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Smallpox vaccine with integrated IL-15 demonstrates enhanced in vivo viral clearance in immunodeficient mice and confers long term protection against a lethal monkeypox challenge in cynomolgus monkeys

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Abstract
Despite the eradication of smallpox, there is heightened concern that it could be reintroduced as a result of intentional release of Variola major virus through an act of bioterrorism. The live vaccine that was pivotal in the eradication of smallpox though considered a gold standard for its efficacy still retains sufficient residual virulence that can cause life-threatening sequelae especially in immune deficient individuals. Therefore, a safer smallpox vaccine that can match the efficacy of first generation vaccines is urgently needed. We previously reported that the integration of human IL-15 cytokine into the genome of Wyeth strain of vaccinia (Wyeth/IL-15), the same strain as the licensed vaccine, generates a vaccine with superior immunogenicity and efficacy in a mouse model. We now demonstrate that Wyeth/IL-15 is non-lethal to athymic nude mice when administered intravenously at a dose of 10^7 plaque forming units and it undergoes enhanced in vivo clearance in these immune deficient mice. Furthermore, a majority of cynomolgus monkeys vaccinated with vaccinia viruses with integrated IL-15, when challenged 3 years later with a lethal dose of monkeypox virus displayed milder clinical manifestations with complete recovery supporting the utility of Wyeth/IL-15 for contemporary populations as a safer and efficacious smallpox vaccine.

1. Introduction
Global eradication of smallpox in 1977 as a result of a sustained word-wide campaign under the auspices of the World Health Organization represents one of the most significant medical triumphs of the 20th century [1]. Pivotal in this eradication endeavor was the availability of a live vaccine derived from a related Orthopoxvirus vaccinia (Wyeth strain in the US, Lister/Elstree strain in Britain, EM63 strain in Russia, Ikeda strain in Japan and Tian Tan strain in China) with stellar efficacy against smallpox [1,2]. Despite the elimination of smallpox as a natural disease, the etiological agent of smallpox, Variola major virus still remains in more than one designated high security repositories in the world. In addition, the possibility of undocumented existence of V. major virus has long been suspected, fuelling concerns that smallpox could reemerge due to malicious release of this virus in an act of bioterrorism with devastating consequences. In recognition of this potential threat, the US Department of Defense has vaccinated more than 1.5 million individuals in the military vaccination program since December of 2002, and the Department of Health and Human Services has vaccinated close to 40,000 in the civilian first-responder program, along with the establishment of a strategic national stockpile of smallpox vaccines by the US government and many other nations [reviewed in [3]].

The Dryvax vaccine that was used in the smallpox eradication campaigns, despite being efficacious against smallpox still retained residual virulence that contributed to serious adverse complications in a small proportion of vaccinees or their close contacts especially with underlying immunological deficiencies or atopic skin disease [4]. Although, the production of Dryvax vaccine has been discontinued and its license withdrawn, the currently licensed, cell culture grown ACAM 2000 vaccine (Wyeth strain) is
a derivative of the original Dryvax vaccine and has the same con-
traindications as Dryvax and is not recommended for individuals
with immune deficiencies, atopic skin diseases, cardiac disorders or
pregnancy [5]. Based on these recommendations, should mass vac-
cination against smallpox be required as a consequence of bioterror
attack involving smallpox, nearly 25% of the contemporary pop-
ulation would be ineligible for vaccination with the ACAM 2000
smallpox vaccine. Therefore, the development of a smallpox vac-
cine that can match the efficacy and immunogenicity of Dryvax
and yet be devoid of its residual virulence and thus better suited
for contemporary populations with greater numbers of immuno-
deficient individuals, either due to HIV infections or iatrogenic
consequences such as therapy for autoimmune disorders or organ
transplantations, remains a priority.

We recently reported that the integration of IL-15, a cytokine
with pleiotropic immune modulatory activities into the Wyeth
strain of vaccinia virus derived from the Dryvax vaccine resulted
in the development of a smallpox vaccine candidate with supe-
erior efficacy and immunogenicity that out-performed the parental
vaccine in protecting mice when lethally challenged with the neu-
rotropic WR strain of vaccinia virus intranasally [6]. Similarly,
the integration of IL-15 into the modified vaccinia Ankara (MVA),
a weakened vaccinia strain that is under consideration for licen-
sure also displayed enhanced immunogenicity and efficacy [6]. In
continuation with further preclinical development of these IL-15
integrated vaccines, we now demonstrate with in vivo imaging
techniques that the replication competent Wyeth/IL-15 vaccine
we generated, undergoes enhanced in vivo clearance in T-cell defi-
cient nude mice without causing any mortality when administered
intravenously at a dose of 10^2 pfu (plaque forming units), unlike
the wild-type Wyeth strain that causes a progressively fatal infec-
tion in these immune deficient nude mice. Furthermore, when
cyonomolgus macaque (Macaca fascicularis) monkeys were vacci-
nated with a single dose of Wyeth/IL-15 vaccine, the vaccine not
only prevented the death of vaccinated monkeys when challenged
intravenously with a high lethal dose of monkeypox virus (Zaire
79 strain) after a period of three years following vaccination, but
two challenged animals out of three developed fewer than 15
skin lesions, whereas animals vaccinated with wild-type Wyeth,
Wyeth/IL-2 (Wyeth strain of vaccinia with integrated human IL-
2), MVA, MVA/IL-15 or MVA/IL-2 displayed much greater numbers
of pock lesions on their skin. These results further strengthen the
notion that our Wyeth/IL-15 vaccine is superior in both efficacy and
safety than the currently licensed smallpox vaccine and is a better
suited alternative for contemporary populations.

