

**Enhancing Cellular Immunogenicity of MVA-vectored Vaccines by Utilizing the *F11L* Endogenous Promoter**

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## Abstract

Modified vaccinia virus Ankara (MVA)-vectored vaccines against malaria, influenza, tuberculosis and recently Ebola virus are in clinical development. Although this vector is safe and immunogenic in humans, efforts remain on-going to enhance immunogenicity through various approaches such as using stronger promoters to boost transgene expression. We previously reported that endogenous MVA promoters such as pB8 and pF11 increased transgene expression and immunogenicity, as compared to the conventional p7.5 promoter. Here, we show that both promoters also rivalled the mH5 promoter in enhancing MVA immunogenicity. We investigated the mechanisms behind this improved immunogenicity and show that it was a result of strong early transgene expression *in vivo*, rather than *in vitro* as would normally be assessed. Moreover, keeping the TK gene intact resulted in a modest improvement in immunogenicity. Utilizing pB8 or pF11 as ectopic promoters at the TK locus instead of their natural loci also increased transgene expression and immunogenicity. In addition to a reporter antigen, the pF11 promoter was tested with the expression of two vaccine antigens for which cellular immunogenicity was significantly increased as compared to the p7.5 promoter. Our data support the use of the pF11 and pB8 promoters for improved immunogenicity in future MVA-vectored candidate vaccines.

## Introduction

Modified vaccinia virus Ankara (MVA) has been used as a viral vector to develop many vaccines against cancer and infectious diseases such as malaria, HIV/AIDS, influenza, and tuberculosis [1-4] and recently Ebola virus infection [5]. It is a highly immunogenic replication-deficient virus with an excellent safety profile that has been evaluated in multiple clinical trials [3]. This vector has been used mainly as a boosting agent in heterologous prime-boost vaccination regimens, to expand primed T cells specific to recombinant antigens [5-7]. Improving MVA immunogenicity has been the goal of many researchers as this would allow dose-sparing with concomitant cost reduction, or potentially allow for single dose rather than prime-boost vaccination [8]. One approach entails using stronger promoters to enhance recombinant antigen expression [9-12]. These must be poxviral promoters because poxviruses have evolved as cytoplasmic viruses, using virally-encoded transcription machinery to recognize their unique promoter sequences [13]. Poxviral promoters used in VACV or MVA vectors have both early and late elements. Indeed, only early gene expression in recombinant MVA (rMVA) correlates with CD8<sup>+</sup> T cell responses towards encoded antigens [9, 14-17], and the majority of CD8<sup>+</sup> T cell-specific epitopes in VACV are products of early genes, controlled by early promoters, in both humans [18] and mice [19]. Therefore, utilizing a strong early promoter has been our focus to improve rMVA as a T cell-inducing viral vector [12].

We have previously reported the use of MVA endogenous early promoters that naturally control the expression of the *B8R* and *F11L* ORFs. Replacing these non-essential ORFs, exactly at their ATG start codons, with a transgene revealed that their respective promoters (pB8 and pF11) were able to drive transgene expression *in vitro* with *in vivo* immunogenicity that was similar to, or higher than that of the

p7.5 promoter [12]. Moreover, an indirect comparison of these promoters with the mH5 promoter suggested they might rival this “benchmark” promoter. Utilizing endogenous promoters is also linked to using their respective ORFs as insertion sites whereas the mH5 promoter, in our previous study, was inserted with a transgene into the thymidine kinase (TK) locus, thereby disrupting the TK gene [12].

