1 **Title: Baculovirus-inducing fast-acting innate immunity kills *Plasmodium* liver**
2 **stages**

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4 **Running title:** Baculovirus acts as an anti-malaria liver-stage agent

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ABSTRACT

Baculovirus (BV), an enveloped insect virus with a circular double-stranded DNA genome, possesses unique characteristics that induce strong innate immune responses in mammalian cells. Here, we show that BV administration not only sterilely protects BALB/c mice for at least 7 days from subsequent *Plasmodium berghei* sporozoite infection but also eliminates existing liver-stage parasites completely, effects superior to those of primaquine, and does so in a TLR9-independent manner. Six hours post-BV administration, IFN-α and IFN-γ were robustly produced in serum, and RNA transcripts of interferon-stimulated genes were drastically upregulated in the liver. The *in vivo* passive transfer of post-BV administration serum effectively eliminated liver-stage parasites, and IFN-α neutralization abolished this effect, indicating that the BV liver-stage parasite killing mechanism is downstream of the type I IFN signaling pathway. Our results demonstrate that BV is a potent IFN-inducing prophylactic and therapeutic agent with great potential for further development as a new malaria vaccine and/or anti-hypnozoite drug.

INTRODUCTION

Malaria remains a severe public health problem and causes significant economic losses worldwide. In 2016, there were approximately 216 million malaria cases and an estimated 445,000 malaria deaths, mainly in children under five (1). Malaria infection is initiated following injection of *Plasmodium* sporozoites into the skin during the taking of a blood meal by *Anopheles* mosquitoes. The sporozoites migrate to the liver and invade hepatocytes. Before clinical symptoms of malaria occur during the blood stage of infection, *Plasmodium falciparum* in the liver develop into exoerythrocytic schizonts for 5 to 6 days. *P. vivax* and *P. ovale* can develop dormant liver-stage forms, known as hypnozoites, which cause relapsing blood-stage infections months or years after the primary infection. Currently, the only licensed drug for the radical cure of *P. vivax* hypnozoites is primaquine (PQ), and artemisinin-based combination therapies are recommended by the World Health Organization (WHO) as the first-line treatment for blood-stage *P. falciparum* malaria. However, PQ has a high associated risk of life-threatening haemolytic anaemia in people with glucose-6-phosphate-dehydrogenase enzyme (G6PD) deficiency (2). For future

malaria eradication strategies, safer radical curative compounds that efficiently kill hypnozoites are required.

A series of studies performed by Nussenzweig and colleagues in 1986–1987 revealed that exogenously administered interferon (IFN)-γ effectively inhibits development of liver-stage parasites *in vitro* and *in vivo* (3-6). Recently, Boonhok *et al.* reported that IFN-γ-mediated inhibition occurs at least partially in an autophagy-related protein-dependent manner in infected hepatocytes (7). Additionally, Liehl *et al*. reported that hepatocytes infected with liver-stage parasites induce a type I IFN secretion via the host cells sensing *Plasmodium* RNA, resulting in reduction of the liver-stage burden (8). These findings suggest that IFN-mediated immunotherapy against liver-stage parasites might be effective. However, new anti-hypnozoite drugs (e.g. recombinant IFNs or appropriate IFN inducers) have not been developed yet.

Autographa californica nucleopolyhedrosis virus (AcNPV), a type of baculovirus (BV), is an enveloped, double-stranded DNA virus that naturally infects insects. BVs possess unique characteristics that activate dendritic cell (DC)-mediated innate immunity

66 through MyD88/Toll-like receptor 9 (TLR9)-dependent and -independent pathways (9).
67 Takaku and colleagues reported that BV also directly activates murine natural killer (NK)
68 cells through the TLR9 signalling pathway (10, 11), which leads to induction of NK
69 cell-dependent anti-tumour immunity. Based on the unique adjuvant properties of BV that
70 induce DC maturation and NK cell activation, which are prerequisites for generating robust
71 and long-lasting adaptive immune responses, we have developed BV-based malaria
72 vaccines effective for all three parasite stages, the pre-erythrocytic stage (12-14), asexual
73 blood stage (15, 16), and sexual stage (17, 18).

74 Here, we investigated BV-mediated innate immunity against the pre-erythrocytic stage
75 parasites. Our results clearly demonstrate that BV intramuscular administration not only
76 elicits short-term sterile protection against *Plasmodium* sporozoite infection but also
77 eliminates liver-stage parasites completely through the type I IFN signalling pathway. We
78 propose that, due to its potent IFN-inducing function, BV has great potential for
79 development into not only a new malaria vaccine platform capable of protecting
80 vaccinators for a short period before and after malaria infection but also a new

non-haemolytic single-dose alternative to PQ.

RESULTS

BV administration induce transgene expression and innate immune responses

This study investigated the effects induced by BV on innate immune responses relating to malaria infection. We used BES-GL3 harbouring two gene cassettes consisting of the *luciferase* gene under the control of the *CMV* promoter and the *DAF* gene under the control of the *p10* promoter, which were designed to express luciferase as a transducing marker and to display DAF as protection for BV from complement attack, respectively (19). BES-GL3 intramuscular administration into the left thigh muscle of mice initially increased the luciferase expression levels robustly, but these levels gradually decreased to 2% on day 28 (Fig. 1A), which is consistent with previous studies (19, 20). Among various cell types tested *in vitro*, hepatocytes were found to take up BV most effectively (21), suggesting a potential use for BV as a vector for liver-directed gene transfer. However, direct evidence of *in vivo* liver-directed gene transfer has not been reported because BV-mediated gene transfer into hepatocytes via intravenous injection is severely hampered by serum

96 complement (22). We found for the first time that intravenous administration of BV
97 effectively transduces hepatocytes *in vivo* (Fig. 1B).

98 We next examined the kinetics of proinflammatory cytokines, ALT, and AST in sera
99 following BES-GL3 intravenous administration. IFN-γ and TNF-α levels rapidly reached
100 their peaks at 6 h and decreased to baseline by 24 h (Fig. 1, C and D). Similarly, the ALT
101 and AST levels rapidly reached their peaks at 12 h and decreased to baseline by 48 h (Fig. 1,
102 E and F). Compared with intravenous administration, intramuscular administration did not
103 affect the ALT levels; although the AST level trended higher, this difference did not reach
104 statistical significance (Fig. 1, G and H). ALT is a sensitive indicator of liver damage, so
105 these results suggest that, for BV, intramuscular administration may be less destructive than
106 intravenous administration.

107

108 **BV administration elicits sterile protection against sporozoite**

109 Table 1 summarizes the protective efficacy results for BV administration against malaria
110 sporozoite challenge. First, to examine the effects of BV intravenous administration, mice

were intravenously administered 10^7 pfu of BES-GL3. At 6 h post-BV injection, which coincides with peak IFN-γ production, the mice were intravenously challenged by 1,000 Pb-conGFP sporozoites, which are transgenic *P. berghei* constitutively expressing GFP. All BV-injected mice were protected, whereas all PBS- and AdHu5-injected mice treated similarly became infected. Next, we investigated the effects of BES-GL3 intramuscular administration (10^8 pfu) followed by sporozoite challenge at various intervals post-BV injection. After intramuscular administration of BES-GL3, all mice were protected for at least 7 days. However, there was a complete loss of protection by 14 days post-BES-GL3 intramuscular administration, and no delay of parasitaemia was observed in these mice. Additionally, no protection was observed in mice treated intranasally with BES-GL3.

