Systems vaccinology analysis of a recombinant vaccinia-based vector reveals diverse innate immune signatures at the injection site

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Keywords: Poxvirus vaccine vector, vaccinia, systems vaccinology, Sementis Copenhagen Vector.

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Abstract

Poxvirus systems have been extensively used as vaccine vectors. Herein a systems vaccinology analysis of intramuscular injection sites provides detailed insights into host innate immune responses, as well as expression of vector and recombinant immunogen genes, after vaccination with a new multiplication defective, vaccinia-based vector, Sementis Copenhagen Vector. Chikungunya and Zika virus immunogen mRNA and protein expression was associated with necrotic, skeletal muscle cells surrounded by mixed cellular infiltrates. Adjuvant signatures at 12 hours post-vaccination were dominated by TLR3, 4 and 9, STING, MAVS, PKR, and the inflammasome. Th1 cytokine signatures were dominated by IFNγ, TNF and IL1β, and chemokine signatures by CCL5 and CXCL12. Multiple signatures associated with dendritic cell stimulation were evident. By day seven, vaccine transcripts were absent, and cell death, neutrophil, macrophage and inflammation annotations had abated. No compelling arthritis signatures were identified. Such innate systems vaccinology approaches should inform refinements in poxvirus-based vector design.

Introduction

A range of vaccine vector systems based on vaccinia virus (VACV) and other poxviruses have been developed, with several sold as products and many more in development and in human clinical trials (Prow et al., 2018a). These include Modified Vaccinia Ankara (MVA) (Koch et al., 2020; Sutter, 2020), NYVAC (Pantaleo et al., 2019), ALVAC (Laher et al., 2020), fowlpox (Gatti-Mays et al., 2019), LC16m8 (Omura et al., 2018), and raccoonpox (Stading et al., 2017). A large series of recombinant MVA (rMVA) vaccines have been evaluated in non-human primate (NHP) studies (Nagata et al., 2018) and in human clinical trials (Pittman et al., 2019; Prow et al., 2018a), with MVA licensed as a smallpox vaccine (sold as Imvanex/Imvamune). Recombinant poxvirus vector systems have a number of attractive features for vaccine development including a large payload capacity (at least 25,000 base pairs), potential for cold chain-independent distribution, lack of vaccine DNA integration and induction of both cellular and humoral immunity (Prow et al., 2018a). Nevertheless, a range of strategies are being sought to improve immunogenicity and reduce reactogenicity (Albarnaz et al., 2018; Chea et al., 2019; Izzi et al., 2014; Joachim et al., 2020; Koch et al., 2020; Marin et al., 2018). Both these key characteristics of vaccines are largely dictated by the early behavior of the vaccine at the injection site. However, a comprehensive systems vaccinology approach to characterize the post-inoculation injection site responses has not been undertaken for a recombinant poxvirus vaccine.

The Sementis Copenhagen Vector (SCV), derived from the Copenhagen strain of VACV, was recently described (Eldi et al., 2017; Prow et al., 2018b). SCV can replicate its DNA but is rendered unable to generate viral progeny in vaccine recipients by virtue of a targeted deletion of the D13L gene that encodes the essential viral assembly protein, D13. Recombinant SCV vaccines are produced in Chinese Hamster Ovary (CHO) cells modified to express D13 and the host range protein, CP77 (Eldi et al., 2017). A single construct recombinant SCV vaccine encoding the structural gene cassettes of both chikungunya virus (CHIKV) and Zika virus (ZIKV) (SCV-ZIKA/CHIK) was constructed with each polyprotein immunogen driven by the same synthetic strong early late promoter (Alharbi, 2019), but from two distinct distant loci from within the SCV genome (Prow et al., 2018b). A dual ZIKV and CHIKV vaccine was deemed attractive as these virus co-circulate in overlapping geographic regions, and can co-infect both mosquitoes and humans (Prow et al., 2020; Schrauf et al., 2020; Suhrbier, 2019). SCV-ZIKA/CHIK was shown to protect against CHIKV and ZIKV in a series of mouse models (Prow et al., 2018b). In NHPs the vaccine also induced neutralizing antibodies against VACV, CHIKV and ZIKV and provided protection against ZIKV viremia (Prow et al., 2020).

Systems vaccinology uses mRNA expression profiling to gain a detailed molecular understanding of the behavior of vaccines in vivo, thereby informing design and development (Sharma et al., 2019). The
approach has been used to understand and predict immunogenicity (Matthijs et al., 2019; Natrajan et al., 2019), reactogenicity/safety (Gonzalez-Dias et al., 2020; McKay et al., 2019) and adjuvant activity (Ng et al., 2019b; Sarkar et al., 2019). Most systems vaccinology studies have analyzed peripheral blood post vaccination, as this is readily accessible in humans. However, herein we described RNA-Seq and bioinformatics analyses of injection sites after intramuscular (i.m.) vaccination. Adult wild-type were vaccinated with SV-ZIKA/CHIK and muscles were harvested at 12 hours post vaccination to characterize early injections site innate responses and identify adjuvant signatures. As vector-induced cytopathic effects (CPE) are only just beginning (at least in vitro) at 12 hours post infection (Eldi et al., 2017), this was also deemed a suitable time to investigate expression in vivo of both viral vector genes and expression of the recombinant immunogens. Muscle tissue was similarly analysed on day 7 post i.m. vaccination to determine the persistence of vaccine transcripts and characterize the evolution of injection site inflammatory responses at a time when vaccine-induced adaptive immune responses are being generated. This is also the time when inflammatory lesions develop after VACV vaccination (Darling et al., 2014; Frey et al., 2002; Fulginiti et al., 2003; He et al., 2014; Parrino et al., 2007; Talbot et al., 2006; Tian et al., 2017). Finally, feet were harvested on day 7 post vaccination to determine whether SCV-ZIKA/CHIK vaccination might be associated with an arthritic signature, given that a previous live-attenuated CHIKV vaccine induced arthralgia (Edelman et al., 2000). The characterization provided herein of vaccine gene expression and innate host responses at the injection site provide both a process and insights that may inform future endeavors to improve immunogenicity whilst limiting reactogenicity of poxvirus-based vaccine vectors.

Results

RNA-Seq and differential gene expression

Mice were vaccinated i.m with SCV-ZIKA/CHIK or were mock vaccinated with PBS; feet and quadriceps muscles were then harvested at 12 hours and 7 days post vaccination (S1A Fig). Each of the 3 biological replicates comprised pooled RNA from 4 feet or 4 quadriceps muscles from 4 different mice (S1B Fig). Poly-adenylated mRNA was sequenced using the Illumina HiSeq 2500 Sequencer. Per base sequence quality for >93% bases was above Q30 for all samples. The mean total paired-end reads per group ranged from ~19 to 24 million, with >91.6% of reads mapping to the mouse genome (S1C Fig). Five groups were analyzed in triplicate (i) quadriceps muscles from mock vaccinated mice (MQ), (ii) quadriceps muscles from mice vaccinated with SCV-ZIKA/CHIK taken 12 hours post vaccination (SCV12hQ), (iii) quadriceps muscles from mice vaccinated with SCV-ZIKA/CHIK and taken 7 days post vaccination (SCVd7Q), (iv) feet taken from mice mock vaccinated i.m. (MF) and (iv) feet from SCV-ZIKA/CHIK vaccinated mice taken 7 days post vaccination (SCVd7F). Reads were mapped to the Mus musculus genome (mm10) using STAR aligner, with a similar distribution of read counts observed for all samples (S1D Fig). Differentially expressed genes were generated for MQ vs SCV12hQ (for early post-vaccination injection site responses), MQ vs SCVd7Q (for injection site responses on day 7 post-vaccination) and MF vs SCVd7F (to evaluate potential arthritogenic side effects associated with vaccination) (Smear plots are provided in S1E Fig).

Read alignments to the SCV-ZIKA/CHIK vaccine genome

Expression of the vaccine vector and recombinant ZIKA and CHIK immunogen genes was analysed by aligning reads to a combined reference that included mouse, VACV, ZIKV and CHIKV genomes. Given vaccine transcripts can only be expressed in host cells, SCV-ZIKA/CHIK vaccine reads were expressed as a percentage of total RNA sequencing reads mapping to the mouse genome (Fig. 1A; S1A Table). The overall expression profile remained similar when an alternative aligner was used (S2 Fig). The only time at which significant vaccine-derived reads were evident was in quadriceps muscles at 12 hours post vaccination (Fig. 1A; S1A,B Table), suggesting that the vaccine had largely been cleared.
from the injection site by day 7. This is consistent with in vitro data showing that SCV induces
cytopathic effects (CPE) in infected cells within a few days and that SCV is unable to produce viral
progeny (Eldi et al., 2017; Prow et al., 2018a; Prow et al., 2018b). The paucity of vaccine reads in the
feet 7 days post vaccination (Fig. 1A, SCVd7Q) also illustrates that the vaccine does not disseminate to
and/or persist in joint tissues (a potential safety concern; see below). The percentage of reads mapping
to a murine house-keeping gene, RPL13A (Schroder et al., 2010), was similar for the 3 samples from
quadriiceps muscles, and for the two samples from feet (Fig. 1B), illustrating that the low vaccine read
counts for mock and day 7 samples (Fig. 1A) were not due to low read counts generally for those
samples.