2. Materials and methods

2.1. Recombinant vaccinia viruses

The Wyeth New York Board of Health strain of vaccinia was
obtained from Wyeth Ayerst Laboratories (Marietta, PA). Modi-
ified vaccinia virus Ankara was kindly provided by Dr. Bernard
Moss at the National Institute of Allergy and Infectious Diseases.
The creation of recombinant viruses that express human IL-15
(Wyeth/IL-15 and MVA/IL-15) has been described previously [6].
To create recombinant vaccinia viruses that express human IL-2, a
cDNA clone of human IL-2 was obtained from ATCC (cat# 36673)
and the coding region of the IL-2 gene was excised by digesting with
Pst I enzyme. An 800 bp Pst I fragment carrying the coding segment
of IL-2 was then cloned into a transfer vector that carries vaccinia
hemagglutinin gene segments and E. coli gpt gene derived from the
pTFHA plasmid [7] for the creation of IL-2 expressing recombi-
nant vaccinia in either Wyeth or MVA backbones (Wyeth/IL-2 and
MVA/IL-2 respectively). To create, Wyeth/Luc, the coding region of
luciferase gene was excised from the pGL3 basic vector (Promega
Corp.) and cloned into the same transfer vector described above for
IL-2 recombinants whereas for the creation of Wyeth/IL-15/Luc,
both human IL-15 and luciferase genes (coding segments) with
synthetic vaccinia promoter sequences were placed in a head
to tail configuration in the same transfer vector. In all vaccinia
recombinants used in the present study, heterologous genes were
recombined into the hemagglutinin locus of the respective vaccinia
strain genome. The Wyeth strain of vaccinia and its recombinant
derivatives were grown and titered in a CV-1 monkey kidney cell
line from ATCC while the MVA strain and its recombinant deriva-
tives were grown in a BHK-21 cell line from ATCC.

2.2. Animals and immunizations

Eight to twelve weeks old, female BALB/c and athymic congenic
nude mice (CAnN.Cg-Foxn1nu/Crl) were obtained from the Veteri-
nary Resources Program, National Institutes of Health (Bethesda,
MD) and Charles River respectively. Twenty adult male cynomolgus
macaques (Macaca fascicularis) approximately 10 years of age were
obtained from a NCI sponsored facility colony and were housed
at the NIH primate center during the immunization period, prior
to being transferred to Southern Research Institute, Frederick,
MD for monkeypox challenge experiments. Cell-culture grown vaccine
viruses were purified by sedimentation through a sucrose cushion.
A single dose of vaccine in a volume of 50 μl containing 1 × 10^6 pfu
of virus was administered intradermally in a shaved area between
the two scapulae of the cyonomolgus monkeys. Housing and car-
ing of monkeys were carried out in accordance with the American
Association for Accreditation of Laboratory Animal Care standards
in accredited facilities. The experimental design of this study was
approved by the Institutional Animal Care and Use Committees.

2.3. Intracellular cytokine staining

Freshly isolated, 3 × 10^6 peripheral blood mononuclear cells
(PBMC) were mixed with an equal number of irradiated (3000 rads)
autologous PBMC that had been infected with Wyeth vaccinia at a
multiplicity of infection of 5 for 4 h prior to being irradiated, and co-
cultured in RPMI medium containing 20% fetal calf serum with anti
CD49d (cat# 556634) and anti CD28 (cat# 556620) both at a final
concentration of 0.25 μg/ml. After 4 h of co-culture with irradiated vaccinia
infected cells at 37 °C, Brefeldin A was added (1 μg/ml) to the cultures and incubated for an additional 8 h before stain-
ing for intracellular interferon gamma and surface CD4 and CD8
markers. All antibodies were purchased from Pharmingen and the
background staining was controlled for by using isotype matched
control antibodies. Cells stained with appropriate antibodies were
analyzed on a FACS Calibur instrument (Becton Dickinson).

2.4. Plaque-reduction neutralization test (PRNT)

Pre-immunization and post immunization serum samples were
heat inactivated for 30 min at 56 °C. Anti-vaccinia neutralizing anti-
body titers (80% PRNT) were determined as described previously
[6]. To determine anti-monkeypox virus neutralizing antibody
titers (50% PRNT), serum samples that were serially diluted 2-fold
were mixed (225 μl) with an equal volume of medium containing
2000 pfu of monkeypox virus (Zaire strain) and incubated overnight
at 37 °C, followed by the removal of 100 μl of this mixture and the
addition onto E6 Vero cell monolayers in 24-well culture plates.
After a period of 1 h incubation, cells were overlaid with DMEM con-
taining 2% FBS, 1% carboxy-methylcellulose (Sigma: cat# M-0512).
Each well was then evaluated for the
number of plaques and the reciprocal serum dilution yielding 50% reduction in the plaque count was determined and expressed as the neutralization titer. Each sample was assayed in triplicate.

2.5. In vivo bioluminescence measurements in mice

Vaccinia viruses (1 × 10⁷ pfu) with an integrated luciferase gene were administered via the tail vein in a volume of 50 μl. For imaging of vaccinated infected animals, mice were first lightly anesthetized using isoflurane/O₂ (1.5–5%, v/v) and injected with 100 μl (30 mg/ml solution in phosphate buffered saline) of luciferin intraperitoneally (Caliper Life Sciences, Alameda, CA). Imaging was performed 10 min after the administration of luciferin with an IVIS 100 imager from Xenogen. Overlay images and luminescence measurements were made using Living Image software (version 2.50.1; Xenogen).

2.6. Virus challenge

Monkeypox virus (Zaire 79 MA-104 11/26/03) was the challenge agent used in this study and was obtained from the NIAID Biodefense and Emerging Infections Research Resources Repository. All macaques in Groups 1–7 were administered an intravenous dose of 5 × 10⁷ pfu of monkeypox virus in a final volume of 1 ml.

2.7. Clinical observations

All study animals were observed for clinical illness or changes in behavior twice daily. On study Days 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27, all study animals were monitored for number and quality of pock lesions (lesions were photographed), weight, and body temperature.