BES-GL3 intravenous administration failed to provide protection against challenge with 1,000 parasitized red blood cells (pRBCs) at 6 h post-BV injection, indicating that BV has no residual effect on blood-stage parasites. CpG intramuscular administration at 6 or 24 h prior to challenge conferred protection against sporozoite challenge in 90% or 80% of mice, respectively. This is consistent with previous work showing short-term (2-days) protection

induced by CpG intramuscular administration (50 µg) against challenge with 100 *P. yoelii* sporozoites (23), although only partial protection (50%) was observed when the challenge occurred at 7 days post-CpG intramuscular injection. Thus, the protective efficacy induced by BES-GL3 intramuscular administration is more effective and longer-lasting (7 days) compared with CpG. All PBS-treated control mice developed blood-stage infection within 6 days following an intravenous injection of 1,000 Pb-conGFP sporozoites.

BV administration completely eliminates of liver-stage parasites

Pathways stimulated by type I and II IFNs can lead to the killing of hepatocytes infected with liver-stage parasites (3-8). Because BV is a potent inducer of type I and II IFNs (24, 25), and we observed BV-mediated protection as described above, we next investigated whether BV-induced IFNs could kill liver-stage parasites *in vivo*. To examine the elimination effects on the trophozoite and exoerythrocytic (mature) schizont stages, we administered BES-GL3 intravenously or intramuscularly at two different intervals following sporozoite challenge, 24 and 42 h, respectively. Table 2 summarizes these results

141 on the elimination efficacy of BES-GL3 administration against liver-stage parasites.
142 Blood-stage parasites were completely prevented in all mice intravenously injected with
143 BES-GL3 24 h post-infection; in contrast, the protective effectiveness of BES-GL3
144 intravenous administration was diminished when mice did not receive it until 42 h
145 post-infection. The same results were obtained when mice were intramuscularly injected
146 with BES-GL3.

147 To visualize the parasite elimination by BV, mice were infected with Pb-Luc, which is
148 transgenic *P. berghei* constitutively expressing luciferase, and then examined via IVIS; this
149 is a highly sensitive method for detecting liver- and blood-stage parasites. Parasites were
150 observed in the liver at both 24 h and 42 h post-infection (Fig. 2, A and B, respectively; left
151 panels). BES-GL3 intramuscular administration into the left thigh muscle at 24 h
152 post-infection completely eliminated the liver-stage parasites at 72 h post-infection,
153 whereas the PBS control treatment failed to prevent the development of blood-stage
154 parasites (Fig. 2A; right panel). Although BES-GL3 intramuscular administration into the
155 right thigh muscle at 42 h post-infection also failed to prevent the development of

blood-stage parasites (Fig. 2B; right panel), it caused a significant delay of parasitaemia (Fig. 2C). This result indicates that even for exoerythrocytic schizonts (42 h post-infection), the elimination effect of BV intramuscular administration was invoked in the liver within 2–6 h because the exoerythrocytic merozoites of *P. berghei* are released from infected hepatocytes into the blood stream 44–48 h after the liver stage (26). Lower doses (10^4 and 10^6 pfu) of BES-GL3 administered at 24 h post-infection failed to prevent blood-stage parasites. However, a significant delay of parasitaemia was observed for the dose of 10^6 pfu of BES-GL3 (Fig. 2D), indicating that the elimination effect is dependent on the amount of BV that is intramuscularly administered.

As PQ is the only licensed drug for the radical cure of *P. vivax* hypnozoites, we compared the elimination effects of BV with those of PQ. Two different doses of PQ, high dose (2 mg/mouse) and low dose (0.1 mg/mouse), were intraperitoneally administered; these doses are 533 and 27 times, respectively, higher than the WHO recommended dose per weight for people. A single administration of high dose PQ completely eliminated the liver-stage parasites (Table 2), whereas low dose PQ was suboptimal, producing only a

171 reduction in parasite burden in the liver and significant delay of parasitaemia (Fig. 2C). The
172 WHO-recommended treatment schedule for PQ is 15 mg/day for 14 days, but because high
173 doses of PQ often cause side effects like nausea, vomiting, and stomach cramps, these side
174 effects can limit patient compliance, potentially resulting in PQ resistance (27, 28). Thus,
175 BV intramuscular administration may have important advantages of over PQ.
176
177 **BV-mediated liver-stage parasite killing is due to TLR9-independent pathways**
178 CpG intramuscular administration completely eliminated early liver-stage parasites (6 h
179 post-infection) (Table 3); however, although it caused a significant delay of parasitaemia, it
180 had little effect on mature schizonts (24 h post-infection) (Fig. 2C). BV possesses unique
181 characteristics that activate DC-mediated innate immunity through
182 MyD88/TLR9-dependent and -independent pathways (9). Therefore, we next investigated
183 whether TLR9 plays an important role in BV-mediated parasite killing in the liver. A single
184 dose of intramuscularly administered BES-GL3 completely prevented blood-stage parasites
185 in all TLR9$^{-/-}$ mice previously infected with liver-stage parasites. In contrast, no elimination

effect or parasitaemia delay was observed following CpG intramuscular administration in TLR9$^{-/-}$ mice (Table 3). These results clearly demonstrate that BV-mediated parasite killing occurs via TLR9-independent pathways.

BV intravenous administration was reported to produce type I IFNs through TLR-independent and IRF3-dependent pathways in mice (9). To further investigate IFN production following BV intramuscular administration, the IFN serum levels were measured in WT and TLR9$^{-/-}$ mice 6 h after BES-GL3 intramuscular administration. As with intravenous administration, BES-GL3 intramuscular administration produced IFN-α in not only WT mice (6,311 ± 2,363 pg/ml) but also TLR9$^{-/-}$ mice (1,590 ± 737 pg/ml), whilst mice intramuscularly injected with PBS or CpG did not produce detectable IFN-α (< 20.0 pg/ml) (Fig. 3A). IFN-γ, a type II IFN, was also produced in both WT mice (1,367 ± 1,303 pg/ml) and TLR9$^{-/-}$ mice (488 ± 132 pg/ml) (Fig. 3B). CpG intramuscular administration induced much less IFN-γ compared with BV, but it induced a robust IL-12 response (Fig. 3C). Notably, CpG intravenous administration induced a high level of IFN-γ with considerable systemic side effects (29, 30).

201

202 **Liver-stage parasites are killed by IFN-mediated immunity**

203 To determine whether the serum cytokines act as effectors against liver-stage parasites, a
204 serum transfer assay was performed. Pooled sera were collected from donor mice 6 h after
205 they were intramuscularly injected with BES-GL3 or PBS. An aliquot of the pooled sera
206 (100 µl/animal) was transferred to each recipient mouse 24 h after intravenous injection
207 with 1,000 sporozoites. One of the five recipient mice effectively eliminated the liver-stage
208 parasites, and the other four infected recipient mice showed a significant delay in the time
209 to 1% parasitaemia (mean delay of 3.54 days; p = 0.0008, compared with the PBS sera
210 group) (Fig. 3D).