A criticism of virally vectored vaccines has been that viral vector transcripts can be so much more
abundant than recombinant immunogen transcripts, resulting in vaccine responses excessively biased
towards vector proteins (Harrington et al., 2002; Tscharke et al., 2005; Wyatt et al., 2017). However,
≈20% of all the SCV-ZIKA/CHIK vaccine reads mapped to the two recombinant immunogens, even
though the ZIKV and CHIKV structural protein sequences in the vaccine genome were relative small
(2067 bp and 3747 bp, respectively), when compared to the large SCV genome (≈190,000 bp). This
perhaps attests to the strength of the poxvirus synthetic strong early late promoter (Alharbi, 2019) used
for CHIKV and ZIKV immunogens in the SCV-ZIKA/CHIK vaccine (Prow et al., 2018b).

Expression of two immunogens in a single poxvirus vector construct carries the risk that one
immunogen is expressed significantly better than the other, a problem encountered in a variety of
settings (Prow et al., 2018a). A comparable number of reads mapped to the recombinant CHIK and
ZIKV inserts (Fig. 1A), with these two inserts distantly separate from each other in the SCV genome
and driven from the same promoter (Prow et al., 2018a). This approach would seem largely to ensure
(at least in SCV-ZIKA/CHIK) that comparable levels of mRNA are produced for each of the two
immunogens.

Reads aligned to the CHIKV and ZIKV genomes were viewed using Integrative Genome Viewer
(IGV) (Robinson et al., 2011). As expected, reads mapped to prME and C-E3-E2-6K-E1, which are
encoded by SCV-ZIKA/CHIK; but not ZIKV capsid nor the non-structural proteins from both
arboviruses (NS1-5 and nsP1-4), which are not encoded by SCV-ZIKA/CHIK (Fig. 1C). Premature
immunogen termination has been described previously for a VACV-based vaccine (Earl et al., 1990),
with VACV transcription occurring in the cytoplasm (Moss et al., 1991). No evidence for premature
termination of SCV-ZIKA/CHIK immunogen transcription was apparent (Fig. 1C).

Read alignments to genes encoded by SCV are described and annotated in detail in S1B Table, with
immune-modulation and cell-death modulating proteins highlighted, along with annotations regarding
their activity in mice and their activity in the Copenhagen strain of VACV, from which SCV was
derived. Many of these genes are referred to below.
Figure 1. Read alignments to the viral genomes. (A) RNA-Seq reads from each of the five groups aligned to the three viral genomes (the vector, SCV, and the two recombinant immunogen inserts from ZIKV and CHIKV); MQ - quadriceps muscles from mock vaccinated, SCV12hQ mice quadriceps muscles from SCV-ZIKA/CHIK vaccinated mice 12 hours post vaccination, SCVd7Q - quadriceps muscles from SCV-ZIKA/CHIK vaccinated mice taken 7 days post vaccination, MF - feet from mock vaccinated mice 7 days post vaccination, and SCVd7F - feet from SCV-ZIKA/CHIK vaccinated mice 7 days post vaccination. The number of viral reads is expressed as a percentage of the number of reads mapping to the mouse genome, with 3 biological replicates providing the SD (Supplementary Fig. 1). The bars plotting to ≈0% had values ranging from 0 to 3.5x10^{-5}%. (B) RNA-Seq reads from each of the five groups aligned to the housekeeping gene, RPL13A, also expressed as a percentage of the number of reads mapping to the mouse genome. (C) IGV visualization of reads aligned to the recombinant structural polyprotein immunogens of ZIKV (prME) and CHIKV (C-E3-E2-6K-E1), which are encoded in the SCV-ZIKA/CHIK vaccine. All reads from all replicates are shown (for details see (S1A Table). As expected, no reads mapped to the non-structural genes of ZIKV or CHIKV (NS1-5 and nsP1-4, respectively), as these are not encoded in SCV-ZIKA/CHIK. (Vertical purple lines for ZIKV indicate base call errors after a string of Gs). (Reads mapping to the SCV genome are shown in S1B Table). (D) H&E staining of injection site 12 hours post vaccination. Dotted ovals indicate muscle cells in early stages of necrosis (pink staining). (E) Top: IHC with anti-Ly6G staining for neutrophils (parallel section to D focusing on area of infiltrates). Bottom: Apoptag staining of the same area, illustrating apoptosis within areas of infiltrating cells. (F) Top: IHC for CHIK capsid protein. Bottom: parallel section showing IHC for ZIKV E protein. (G) Cell death annotation from Cytoscape analysis of up-regulated DEGs at 12 hours post vaccination (MQ vs SCV12Q) (S2C Table) divided into non-apoptotic signatures (left) and apoptotic signatures (right). KEGG Pathways (purple), Go process (black), Reactome Pathways (brown), UniProt Keywords (green).
**Injection site histology and immunohistochemistry at 12 hours post vaccination**

H&E staining of the intramuscular injection sites showed that some skeletal muscle cells displayed fragmented pale cytoplasm with loss of striation and small condensed pyknotic nuclei indicative of necrosis (Fig. 1D, dotted ovals). These necrotic cells were partially surrounded by mixed inflammatory cells infiltrates (high densities of purple nuclei) and some cellular debris (Fig. 1D).

Immunohistochemistry (IHC) with a neutrophil-specific marker, anti-Ly6G (Poo et al., 2014; Prow et al., 2019), illustrated that the infiltrates contained abundant neutrophils (Fig. 1E, top panel, Ly6G).

Interestingly, neutrophils have recently been shown to contribute to adjuvant activity (Stephen et al., 2017). The infiltrates also contained areas staining with Apoptag indicating apoptosis (Fig. 1E, parallel section, bottom panel, Apoptag).

IHC with monoclonal antibodies recognizing CHIKV capsid (5.5G9 (Goh et al., 2015) and ZIKV envelope (4G2) (Hobson-Peters et al., 2019), clearly illustrated expression of vaccine antigens in skeletal muscle cells 12 hours post infection (Fig. 1F). The spherical/oval cytoplasmic staining patterns likely reflect the well described cytoplasmic factories wherein the poxvirus coordinates protein expression and subjugates host functions (Katsafanas et al., 2007; Kieser et al., 2020; Paszkowski et al., 2016). These results illustrated that the immunogen mRNA expression seen in Fig. 1A and C translates into protein expression in muscle cells in vivo.

**Injection site host cell death signatures at 12 hours post vaccination**

The mode of cell death for a host cell expressing vaccine immunogens can have important implications for immunogenicity, with necrosis often favored over apoptosis (Chea et al., 2019; Gargett et al., 2014). RNA-Seq analysis of the mouse i.m. injection sites 12 hours post vaccination (MQ vs SCV12hQ; full gene list in S2A Table) provided a set of differentially expressed genes (DEGs) (S2B Table; FDR or q ≤0.01, fold change ≥2 and sum of all counts across the six samples >6). The upregulated DEGs (n=1390; S2B Table) were analyzed by Cytoscape (S2C Table), with cell death terms suggesting the presence of apoptosis, necrosis and necrosis (Fig. 1G). Skeletal muscle cells are generally resistant to apoptosis (Schwartz, 2008, 2018) and VACV’s apoptosis inhibitor, B13R (Chea et al., 2019), was also expressed at the injection site (S1B Table). Skeletal muscle cells have recently been shown to be able to undergo necroptosis (Morgan et al., 2018), with skeletal muscle necrosis well described (Lentscher et al., 2020; Szugye, 2020). The apoptosis signatures (Fig. 1G) may largely be associated with infiltrating leukocytes such as neutrophils, which are highly prone to apoptosis (Soehnlein et al., 2010), as Apoptag staining was clearly present in the aforementioned infiltrates (Fig. 1E, Apoptag). MVA can induce apoptosis in vitro and in certain settings in vivo (Chea et al., 2019; Torres-Dominguez et al., 2019) and SCV can induce apoptosis (at least in vitro, S3 Fig); however, the mode of cell death elucidated in vitro may not be recapitulated in primary skeletal muscle cells in vivo.

**Large Toll-like receptor signatures at 12 hours post vaccination**

The up-regulated DEGs (for MQ vs SCV12hQ; S2B Table) analyzed as above by Cytoscape returned multiple terms associated with innate immune responses (S2C Table). To provide insights into the early innate host immune responses and potential adjuvant signatures induced by SCV-ZIKA/CHIK vaccination, the full DEG list (1608 genes) for MQ vs SCV12hQ (S2B Table) was analyzed by Ingenuity Pathway Analysis (IPA) using the Up-Stream Regulator (USR) function and the direct and indirect interaction option. The list of USRs (S2D Table) illustrated a highly significant Toll-like receptor (TLR) signature, dominated by TLR3 and 4, followed by TLR9, 7 and 2 (Fig. 2A). Although other TLRs (TLR1, 5, 6, 7/8, 8) were also identified, the number of unique DEGs responsible for these annotations was low (Fig. 2A, numbers in brackets), arguing these were less reliable USRs as they...
arose from subsets of DEGs already used in the annotations for TLR3, 4, 9, 7 and/or 2 (S2D Table, Target molecules in dataset). Given the common signaling pathways used by all TLRs, primarily involving MyD88 and/or TICAM1/TRIF, overlap in genes induced via the different TLRs would be expected.