2.8. Quantitation of plasma monkeypox viral loads

On study days −1, 0 (day of challenge), 3, 6, 9, 12, 15, 18, 21, 24, and 27, blood samples were collected from all study animals for determination of viral load by Real-Time PCR. DNA was extracted from 200 μl of whole blood using Qiagen DNA mini kits according to the manufacturer’s instructions. Monkeypox virus genomic DNA was measured with the LightCycler Quantitative Panorthopox HA PCR Assay. Primers and probes used for targeting the HA gene are listed below: forward primers: OPHA F89 5′-ATGTACTATCTCAACGTAGTAG-3′, reverse primer: OPHA R219 5′-CTGCAGAACATAAAACTATTAATATG-3′, probe: OPHA-P143S-MGB6FAM AGTGCTTGGTATAAGGAG MGBNFQ. Viral load data are reported as the number of monkeypox virus DNA genome copies per milliliter of blood. The limit of detection for the assay was 5000 viral DNA genome copies per milliliter.

2.9. Statistical analysis

Analysis of variance was used to determine the effect of different immunization protocols on the magnitude of cellular and antibody responses. Student’s t test was used to compare immunization protocols and significance levels were set at a P value of 0.05.

3. Results and discussion

Because the second generation cell-culture grown smallpox vaccine ACAM 2000 displays similar reactivity and residual virulence profiles as the calf lymph derived Dryvax vaccine [5], many approaches are being explored to develop a smallpox vaccine that can match the immunogenicity of Dryvax, but without its residual virulence. The use of strains with deleted putative virulence genes from the genome of the vaccine strain as in NYVAC or the use of spontaneous deletant mutants with attendant attenuation such as the modified virus Ankara (MVA) has been proposed and considered as potential replacements that are more suited for contemporary populations. However, several immunogenicity studies conducted either in non-human primates and/or human volunteers indicate that both NYVAC and MVA are less immunogenic than Dryvax [8,9]. Although, the efficacy of NYVAC or MVA against now eradicated smallpox cannot be determined in humans, challenge studies with monkeypox in vaccinated non-human primates, which is considered as a reasonable model of smallpox in humans have revealed that both NYVAC and MVA are inferior to the protection conferred by Dryvax in this model, thus raising concerns whether these attenuated deletant viruses could confer sufficient protection against smallpox in a situation, where deliberate release of V. major virus in a concentrated fashion occurs as a result of bioterrorism.

3.1. Impact of IL-15 on the replication and clearance of vaccinia virus in immune competent mice

We are of the view that approaches to enhance the engagement of the host immune system to attenuate the residual virulence of the Wyeth strain of vaccinia without reducing the genetic content of the viral genome is more likely to yield a reliable vaccine candidate that can match the efficacy/immunogenicity of the Dryvax vaccine. Recently, we reported the generation of a smallpox vaccine candidate (Wyeth/IL-15) with superior immunogenicity and efficacy as determined by protection against a lethal intranasal challenge with WR vaccinia in vaccinated mice by integrating the human IL-15 cytokine into the hemagglutinin locus of the Wyeth strain of vaccinia derived from the Dryvax vaccine [6]. The only vaccinia gene disrupted in creating this Wyeth/IL-15 vaccine is the hemagglutinin gene, which does not appreciably affect the replication or pathogenicity of the virus unlike the thymidine kinase gene locus that has been widely used for constructing an array of recombinant vaccinia viruses [10]. However, when we assessed the lethality of Wyeth/IL-15 in athymic nude mice, the lethal dose 50 (LD₅₀) of Wyeth/IL-15 was approximately 500–1000-fold higher than the LD₅₀ of parental Wyeth vaccinia virus (data not shown) suggesting that the integration of IL-15 considerably attenuates the in vivo virulence of Wyeth vaccinia. In order to delineate whether the greater LD₅₀ seen with our Wyeth/IL-15 is reflective of diminished in vivo viral replication or alternatively, an indication of enhanced viral clearance, we utilized in vivo imaging to monitor the progression of intravenously inoculated virus by integrating the firefly luciferase gene into the viral genome. In Wyeth/Luc virus, the luciferase gene is integrated into the hemagglutinin locus of wild-type Wyeth virus derived from the Dryvax vaccine, whereas in the Wyeth/IL-15/Luc, the luciferase and human IL-15 genes are integrated into the hemagglutinin locus of Wyeth vaccinia virus in a head to tail configuration. To evaluate in vivo replication and subsequent clearance of these two viruses in immune competent BALB/c mice, 10⁷ pfu were administered intravenously to a group of five mice and in vivo imaging performed longitudinally starting 30 min post administration of the virus at which time point no appreciable signal emanated from the infected mice (see Fig. 1). Of note, it is the luminescence elicited by virus infected cells expressing luciferase, but not free virus in the plasma that gets detected by in vivo imaging. In contrast, 12 h post infection images revealed rampant viral activity in multiple organs. At this period of peak viral activity, the robustness of Wyeth/IL-15/Luc replication was similar if not slightly higher than that of Wyeth/Luc in these immune competent mice. The duration of infection in immune competent BALB/c mice with both Wyeth/Luc and Wyeth/IL-15/Luc vaccinia virus was rather abbreviated with the viruses being cleared almost
with Wyeth/Luc. It should be mentioned that a dose of $10^7$ pfu of vaccinia in mice inoculated with Wyeth/IL-15/Luc over animals inoculated $38$ h post inoculation revealed a clear cut reduction in viral activity that continued for the first $26$ h. But, imaging analysis performed at $72$ h post inoculation in these nude mice and this high viral replicative activity was apparent in the brains of all animals and the viral clearance of the virus from the other organs continued such that at the end of the experiment viral activity was no longer detectable in any of the inoculated animals and only a solitary animal displayed some viral activity in the brain by Day 13 post inoculation (Fig. 2). Yet, even this animal subsequently cleared the viral infection and survived.