211 We next examined whether neutralization of IFN-α or IFN-γ in the sera altered the effect
212 of the sera on liver-stage parasites. Either anti-IFN-α or anti-IFN-γ antibody was incubated
213 with 100 µl of the sera, which contained 8,619 pg/ml of IFN-α and 4,705 pg/ml of IFN-γ.
214 Complete neutralization of IFN-α was confirmed by ELISA (see Fig. S1 in the
215 supplemental material). The IFN-α- or IFN-γ-neutralized sera (100 µl) was intravenously

administered to recipient mice that had been intravenously injected with 1,000 sporozoite 24 h before. The anti-IFN-α antibody treatment completely abrogated the serum-induced delay of parasitaemia, whereas the anti-IFN-γ antibody treatment only partially impaired the serum-induced elimination effect (Fig. 3D). To assess the effects of exogenous IFN-α and IFN-γ on elimination of liver-stage parasites, recombinant IFN-α (8,619 pg/mouse) or recombinant IFN-γ (4,705 pg/mouse) was intravenously administered to mice that had been intravenously injected with 1,000 sporozoites 24 h before. IFN-α administration completely eliminated the liver-stage parasites, whereas IFN-γ administration only partially eliminated them but caused a significant delay in the time to 1% parasitaemia (mean delay of 4.82 days; $p = 0.0082$, compared with the PBS group) (Fig. 3E). The IFN-α-mediated parasite elimination may be mediated via an effector mechanism distinct from that activated by IFN-γ. It is also possible that the effector mechanisms induced by IFN-α and IFN-γ may still be synergistically operative but that an alternate protective mechanism may be activated by BV. Similarly, Miller *et al.* showed that IFN-γ produced by NKT cells following type I IFN signalling from infected hepatocytes play an important role on

231 elimination of liver-stage parasites (31). Table S1 summarizes the results on the elimination
232 efficacy against liver-stage parasites of serum transfer and IFN administration.
233
234 **IFN-stimulated genes (ISGs) are upregulated in the liver post-BV intramuscular**
235 **administration**
236 Signal transduction of type I IFNs results in the induction of numerous ISGs (32). Some
237 ISGs participate in direct antimicrobial activities, such as apoptosis induction and
238 post-transcriptional event regulation for microbial killing, mainly acting as antiviral
239 responses. Gene targeting studies have distinguished four effector pathways of the
240 IFN-mediated antiviral response: the Mx GTPase pathway, 2′-5′ oligoadenylate-synthetase
241 (OAS)-directed ribonuclease L pathway, protein kinase R (PKR) pathway, and ISG15
242 ubiquitin-like pathway (33). Additionally, several ISGs, such as IFN-induced proteins with
243 tetratricopeptide repeats (IFITs), as well as the transcription factors IRF3 and IRF7 are
244 responsible for sensing the liver-infection by *Plasmodium* sporozoites (8). To confirm the
245 involvement of ISGs, the gene expression in the livers of mice intramuscularly injected

246 with BES-GL3 were measured by quantitative RT-PCR (qRT-PCR). BES-GL3 significantly
247 induced the gene expression of antiviral proteins (*Isg15*, *Mx1*, *Oas1a/b*, *Oasl1*, and *Pkr*) in
248 wildtype (WT) mice (Fig. 4A). All these genes, except Oas1a/b, possibly due to the gene
249 locus, were also upregulated by BV in TLR9$^{-/-}$ mice. Gene expression of IFIT proteins, such
250 as Ifit1, Ifit3, and Ifit44, were markedly and significantly enhanced in both WT and TLR9$^{-/-}$
251 mice (Fig. 4B). Gene expression of the transcription factors Irf3 and Irf7 were also induced
252 by BV in the same manner (Fig. 4C). These results indicate that systemic type I IFN
253 secretion following BV intramuscular administration in the thigh muscle strongly induced
254 ISGs in the liver.
255
256 **AdHu5-prime/BDES-boost heterologous immunization regimen confers sterile**
257 **protection and complete elimination**
258 To evaluate our newly developed malaria vaccine in an AdHu5-prime/BDES-boost
259 heterologous immunization regimen (14), mice were challenged twice (before and after)
260 BDES-sPfCSP2-boost following AdHu5-prime. All mice survived without any symptoms

following both the first and second challenges (Table S2, Group 2). In contrast, AdHu5-prime immunization alone did not confer protection (Group 1). All control mice intramuscularly injected with PBS became infected (Groups 3 and 4). Thus, BDES-PfCSP boosting was able to exert not only a therapeutic effect on liver-stage parasites but also a prophylactic effect on sporozoites. The animal experimental designs are illustrated in Fig. S2, E and F.

DISCUSSION

Here, we show that BV intramuscular administration not only elicits short-term sterile protection against sporozoite infection but also eliminates liver-stage parasites completely. For liver-stage parasites proliferating vigorously at 24 h post-infection, the BV-induced fast-acting innate immune responses completely kill them within the following 20 h and prevent blood-stage parasite development in the absence of any clinical symptoms, which is more effective than PQ in a mouse model with early liver-stage *P. berghei*. The *P. berghei* liver-stage model is thought to correlate with anti-hypnozoite activity in primates (34). PQ is currently the only available drug that kills the dormant hypnozoites of *P. vivax*, but its severe side effects in G6PD-deficient people prevent the widespread use of this drug (35). The presence of hypnozoites and their drug-insensitivity form a major hurdle for malaria elimination programmes. Although BV has not been tested in a clinical setting yet, our previous study showed that the BV-based vaccine vector is safe and well-tolerated with acceptable reactogenicity and systemic toxicity in a primate model (13). Thus, BV offers a promising new non-haemolytic single-dose alternative to PQ for first-in-human clinical

283 trials. Further experiments to determine optimum BV administration routes and dosages are
284 needed.

285 BV possesses attractive attributes as a new vaccine vector (e.g., its low cytotoxicity,
286 inability to replicate in mammalian cells and absence of pre-existing antibodies against it).
287 This study demonstrates the further unique advantage of BV as a therapeutic vaccine vector
288 with short-term protection via an intrinsic potent immunostimulatory property. In a Phase
289 II–III malaria vaccine trial, all volunteers are presumptively treated with three daily doses
290 of anti-malaria drug one week before final vaccination and rechecked for asexual *P.*
291 *falciparum* parasitaemia one week after the final vaccination. Any subject who tests
292 parasite-positive is treated with a second line drug or excluded from the trial (36). Thus,
293 clinical trials aim to test vaccine efficacy after all vaccine schedules are completed to assess
294 the maximum effect. For clinical application, however, vaccinators are still in danger of
295 infection until the full vaccination schedule is completed, even though improved effective
296 vaccine would be developed. This study shows that our newly developed heterologous
297 AdHu5-PfCSP-prime and BDES-PfCSP-boost vaccine eliminated liver-stage parasites that

had infected the mice 24 h before administration of the BDES-boost and also elicited sterile protection against sporozoite challenge 21 days post-boost. We propose that BV-based vaccines can not only minimize the risk of infection for vaccinators during the vaccination schedule but also generate robust and long-lasting adaptive immune responses via stimulation of the innate immune system. Alternatively, BV itself may also be used as an additive to eliminate liver-stage parasites and impart this short-term protection to RTS,S or other licensed vaccines.

This study showed that IFN-α and IFN-γ were rapidly and robustly produced in serum 6 h post-BV administration. Interestingly, the prophylactic effect against sporozoite infection lasted for at least 7 days, even after IFN levels returned to baseline. In the case of 'natural' *Plasmodium* liver-stage infection, the infected hepatocytes induce IFN-α, resulting in a reduction of the liver-stage burden (8). However, the parasite-induced IFN-α responses fail to eliminate every parasite. This implies that the endogenous innate immune responses may be not strong enough for complete elimination and/or that the innate immune response peak occurs at the end of liver-stage development, just prior to or concurrent with

313 exoerythrocytic merozoite release (37). Compared with the type I IFN induced by host
314 sensing of parasites, the quantity of BV-induced type I IFN and its speedy induction may
315 make it more effective, resulting in its potent therapeutic and prophylactic effects. A better
316 understanding of the molecular mechanisms by which BV administration confers both
317 protection and elimination of pre-erythrocytic parasites will provide new strategies for
318 malaria drug and vaccine development.