Here, we have extended our previous study to investigate the pB8 and pF11 promoters in direct comparison with the mH5 promoter as a benchmark comparative control. We have also investigated the mechanisms behind the improved immunogenicity achieved by using the pB8 and pF11 promoters, in spite of their *in vitro* expression profiles that were relatively lower than p7.5 (in the previous study) and mH5 (in the current study). Notably, our previous studies had compared the use of pF11 or pB8 expressing a recombinant antigen in a TK+ genome compared to p7.5 or mH5 promoters expressing the same antigen in a TK- genome. The TK is involved in MVA genome replication by phosphorylating the 2'deoxy-thymidine (dTd) to produce thymidine 5'-monophosphate (dTMP), which is then converted by thymidylate kinase to thymidine 5'-diphosphate (dTDP), leading eventually to pyrimidine deoxyribonucleotide triphosphate (dTTP) and DNA synthesis [20]. Disruption of TK gene by insertion of a recombinant antigen within the TK locus could reduce genome replication and therefore the overall gene expression, leading to a lower level of recombinant antigen expression in TK- viruses in comparison to TK+ viruses. We therefore sought to test the expression profiles and immunogenicity of rMVAs with endogenous promoters, with intact versus disrupted TK. We also sought to extend our study to test rMVAs with endogenous promoters, which have disrupted TK, and compared these to rMVAs with endogenous promoters inserted at the TK locus (as ectopic promoters). Moreover, given we had noticed a lack of correlation between *in vitro* transgene expression and *in vivo* immunogenicity, we have now evaluated the promoter activity *in vivo* as a better predictor of *in vivo*

79 immunogenicity. We demonstrate higher *in vivo* expression and immunogenicity using the pF11  
80 promoter, as compared to the conventional p7.5 promoter, for two vaccine antigens that are currently  
81 in clinical trials.

82

## Results

### Generation of recombinant MVA viruses

MVA-BAC recombineering technology [21] was used to make two panels of rMVAs, utilizing either the pB8 or pF11 promoters, driving the expression of the tPA-pb9-rLuc transgene. This transgene consists of tPA (human tissue plasminogen activator, as a leader sequence), fused to pb9 (a murine malaria immunodominant H2<sup>d</sup>-restricted peptide from *Plasmodium berghei* circumsporozoite protein), then fused to rLuc (chemoluminescence luciferase protein from *Renilla reniformis*) (Fig. 1). Although Western blot was not performed to evaluate the fused transgene expression, the measurement of luciferase activity would in turn be an assessment of promoter activity while the evaluation of pb9-specific cellular immune responses would detect any enhancement achieved by using different promoter. Taking the pF11 promoter as an example, its panel consists of, first, a rMVA with the endogenous pF11 promoter at its natural context (replacing the *F11L* ORF with the transgene) with an intact TK gene (F11-MVA). Second, the same rMVA but with an inactive TK gene, disrupted by insertion of mCherry gene, a red fluorescent protein (F11-MVA( $\Delta$ TK)). Third, a rMVA with the pF11 promoter inserted at the TK locus as an ectopic promoter plus the transgene, simultaneously disrupting the TK gene (eF11-MVA( $\Delta$ TK)). The same panel of rMVAs was made using the pB8 promoter. These rMVAs were all compared to mH5-MVA, the benchmark control that utilizes the mH5 promoter to control the expression of the transgene at the TK locus (disrupting the TK gene).

### Thymidine kinase effect on rMVA immunogenicity

To determine whether the absence of thymidine kinase could potentially decrease transgene expression, the transgene expression was assessed by luciferase assay. Pairs of rMVAs with the same promoter that differed only in the presence of an intact TK gene were compared to each other, and to the mH5-MVA. pB8 and pF11 are early promoters, unlike the early/late mH5 promoter. Arabinose cytosine (AraC) was thus also used to block DNA replication and assess the early expression only. Supernatants and cell lysates from infected BHK-21 cells were collected, pooled together, and the total luciferase signal was measured to determine the strength of promoter activity. The luciferase expression profiles, when using either pB8 or pF11, were not affected by the disruption of the TK gene (Fig. 2a). The early expression (AraC treated cells) of those two promoters was very similar to their overall activity, confirming they are very strong early promoters. In addition, the early activity of the pB8 was similar to that of mH5. As expected, luciferase expression from mH5-MVA with AraC treatment dropped due to inactivation of its late element. These data confirmed the expected early/late expression activity of each promoter, and showed no impact of TK locus disruption.