The identification herein of TLR4, TLR3, TLR9, TLR7 and TLR2 signatures (Fig. 2A) is notably consistent with the literature on poxvirus infections in GMO mice (summarized in S2E Table). TLR4 is stimulated by an unknown ligand present in/on VACV particles, with TLR4 required for effective antiviral activity and protection against mortality in mice after VACV infection (Hutchens et al., 2008b). TLR3 stimulation is likely mediated by dsRNA derived from the abundant complementary RNA transcripts produced late in the VACV infection cycle (Wolferstatter et al., 2014). TLR3 stimulation in VACV-infected mice promotes inflammatory cytokine production and immunopathology (Hutchens et al., 2008a). TLR7 (which detects ssRNA) is expressed on plasmacytoid dendritic cells and B cells, with TLR7 and TLR9 important for type I interferon secretion by dendritic cells following fowlpox infection (Lousberg et al., 2011). TLR9 is required for survival of mice following ectromelia virus infections (Samuelsson et al., 2008; Sutherland et al., 2011) and is likely stimulated by viral unmethylated ssDNA containing CpG motifs (Li et al., 2010) and/or mitochondrial DNA (Wang et al., 2017) released by viral CPE. TLR2 stimulation during VACV infection in mice has minimal impact on viral replication (Davies et al., 2014), but does promote NK activation and CD8 T cell expansion and memory (Martinez et al., 2010; Quigley et al., 2009). Thus both SCV and VACV would appear to stimulate TLR2, whereas MVA is reported not to do so (Price et al., 2014). To the best of our knowledge, there is no literature suggesting a role for TLR1, 5, 6 or 7 in poxvirus infections, consistent with the low number of unique DEGs for these annotations (Fig. 2A). The role of TLR8 in VACV infections remains controversial (Bauer et al., 2010), with TLR8 nonfunctional in mice (Ng et al., 2019a).

VACV produces an inhibitor of TRIF, MYD88, TRAM and MAL, called A46 or VIPER (encoded by A46R), a protein reported to be active in murine systems (Lysakova-Devine et al., 2010). However, A46 is expressed in the cytoplasm of SCV-infected cells and not in neighboring uninfected cells that may also express TLRs. Such cells might sense TLR agonists comprising viral pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) released by SCV infection-induced CPE (Eldi et al., 2017; Ink et al., 1995; Tsung et al., 1996).
Figure 2. TLR and cytosolic sensor signatures at 12 hours post vaccination. (A) TLR signatures identified by IPA USR analysis (S2D Table) of 1608 DEGs identified in quadriceps muscles 12 hours post SCV-ZIKA/CHIK vaccination (MQ vs SCV12hQ; S2B Table). Circle diameters reflect the number of DEGs associated with each IPA USR annotation. Numbers in brackets indicate the number of unique DEGs associated with each annotation over the total number of DEGs associated with the TLR annotation; circles with colored fills contain >3 DEGs uniquely associated with the indicated TLR annotation. A46R is expressed in the cytoplasm of infected cells. (B) Cytosolic sensor signatures identified by IPA USR analysis (S2D Table). Sensors divided into 3 categories associated with dsRNA (red circles), DNA (blue circles) and inflammasome activation (black circles). Circle diameters reflect the number of DEGs associated with each annotation. VACV genes encoding cytoplasmic inhibitors are shown in blue, with the black cross indicating that the gene/protein is not present or not functional in SCV (or in the Copenhagen strain of VACV), the red cross indicating that the gene was not detected by RNA-Seq of MQ vs SCV12hQ, the green cross indicate that the activity in mice is unknown (see S1B Table).

Multiple cytoplasmic sensor signatures at 12 hours post vaccination

The IPA analysis of DEGs for MQ vs SCV12hQ (S2D Table, direct and indirect) produced a series of USRs associated with (i) detection of cytoplasmic dsRNA (Fig. 2B, red circles) via MAVS, MDA5 and PKR, (ii) detection of cytoplasmic DNA (Fig. 2B, blue circles) dominated by STING/IFI16/cGAS, and (iii) activation of the inflammasome (Fig. 2B, black circles). These results again show marked consistency with existing poxvirus literature on KO mouse infections (summarized in S2E Table). dsRNA from complementary VACV RNA transcripts stimulates MAVS signaling (Myskiw et al., 2011), likely via MDA5 (Deng, 2017). Stimulation of MDA5 or RIG-I and PKR by VACV in vitro has been reported previously (Myskiw et al., 2011; Pichlmair et al., 2009), with both MAVS and MDA5 reported to contribute to host defense against VACV infection (Deng, 2017). PKR activation is also enhanced by MDA5 (Pham et al., 2016). Like SCV, the canarypox virus vector, ALVAC, also...
stimulates the cGAS/IFI16/STING pathway (Liu et al., 2017). Activation of the proteases Caspase 1 (gene CASP1) (canonical) and Caspase 11 (gene CASP4) (non-canonical) represent the central outcomes of inflammasome activation, with VACV stimulation of the inflammasome well described (Amsler et al., 2013). ALVAC is also reported strongly to stimulate the inflammasome via AIM2 in both human and mouse cells (Liu et al., 2017). Viron assembly is arrested at the viroplasma stage in SCV-infected host cells due to the deletion of D13L (Eldi et al., 2017), which may limit inflammasome activation.

Poxviruses encode a number of proteins that seek to limit the activity of host immune responses (S1B Table, yellow highlighting), with some of these inhibiting the activities of cytoplasmic sensors (Fig. 2B, blue text). VACV’s decapping enzymes (D9 and D10, encoded by D9R and D10R) are expressed at the vaccination site (S1B Table). Both proteins inhibit dsRNA accumulation, and D10 is functional in mice (Liu et al., 2015); whether D9 is functional in mice is unknown (Fig. 2B, green cross). DHX9 is involved in both DNA and RNA sensing and is targeted by VACV’s E3 protein (encoded by E3L), with PKR inhibition the best defined activity of E3 (Brandt et al., 2001; Dempsey et al., 2018; Langland et al., 2006). VACV DNA is usually shielded from cytoplasmic sensors during replication in viral factories via wrapping in ER membranes; however, this wrapping is lost during viron assembly (Tolonen et al., 2001). The DNA sensor PRKDC/DNA-PK has a z-score of zero, perhaps due to the inhibitory activity of C4 (encoded by C4L) (Scutts et al., 2018), with C16L transcripts not detected by RNA-Seq in SCV12hQ (Fig. 2B, red cross; S1B Table). CrmA (from cowpox) and VACV’s homologue, B13R/SPI-2, inhibit caspase 1 (and other caspases), but are not functional in the Copenhagen strain of VACV (Smith et al., 2013) (Fig. 2B, black cross). NLRP1 was not identified by the IPA USR analysis, potentially due to the expression of F1L (S1B Table) (Gerlic et al., 2013).

**Dominant TLR-signaling associated signatures at 12 hours post vaccination**

Following stimulation of TLR4, 9, 7 and 2 (but not TLR3) (Fig. 2A), a series of signaling events are initiated via the Myddosome, which contains MyD88/IRAK2/IRAK4 and signals to IRAK1 and TRAF6, with TRAF3 acting as a negative regulator (Kawasaki et al., 2014; O’Neill et al., 2010). TRAF5 (Buchta et al., 2014), TRAFD1/FLN29 (Sanada et al., 2008) and IRAK3 (aka IRAK-M) (Kobayashi et al., 2002) are also negative regulators of TLR signaling. TLR3 signaling also involves TRAF6 and TRAF3. All the aforementioned signaling molecules were identified by the IPA USR analysis, with negative regulators having negative z-scores and the rest positive z-scores (Fig. 3A; S2D Table, direct and indirect). These results are entirely consistent with the dominant TLR signatures illustrated in Fig. 2A. IL-1 receptor signaling (see below) also involves many of the same signaling molecules as TLR signaling (Rhyasen et al., 2015). The dominance of TBK1 may reflect its involvement in a series of signaling pathways; specifically, TLRs (including TLR3), STING and MDA5/MAVS (Zhao et al., 2019) that were illustrated in Fig. 2. C6 (encoded by C6L) inhibits TBK1 via binding to TBK1 adaptors (such as TANK) (Fig. 3A) thereby inhibiting activation of IRF3 and IRF7 (Smith et al., 2018; Unterholzner et al., 2011). K7 (encoded by K7R) binds DDX3 (Oda et al., 2009), an adaptor protein for the TBK1/IKKe complex that promotes IRF3 phosphorylation (García-Arriza et al., 2013; Smith et al., 2018). TLR signaling is also inhibited by K7 and A52 (encoded by A52R), which bind to IRAK2 and TRAF6 (Carty et al., 2010; Smith et al., 2013; Yokota et al., 2010). N1 (encoded by N1L) binds TBK1 and inhibits NF-xB and IRF3 signaling pathways (Dai et al., 2014; DiPerna et al., 2004; Maluquer de Motes et al., 2011) (Fig. 3A).
Figure 3. Secondary messenger signatures at 12 hours post vaccination. (A) Secondary messenger signatures. VACV encodes a series of cytoplasmic inhibitors, which are indicated in blue text. (B) Interferon response factors (IRFs). (C) NF-κB signatures. Green fill indicates canonical pathways, magenta fill non-canonical pathway, blue fill - not assigned to canonical or non-canonical. VACV-encoded cytoplasmic inhibitors are shown in blue text. K1L and A49R (black text) enhance the activity of NFKBIA, an inhibitor of the canonical pathway. Blue cross means the inhibitor is not active in mice.

