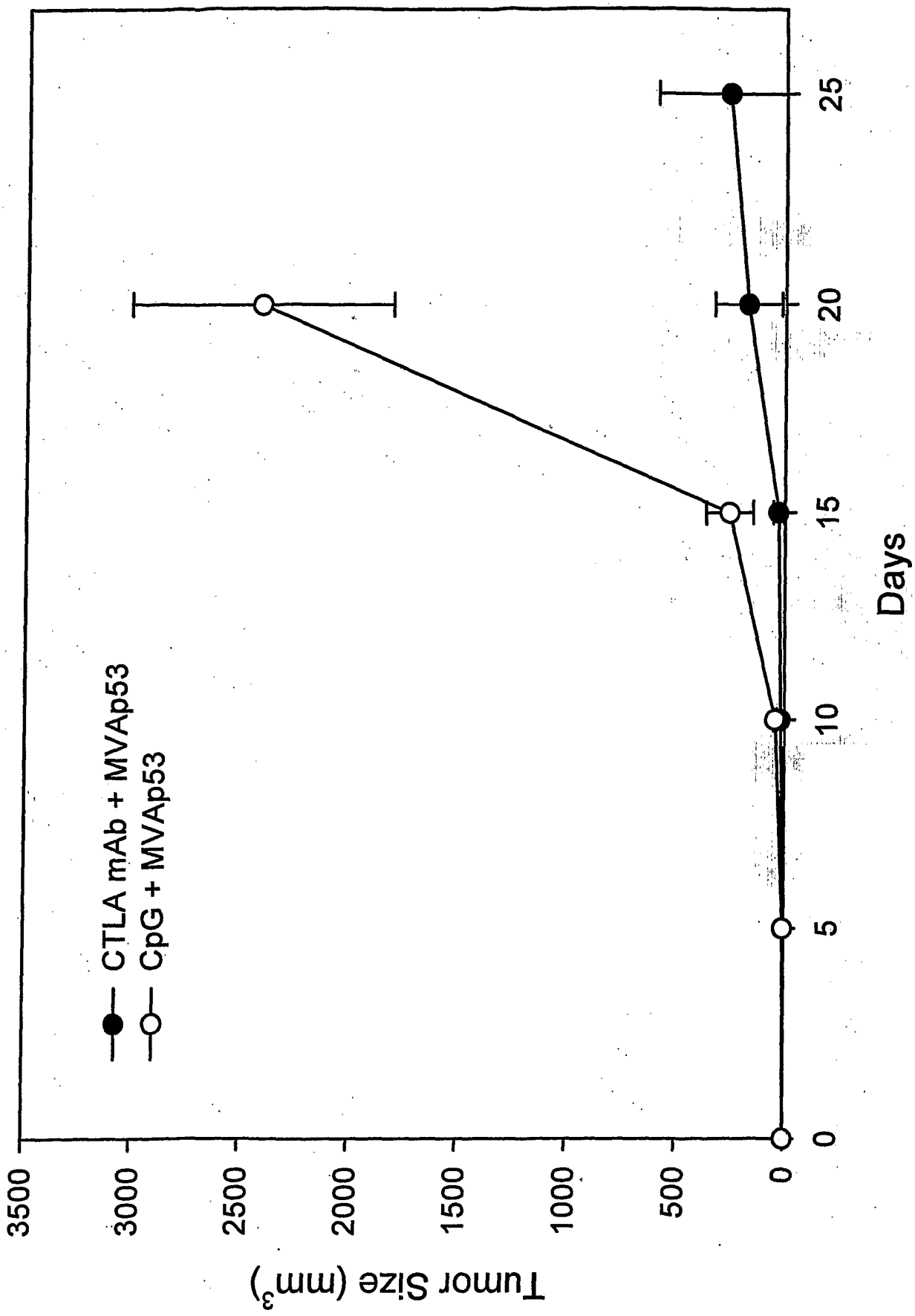

Tumor Size (mm³)
3500
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
Days
CTLA mAb + MVAp53
CpG + MVAp53

Fig. 17

Fig. 18

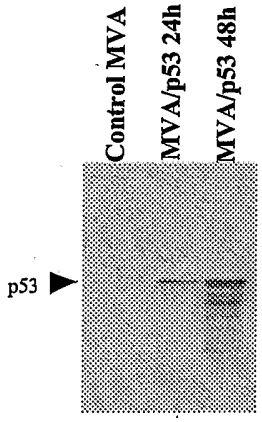
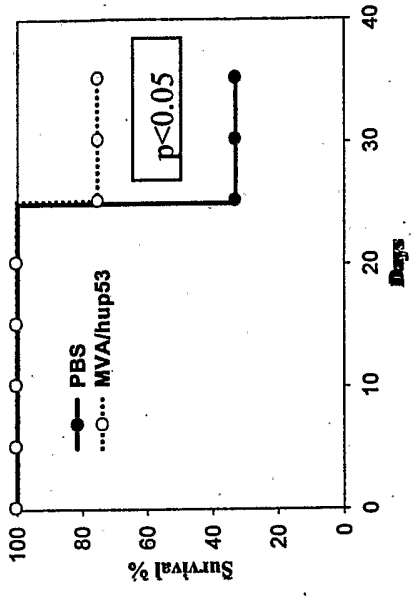


Fig. 19



SEQUENCE LISTING

<110> Ellenhorn, Joshua D.I.
Diamond, Don J.

<120> Modified vaccinia Ankara expressing p53 in cancer immunotherapy

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<150> 06/436,268
<151> 2002-12-23

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