319 IFN-α has been extensively explored for its efficacy in various disease conditions and is
320 currently used as a standard treatment in several illnesses. However, its use is accompanied
321 by a wide variety of possible side effects (38), such as autoimmune thyroiditis. This study
322 found that BV intramuscular administration, which induced 8,619 pg/ml of IFN-α in mouse
323 sera with normal levels of ALT, completely killed liver-stage parasites. The manufacturing
324 cost of BV would be much lower than that of recombinant IFN-α. Thus, BV intramuscular
325 administration also has great potential for use as an alternative IFN-α-based
326 immunotherapy; its high biological activity, cost-effectiveness, non-invasive nature, and
327 minimal adverse effects make it superior to the current IFN-α therapy using recombinant

328 IFN-α via intravenous administration.

329 In conclusion, BV effectively induces fast-acting innate immune responses that provide
330 powerful first lines of both defensive and offensive attacks against pre-erythrocytic
331 parasites. Our results illustrate the great potential of baculovirus as a new potent
332 prophylactic and therapeutic immunostimulatory agent against preerythrocytic-stage
333 parasites. We propose that these baculovirus-based vaccine and drug strategies could be
334 applicable not only for malaria but also for other infectious diseases.
335

MATERIALS AND METHODS

Animals, cell lines, parasites, and mosquitoes

Female inbred BALB/c (*H-2*^{d}) mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) and used in all experiments at 7–8 weeks of age. TLR9-deficient (TLR9$^{-/-}$) mice on a BALB/c background were kindly provided by S. Akira (University of Osaka, Suita, Japan). *Spodoptera frugiperda* and HepG2 cells were maintained as described previously (13). Three transgenic *P. berghei* ANKA parasites were used in this study: GFP-*P. berghei* (Pb-conGFP) (39), luciferase-*P. berghei* (Pb-Luc) (40), and PfCSP-*P. berghei* (PfCSP-Tc/Pb) (41). These transgenic parasites were maintained by cyclical passaging through BALB/c mice and *Anopheles stephensi* (SDA 500 strain) at the Kanazawa University and Jichi Medical University according to a standard protocol (12, 42).

Ethics statement

All animal care and handling procedures were approved by the Animal Care and Ethical Review Committee of Kanazawa University (no. 22118–1) and Jichi Medical University

(no. 09193) Japan. For animal experiments, all efforts were made to minimize suffering in the animals. Mice were anesthetized with ketamine (100 mg/kg; intramuscular; Daiichi Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; intramuscular; Bayer, Tokyo, Japan) when necessary.

Recombinant viruses

The recombinant baculoviruses BES-GL3 and BDES-sPfCSP2-WPRE-Spider have been described previously (19). The purified baculovirus particles were free of endotoxin (<0.01 endotoxin units/10^9 pfu), as determined by the Endospecy® endotoxin measurement kit (Seikagaku Co., Tokyo, Japan). The recombinant adenoviruses AdHu5-Luc and AdHu5-sPfCSP2 have been described previously (14). In this paper, BDES-sPfCSP2-WPRE-Spider and AdHu5-sPfCSP2 are described as BDES-PfCSP and AdHu5-PfCSP, respectively.

Collection of sporozoites

366 *An. stephensi* mosquitoes were infected by feeding on infected mice using standard
367 methods of mosquito infection. On days 21–24 post-infection, the salivary glands of the
368 mosquitoes were collected by hand-dissection. Salivary glands were collected in DMEM
369 (Thermo Fisher Scientific K.K., Tokyo, Japan) and homogenized in a plastic homogenizer.
370 The free sporozoites were counted in a disposable haemocytometer counting chamber using
371 phase-contrast microscopy.
372
373 **Analysis of protective effects against sporozoite parasites**
374 BALB/c mice were intravenously, intramuscularly, or intranasally administered 10^4–10^8 pfu
375 of BES-GL3. Alternatively, instead of BES-GL3, BALB/c mice were intramuscularly
376 injected with 50 μg of CpG ODN 1826 (TCCATgACgTTCCTgACgTT, Fasmac Inc.,
377 Tokyo, Japan). The mice were intravenously challenged with 1,000 Pb-conGFP sporozoites
378 or 1,000 pRBCs at various time intervals (6 h–14 days). The mice were checked for *P.*
379 *berghei* blood-stage infection by microscopic examination of Giemsa-stained thin smears of
380 their tail blood, prepared on days 5, 6, 7, 8, 11, and 14 post-challenge. The time required to

reach 1% parasitaemia was determined as described previously (43). A minimum of 20 fields (magnification, × 1,000) were examined before a mouse was deemed to be negative for infection. Protection was defined as the complete absence of blood-stage parasitaemia on day 14 post-challenge.

Analysis of elimination effects on liver-stage parasites

BALB/c mice were intravenously injected with 1,000 Pb-conGFP sporozoites and subsequently intravenously (10^7 pfu) or intramuscularly (10^8 pfu) injected with BES-GL3 at various time intervals (6, 24, or 42 h post-infection). Alternatively, instead of BV, a single high (2 mg) or low (0.1 mg) dose of PQ (primaquine diphosphate 98%, Sigma-Aldrich, St. Louis, MO, USA), with corresponding concentrations of roughly 40 mg/kg body weight and 20 mg/kg body weight respectively, was intraperitoneally administered 24 h after injection of 1,000 Pb-conGFP sporozoites. The mice were checked for *P. berghei* blood-stage infection and evaluated for 1% parasitaemia as described above.

396 ***In vivo* bioluminescent imaging**

397 BALB/c mice were intravenously or intramuscularly injected with BES-GL3 on day 0, and
398 D-luciferin (15 mg/ml; OZ Biosciences, Marseille, France) was then intraperitoneally
399 administered (150 µl/mouse) to these mice at various time points. The animals were
400 anesthetized with a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture 10 min later, and
401 the luciferase expression was detected with an IVIS® Lumina LT *in vivo* imaging system
402 (PerkinElmer, Waltham, MA, USA). Alternatively, BALB/c mice were intravenously
403 injected with 1,000 Pb-Luc sporozoites followed by BES-GL3 (10^8 pfu) intramuscular
404 administration into the left thigh muscle 24 or 42 h later. At 72 h after the sporozoite
405 injection, the luciferase expression was detected as described above. At days 5–14
406 post-infection, the same mice were analysed for blood-stage infections by determination of
407 the course of parasitaemia in Giemsa-stained thin blood films of tail blood.

408

409 **Cytokine, AST, and ALT assays**

410 BALB/c mice were intravenously or intramuscularly injected with BV, and sera were

411 subsequently harvested from whole blood obtained by cardiopuncture at various times and
412 stored at −20 °C until analysis. The concentrations of cytokines in the sera were determined
413 by sandwich ELISA using a Mouse IFN-γ ELISA MAX™ standard kit (Biolegend Inc., San
414 Diego, CA, USA), mouse IL-12/IL-23 (p40) ELISA MAX™ standard kit (Biolegend Inc.),
415 or mouse TNF-α ELISA MAX™ deluxe kit (Biolegend Inc.) according to the
416 manufacturer's instructions. The concentration of IFN-α was determined by sandwich
417 ELISA as described previously. In brief, rat monoclonal antibody against mouse IFN-α
418 (PBL Biomedical Laboratories clone RMMA-1, Piscataway, NJ, USA) was used as the
419 capture antibody (2 μg/ml for coating), rabbit polyclonal antibody against mouse IFN-α
420 (PBL Biomedical Laboratories) was used at 80 neutralizing units per ml for detection, and
421 HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) was used as the
422 secondary reagent. Recombinant mouse IFN-α (PBL Biomedical Laboratories) was used as
423 the standard. The lower detection limits for the IFN-γ and the IFN-α immunoassays were
424 each <20 pg/ml, whereas those for the IL-12 and the TNF-α immunoassays were each <10
425 pg/ml. The concentrations of ALT and AST in the sera were determined by using a

GPT/GOT assay kit (Transaminase CII-test; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) according to manufacturer's instructions.