To determine the cellular immunogenicity of these rMVAs, mice were immunized and interferon-gamma (IFN- $\gamma$ ) secreting CD8<sup>+</sup> T cells specific to the pb9 peptide were measured by intracellular cytokine staining (ICS). Responses towards MVA-specific F2(G) and E3 peptides were also measured as disruption of TK could be expected to reduce the overall rMVA immunogenicity, not just that of the transgene. First, and unexpectedly, rMVAs utilizing the endogenous pB8 or pF11 promoters elicited significantly higher pb9 transgene-specific CD8<sup>+</sup> T cell magnitudes than mH5-MVA (Fig. 2b). These findings were in stark contrast to the *in vitro* expression profiles (comparing Figs. 2a and 2b), especially for the viruses using pF11, which exhibited lower early transgene expression than mH5. This raised the question of whether *in vivo* transgene expression levels might be the best correlate of immunogenicity,

(investigated later). Second, disruption of the TK gene seemed to modestly reduce the overall immunogenicity of rMVA, but this did not reach significance, measuring the responses to the transgenic pb9 epitope (Fig. 2b) or the MVA-specific epitopes F2(G) and E3 (Figs. 2c,d). However, overall this effect did not reduce immunogenicity levels of rMVA with endogenous promoters to the level of mH5-MVA, suggesting that endogenous promoters *per se* are likely the major driver of improved immune responses to the encoded transgene.

### **Comparing endogenous and ectopic promoters**

To evaluate transgene expression and immunogenicity when using the pB8 and pF11 endogenous promoters outside of their natural contexts (ectopic promoters at the TK locus), rMVAs with ectopic promoters (eB8-MVA( $\Delta$ TK) and eF11-MVA( $\Delta$ TK)) were compared to their counterparts with endogenous promoters (B8-MVA( $\Delta$ TK) and F11-MVA( $\Delta$ TK)) which also have a disrupted TK gene to rule out any effect of TK expression. The comparisons were made with the *in vitro* luciferase expression assay (Fig. 3a) as well as *in vivo* immunogenicity (Fig. 3b). First, F11-MVA( $\Delta$ TK) and B8-MVA( $\Delta$ TK) again showed significantly improved CD8<sup>+</sup> T cell responses against pb9, in comparison to mH5-MVA despite their relatively low activity *in vitro* (especially for pF11 early activity). Second, ectopic promoters showed similar *in vitro* activity and *in vivo* cellular immunogenicity to their endogenous counterparts. These findings implied the usefulness of pB8 and pF11 promoters when used exogenously outside of their native location within the genome. The data also suggested that deletion of the non-essential *B8R* or *F11L* ORFs did not contribute to the improved immunogenicity against the transgene, given these ORFs are intact in rMVA with ectopic promoters (e.g. eF11-MVA( $\Delta$ TK)), but deleted in rMVA with endogenous promoters (e.g. F11-MVA( $\Delta$ TK)) due to their utilization as insertion sites. Importantly, the transgene insertion site did also not appear to have an effect on rMVA immunogenicity. Taken together, these



results suggested that the pB8 and pF11 promoters could result in significantly greater CD8<sup>+</sup> T cell immunogenicity against a transgene than the mH5 promoter, regardless of any other factors discussed above.