This observation underscores the fact that despite the integration of IL-15, Wyeth/IL-15 is not totally avirulent in immune compromised host and the possible existence of host associated variability in the reduction of virulence.

The imaging data shown in Figs. 1 and 2 collectively demonstrate that the integration of IL-15 and its expression had no impact on the initial vaccinia virus replication in vivo. Because of the relatively short and self-limiting infection of Wyeth virus in immune competent mice, the impact of virally expressed IL-15 on the clearance of vaccinia virus was not apparent. However, the progressive course of infection in T-cell deficient nude mice, clearly revealed the impact of virally expressed IL-15 on the course of virus replication allowing the host to limit the progressive spread of the virus and facilitate its clearance along with the resolution of an otherwise fatal vaccinia virus infection in these immune deficient mice.

IL-15 is a cytokine with profound pleiotropic effects on both innate and adaptive immune systems. It is involved in the activation, proliferation and differentiation of CD8+ T-cells and the maintenance of CD8+ memory T cells, in addition to supporting the survival of mature dendritic cells ([12,13]). IL-15 is also pivotal in NK cell function and differentiation, an innate cell component that is implicated in the control and clearance of vaccinia virus ([14,15]).

3.2. Impact of IL-15 on the replication and clearance of vaccinia virus in T-cell deficient nude mice

However, when we administered Wyeth/Luc or Wyeth/IL-15/Luc to T-cell deficient immunocompromised nude mice the course of infection was dramatically different as shown in Fig. 2. Widespread intense viral activity was demonstrable within $2$ h post inoculation in these nude mice and this high viral replicative activity, without any discernible differences between the two viruses continued for the first $26$ h. But, imaging analysis performed at $38$ h post inoculation revealed a clear cut reduction in viral activity in mice inoculated with Wyeth/IL-15/Luc over animals inoculated with Wyeth/Luc. It should be mentioned that a dose of $10^7$ pfu of Wyeth/Luc by intravenous route was lethal to athymic nude mice although the occurrence of death varied from $5$ to $21$ days with loss of body weight and emaciation. Another important finding to emerge from the imaging studies was that in immune competent mice, no appreciable viral activity was detectable in the brain after intravenous administration of the Wyeth/Luc or Wyeth/IL-15/Luc vaccinia throughout the course of infection. In striking contrast, images taken from T cell deficient nude mice $114$ h following the intravenous administration of Wyeth/Luc, but not before, abundant viral activity was apparent in the brains of all animals and the viral replication in the brain continued to intensify although the clearance of the virus from the other organs continued such that at the moribund stage viral activity was almost exclusively confined to the brain in animals given Wyeth/Luc. But, in the Wyeth/IL-15/Luc group, no loss of body weight or deaths occurred in any of the inoculated animals and only a solitary animal displayed some viral activity in the brain by Day 13 post inoculation (Fig. 2). Yet, even this animal subsequently cleared the viral infection and survived.

Despite the eradication of smallpox with a highly efficacious vaccine, the correlates of vaccine mediated protection yet remain to be fully defined. The efficacy assessment and subsequent licensure of a safer smallpox vaccine more suited for our present day populations in not exist as a natural disease any more. The U.S. Food and Drug Administration (FDA) animal efficacy rule that provides a pathway for licensure of vaccines for such diseases mandates testing of a candidate vaccine in more than one sufficiently well characterized animal model that recapitulates the pathophysiology and protective endpoints in humans ([16,17]). Having confirmed the superior immunogenicity, efficacy (see ref [6]) and safety of our Wyeth/IL-
15 vaccine in mice, we proceeded to evaluate the efficacy of this vaccine in a cohort of cynomolgus macaque monkeys (*Macaca fascicularis*) against a high dose lethal intravenous monkeypox virus (Zaire 79 strain) challenge that is considered a valid non-human primate model of smallpox.

A cohort of 20 cynomolgus macaque monkeys were assigned to 7 groups. As shown in Table 1, for vaccination with 6 different vaccine candidates, namely, the wild type Wyeth, Wyeth/IL-2, Wyeth/IL-15, MVA, MVA/IL-2, MVA/IL-15. Three animals that were not vaccinated with any virus but included in the challenge study served as controls. Because MVA is under consideration for licensure and the integration of IL-15 significantly improved the immunogenicity and efficacy of parental wild-type MVA as a vaccine in a mouse model of smallpox as we reported previously[6], we included the wild-type

| Study group | Monkey ID | Anti-vaccinia titer (PRNT 80%) | Anti-monkeypox titer (PRNT 50%) |
|-------------|-----------|-------------------------------|---------------------------------|
|             | 6 Weeks post vaccination | 3 Years post vaccination | Pre-challenge | Post-challenge |
| Wyeth       | CY1990    | 1:25                           | <1:10                         | <1:10          | 1:4593              |
|             | CY1992    | 1:25                           | <1:10                         | <1:10          | 1:1600              |
|             | CY128175  | 1:25                           | <1:10                         | <1:10          |                    |
| Wyeth/IL-2  | CY3856    | 1:50                           | <1:10                         | <1:10          | 1:10,240            |
|             | CY10619   | 1:50                           | <1:10                         | <1:10          | 1:10,240            |
|             | CY66400   | 1:50                           | <1:10                         | <1:10          | 1:10,240            |
| Wyeth/IL-15 | CY19248   | 1:50                           | <1:10                         | <1:10          | 1:7680              |
|             | CY19249   | 1:50                           | <1:10                         | <1:10          | 1:10,240            |
|             | CY19251   | 1:50                           | <1:10                         | <1:10          | 1:10,240            |
| MVA         | CY66393   | 1:200                          | <1:10                         | <1:10          | 1:7314              |
|             | CY128497  | 1:200                          | <1:10                         | <1:10          | 1:10,240            |
| MVA/IL-2    | CY10621   | 1:200                          | <1:10                         | <1:10          | N/A                 |
|             | CY19279   | 1:200                          | <1:10                         | <1:10          | 1:7447              |
|             | CY48447   | 1:200                          | 1:25                          | 1:40            | >1:10,240           |
| MVA/IL-15   | CY3887    | 1:200                          | <1:10                         | <1:10          | 1:1173              |
|             | CY10627   | 1:200                          | 1:25                          | 1:40            | >1:10,240           |
|             | CY66406   | 1:200                          | <1:10                         | <1:10          | >1:10,240           |