Serum transfer and IFN administration analysis

Pooled sera were obtained from blood harvested by cardiopuncture from 5 BALB/c mice that had been intramuscularly injected with BES-GL3 6 h previously (at −6 h), and the concentrations of IFN-α and IFN-γ were measured immediately. On the same day, the IFN-α and IFN-γ in 100-µl aliquots of the pooled sera were neutralized by incubation with sufficient amounts of anti-IFN-α (anti-mouse interferon alpha, rabbit serum; PBL Biomedical Laboratories) and anti-IFN-γ (Ultra-LEAF^{TM} purified anti-mouse IFN-γ antibody; BioLegend Inc.) antibodies, respectively, on ice for 6 h according to the manufacturer's instructions. At 24 h after being intravenously injected with 1,000 Pb-conGFP sporozoites, BALB/c mice were then intravenously injected with 100 µl of the sera that had been treated with either anti-IFN-α or anti-IFN-γ. For the IFN administration experiment, BALB/c mice that had been intravenously injected with 1,000 Pb-conGFP

441 sporozoites 24 h before were then intravenously administered either 8,619 pg of IFN-α or
442 4,705 pg of IFN-γ. For each experiment, the mice were checked for *P. berghei* blood-stage
443 infection and evaluated for 1% parasitaemia as described above.
444
445 **RNA isolation from livers and qRT-PCR quantification**
446 BALB/c (WT or TLR9$^{-/-}$) mice were intramuscularly injected with 10^8 pfu of BES-GL3.
447 Alternatively, 50 µg of CpG ODN1826 were administered intramuscularly. Six hours later,
448 whole livers were obtained by dissection of the treated mice. Each whole liver was placed
449 in a 5-ml plastic tube with a cap containing 4 ml of buffer RLT (Qiagen, Valencia, CA,
450 USA) containing 1% 2-mercaptoethanol. Two stainless beads (5-mm external diameter)
451 were added to the mixture. The tube was capped, attached to an µT-12 Beads Crusher
452 (TAITEC, Saitama Japan), and vigorously shaken at 2,500 rpm for 3.5 min. Total RNA was
453 isolated from 100-µl aliquots of the homogenates by using the RNeasy kit (Qiagen). cDNA
454 was synthesized by using random hexamers and Multiscribe reverse transcriptase (Applied
455 Biosystems, Foster City, CA, USA). Quantitative analysis of RNA transcripts was

456 performed by real-time PCR with SYBR® Green Premix Ex Taq™ (Takara, Tokyo, Japan).
457 All oligonucleotide primers used for the real-time PCR are detailed in Table S3 in the
458 supplemental material. Amplification of the *gapdh* gene was performed in each experiment.
459 Each Ct value of the samples was standardized based on the *gapdh* Ct value (ΔCt), and
460 each ΔCt value was normalized to that of the ΔCt value from PBS-treated control WT mice
461 (ΔΔCt). Results are shown as the relative expression ($1/2^{\Delta\Delta Ct}$).
462
463 **Immunization and challenge infections**
464 **Protective efficacy of heterologous AdHu5-prime/BDES-boost immunization against**
465 **sporozoite challenge**
466 BALB/c mice were intramuscularly immunized with a heterologous AdHu5-prime and
467 BDES-boost regimen with a 3-week interval as described previously (14). Alternately,
468 BALB/c mice were intramuscularly immunized with an AdHu5-prime and PBS-boost
469 regimen with a 3-week interval. The 100-μl vaccine doses contained 5×10^7 pfu of
470 AdHu5-PfCSP or 1×10^8 pfu of BDES-PfCSP. The mice were intravenously challenged

with 1,000 PfCSP-Tc/Pb sporozoites at 24 h after the boost was administered, as described previously (44). The mice were checked for *P. berghei* blood-stage infection and evaluated for 1% parasitaemia as described above. Protected mice were intravenously re-challenged with 1,000 PfCSP-Tc/Pb sporozoites, and protection was defined as described above.

Statistical analysis

Information on the study outline, sample size, and statistical analysis is shown in the main text, figures, and figure legends. A two-tailed Fisher's exact probability test was performed to determine the significance of differences in the protective efficacies of the vaccines, using SPSS software (version 19, Chicago, IL, USA). In all other experiments, statistical differences between the experimental groups were analysed by the methods described in the individual figure legends; p values of <0.05 were considered statistically significant. Statistical analyses were performed with either Prism version 7.0a (GraphPad Software Inc., La Jolla, CA, USA) or Microsoft® Excel (Radmond, WA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://XXXXXX.

SUPPLEMENTAL FILE 1, PDF file, XXX MB.

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668 **FIGURE LEGENDS**

669 **Figure 1. BV administration via intravenous vs intramuscular routes for transgene**

670 **expression and innate immune responses.** (A, B) Luciferase expression at different time

671 points after intramuscular (10^8 pfu) (A) or intravenous (10^7 pfu) (B) administration of

672 luciferase-expressing BES-GL3 (described as BV), detected by using the IVIS® Lumina LT

673 *in vivo* imaging system. The heat map image visible in the mice represents the total flux of

674 photons (p/sec/cm2) in that area. Rainbow scales are expressed in radiance (p/s/cm^2/sr). (C,

675 D) Kinetics of proinflammatory cytokines, IFN-γ (C) and TNF-α (D), in the sera at

676 different time points post-BV intravenous administration (10^7 pfu) (n = 6). (E, F) Kinetics

677 of liver damage markers. ALT (E) and AST (F) in the sera after BV intravenous

678 administration (10^7 pfu) (n = 6). (G, H) Comparison of ALT (G) and AST (H) in the sera at

679 6 h after intramuscular (10^8 pfu) or intravenous (10^7 pfu) administration of BV (n = 6).

680 (C-H) Bars or points are the mean ± SD. (G, H) The difference from the PBS group was

681 assessed by a Kruskal-Wallis test with Dunn's correction. ***p < 0.001. i.m.,

682 intramuscular; i.v., intravenous.

683

684 **Figure 2. BV intramuscular administration completely eliminates liver-stage parasites.**

685 (A, B) Challenge infection with Pb-Luc sporozoites at 0 h, followed by BES-GL3

686 (described as BV) intramuscular administration (10^8 pfu) at indicated time points.

687 Luminescence in the liver shows parasite growth, whereas that in the thigh originates from

688 the BV injection. The heat map images visible in the mice represent the total flux of

689 photons (p/s/cm^2) in that area. Rainbow scales are expressed in radiance (p/s/cm^2/sr). (C, D)

690 Delay of parasitaemia in infected mice. Parasitaemia of groups of infected mice shown in

691 Table 2 (10^6 or 10^4 pfu of BV injected intramuscularly 24 h post-infection, 10^8 pfu of BV

692 injected intramuscularly 42 h post-infection, and PQ low dose administered 24 h

693 post-infection) and Table 3 (CpG administered 24 h post-infection). Bars or points are the

694 mean ± SD. The difference from the PBS group was assessed by a two-way ANOVA. *p <

695 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. i.m., intramuscular; i.v., intravenous.