Interferon response factor signatures at 12 hours post vaccination

Interferon response factors (IRFs) are key transcription factors triggered by PAMPs, with IRF3, IRF7 and IRF1 dominating (Fig. 3B) in the IPA USR analysis (S2D Table, direct and indirect) of the DEGs from MQ vs SCV12hQ (S2B Table). These 3 IRFs are also in the top 5 USRs (sorted by p value) when the “direct” only option was used for the IPA USR analysis (S2D Table, direct only).

IRF3 and IRF7 are activated via multiple PAMP sensors described in Fig. 2 (Bakshi et al., 2017; Pham et al., 2016; Zhao et al., 2019), are intimately involved in driving antiviral responses (Jefferies, 2019; Rudd et al., 2012; Wilson et al., 2017), are also activated by MVA (Dai et al., 2014) and ALVAC (Harenberg et al., 2008), and have been shown to promote adaptive immune responses in a number of settings (Hatesuer et al., 2017; James et al., 2018; Suschak et al., 2016). SCV also encodes N2L, with N2 inhibiting IRF3 activation (Smith et al., 2013). IRF1 has a role in positive feedback maintenance of ISG expression (Michalska et al., 2018; Wilson et al., 2017) and has been shown to promote adaptive immunity in certain settings (Forero et al., 2019; Yang et al., 2018). IRF8 has a critical role in development and maturation of myeloid cells such as dendritic cells (Salem et al., 2020) and IRF5 is predominantly expressed by myeloid cells and regulates inflammatory responses, generally downstream of TLR-MyD88 pathways (Forbester et al., 2020). IRF4 is a negative regulator of TLR
signaling (Negishi et al., 2005) (Fig. 3B).

**Conical NF-κB family signatures at 12 hours post vaccination**

The NF-κB family of transcription factors play key roles in immunity, with the IPA USR analysis (S2D Table, direct and indirect) illustrating a dominant canonical NF-κB signature (Fig. 3C, green circles), consistent with the TLR signaling USRs described above. The dominance of the NFKBIA, but not another NF-κB inhibitor NFkBIB, likely reflects the activities of the K1L and A49R genes; both K1 and A49 proteins prevent degradation of NFKBIA (Smith et al., 2013), with A49 binding the ubiquitin ligase B-TrCP (Neidel et al., 2019). B14 (encoded by B15R in the Copenhagen strain) binds and inhibits IKKβ (Tang et al., 2018), and intracellular M2 (encoded by M2L) inhibits RelA (p65) nuclear translocation, but is not active in mice (Smith et al., 2013) (Fig. 3C, blue cross). A55 (encoded by A55R) dysregulates NF-κB signaling by disrupting p65-importin interaction, is active in mice (Pallett et al., 2019) and is expressed by SCV at the injection site (S1B Table).

**Th1 cytokine signatures at 12 hours post vaccination**

The cytokine USR profile at 12 hours post vaccination is dominated by cytokine signatures generally associated with Th1 responses (Fig. 4A, red circles), in particular TNF, IL-1β and IFNγ, with in vivo induction of these cytokines by VACV suggested by previous studies (Carpenter et al., 1994; Tian et al., 2012; Tian et al., 2017). The Th1 dominance is consistent with studies on recombinant MVA vaccines (Bohnen et al., 2013; Tameris et al., 2014). TNF is required for optimal adaptive immune responses to VACV and other immunogens (So et al., 2019; Tian et al., 2017). IL-1 is important for host immune responses to VACV (Tian et al., 2012), with many vaccine adjuvants also inducing the release of IL-1 (Munoz-Wolf et al., 2018). Finally, IFNγ has anti-VACV activity (Kohonen-Corish et al., 1990) and has adjuvant properties in a range of settings (Nimal et al., 2005; Playfair et al., 1987; van Slooten et al., 2000). Although IL-27 was initially associated with Th1 responses, it is now recognized as a promoter of T regulatory cells (Yoshida et al., 2015) (Fig. 4A, IL27).

VACV encodes a number of soluble inhibitors of several cytokines (Fig. 4A, purple text). C10L encodes C10, which blocks interaction of IL-1 with its receptor (Kluczyk et al., 2002), but its activity in mice is unknown (Fig. 4A, green cross). B16 (encoded by B16R) (B15R in Western Reserve) is a secreted IL-1β decoy receptor (Alcami et al., 1992; Perdiguero et al., 2009), but appears to be truncated in the Copenhagen strain of VACV (Uniprot; GCA 006458465.1). B8 (encoded by B8R) is a secreted IFNγ receptor homologue, and ZIKA prME was inserted into the B7R-B8R locus in SCV-ZIKA/CHIK thereby inactivating these genes (Prow et al., 2018b). B28R (C22L) and A53R encode TNF receptor homologues that are not active in the Copenhagen strain of VACV (Alcam et al., 1999). C12 (encoded by C12L) inhibits IL-18 (Symons et al., 2002). B19 (encoded by B19R) (also known as B18R in other VACV strains) is a decoy receptor for soluble IFNαs (Colamonici et al., 1995; Waibler et al., 2009). B19 mRNA is well expressed at the injection site (S1B Table) and is potentially responsible for the relatively low z-scores of the IFNα USRs (Fig. 4A). A35 (encoded by A35R) is an intracellular VACV protein that also inhibits the synthesis of a number of chemokines and cytokines (including IFNα, MIP1α, IL-1β, IL-1α, GM-CSF, IL-2, IL-17, GRO1/KC/CXCL1, RANTES, TNFα) by VACV-infected cells (Rehm et al., 2010) (not shown).
Figure 4. Cytokine, chemokine, dendritic cell and STAT signatures at 12 hours post vaccination. (A) Cytokine signatures. VACV genes encoding secreted inhibitors are shown in purple text. Black crosses indicate the inhibitors are not present or functional in SCV. Green crosses indicate that the activity in mice is unknown. Red crosses indicate the RNA was not detected in our RNA-Seq analysis. (B) Chemokine signatures. Purple text and crosses as in A. (C) STAT signatures. VACV encoded cytoplasmic inhibitors are shown in blue text. Crosses as in A. (D) IPA USRs associated with stimulation of dendritic cells. * indicates that the mediators have more than one of the four dendritic activities indicated. (The figure includes some USRs present in previous bubble graphs). For references see S2F Table. (E) GSEAs for the Blood Transcript Modules (right to left) M43.0 and M43.1 (gene sets combined, n=21); M95.0, M95.1, M71 and M200 (gene sets combined, n=49); M64, M67, M119 and M165) (gene sets combined, n=71); M168 (n=19). For gene set details see S2F Table.
Chemokine signatures at 12 hours post vaccination

The chemokine signatures (Fig. 4B) are dominated by (i) CXCL12, which is made by many cell types and is strongly chemotactic for lymphocytes, (ii) CCL5 (RANTES), which is inter alia chemotactic for T cells and (iii) CXCL2, a neutrophil chemoattractant (consistent with Fig. 1E). CXCL12 and CCL5 are also involved in dendritic cell (DC) recruitment (Chabot et al., 2006; Lopez et al., 2018). CCL2 is also induced by MVA (Lehmann et al., 2009; Lehmann et al., 2016) and is involved in DC maturation and induction of T cell immunity (Gomes et al., 2019).

VACV infected cells secrete a number of proteins that bind and inhibit certain chemokines, although only A41 (encoded by A41L) is active in the Copenhagen strain of vaccinia (Fig. 4B, purple text). A41L inhibits CCL21 (Smith et al., 2013), perhaps consistent with its low z-score (Fig. 4B). B29, encoded by B29R (C23L) inhibits multiple CC chemokines, but is inactive in the Copenhagen strain (Alcamí et al., 1998). CCI inhibits a series of chemokines (Fig. 4B), but is not expressed on the Copenhagen strain of VACV (Burns et al., 2002; Reading et al., 2003).

STAT signatures at 12 hours post vaccination

Cytokines and chemokines bind to their receptors and activate transcription via STATs. The dominant STAT signatures (from S2D Table, IPA direct and indirect) were STAT1, STAT4 and STAT3 (Fig. 4C), with STAT1 and STAT3 representing the top USRs by p value and STAT1 also the topUSR by z-score when analyzed by IPA using direct only interaction (S2D Table, direct only). STAT1 forms complexes primarily STAT1-STAT1 homodimers (stimulated by IFNγ signaling) and STAT1-STAT2-IRF9 (ISGF3) (stimulated by type I IFN signaling). Using Interferome to interrogate the “Target molecules in dataset” listed for the STAT1 signature (S2D Table, direct and indirect), nearly all the target molecules were deemed IFNγ inducible (with most also inducible by type I IFNs). The relatively low p values and z-scores for STAT1 dimers would thus appear to be an under-annotation within IPA. The dominant STAT1 signature (Fig. 4C) is thus consistent with the dominant IFNγ signature in Fig. 4A. The cytoplasmic VACV-expressed H1 (encoded by H1L) inhibits STAT1 and STAT2 (Mann et al., 2008), but clearly only in cells infected with VACV. C6 (encoded by C6L), as well as the aforementioned binding of TBK1 adaptors, also binds the TAD domain of STAT2 (Stuart et al., 2016).