#### **Evaluating MVA promoter activity by *in vivo* transgene expression and immunogenicity**

Despite the lack of correlation between *in vitro* expression profiles and *in vivo* immunogenicity, results above suggested that the pB8 or pF11 promoters could induce higher levels of CD8<sup>+</sup> T cells against a transgene than the mH5 promoter. The data also ruled out the effect of non-essential ORF deletion, insertion sites, and the context of endogenous promoters, and we confirmed that presence or absence of TK has only a limited effect on immunogenicity. Therefore we continued to address the question as to why there is a lack of correlation between *in vitro* transgene expression and *in vivo* CD8<sup>+</sup> T cell immunogenicity when using early promoters. An *in vivo* imaging system was thus set up (see materials and methods, supplemental files) to simultaneously assess transgene expression and immunogenicity in mouse models (Fig. 4a). *In vivo* luciferase signals were significantly higher ( $P < 0.05$  by *t*-test) in the F11-MVA vaccinated group than in the mH5-MVA group (Fig. 4b). ICS carried out on spleens from the same animals 7 days post vaccination and imaging revealed a strong correlation between F11-MVA *in vivo* transgene expression, in the first 24 hours post-vaccination, and immunogenicity as compared to those of mH5-MVA (Fig. 4c,d). In contrast to pb9, similar results were again seen in all mice groups for the MVA-specific peptides E3 and F2(G) confirming the effect was specific to the pF11 controlled transgene. The TK role in reducing *in vivo* transgene expression or immunogenicity was not significant, comparing F11-MVA and F11-MVA( $\Delta$ TK) (Figs. 4b,c). Overall, the pF11 promoter proved stronger than mH5 promoter in enhancing *in vivo* transgene expression and immunogenicity.

## Evaluating the pF11 promoter activity with different antigens

Although pF11 promoter enhanced the CD8<sup>+</sup> T cell response as compared to the mH5 promoter, its activity was higher than the mH5 only when they were measured *in vivo*, not *in vitro*. Therefore, we continued to assess the utility of pF11 by evaluating the *in vivo* immunogenicity of different antigens. We selected two advanced candidate vaccine antigens, which have recently been tested in clinical trials; the nucleoprotein-matrix 1 (NP-M1) fusion protein, designed to boost T cell responses to conserved influenza antigens [22]; and the *Plasmodium falciparum* liver-stage malaria antigen METRAP – a multi-epitope string fused to the thrombospondin-related adhesion protein [23, 24]. We evaluated the pF11 activity in comparison to the p7.5, instead of the mH5, because the vaccines in clinical development utilize the p7.5. Immunogenicity was assessed by IFN- $\gamma$  ELISpot performed on splenocytes, collected 14 days post-vaccination in mice. A third group of mice was vaccinated with rMVA, encoding the GFP gene under the control of p7.5 only. The results showed that the F11-NP-M1-MVA induced significantly higher CD8<sup>+</sup> T cell magnitudes than the p7.5-NP-M1-MVA, when measuring the responses to a peptide pool spanning the fused NP-M1 antigens, as used to assess immunogenicity in clinical trials for this vaccine (Fig. 5a). Similarly, immunogenicity of the vaccine candidate p7.5-METRAP-MVA was measured by ELISpot ten days after vaccination of mice. F11-METRAP-MVA induced significantly higher CD8<sup>+</sup> T cell responses as compared to p7.5-METRAP-MVA, as measured using the response to the pb9 epitope, which is part of the multi-epitope string in METRAP. These findings confirmed that the use of pF11 could enhance the transgene immunogenicity of these two leading clinical vaccine candidates.

## Discussion

In this study we aimed to improve MVA immunogenicity by enhancing transgene expression and hence transgene cellular immunogenicity by utilizing strong early poxviral promoters. We have previously shown that endogenous MVA promoters, driving non-essential ORFs such as *F11L* and *B8R*, could drive transgene expression and immunogenicity to similar levels that were achieved with the conventional p7.5 promoter [12]. We have also reported that the mH5 promoter is stronger than the p7.5 for both transgene expression and immunogenicity [12]. Moreover, we have suggested in an indirect comparison that the pF11 promoter, and to a lesser extent the pB8 promoter, might rival the mH5 promoter [12]. In this study, we selected the pB8 and pF11 promoters for further investigation using the mH5 promoter as a benchmark comparative control.