696

697 **Figure 3. IFN-α induced by BV intramuscular administration contributes to**

698 **elimination of liver-stage parasites.** (A-C) Levels of IFN-α (A), IFN-γ (B), and IL-12 (C)
699 in sera from WT or TLR9$^{-/-}$ mice 6 h after intramuscular administration of BES-GL3
700 (described as BV) (10^8 pfu), CpG, or PBS (n = 9–10). Bars are means ± SD. (D, E)
701 Prediction of the time to reach 1% parasitaemia. (D) Results of serum transfer assay to
702 determine the role of IFN-α and IFN-γ in the elimination of liver-stage parasites. Sera
703 collected from mice 6 h after BV administration were neutralized by either anti-IFN-α or
704 anti-IFN-γ antibody. Passive transfer of antibody-treated sera, non-treated sera, or PBS was
705 conducted 24 h after sporozoite infection (n = 5). (E) Effect of exogenous IFN-α or IFN-γ
706 on the elimination of liver-stage parasites. Recombinant mouse IFN-α or IFN-γ was
707 intravenously administered 24 h after sporozoite infection (n = 5). (A-E) The difference
708 from the PBS group was assessed by a Kruskal-Wallis test with Dunn's correction. *p <
709 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
710
711 **Figure 4. BV intramuscular administration induces ISGs in the liver.** Gene expression
712 of antiviral proteins (A), IFITs (B), and IRFs (C) in the livers of WT and TLR9$^{-/-}$ mice at 6

713 h after intramuscular administration of BES-GL3 (described as BV) (10^8 pfu) was

714 measured by real-time RT-PCR (n = 5–7). Bars are means ± SEM. The difference from the

715 PBS group was assessed by a Mann-Whitney's U test. *$p < 0.05$, **$p < 0.01$.

716

717

718

Table 1. Protective efficacy of BV injection against *P. berghei* sporozoite challenge[a, b]

Treatment[a]	Dose (Route[c])	Time interval of challenge after administration	%Protection (protection/total)[d]
PBS	(i.v.)	6 h	0 (0/8)[e]
BV	1 x 10^7 pfu (i.v.)	6 h	100 (8/8)
AdHu5-luc	5 x 10^7 pfu (i.v.)	6 h	0 (0/3)
PBS	(i.m.)	12 h – 14 days	0 (0/20)[e]
BV	1 x 10^8 pfu (i.m.)	12 h	100 (5/5)
BV	1 x 10^8 pfu (i.m.)	24 h	100 (5/5)
BV	1 x 10^8 pfu (i.m.)	3 days	100 (5/5)
BV	1 x 10^8 pfu (i.m.)	5 days	100 (5/5)
BV	1 x 10^8 pfu (i.m.)	7 days	100 (5/5)
BV	1 x 10^8 pfu (i.m.)	14 days	0 (0/5)
BV	1 x 10^8 pfu (i.n.)	6 h	0 (0/3)
BV	1 x 10^7 pfu (i.v.)	6 h/1,000 pRBC[f]	0 (0/5)
CpG	50 μg (i.m.)	6 h	90 (9/10)[e]
CpG	50 μg (i.m.)	24 h	80 (4/5)

[a]BALB/c mice were injected with BES-GL3 (described as BV) by the indicated route. After the indicated interval, mice were intravenously challenged with 1,000 Pb‑conGFP sporozoites. Parasitaemia was monitored on days 5-8, 11, and 14 after sporozoite challenge. Once parasites appeared in the blood, all mice died.

[b]Scheme of the experimental design is shown in Fig. S2A.

[c]i.v., intravenous; i.n., intranasal; i.m., intramuscular

[d]Protection is defined as the complete absence of blood-stage parasitaemia on day 14 post-challenge.

[e]Cumulative data from two or four experiments

[f]BALB/c mice were intravenously challenged with 1,000 Pb-conGFP-pRBC.

Table 2. Elimination of liver-stage parasites by BV administration[a, b]

Treatment[a]	Dose (Route[c])	Time interval of administration after challenge	%Elimination (uninfected/total)
PBS	(i.v.)	24 h	0 (0/12)[e]
BV	1 x 10^7 pfu (i.v.)	24 h	100 (13/13)[e]
BV	1 x 10^7 pfu (i.v.)	42 h	0 (0/3)
PBS	(i.m.)	24 h	0 (0/9)[e]
BV	1 x 10^8 pfu (i.m.)	24 h	100 (7/7)
BV	1 x 10^6 pfu (i.m.)	24 h	0 (0/5)[f]
BV	1 x 10^4 pfu (i.m.)	24 h	0 (0/5)[f]
BV	1 x 10^8 pfu (i.m.)	42 h	0 (0/3)[f]
PQ (High)[d]	2 mg (i.p.)	24 h	100 (5/5)
PQ (Low)[d]	0.1 mg (i.p.)	24 h	0 (0/5)[f]

[a]BALB/c mice were intravenously injected with 1,000 Pb-conGFP sporozoites. After the indicated interval, mice were administrated either with BES-GL3 (described as BV) or PQ. Parasitaemia was monitored on days 5-8, 11, and 14 after sporozoite injection. Once parasites appeared in the blood, all mice died.

[b]Scheme of the experimental design is shown in Fig. S2B.

[c]i.v., intravenous; i.m., intramuscular; i.p., intraperitoneal

[d]The two different doses of PQ were administrated to eliminate liver-stage parasites. High dose (2 mg/100 μl) and low dose (0.1 mg/100 μl) were 533 and 27 times, respectively, higher than WHO recommended dose for human per weight.

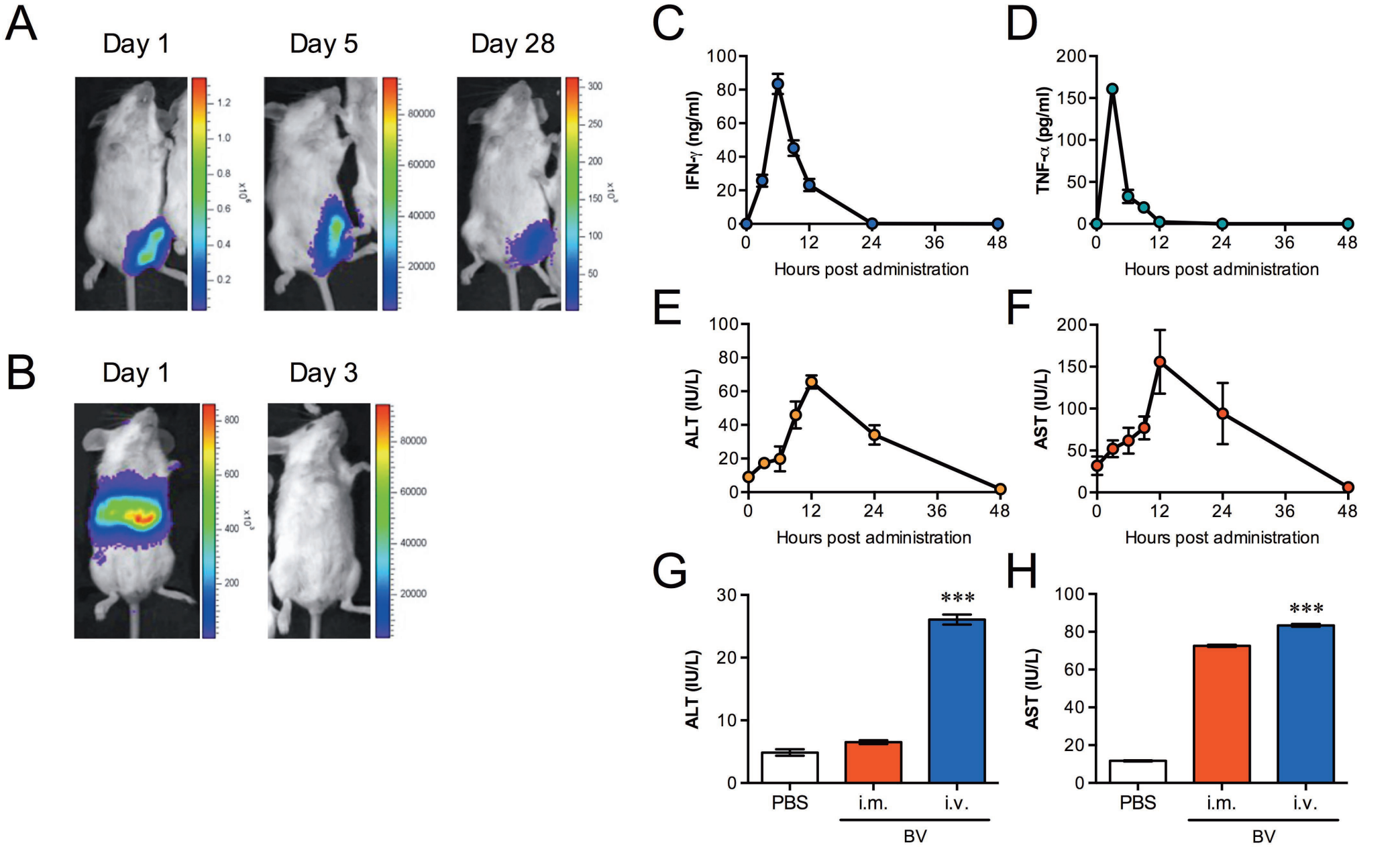
[e]Cumulative data from three experiments.