STAT4 signaling is induced by a number of cytokines including IL-12 and IL-2 (Yang et al., 2020) and is critical for IFNγ production during generation of Th1 responses (Varikutì et al., 2016). STAT3 signaling is induced by a number of cytokines including IL-6 and OSM (and growth factors such as GM-CSF), with BCG vaccination recently shown to cause STAT3 phosphorylation in antigen presenting cells (Copland et al., 2019). STAT6 is involved in driving Th2 responses (Gaylo-Moynihan et al., 2019) and has a negative z-score (Fig. 4C), consistent with the Th1 dominance illustrated in Fig. 4A.

Dendritic cell associated signatures

A range of mediators affect dendritic cell activities, with many of these identified as USRs by IPA analysis of DEGs for MQ vs SCV12hQ (Fig. 4D; S2D Table; for references see S2F Table). The VACV protein A35 inhibits a number of these mediators (see above), as well as inhibiting class II antigen presentation (Rehm et al., 2010). Multiple key mediators needed for induction of adaptive immune responses by dendritic cells would thus appear to be active at the injection site 12 hours post vaccination.

Extensive bioinformatics treatments of >30,000 peripheral blood transcriptomes from >500 human studies of 5 vaccines provided 334 publically available gene sets in the form of Blood Transcription Modules (BTMs). BTM gene sets are associated with specific subsets of cells and/or their activities (Li et al., 2014). BTM gene sets associated with dendritic cells and dendritic cell activities (S2F Table) and Gene Set Enrichment Analyses (GSEAs) were used to determine whether genes from dendritic cell
BTMs were significantly represented in the MQ vs SCV12hQ gene list (S2A Table). The GSEAs provided highly significant results (Fig. 4E), illustrating that signatures associated with dendritic cells and their activities can be readily identified at the injection site 12 hours post vaccination. Such signatures likely underpin the immunogenicity of the vector system.

Injection site signatures at day 7 post vaccination

The most common side effects reported for MVA (licensed as a small pox vaccine in Europe as IMVANEX) were at the site of subcutaneous injection; most of them were mild to moderate in nature and resolved without any treatment within seven days. To gain insights into the injection site responses after SCV vaccination, RNA-Seq of muscles on day 7 post-vaccination was undertaken to provide a gene list (MQ vs SCVd7Q, S2G Table), from which a DEG list (n=1413 genes) was generated (S2H Table) by applying the same filters as above (q ≤0.01, FC ≥2 and sum of all counts across the six samples >6). Of the 1413 DEGs, 1337 were up-regulated, with 633 (47%) of these also up-regulated DEGs for MQ vs SCV12hQ.

Cytoscape analyses of up-regulated DEGs from MQ vs SCV12hQ (S2C Table) were compared with MQ vs SCVd7Q (S2I Table). Multiple top signatures (by FDR) associated with T cells and B cells were substantially more significant on day 7 than at 12 hours (Fig. 5A; Table S2J). For instance, FDR values associated with the GO Process terms “positive regulation of T cell activation” and “T cell differentiation” were ≈9 logs more significant by day 7, when compared with 12 hours post vaccination (Fig. 5A; S2J Table). T cell receptor associated KEGG Pathways and GO Component terms were also more significant on day 7 (Fig. 5A; S2J Table). “T cell receptor complex” was also the top “GO Cellular Component” term by p value for day 7 up-regulated DEGs (S2J Table, Enrichr). A similar pattern emerged for B cell terms (Fig. 5A; S2J Table).

GSEAs (as in Fig. 4E) using genes from BTMs (Li et al., 2014) associated with T cell differentiation and division, and B cell differentiation into plasma cells, showed significance for SCVd7Q, but not SCV12hQ (Fig. 5B). Thus remarkably, these BTMs were able to identify signatures at the injection site on day 7 that were associated with the development of adaptive immune responses. (IgG responses are known to be induced after SCV-ZIKA/CHIK vaccination (Prow et al., 2018b)).

Vaccination site lesions are well described for VACV vaccination (Frey et al., 2002), with skin lesions reported days 6-11 after vaccination with the Lister strain (Talbot et al., 2006) and days 3-19 after Dryvax vaccination (Parrino et al., 2007). Such lesions are associated with cell death (He et al., 2014), tissue damage (Fulginiti et al., 2003) and recruitment of neutrophils, with neutrophil recruitment also a feature of eczema vaccinatum, a complication of smallpox vaccination (Darling et al., 2014; Frey et al., 2002; Tian et al., 2017). Cytoscape analyses (S2C and S2I Table) illustrated that the cell death pathway annotations identified at 12 hours (Fig. 1G) were considerably less significant or absent for day 7 (Fig. 5C, S2K Table). Analysis of the 1413 DEGs from MQ vs SCVd7Q (S2H Table) with IPA Diseases and Functions feature (S2L Table), showed a significant reduction in the z-scores of neutrophil-associated annotations on day 7 when compared to 12 hours (Fig. 5D; S2M Table). (The Cytoscape analysis also showed a highly significant reduction in FDR values for neutrophil terms, S2I Table, graph on right). These analyses indicate that progression of cell death and neutrophil infiltration is not a feature of SCV vaccination, likely consistent with the inability of SCV to produce viral progeny (Eldi et al., 2017). SCV does not cause a spreading infection, with vaccine-derived mRNA lost by day 7 (Fig. 1A). A similar IPA Diseases and Functions analysis of macrophage-associated annotations also illustrated a significant reduction by z-scores (Fig. 5D, S2M Table), further indicating that injection site inflammatory responses were abating by day 7 (Soehnlein et al., 2010).

The dominant pro-inflammatory cytokine USRs identified at 12 hours post vaccination (Fig. 4A) were substantially lower by day 7 post vaccination with respect to both log10 p values and z-scores (Fig. 5E; S2D vs S2N Table). Fold changes in cytokine mRNA expression levels relative to MQ were
also substantially lower on day 7 (Fig. 5E), with the exception of IFNγ, which had a fold change relative to MQ of 4.17 at 12 h and a fold change of 5.26 relative to MQ on day 7, perhaps due to the emerging Th1 T cell responses (see above). IPA Diseases and Functions also showed reduced significance and z scores on day 7 for Inflammatory response (-log10 p value 110.7 to 59.8, z-score 7.7 to 5.5) and Chronic inflammatory disorder (-log10 p value 70.1 to 38.1, z-score -0.23 to -2.2) (S2L Table). These analyses again argue that inflammation at the injection site is abating on day 7, with persistent inflammation at the injection site generally deemed undesirable in most vaccination settings (Clarke et al., 2020; Lee et al., 2008; Wang et al., 2014).
Figure 5. The injection site day 7 post vaccination. (A) Cytoscape analysis of up-regulated DEGs from MQ vs SCV12hQ and MQ vs SCVd7Q, illustrating the upward trend in significance of top B and T cell associated annotations. Black line – GO Process, Blue line GO - Component, Purple line - KEGG Pathways. For full lists and descriptions of annotations see S2J Table; statistics by paired t test for the full lists (parametric data distribution). (B) GSEAs use to interrogate MQ vs SCV12hQ and MQ vs SCVd7Q gene lists using T cell and B cell BTMs (left to right) M14 (n=12); M4.5 (n=35); M156.0 and M156.1 (gene sets combined, n=56) (for details of BTM gene sets see S2J Table). (C) As for A illustrating the downward trend of cell death annotations. Annotions not identified by the IPA analysis were nominally given a -log₁₀ FDR value of zero (y axis). Color coding as for A, but also Green line – UniProt Keywords, Brown line – Reactome Pathways. For descriptions of annotations see S2K Table. (Statistics by Wilcoxon Signed Rank tests; non-parametric data distribution). (D) IPA Diseases and Functions analysis of DEGs (up and down-regulated) from MQ vs SCV12hQ and MQ vs SCVd7Q, illustrating the downward trend in z-scores for macrophage and neutrophil annotations (for description of annotations see Table S2L and S2M). (Statistics by Wilcoxon Signed Rank tests). (E) Major IPA USR pro-inflammatory cytokine annotations identified at 12 hours (Fig. 4A; S2D Table) had much lower z-scores and p values on day 7 post vaccination (S2N Table). Numbers in the circles represent the log₂ fold change for that cytokine relative to MQ. (F) H&E staining of intramuscular injection site lesions on day 7 post infection. (G) Neutrophil Ly6G staining of lesions from day 7 post infection. Arrow in insert shows positive staining of a neutrophil in a blood vessel capillary. (H) Eosinophils in the intramuscular injection site lesions on day 7 post vaccination. White arrow heads - mature segmented eosinophils. Yellow arrowheads – immature band eosinophils. (I) Expression of skeletal muscle genes from MQ vs SCV12hQ (S2a Table) and MQ vs SCVd7Q (S2G Table); bars with red outline indicate significant fold change (q<0.05).
**Loss of neutrophils and presence of eosinophils on day 7 post vaccination**

H&E staining of the injection sites day 7 post vaccination supports the bioinformatics results described in the previous section. When compared with 12 hours (Fig. 1D), necrotic muscle lesions were largely absent, with the cellular infiltrates less disseminated and more focal (Fig. 5F). In addition, in contrast to 12 hours (Fig. 1E), neutrophils (stained with anti-Ly6G) were not observed in the day 7 cellular infiltrates (Fig. 5G), although the occasional neutrophil could be seen in blood vessels, illustrating that the staining had worked (Fig. 5G, insert, arrowhead). In contrast to Fig. 1E, ApoTag staining was also largely negative on day 7 (not shown). Loss of neutrophils is consistent with inflammation resolution (Soehnlein et al., 2010).