Table 1
Study groups with post vaccination vims neutralizing antibody titers.
MVA and its cytokine integrated derivatives in the present non-human primate study for a direct comparison with the replication competent Wyeth virus and its derivatives. The rationale to include IL-2 integrated vaccinia viruses in the present study was based on previous reports indicating that the integration of IL-2 gene into the WR strain of vaccinia results in dramatic attenuation of the virulence of this neurotropic virus [18–21] and the fact that both IL-2 and IL-15 share many biological functions including the augmentation of NK cell activation with enhanced interferon gamma secretion that is pivotal for the control of vaccinia infection [12–15].

IL-2 integrated, attenuated vaccinia viruses have not been evaluated for their potential use as safer smallpox vaccines and therefore we generated two new vaccine candidates in the Wyeth and MVA backbone, Wyeth/IL-2 and MVA/IL-2 by integrating the human IL-2 gene into the hemagglutinin locus of the respective genomes so that we could make direct comparisons between the IL-15 integrated versions versus IL-2 integrated versions for their efficacy and suitability. The key elements in the design of the study that enable us to make direct comparisons among the 6 vaccine candidates included:

(i) a single dose of vaccine containing $10^8$ pfu of virus in a volume of 50 μl given intradermally for all six vaccine candidates tested, (ii) an intravenous challenge with a dose of $5 \times 10^7$ pfu of monkeypox virus (Zaire 79 strain) administered 3 years after the initial vaccination so that vaccine induced immune responses are in the waning phase and thus likely to permit us to detect any subtle differences in the protective responses induced by the respective vaccines.

In animals inoculated with Wyeth and Wyeth/IL-15, the site of inoculation became erythematous and slightly raised around 48 h post vaccination and progressed into classic vesicular lesions with swelling and erythema by Day 4. The lesions became erosive by Day 10 with a diameter of approximately 10 mm with some scab formation as shown in Fig. 3. However, in animals vaccinated with Wyeth/IL-2, the lesions were much milder in intensity with less erythema and swelling consistent with an earlier report indicating a significant reduction in the area of induration in Patas monkeys vaccinated with a WR recombinant virus expressing IL-2 [16]. Complete healing and resolution of the lesions were slightly faster for cytokine integrated versions of Wyeth virus and shown in Fig. 3, are the healed lesions of three macaques vaccinated with Wyeth/IL-15 thirty days post vaccination. Surprisingly, we also noted some induration and erythema at the site of intradermal vaccination with MVA or its cytokine integrated derivatives which appeared within 24 h post vaccination, but lacked the typical characteristics of vaccinia skin lesions with bullae or vesicle formation. These lesions...
resolved within a few days and are likely due to some host inflammatory response to BHK-21 cell components present in the vaccine preparation rather than due to the non-replicating MVA virus itself (data not shown).

Previous reports indicate that monkeys vaccinated with either the Wyeth strain or MVA are protected against a lethal monkeypox virus infection [22–28], although it should be noted that in some of those studies, the lethal monkeypox challenge dose employed was relatively low \((1 \times 10^5\text{ pfu})\) compared to the dose of \(5 \times 10^7\text{ pfu}\) used in the present study. Therefore, to determine an optimal time to challenge the vaccinated monkeys that would permit us to discern any differences, if any, among the six vaccine candidates in their protective efficacy against a lethal monkeypox virus challenge, we considered the data from our own work in mice with these vaccine candidates [6], as well as published reports [22,28] and reached the view that in the late phase of post vaccination when vaccine induced immune responses are waning is most likely to reveal any impact of cytokine mediated enhancement of protective efficacy especially considering the effects of IL-15 on CD8+ memory cells [12,29]. In addition, during the smallpox eradication campaigns in the 1960s, the WHO recommendation was to revaccinate individuals every three years because of the waning immunity at this time [1]. Therefore, we decided to challenge the vaccinated monkeys three years after they have been vaccinated with a single inoculation of the respective vaccine. In the interim, vaccinated monkeys were periodically monitored for their humoral and cell mediated immune responses against vaccinia virus to assess the long term persistence of their immune status.

As shown in Table 1, at 6 weeks post vaccination, vaccinia plaque reduction neutralizing antibody titers (PRNT 80%) were approximately 4-fold higher in the monkeys vaccinated with MVA or MVA derivatives with integrated cytokines compared to their counterparts vaccinated with Wyeth or Wyeth derivatives with integrated cytokines. Interestingly, among the MVA vaccines, the integration of IL-2 or IL-15 had no measurable impact on the antibody levels induced in vaccinated monkeys. In contrast, with the Wyeth vaccines, the integration of IL-2 or IL-15 resulted in a slight increase (2-fold) in the antibody levels that was consistent and reproducible in the early phase of post vaccination. When the serum vaccinia neutralizing antibody levels were re-assessed three years later, except for three animals that displayed low yet detectable levels (a titer of 50 in animal #19251 vaccinated with Wyeth/IL-15, a titer of 25 in animal #48447 vaccinated with MVA/IL-2 and a titer of 25 in animal #10627 vaccinated with MVA/IL-15) in all other 14 animals no vaccinia neutralizing antibodies were detectable at the lowest serum dilution tested (1:10). Similarly, as shown in Table 1, except for the three animals with detectable anti-vaccinia neutralizing antibodies, all other 14 animals also lacked any detectable neutralizing antibodies against monkeypox virus, a closely related orthopoxvirus at 3 years post vaccination, just prior to being challenged with monkeypox virus.