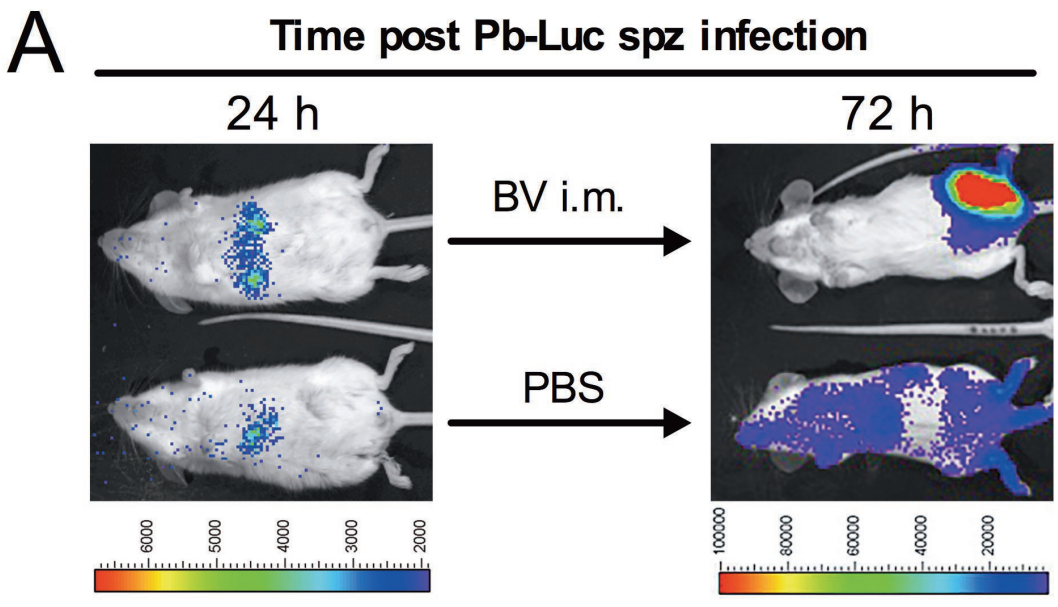
Table 3. Elimination of liver-stage parasites by BV injection in TLR9$^{-/-}$ mice[a]

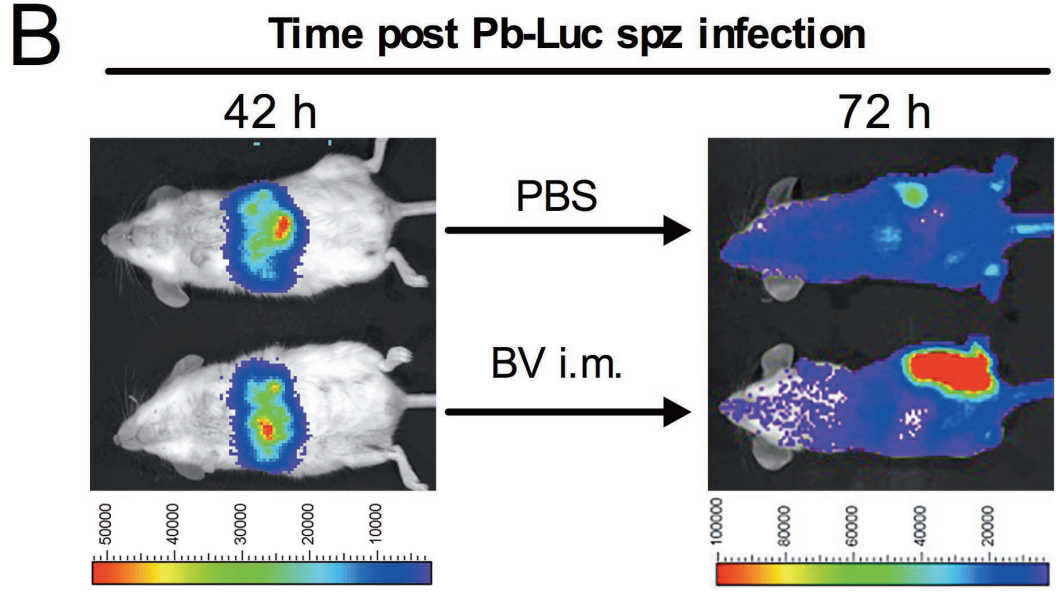
Treatment[a]	Mouse strain	Dose	Time interval of administration after challenge	%Elimination (uninfected/total)
PBS	TLR9$^{-/-}$	-	24 h	0 (0/7)
BV	TLR9$^{-/-}$	1 x 10^8 pfu	24 h	100 (7/7)
BV	WT	1 x 10^8 pfu	24 h	100 (5/5)
CpG	WT	50 μg	6 h	100 (5/5)
CpG	WT	50 μg	24 h	0 (0/4)[b]
CpG	TLR9$^{-/-}$	50 μg	24 h	0 (0/5)