Another feature of the resolving infiltrates on day 7 post vaccination (clearly evident from H&E staining) was the presence of eosinophils (Fig. 5H), despite the retention of a dominant Th1 signature (Fig. 5E). Many of these cells showed the morphological features of immature band eosinophils, as distinct from segmented mature eosinophils (Fig. 5H).

At 12 hours post vaccination, genes specific to skeletal muscle were generally slightly down-regulated (Fig. 5I; S2A Table), consistent with the SCV infection-associated necrosis or pyroptosis (Fig. 1D). On day 7 post vaccination, Myh3 and Myh8 were significantly up-regulated (Fig. 5I; S2G Table), with these genes transiently up-regulated after muscle injury (Yoshimoto et al., 2020). Tnni1, a skeletal myogenesis marker (Park et al., 2016), was also up-regulated (Fig. 5I). However, stable expression of Tnni2, Myoz1, Myoz3 and Dmd by day 7 (Fig. 5I), argues that muscle regeneration had been largely completed at this time (Yoshimoto et al., 2020); consistent with the H&E staining (Fig. 5F).

**No compelling arthritic signature in feet on day 7 post vaccination**

An early attenuated CHIKV vaccine tested in a phase II human clinical trial caused a transient arthralgia in 5 out of 58 vaccine recipients (Edelman et al., 2000). Using RNA-Seq we have previously characterized the molecular signatures associated with hind foot arthritis 7 days after infection of mice with CHIKV (Prow et al., 2019; Wilson et al., 2017). Many features identified in mice recapitulated those seen in human patients (Michlmayr et al., 2018; Suhrbier, 2019; Wilson et al., 2017). We reanalyzed the FASTQ files (Wilson et al., 2017) (deposited in NCBI BioProject PRJNA431476) using STAR aligner and the more recent mouse genome build (GRCm38 Gencode vM23). The complete gene list for day 7 feet (peak CHIKV arthritis) is provided in S2O Table.

To determine whether SCV-ZIKA/CHIK vaccination is associated with the induction of an arthritic signature on day 7 post vaccination, feet were collected (by severing at the bottom of the tibias after euthanasia) and analyzed by RNA-Seq (MF vs SCVd7F) (S2P Table). A DEG list was generated after application of two filters q<0.05 and the sum of counts across all 6 samples >6, resulting in only 22 genes, of which 8 were up-regulated (S2Q Table). Three of these genes (Daglb, Tgtp2 and Gbp3) were also present in the up-regulated DEGs for day 7 feet of CHIKV infected mice (S2O Table), although the fold change of the latter two were substantially higher after CHIKV infection than after SCV vaccination (Fig. 6A). Of the 8 up-regulated DEGs, Tnfaip6 and Crispld2 have anti-inflammatory activities (Day et al., 2019; Zhang et al., 2016) and Thbs4 and Daglb have pro-inflammatory activities (Hsu et al., 2012; Rahman et al., 2020), with Gbp3 and Spon2 associated with antiviral responses (Li et al., 2009b; Nair et al., 2017) and Mrgprf associated with the itch response (Meixiong et al., 2017).

IPA Diseases and Functions analysis of the 22 DEGs returned a significant “Inflammatory response” with a negative z-score, and cellular infiltrate terms with low z-scores (Table S2Q). Importantly, the z-scores and p-values for these annotations were very much lower for SCVd7F than they were for day 7 feet from CHIKV infected mice (Fig. 6B). Comparison of the 22 DEGs with those reported for collagen induced arthritis (CIA) (GSE13071) (Geurts et al., 2009) also identified no obvious concordance (S2Q Table), although with only 22 DEGs such a comparison is somewhat underpowered.
Overall these results suggest that SCV vaccination was not associated with a compelling arthritic signature, even though the injection sites (quadriceps muscles) were in the same legs as the feet that were used to generate the MF vs SCVd7F gene set.

**Figure 6. No compelling arthritic signature after SCV vaccination.** (A) MF vs SCVd7F provided 22 DEGs (with 2 filters applied q<0.05 and count sum >6) of which 8 were up-regulated (Table S2Q). Three of these were also up-regulated DEGs for CHIKV arthritis day 7 post infection (Table S2O; q<0.05). (B) The 22 DEGs analyzed by IPA Diseases and Functions feature (direct and indirect) (S2Q Table) and compared with the same annotations identified by IPA analysis of DEGs for CHIKV arthritis (S2O Table).

**Discussion**

We provide herein a detailed systems vaccinology analysis of a recombinant SCV vaccine in mouse muscle, to provide insights into vaccine gene expression, and host adjuvant signatures and immune responses. Of all the reads mapping to the SCV-ZIKA/CHIK vaccine, ~20% mapped to the recombinant immunogens. IHC illustrated immunogen protein expression in skeletal muscle cells, with these cells showing histological signs of necrosis (Lentscher et al., 2020; Szugye, 2020) and bioinformatics indicating the presence of necrosis and necroptosis (Morgan et al., 2018). Adjuvant signatures were driven by TLRs, cytoplasmic RNA and DNA sensors and the inflammasome, with neutrophils potentially also contributing (Stephen et al., 2017). By day 7 vaccine transcripts and neutrophils were largely absent, and inflammation was abating, with the presence of what appeared to be tissue repair-associated eosinophils (Coden et al., 2020; Heredia et al., 2013; Lloyd et al., 2018; Toor, 2019; Weller et al., 2017). Although a previous live-attenuated CHIKV vaccine was associated with some arthropathy (Edelman et al., 2000), no compelling arthritic signature was evident after SCV vaccination.

The immunogenicity of SCV, and likely poxvirus vector systems generally, would appear to be underpinned by the ability to stimulate a broad range of adjuvant pathways. Multiple TLR signatures were identified at 12 hours post vaccination (Fig. 2A), with the TLR4 agonist monophosphoryl lipid A a component of Fendrix (hepatitis B vaccine) and Cervarix (human papilloma virus vaccine) (which are formulated in ASO4 adjuvant), as well as Shingrix (herpes zoster vaccine) (formulated in AS01 adjuvant). The dsRNA TLR3 agonists, Ampligen (Rintatolimod) and Hiltonol, also have well described adjuvant properties (Martins et al., 2015), with Hiltonol being tested in therapeutic cancer vaccine trials (ClinicalTrials.gov Identifier: NCT04345705 and NCT02423863). The TLR9 agonist, CpG oligonucleotide (CpG 1018), was recently approved as an adjuvant in Heplisav-B (hepatitis B vaccine). A range of cytoplasmic sensor signatures with known adjuvant activity were also identified (Fig. 2B), including multiple inflammasome signatures; the best known human adjuvant, alum, is believed to mediate its activity via activation of the inflammasome (Kelley et al., 2019). Stimulation of
cytoplasmic dsRNA sensors represents a key adjuvant activity for replicon-based RNA vaccines (Fuller et al., 2020; Pijlman et al., 2006) and the utility of STING-activating adjuvants is being actively explored (Jiang et al., 2020; Luo et al., 2019). Although SCV does not generate viral progeny, it does replicate its DNA (Eldi et al., 2017), perhaps explaining the dominant STING signature (Fig. 2B).

Virulent poxviruses inhibit STING activation via unknown factors, an inhibitory activity not found for MVA (Georgana et al., 2018). This activity is perhaps similarly absent for SCV or is inactive in mice.