We also evaluated vaccinia-specific CD8+ T cell responses in vaccinated monkeys longitudinally following vaccination. As shown in Fig. 4, all animals vaccinated with the Wyeth viruses displayed measurable vaccinia-specific CD8+ T cell responses and the 3 animals vaccinated with Wyeth/IL-15 as a group had the most robust response 10 days post vaccination. This superior vaccinia-specific CD8+ T cell response induced by Wyeth/IL-15 was statistically significant at 1-month, 2-month and 3-month post vaccination over the parental Wyeth vaccine-induced CD8+ T cell response \((p < 0.05)\). However, when tested at 6 months post vaccination we were unable to detect vaccinia specific CD8+ cells by this assay in any of the vaccinated animals. Of interest, we were also unable to detect any vaccinia-specific CD8+ T cell responses in any of the monkeys vaccinated with the MVA vaccinia viruses, starting at Day 10 post vaccination or anytime thereafter. Our observation of lack of any demonstrable anti-vaccinia CD8+ response following a single dose of MVA administration is consistent with a similar observation in humans who had received a single dose of MVA or a much attenuated response in monkeys [26,30].

Recent reports have indicated long term persistence of both cellular and humoral immune responses in Dryvax vaccinees for decades even after a single vaccination which is in contrast to what we observed here in cynomolgus monkeys perhaps reflecting species-specific differences in the longevity of vaccinia induced immune responses (reviewed in [31,32]). Despite having very low (three animals with low yet detectable anti-monkeypox antibody titers) or no measurable immune activity against vaccinia or monkeypox, at three years post vaccination, when we challenged these monkeys intravenously with a high lethal dose of monkeypox virus \((5 \times 10^7\text{ pfu} \text{ of Zaire 79 strain})\) in a blinded study, a rapid rise (1000–10,000-fold) in serum anti-monkeypox antibody titers was observed in all vaccinated animals (sera collected 21 days post-challenge) except macaque #CY10621 (vaccinated with MVA/IL-2) that succumbed to monkeypox infection before the collection of serum reflecting a robust recall response in the cohort of vaccinated animals in our study (see Table 1). A commercial vaccinia immunoglobulin (Vig) preparation which displayed a PRNT 50% titer between 3413 and 6784 against monkeypox was included in the assay for comparison.

3.4. Post-challenge clinical observations

Post-challenge body temperatures for all study animals were monitored and recorded for the duration of the study as indicated in Table 2. In general, the majority of the animals did not have temperatures greater than 103 F with the exception of macaques #A1345 and #A13465 (both unvaccinated controls). These two animals had temperatures above 103 F at Day 3 post-challenge and returned to acceptable levels by Day 6 post-challenge. Macaque #A13465 (unvaccinated control) did have a slightly elevated temperature at the time of euthanasia (Day 11 post-challenge). But all macaques that progressed to fatal monkeypox disease [macaque #CY10621
3.5. Monkeypox virus lesion counts during the study to overcome dehydration. It should be noted that several animals were placed on fluid support no apparent negative impact on the outcome of the disease course. The end of the study. Even with this pattern of weight loss, there was gained weight at the time of euthanasia. Interestingly, six other animals with lesion counts greater than 200 (TNTC lesions) due to complications from monkeypox virus infection. By Day 27, all vaccinated animals (94%) except one macaque vaccinated with MVA/IL-2 (#CY10621) had resolved their lesions and were protected from monkeypox virus infection, whereas all three unvaccinated control animals succumbed to monkeypox during the study period.

3.6. Monkeypox viral loads in plasma of challenged macaques

Post-challenge quantitative pan-orthopoxvirus DNA genome data are provided in Table 5. The lower limit of detection of viral loads was 5000 DNA genome copies per milliliter of blood. By Day 3 post-challenge, all animals (25%) had detectable viral loads that ranged from \(1.4 \times 10^4\) to \(1.8 \times 10^6\) genome copies/milliliter. It was observed that by Day 6, only four animals [CY19249 (Wyeth/IL-15), CY19251 (Wyeth/IL-15), CY1990 (Wyeth), CY128497 (MVA)] had virus levels below the detection threshold. Interestingly, three of these four animals (CY19249, CY1990, and CY128497) did not have detectable viral loads during the entire course of the study even though they were vaccinated over three years earlier. Four animals were euthanized on the following days: A13345 and CY10621 at Day 9 (viral load: \(7.2 \times 10^6\) at Day 15 and was euthanized due to moribund monkeypox disease. By Day 15 all surviving animals had undetectable plasma viral loads with the exception of #A13778. This animal had DNA copies of \(5.1 \times 10^6\) at Day 15 and was euthanized due to complications from monkeypox virus infection. At Day 27, all surviving animals had subsequently cleared the virus and were euthanized humanely at the termination of the study. An important unanticipated observation was that the lesion count did not exactly correlate with the magnitude of the circulating viremia as manifested by animals #A13778 (Wyeth) and CY128497 (MVA) in whom repeated longitudinal assessment of plasma monkeypox viral DNA revealed no detectable viral DNA above the base-line threshold despite both animals developing more than 200 (TNTC) skin lesions during the post-challenge observation period. Although there are not many reported studies where

| Table 2 |
| Post-challenge body temperature observations (F) for all study animals. |