In a direct comparison with the mH5 promoter, both pB8 and pF11 endogenous promoters drove transgene (luciferase) expression to levels similar to that of mH5 at the early expression stage, controlled by AraC treatment. In the untreated cells, the mH5 overall activity (early and late) was higher than either of the endogenous promoters. *In vivo* cellular immune responses were enhanced when pB8 or pF11 endogenous promoters were utilized, suggesting that the CD8<sup>+</sup> T cell responses is more closely associated with early promoter activity, measured by *in vitro* expression, not to the overall (early and late) activity. However, the pF11 in fact drove lower transgene expression *in vitro* but resulted in higher T cell immunogenicity, when compared to the mH5 promoter, thus there remained a distinct lack of correlation between *in vitro* and *in vivo* measurements of promoter activity and immunogenicity respectively. This result led us to hypothesize that there might be other factors in F11-MVA that contribute to the improved immunogenicity, other than the promoter itself. First, we investigated the effect of the TK gene that is present in F11-MVA and B8-MVA, but is disrupted in the mH5-MVA, by

inserting mCherry gene into the TK locus of rMVAs with the endogenous promoters. There were no detectable effects of the TK presence on transgene expression *in vitro*, but our data suggested that the intact TK tended to improve *in vivo* immune responses, presumably by providing more genomic DNA that could serve as template for more transgene expression. This might explain the small decrease in pb9-specific cellular immunogenicity of F11-MVA( $\Delta$ TK) and B8-MVA( $\Delta$ TK) as compared to their TK intact counterparts although this did not reach significance. Second, disrupting the TK gene did not seem to explain the improved immunogenicity of F11-MVA, and to a lesser extent B8-MVA. TK disruption with mCherry would not affect overall MVA vector-specific immunity as our previous work showed that insertion of *Galk* marker, GFP, or BAC DNA did not alter immune responses to MVA-specific E3 or F2(G) peptides (Cottingham, M et al (2008). The insertion of mCherry did not affect MVA immunogenicity either (Cottingham, M.G. and Spencer, A.J., unpublished data). The disruption was preferred to deleting the whole TK gene to mimic the normal utilization of this locus in MVA-based vaccines. We therefore proceeded to evaluate pB8 and pF11 as ectopic promoters inserted into the TK locus. Both transgene expression and immunogenicity of rMVAs with ectopic promoters were similar to their endogenous counterparts. This revealed that the insertion sites (e.g. *F11L* vs. TK) should not affect the immunogenicity of potential vaccines delivered by a rMVA. The use of pB8 or pF11 promoters out of their natural contexts should also not affect the potential utility of MVA endogenous promoters. Moreover, utilizing endogenous promoters in rMVA simultaneously created deletion mutants; for example, the F11-MVA utilizes the pF11 promoter to drive the transgene, which is inserted as a replacement of the *F11L*, thus also generating a *F11L* deletion MVA mutant. Therefore, comparing the F11-MVA( $\Delta$ TK), which lacks the *F11L* ORF, to the eF11-MVA( $\Delta$ TK), which has intact *F11L* ORF should also reveal the effect of deleting these non-essential ORFs on rMVA immunogenicity. Our results suggested that deletion of *F11L* or *B8R* ORFs from rMVA did not significantly affect transgene immunogenicity.

Overall, improved transgene cellular immune responses with the pF11 promoter did not correlate with *in vitro* transgene expression levels. TK deletion had a marginal effect but none of the tested hypotheses fully explained the lack of correlation between *in vitro* transgene expression and *in vivo* immunogenicity of endogenous promoters. However *in vivo* imaging demonstrated that CD8<sup>+</sup> T cell responses correlated perfectly with transgenic protein expression when measured within the same animal (Fig. 4d). Additionally these experiments also confirmed that disrupting the TK gene reduced both transgene expression and immunogenicity although not to significant levels.