A desirable feature for any vaccine is the avoidance of reactogenicity, a term describing a series of post-vaccination adverse events often associated with excessive injection site inflammation and systemic reactions such as fever (Herve et al., 2019). A potential goal of systems vaccinology is the identification of reactogenic signatures; however, consensus regarding the composition of such signatures, and/or when and where best to sample to obtain such signatures, has yet to be established (Gonzalez-Dias et al., 2020). CCL2 and CXCL10 up-regulation in peripheral blood was identified as potential biomarkers of vaccine-elicited adverse inflammation in mice after a number of different vaccines given i.m. ( McKay et al., 2019). These chemokines featured prominently at the injection site 12 hours after SCV vaccination (Fig. 4B), although fold-change had reduced substantially by day 7 (log2 4.12 to -0.73, and 8.35 to 3.17, respectively) (S2A and S2G Table). CCL2 is also induced by MVA (Lehmann et al., 2016), can be important for avoiding immunopathology (Poo et al., 2014), and is induced by the licensed adjuvants, Alum and MF59 (Seubert et al., 2008). CXCL10 is also induced by MVA (Flechsig et al., 2011) and by the licensed adjuvant, MF59 (Schifanella et al., 2019). Given the extensive clinical safety record of MVA vaccination (Overton et al., 2018) and the lack of overt injection site reactogenicity or fever observed in NHPs after SCV vaccination (Prow et al., 2020), CCL2 and CXCL10 up-regulation at the injection site would thus not appear to be compelling biomarkers for adverse events after MVA or SCV vaccination. Similarly, a whole-blood systemic adverse event signature for yellow fever 17D vaccination has been reported, with 32 up-regulated genes on day 1 (but not day 3) associated with a range of systemic adverse events (either within 24 hours or a median time post vaccination of 6 days) (Chan et al., 2017). GSEAs illustrate that this signature is highly significantly present in the SCV12hQ gene list, and is also significantly present in the SCVd7Q gene list (S2R Table). All the core enriched genes (S2R Table) were type I IFN stimulated genes (by Interferome), with type I IFN stimulated gene induction clearly present at the SCV injection site (Fig. 4A). However, MVA vaccination is also associated with short term (i) increases in local type I IFN responses (Dai et al., 2014; Wolferstatter et al., 2014) and (ii) elevated serum IFNa levels (Lopez-Gil et al., 2013; Waibler et al., 2007). Of note, SCV encodes B19R/B18R, an inhibitor of type I IFN responses (Waibler et al., 2009). Unlike Yellow fever 17D, MVA and SCV vaccinations are not associated with significant viremias or viral dissemination, reducing the probability of excessive systemic type I IFN responses and systemic adverse events; although pyrexia, headache, myalgia, nausea, fatigue and/or chills are seen in a small percentage of MVA vaccine recipients (Överton et al., 2018). Clearly, adverse event signatures identified in peripheral blood, may not to be overly informative for understanding adverse events at the injection site. In addition, not only the presence of specific gene transcripts but also the magnitude of gene induction are likely to be important, with the latter not fully taken into account by GSEAs. In humans, sampling injection sites is difficult, although emerging micro-sampling techniques may provide new avenues (Lei et al., 2019).

Ultimately RNA-Seq of injection sites in animal models should be able to provide early warnings in the vaccine development process of potential reactogenicity issues. Herein we show that, although SCV (unlike MVA) retains the ability to replicate its DNA (Eldi et al., 2017), the injection site reactogenicity (like IMVANEX) has largely resolved by day 7 post vaccination.

How might the information provided herein find utility for poxvirus vaccine design? The ZIKA and CHIK immunogens are inserted into B7R/B8R and A39R, respectively (Prow et al., 2018b). The 12 vector genes that were not expressed in vivo post-vaccination (S1B Table) offer other potential
insertion sites for recombinant immunogens that would ostensibly have minimal impact on vaccine behavior. However, expression of these genes in human muscle might be checked, perhaps via use of human skeletal muscle organoids (Gholobova et al., 2019). The multiple adjuvant pathway stimulated by SCV (Fig. 2) might argue for a certain level of redundancy (Waibler et al., 2007), which might allow certain inhibitors to be reintroduced with the aim of reducing reactogenicity, without compromising immunogenicity. For instance, B13R (also known as SPI-2) is absent in the Copenhagen strain of VACV and in SCV, and inhibits caspase I, a key protease for generation of bioactive IL1-β (Martin-Sanchez et al., 2016). Reintroducing SPI-2, or B16R that encodes an IL-1 receptor (Jackson et al., 2005), into the vector might have minimal effects on immunogenicity (Legrand et al., 2004), whilst reducing generation of this potent pyrogen and thereby potentially the risk of adverse events such as fever (Alcami et al., 1992). Recent sequencing of ancient Variola viruses from Viking corpses perhaps supports such strategies, as active expression of immune modulating genes may be associated with reduced pathogenicity (Alcami, 2020; Muhlemann et al., 2020). Co-formulation of SCV with adjuvants (Barrera et al., 2018; Magnusson et al., 2018) or encoding genetic adjuvants within SCV (Matchett et al., 2020) might appear superfluous, given the large number of adjuvant pathways already being activated. Introducing apoptosis inhibitors to improve immunogenicity (Chea et al., 2019) may have minimal impact for i.m. injections of SCV (or other pox vectors) as skeletal muscle does not appear readily to undergo apoptosis (Schwartz, 2008, 2018), with B13R also well expressed at the injection site (S1B Table). The absence of the chemokine inhibitors B29R (C23L) (not expressed) and CCI (not present/functional) may contribute inter alia to effective recruitment of dendritic cells. Deletion of A41L might be tested to determine whether this would increase immunogenicity, given CCL21 recruits T cells and enhances T-cell responses (Gray et al., 2018). Complement control proteins VCP (encoded by B27R) and C3 (encoded by C3L) were expressed at the mRNA level, with VCP deletion from VACV increasing anti-VACV antibody responses (Albarnaz et al., 2018), suggesting deletion of these genes might enhance immunogenicity. One might consider deletion of N2L (an inhibitor of IRF3) as this was shown to improve the immunogenicity of a recombinant MVA vaccine (Garcia-Arriaza et al., 2014) and also reduced the virulence of VACV (Ferguson et al., 2013). However, the IRF3 signature is already very dominant (Fig. 3B), so additional IRF3 activation (if possible) may not translate to significant improvements in immunogenicity. Deletion of C6L increased the immunogenicity of a recombinant MVA vaccine (Marin et al., 2018), presumably by relieving IRF3, IRF7 (Smith et al., 2018; Unterholzner et al., 2011) and/or STAT2 inhibition (Stuart et al., 2016). However, C6L deletion could risk excessive type I IFN responses and increased reactogenicity (Chan et al., 2017). The presence of eosinophils in the resolving lesion on day 7 was unexpected. Eosinophils have been reported in pruritic papulovesicular eruptions in a case of generalized vaccinia after smallpox vaccination (Beachkofsky et al., 2010) and are well described as drivers of allergic diseases such as eosinophilic asthma (Bhalla et al., 2020; Côté et al., 2020). However, recently a role for eosinophils in tissue repair and wound healing has emerged (Coden et al., 2020; Lloyd et al., 2018), particularly for muscle tissues (Heredia et al., 2013; Toor, 2019; Weller et al., 2017). That the eosinophils in these resolving post-vaccination lesions are distinct from inflammatory eosinophils is supported by the absence of IL-5 mRNA expression (S2G Table), with anti-IL-5 therapy used for eosinophilic asthma (Walsh, 2020). Transcripts for eosinophil cationic protein (Ear1) and eosinophil peroxidase (Epx) (granule components of inflammatory eosinophils) were also not detected. Eotaxins (CCL11, CCL24 and CCL26) were not up-regulated, with IL4, IL13, IL3 and GM-CSF (CSF2) transcripts absent (S2G Table). We were unable to find any eosinophil gene signatures that provided a significant result after GSEAs of the MQ vs SCVd7Q gene set, suggesting that signatures for inflammatory eosinophils are distinct from tissue repair-associated eosinophils, with the signatures for the latter yet to be defined. A limitation of using mice to analyze VACV-based vaccines is that several VACV-encoded
inhibitors are not active in mice (encoded by M2L, A38L) and the activity of others in mice is not known (D9R, H1L, C10L). The activity in mice of B28R and B29R is also not known, but these inhibitors are not active in the Copenhagen strain of VACV. Others were found not to be expressed in muscle or poorly expressed in mice. How critical these genes are to the overall interpretations presented herein is difficult to assess, given the presence of multiple overlapping and potentially cross-compensating pathways.

An extensive history of poxvirus vector development has yet to culminate in the licensing of a recombinant poxvirus vector for human use, although several are in late stage clinical trials. Systems vaccinology approaches such as those described herein may facilitate rationale refinement of pox vector design and contribute to progressing such systems towards registration and licensure.

Methods
Ethics statement
All mouse work was conducted in accordance with the “Australian code for the care and use of animals for scientific purposes” as defined by the National Health and Medical Research Council of Australia. Mouse work was approved by the QIMR Berghofer Medical Research Institute animal ethics committee (P2235 A1606-618M).

Mice and vaccination
Female C57BL/6J mice (6-8 weeks) were purchased from Animal Resources Center (Canning Vale, WA, Australia). Mice were vaccinated once with 50 µl of 0.5 x 10⁶ pfu SCV-ZIKA/CHIK i.m. or Mock vaccinated (with PBS) into both quadriceps muscles as described (Prow et al., 2018b).