| Monkey ID | Study day |
|-----------|-----------|
|           | Day –1 | Day 0 | Day 3 | Day 6 | Day 9 | Day 11 | Day 12 | Day 15 | Day 18 | Day 21 | Day 24 | Day 27 |
|-----------|--------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|
| Wyeth     | CY1990 | 101.5| 103.0| 98.8 | 98.6 | 101.9 | –     | 100.2 | 100.3 | 99.9  | 99.4  | 98.9  | 101.1 |
|           | CY1992 | 100.5| 100.7| 101.4| 102.3| 101.1 | –     | 100.7 | 99.9  | 99.7  | 99.7  | 98.9  | 100.9 |
|           | CY128175| 100.5| 100.7| 98.6 | 100.5| 102.3 | –     | 101.8 | 100.7 | 101.3 | 101.0 | 100.0 | 101.4 |
|           | CY3856 | 99.2 | 101.4| 99.4 | 100.7| 100.8 | –     | 99.6  | 99.9  | 99.2  | 98.8  | 96.8  | 99.4  |
| Wyeth/IL-2| CY10619| 98.6 | 101.9| 101.8| 100.2| 100.5 | –     | 100.1 | 100.9 | 98.5  | 98.5  | 97.7  | 100.0 |
|           | CY66400| 100.3| 100.7| 101.1| 100.8| 95.3  | –     | 100.7 | 99.9  | 101.1 | 100.0 | 100.0 | 101.2 |
|           | CY19248| 101.0| 102.6| 101.1| 100.9| 102.0 | –     | 99.5  | 100.3| 101.1 | 99.0  | 100.2 | 101.3 |
|           | CY19249| 101.0| 101.2| 101.1| 100.5| 101.5 | –     | 100.7 | 101.4| 100.3 | 99.4  | 99.5  | 100.7 |
|           | CY19251| 101.1| 102.1| 100.5| 102.3| 101.9 | –     | 100.9 | 101.4| 99.7  | 100.0 | 99.0  | 101.6 |
| MVA       | CY66393| 100.5| 101.2| 101.5| 100.4| 101.6 | –     | 98.8  | 99.3  | 98.4  | 98.7  | 97.8  | 98.9  |
|           | CY128497| 101.3| 100.8| 100.4| 100.0| 101.8 | –     | 100.0 | 101.3| 100.6 | 99.3  | 98.7  | 98.3  |
|           | CY10621| 98.7 | 98.7 | 100.9| 100.5| 99.8* | –     | –     | –     | –     | –     | –     | –     |
| MVA/IL-2  | CY19279| 102.1| 102.7| 101.6| 101.3| 102.4 | –     | 101.3 | 101.3| 99.4  | 99.7  | 98.2  | 101.1 |
|           | CY48447| 100.1| 100.9| 100.3| 98.0 | 101.4 | –     | 100.0 | 99.7  | 99.0  | 98.6  | 97.8  | 98.9  |
|           | CY3887 | 98.6 | 101.1| 100.1| 101.4| 101.5 | –     | 99.2  | 99.3  | 96.4  | 97.5  | 98.1  | 99.7  |
| MVA/IL-15 | CY10627| 100.4| 101.7| 101.2| 100.4| 101.2 | –     | 100.8 | 100.9| 99.9  | 99.5  | 98.7  | 100.5 |
|           | CY66406| 100.3| 101.1| 101.4| 100.6| 100.7 | –     | 100.9 | 101.1| 99.2  | 98.8  | 98.2  | 100.6 |
| Control   | A13345 | 100.8 | 99.5 | 103.7 | 102.1 | 102.9* | –     | –     | –     | –     | –     | –     | –     |
|           | A13465 | 102.2 | 101.2 | 103.7 | 100.8 | 102.9 | 103.3* | –     | –     | –     | –     | –     | –     |
|           | A13778 | 100.8 | 101.1 | 102.8 | 100.1 | 100.3 | –     | 100.3 | 99.4* | –     | –     | –     | –     |

<sup>*</sup>: Not done.  
<sup>a</sup>: Euthanized.
Table 3
Post-challenge weight observations (kg) for all study animals.

| Monkey ID | Study day | Weight range (kg) ±4% |
|-----------|-----------|----------------------|
| Wyeth     | CY1990    | 6.42–6.66            |
|           | CY1992    | 5.90–6.66            |
|           | CY128175  | 7.06–7.66            |
|           | CY8356    | 5.66–6.14            |
| Wyeth/IL-2| CY10619   | 10.28–11.14          |
|           | CY66400   | 8.35–9.08            |
|           | CY128175  | 7.18                 |
| MVA       | CY66393   | 6.60–7.14            |
|           | CY128497  | 6.60–7.14            |
|           | CY10621   | 7.98                 |
| MVA/IL-2  | CY19279   | 10.46–11.23          |
|           | CY66400   | 6.32–6.84            |
| MVA-IL-15 | CY19248   | 6.82–8.26            |
|           | CY19249   | 6.82–7.38            |
|           | CY19251   | 7.94                 |
| Control   | A13345    | 6.57                 |
|           | A13465    | 6.72                 |
|           | A13778    | 6.72                 |

–: Not done. Bold = weight loss greater than 4% of body weight.
* Euthannized.

Table 4
Monkeypox lesion count observations. Lesions counts >200 were categorized as “Too Numerous To Count” (TNTC).

| Monkey ID | Study day | Lesion count |
|-----------|-----------|--------------|
| Wyeth     | CY1990    | 76 TNTC      |
|           | CY1992    | 34 TNTC      |
|           | CY128175  | 33 TNTC      |
| Wyeth/IL-2| CY3856    | 190 TNTC     |
|           | CY10619   | 90 TNTC      |
|           | CY66400   | 38 TNTC      |
| MVA       | CY19248   | 190 TNTC     |
|           | CY19249   | 140 TNTC     |
|           | CY19251   | 12 Scabs     |
| MVA/IL-2  | CY66393   | 58 Scabs     |
|           | CY128497  | 190 Scabs    |
| MVA-IL-15 | CY10621   | 90 Scabs     |
|           | CY19279   | 93 Scabs     |
|           | CY48447   | 165 Scabs    |
| Control   | A13345    | 100 Scabs    |
|           | A13465    | 23 Scabs     |
|           | A13778    | 39 Scabs     |

–: Not done. * Euthannized.
with a larger cohort is necessary. In contrast, the integration of IL-2 did not seem to enhance the efficacy of the Wyeth vaccine. Because the only vaccine break-through that resulted in the death of a vaccinated animal occurred with an IL-2 integrated vaccine (macaque MVA identically with respect to both route (intradermal) and dose for MVA. In the present study, by administering both Dryvax and live-vaccinia virus under the control of bacteriophage T7 RNA polymerase and the lac repressor. J Virol 1992;66(5):2934–42.