A number of MVA-vectored vaccines have been developed at the University of Oxford that are being tested in clinical trials [3, 6, 25]. Two of these are the influenza vaccine candidate, MVA-NP+M1, and the MVA-METRAP malaria vaccine. Both vaccine candidates utilize the p7.5 promoter to drive vaccine antigens. Therefore, we generated rMVAs with each vaccine antigen under the control of the endogenous pF11 promoter aiming to improve the immunogenicity of these vaccine candidates. CD8<sup>+</sup> T cell responses were enhanced significantly by the use of pF11 promoter.

In support of previous reports [26, 27], our data showed that the pF11 and pB8 promoters have solely early elements and their controlled transgene expression was not reduced by AraC treatment, unlike the mH5 promoter. *In vivo* immunogenicity tended to be in an agreement with early expression *in vitro* (by AraC treatment), and correlated well with *in vivo* transgene expression. This *in vivo* transgene expression revealed that the pF11 activity, which is almost solely early, rivalled the overall mH5 promoter activity (early and late) and correlated with improved CD8<sup>+</sup> T cell responses. Taken together, these data identify the strength of early promoters, *in vivo*, as an important determinant of transgene-specific CD8<sup>+</sup> T cell

262 responses. Moreover, *in vivo* immunogenicity should always be assessed when evaluating promoters as  
263 an approach to improve MVA-based vaccines.

264

265 In conclusion, this study showed the applicability of using pB8 or pF11 promoters either at their natural  
266 loci or in a different location (the TK locus). These promoters drove strong transgene expression  
267 comparable (*in vitro*) to, or higher (*in vivo*) than, the mH5 promoter control, and resulted in higher levels  
268 of CD8<sup>+</sup> T cell immunogenicity than the control. Using these endogenous promoters to drive transgene  
269 expression at their original loci also provides novel insertion sites within the MVA genome, which could  
270 be an advantage for making multivalent MVA-based vaccines.

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283

## 284 **Conflicts of interest**

285 SJD, AVSH and SCG are named on patent applications relating to viral vectored vaccines and prime-boost  
286 immunization regimes.

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## Figure Legends

### Figure 1: Schematic representation of different rMVAs using *F11L* locus and pF11 promoter as examples.

**MVA wt:** MVA wild-type genome with highlighted thymidine kinase (TK) locus and the *F11L* ORF, the latter driven by the endogenous (native) pF11 promoter. **F11-MVA:** MVA-BAC recombineering was performed to replace the *F11L* ORF with the transgene (tPA-pb9-rLuc cassette), leaving the pF11 promoter to drive expression of the transgene at the *F11L* ATG start codon. A red fluorescent marker gene (mCherry) was inserted into the TK locus of the F11-MVA to inactivate the TK activity, resulting in **F11-MVA( $\Delta$ TK)**. The transgene was then amplified from the F11-MVA along with 150 nucleotides upstream of the *F11L* ATG start codon, to include the pF11 promoter, flanked with TK homology sequences, and inserted at the TK locus, resulting in **eF11-MVA( $\Delta$ TK)**. The “e” indicates ectopic location of the promoter. The same approach was used to derive viruses with the pB8 promoter. The genome of **mH5-MVA**, used as a comparative control, is also presented. All rMVAs contain a gene of green fluorescent protein (GFP) driven by FP4b poxviral promoter, and a bacterial artificial chromosome (BAC) inserted into the deletion 3 (DelIII) region of the MVA genome.

### Figure 2: Transgene expression and cellular immunogenicity of rMVAs with different promoters, with an intact or disrupted TK gene.