RNA-Seq
At the indicated times post vaccination, mice were euthanized using CO₂ and quadriceps muscles or feet placed individually into RNAlater (Life Technologies) overnight at 4°C and then homogenized in TRIzol (Invitrogen) using 4 x 2.8 mm ceramic beads (MO BIO Inc., Carlsbad, USA) and a Precellys24 Tissue Homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France) (6000 rpm on ice, 3 times 12 sec for feet and 2 times for 10 seconds for muscle). Homogenates were centrifuged (14,000 g x 15 min) and RNA extracted from the supernatants as per manufacturer's instructions. Following DNase treatment (RNAseq-Free DNase Set (Qiagen)) and RNA purification (RNeasy MinElute Kit), RNA concentration and purity was determined by Nanodrop ND 1000 (NanoDrop Technologies Inc.). RNA samples were pooled so that for each group of 6 mice, 12 sets of quadriceps muscles or feet were used to create 3 biological replicates which contained equal amounts of RNA from 4 different mice. All replicates were then sent to the Australian Genome Research Facility (Melbourne, Australia) for library preparation and sequencing. RNA integrity was assessed using the Bioanalyzer RNA 6000 Nano assay (Agilent) and libraries were prepared from 200 ng of total RNA using TruSeq Stranded mRNA library preparation kit (Illumina). The resulting libraries were assessed by TapeStation D1K TapeScreen assay (Agilent) and quantified by qPCR using the KAPA library quantification kit (Roche). Libraries were normalized to 2 nM and pooled for clustering on an Illumina cBot system using HiSeq PE Cluster Kit v4 reagents followed by sequencing on an Illumina HiSeq 2500 system with HiSeq SBS Kit v4 reagents with 100 bp paired-end reads.

Mouse genome alignments and differential gene expression
Mapping to the mouse genome and differential expression analysis was conducted at AGRF under commercial contract using their in-house pipeline. The quality of the raw sequencing reads were
assessed using FastQC and MultiQC. Adapters were trimmed using the TrimGalore (0.4.4) program and reads with a length <30 bp or quality <10 were removed. Filtered reads were aligned to the *Mus musculus* reference genome (mm10; GTF file GRCm38.6 – annotation release 105) using the STAR aligner (v2.5.3a) with default parameters plus a parameter to restrict multi-mapping reads (‘—outFilterMultimapNmax 2’). Counts per gene were summarized using the featureCounts (v1.4.6-p5) utility in Subread. A counts matrix was generated from the collective samples using in-house scripts and input to R (3.5.0) for differential expression analysis. Differential expression analysis was undertaken using EdgeR (3.22.3) with default settings and no filters, given the importance of key genes with low transcript abundance (Wilson et al., 2017) and the small percentage of cells infected by SCV-ZIKA/CHIK in the quadriceps muscles (with whole quadriceps muscles harvested for RNA-Seq).

Counts were converted to relative counts (CPM) and normalized using the TMM method and modelled using the likelihood ratio test, glmLRT().

**Read alignments to the mouse and viral genomes**

Sequencing reads were assessed using FastQC (Simons, 2010) (v0.11.8) and trimmed using Cutadapt (Martin, 2011) (v2.3) to remove adapter sequences and low-quality bases. Trimmed reads were aligned using STAR (Dobin et al., 2012) (v2.7.1a) to a combined reference that included the GRCm38 primary assembly and the GENCODE M23 gene model (Harrow et al., 2012), VACV Copenhagen (M35027.1; 191737 bp), ZIKV (KU321639.1; 10676 bp), and CHIKV (AM258992.1; 11601 bp). Quality control metrics were computed using RNA-SeQC (DeLuca et al., 2012) (v1.1.8) and RSeQC (Wang et al., 2012) (v3.0.0). SAMtools (Li et al., 2009a) (v1.9) was used to obtain alignments to the coding sequences of mature peptide features of VACV, CHIKV and ZIKV.

**Histology and immunohistochemistry**

H&E staining was undertaken as described previously (Prow et al., 2019). IHC for neutrophils was undertaken as described (Prow et al., 2019) using Ly6G primary antibody (Abcam Anti-Mouse Neutrophil antibody Clone: NIMP-R14 cat. No. ab2557, Cambridge, UK) and Ly6 secondary antibody (Biocare Medical Rat on Mouse HRP Polymer cat. No. RT517L, Concord, CA USA). Apoptag staining used the Millipore Apoptag Peroxidase In Situ Apoptosis Detection kit (cat. No. S7100 Temecula, CA, USA). IHC for CHIKV capsid (monoclonal antibody 5.5G9 (Goh et al., 2015)) and ZIKA envelope (monoclonal antibody 4G2 (Hobson-Peters et al., 2019), was undertaken as described using NovaRed secondary antibody (Vector Laboratories ImmPACT NovaRed Peroxidase Substrate Kit cat. No. SK-4805 Burlingame, CA, USA). Slides were digitally scanned using Aperio AT Turbo (Leica Biosystems).

**GSEAs and Cytoscape**

Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) was performed on a desktop application (GSEA v4.0.3) and the GenePattern Public server (Reich et al., 2006) using the “GSEAPreranked” module. Protein interaction networks of differentially expressed gene lists were visualized in Cytoscape (v3.7.2) (Shannon et al., 2003). Enrichment for biological processes, molecular functions, KEGG pathways and other gene ontology categories in DEG lists was elucidated using the STRING database (Szklarczyk et al., 2019).

**Statistics**

Statistical analysis of experimental data was performed using IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, New York, USA). The paired t-test was used when the difference in variances was <4, skewness was >2 and kurtosis was <2. Otherwise the non-parametric Wilcoxon Signed Rank tests was used.
DATA AVAILABILITY

Datasets and analyses are available in the Supplementary Tables. All raw sequencing data (fastq files) was submitted to the Sequence Read Archive (SRA), BioProject accession: PRJNA610695.

ACKNOWLEDGEMENTS

We thank the animal house staff and Histology and Imaging Services at QIMR B for their assistance. We thank Dr T Larcher (Institut National de Recherche Agronomique, France) for help interpreting histopathology. JEH was supported by a Research Training Program award for her PhD studies via the Faculty of Medicine at the University of Queensland (UQ). JEH also received funding from the Global Change Institute at UQ. NAP was awarded an Advance Queensland Research Fellowship by the Queensland Government, Australia, with co-funding from Sementis. AS is a Leadership Fellowship with the National Health and Medical Research Council of Australia, and is co-director of the AIDRC GVN Center of excellence.

AUTHOR CONTRIBUTIONS

JEH, TD, ASl, SK, A-M P, LG performed the bioinformatics. TTL, NAP performed the experiments. PMH designed and provided access to the SCV-ZIKA/CHIK vaccine. PMH and KY generated supplementary annotation files. LL and JDH characterized and supplied the SCV vaccine. AS supervised the research, conceived the study and wrote the manuscript, with assistance from other authors.

ADDITIONAL INFORMATION

Supplementary information accompanies the paper

Competing interests: NAP, LL and JDH own Sementis shares. JDH is the current CSO of Sementis. AS was a consultant for Sementis. PMH was the previous CEO/CSO of Sementis. LL and NAP have had, and/or currently have, salary and/or project support from funds provided, whole or in part, via Sementis. Sementis had no role in the design and interpretation of the study, or in preparation of the manuscript.
Supplementary figures

(A) SCV-ZIKA/CHK (10^6 pfu) i.m. 50 µl both left and right quadriceps

Harvest quadriceps Harvest quadriceps and feet RNA purified Pool RNA RNA-Seq Gene list generation & bioinformatics

12 hours post vaccination 7 days post vaccination

(B) 6 mice

12 quadriceps 12 quadriceps feet

Q = quadriceps F = feet

Replicate 1 Replicate 2 Replicate 3

(C) Sample | Total paired reads mean of 3 biological replicates ± SD | Mean percent mapping to mm10
---|---|---
12 hour mock quads (MQ) | 19,039,543 ± 2,003,527 | 91.73%
12 hour SCV-ZIKA/CHK quads (SCV12hqQ) | 19,492,582 ± 6,835,162 | 91.63%
Day 7 SCV-ZIKA/CHK quads (SCVd7Q) | 23,919,688 ± 1,062,450 | 91.76%
12 hour mock feet (MF) | 21,292,249 ± 1,095,172 | 93.19%
Day 7 SCV-ZIKA/CHK feet (SCVd7F) | 22,558,810 ± 2,784,124 | 93.72%

S1 Fig. (A) Time line of experiment. (B) Pooling strategy for replicates. (C) Reads and percent of reads mapping to the mouse genome. (D) Boxplot of Log counts (normalized). Boxplots shows similar distributions of read counts amongst samples within and between groups. Boxes are 1st & 3rd quartile; whiskers range (no outliers). (E) Smear plots of the differentially expressed genes for the three comparisons. Red – FDR <0.05 Blue lines represent fold change of 2.
S2 Fig. Bowtie2 alignments to viral genomes. Raw FASTQ files were assessed for quality using FastQC and MultiQC tools. Sequencing adapters were trimmed using Trimmomatic (v0.36.6)\(^1\) where reads with an average quality score over a 4 base sliding window of less than 20 were removed. Trimmed reads were aligned using Bowtie2 (v2.3.4.1)\(^2\) to a combined reference that included the GRCm38 primary assembly and the GENCODE M23 gene model. Vaccinia virus Copenhagen (M55027.1), Zika virus strain ZikaSPH2015 (KU321639.1), and chikungunya virus (AM258392.1) Primary proper pair reads aligned to viral features, including CDS and mature peptide features, were counted using SAMtools (v1.9). A Bar graph of raw data shown in B.

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S3 Fig. A549 cells infected with SCV-ZIKA/CHIK. 48-72 hours after SCV-ZIKA/CHIK infection, morphological features characteristic of apoptosis (condensation of chromatin) were clearly evident (top row) after staining with Hoechst 33342 (Linn et al. J Virol Methods. 1995. 52(1-2):51-4). Bottom image shows uninfected controls.
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