Because of the uncertainty as to how correlative these protective studies with other orthopoxviral infections in animal models will be to smallpox caused by *V. major* virus in humans, our focus has proven to be efficacious against smallpox in both pre-exposure and post-exposure field settings. The integration of IL-15 in to Wyeth vaccinia not only attenuates its virulence as shown in Fig. 2 by *in vivo* imaging studies in immune deficient nude mice, but also improves its efficacy as demonstrated in this study in non-human primates against monkeypox disease. These results combined with our previous observation of its superior efficacy against a lethal intranasal vaccinia infection in mice merit its consideration as an effective and safe smallpox vaccine for contemporary populations.

**References**

[1] Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID. Smallpox and its eradication. History of International Public Health. Geneva, Switzerland: World Health Organization; 1988.

[2] Fenner F. Risks and benefits of vaccinia vaccine use in the worldwide smallpox eradication campaign. Res Virol 1989;140:465–6.

[3] Poland GA, Grabenstein JD, Neff JM, Millard JD. Complications of smallpox vaccination, 1968. N Engl J Med 1969;281(22):1201–8.

[4] Lane JM, Ruben FL, Neff JM, Millard JD. Complications of smallpox vaccination, 1968. N Engl J Med 1969;281(22):1201–8.

[5] Greenberg RN, Kennedy JS. ACAM2000: a newly licensed cell culture-based live vaccinia smallpox vaccine. Expert Opin Investig Drugs 2008;17(4):555–66.

[6] Perez PA, Waldmann TA, Mosk JD, Baldwin N, Berzofsky JA. Oh SK. Development of smallpox vaccine candidates with integrated interleukin-15 that demonstrate superior immunogenicity, efficacy, and safety in mice. J Virol 2007;81(16):8774–83.

[7] Alexander WA, Moss B, Fuerst TR. Regulated expression of foreign genes in vaccinia not only attenuates its virulence as shown in Fig. 2 by *in vivo* imaging studies in immune deficient nude mice, but also improves its efficacy as demonstrated in this study in non-human primates against monkeypox disease. These results combined with our previous observation of its superior efficacy against a lethal intranasal vaccinia infection in mice merit its consideration as an effective and safe smallpox vaccine for contemporary populations.

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**Table 5**

| Monkey ID | Study day |
|-----------|-----------|
|           | Day 1    | Day 0 | Day 3 | Day 6 | Day 9 | Day 11 | Day 12 | Day 15 | Day 18 | Day 21 | Day 24 | Day 27 |
| Wyeth     | CY1990   | <5000 | <5000 | <5000 | <5000 | –     | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 |
|           | CY1992   | <5000 | <5000 | <5000 | <5000 | –     | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 |
|           | CY128175 | <5000 | <5000 | <5000 | 7120  | 12,960 | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 |
| Wyeth/IL-2| CY3856   | <5000 | <5000 | <5000 | 99,600| 19,360 | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 |
|           | CY10619  | <5000 | <5000 | <5000 | 59,200| 38,000 | –     | 11,720 | <5000 | <5000 | <5000 | <5000 |
|           | CY66400  | <5000 | <5000 | 15,580| 26,600| <5000 | –     | <5000 | <5000 | <5000 | <5000 | <5000 |
| Wyeth/IL-15| CY19248 | <5000 | <5000 | <5000 | 21,000| 16,540 | –     | 13,060 | <5000 | <5000 | <5000 | <5000 |
|           | CY19249  | <5000 | <5000 | <5000 | 39,000| <5000 | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 |
|           | CY19251  | <5000 | <5000 | <5000 | <5000 | <5000 | –     | <5000 | <5000 | <5000 | <5000 | <5000 |
| MVA       | CY66393  | <5000 | <5000 | <5000 | 24,400| 34,600 | –     | 91,800 | <5000 | <5000 | <5000 | <5000 |
|           | CY128497 | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 | <5000 |
| MVA/IL-2  | CY10621  | <5000 | <5000 | <5000 | 6300  | 9,240,000 | –     | –     | –     | –     | –     | –     |
|           | CY19279  | <5000 | <5000 | <5000 | 20,800| 29,600 | –     | <5000 | <5000 | <5000 | <5000 | <5000 |
|           | CY48447  | <5000 | <5000 | <5000 | 5620  | 12,680 | –     | 16,920 | <5000 | <5000 | <5000 | <5000 |
| MVA/IL-15 | CY3887   | <5000 | <5000 | <5000 | 11,040| 202,000 | –     | 117,400| <5000 | <5000 | <5000 | <5000 |
|           | CY10627  | <5000 | <5000 | <5000 | 10,460| 592,000| –     | 35,600 | <5000 | <5000 | <5000 | <5000 |
|           | CY66606  | <5000 | <5000 | <5000 | 10,580| 21,000 | –     | <5000 | <5000 | <5000 | <5000 | <5000 |
| Control   | A13345   | <5000 | <5000 | 17,0000| 17,000,000| 72,000,000 | –     | 802,000,000 | –     | –     | –     | –     |
|           | A13465   | <5000 | <5000 | 14,020 | 2,400,000 | 17,740,000 | –     | –     | –     | –     | –     | –     |
|           | A13777   | <5000 | <5000 | 1,792,000| 3,320,000 | 38,800,000 | –     | 116,800,000 | –     | –     | –     | –     |

−: Not done.

a Euthannized.