**(a):** *In vitro* transgene expression. BHK-21 cells were infected with rMVAs with the TK locus intact (closed bars) or disrupted (open bars) at multiplicity of infection (MOI) of 1. The mH5-MVA was included as a comparator. Cells were incubated with or without AraC for 24 h as indicated. Promoter activity was

then determined by the level of luciferase expression. Data are shown as mean + SEM. **(b-d)**: BALB/c mice (n=8) were immunized with rMVAs with different promoters driving the tPA-pb9-rLuc transgene at their endogenous loci, in the presence or absence of an intact TK gene. The mH5-MVA has the mH5 promoter at the TK locus (disrupted TK) and was used as a comparator. Seven days post-immunization, intracellular cytokine staining and flow cytometry were performed to determine the percentage of splenic IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells after *in vitro* re-stimulation with **(b)** pb9 peptide, or **(c,d)** with MVA vector-specific peptides F2(G) and E3. Individual data points are shown with line as the median. Data are representative of two independent experiments. Using Kruskal-wallis with Dunn's Multiple Comparison Test, all groups in panel **b** were statistically significant compared to mH5-MVA group, where promoter effects can be studied, while none of the groups that have pF11 promoter was statistically significant compared to mH5-MVA group in panel **c** and **d**. ns: not significant, \* *P* value < 0.05, \*\* *P* value < 0.001.

**Figure 3: Transgene expression and cellular immunogenicity of rMVA with either endogenous or ectopic promoters.**

**(a)**: BHK-21 cells were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of endogenous promoters at their natural loci (open bars), or the same promoters at the TK locus (ectopic promoters, closed bars) at MOI of 1. Cells were incubated with or without AraC, as indicated, for 24 h. Promoter activity was then determined by the level of luciferase expression. Data are shown as mean of 4 replicates + SEM. **(b)**: BALB/c mice (n=8) were immunized with rMVAs with different promoters (endogenous (closed symbols) vs. ectopic (open symbols)). Seven days post-immunization, intracellular cytokine staining and flow cytometry were performed to determine the percentage of splenic IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells after *in vitro* re-stimulation with pb9 peptide. Individual data points are shown with line as the median. Data are representative of two independent experiments. All groups

were statistically significant compared to the control (mH5-MVA) by Kruskal-Wallis test. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

**Figure 4: The effect of TK gene inactivation on transgene expression and immunogenicity of rMVAs *in vivo*.**

**(a):** BALB/c mice (n=8) were immunized with rMVAs as indicated. Two mice were then injected intravenously with 100  $\mu$ L of VivRen substrate at 2, 4, 6, or 24 hours post-immunization, and imaged via the IVIS machine. **(b):** The total flux of photon per second is plotted, with the mean + SEM. The difference in luciferase expression between mH5-MVA and F11-MVA at either 4 or 6 h was significant ( $P < 0.05$ ) by *t*-test. **(c):** Mice, imaged at 2 and 4 h (n=4), were maintained for 7 days, and splenocytes were then harvested to assess cellular immunogenicity, exactly as performed in Figs. 2b-d. The median of each group is shown with statistical differences presented as applied for Figs. 2b-d. **(d):** Mean of values from graph B are plotted against mean of values from graph C (pb9-specific responses) to test the correlation between promoter activity and cellular immunogenicity *in vivo*. The correlation coefficient (*r*) is 1.000, which indicates perfect correlation using nonparametric Spearman correlation test. Data are representative of two independent experiments.

**Figure 5: Cellular immunogenicity of rMVAs with different antigens driven by p7.5 or pF11 promoters.**

BALB/c mice (n=4-5) were immunized with rMVAs with the conventional p7.5 or the endogenous pF11 promoters driving **(a)** the NP-M1, or **(b)** or the METRAP antigens. ELISpot was performed to determine the percentage of splenic IFN- $\gamma$ -secreting cells after *in vitro* re-stimulation with **(a)** NP-M1 peptide pool,

418 14 days post-immunization, or **(b)** with the pb9 peptide, ten days post-immunization. Data for an  
419 irrelevant control rMVA expressing GFP are shown in (A). Individual data points are shown with median,  
420 and are representative of two independent experiments. *P* values by Mann Whitney test are presented.

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