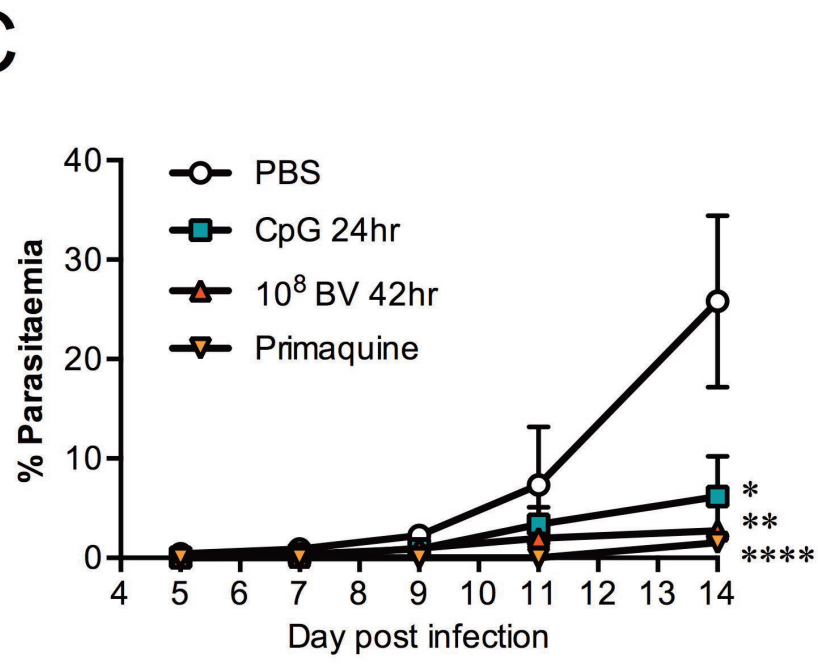
[a]TLR9$^{-/-}$ (BALB/c background) or WT mice were intravenously injected with 1,000 Pb-conGFP sporozoites. After 24 h, mice were intramuscularly administrated either with BES-GL3 (described as BV) or CpG ODN 1826 (described as CpG). Parasitaemia was monitored on days 5-8, 11, and 14 after sporozoite injection. Once parasites appeared in the blood, all mice died.

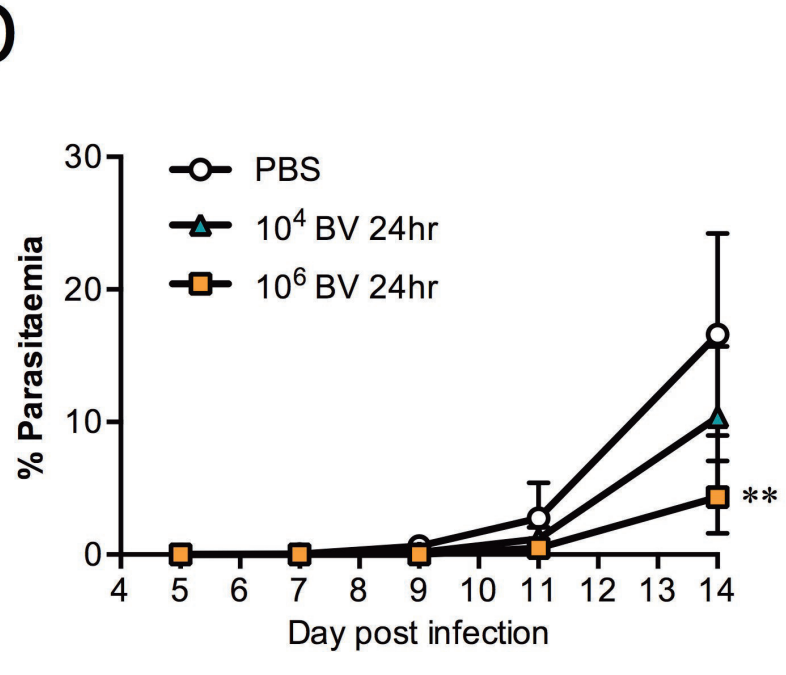
[b]Significant delay of parasitaemia was observed in infected mice, compared with the PBS group as showing in Fig. 2C.

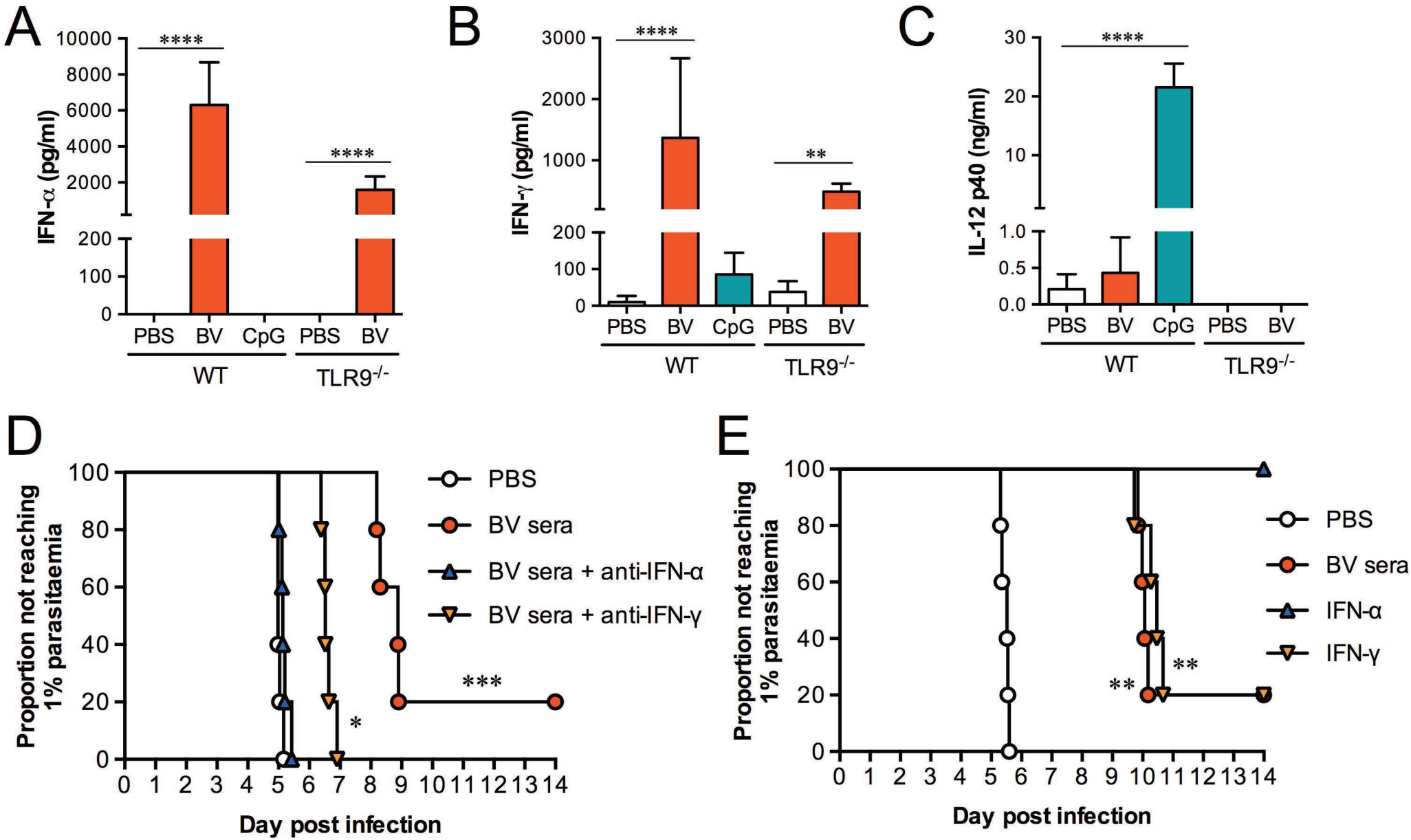

A
Day 1
Day 5
Day 28
B
Day 1
Day 3
C
IFN-γ (ng/ml)
Hours post administration
D
TNF-α (pg/ml)
Hours post administration
E
ALT (IU/L)
Hours post administration
F
AST (IU/L)
Hours post administration
G
ALT (IU/L)
***
PBS
i.m.
i.v.
BV
H
AST (IU/L)
***
PBS
i.m.
i.v.
BV

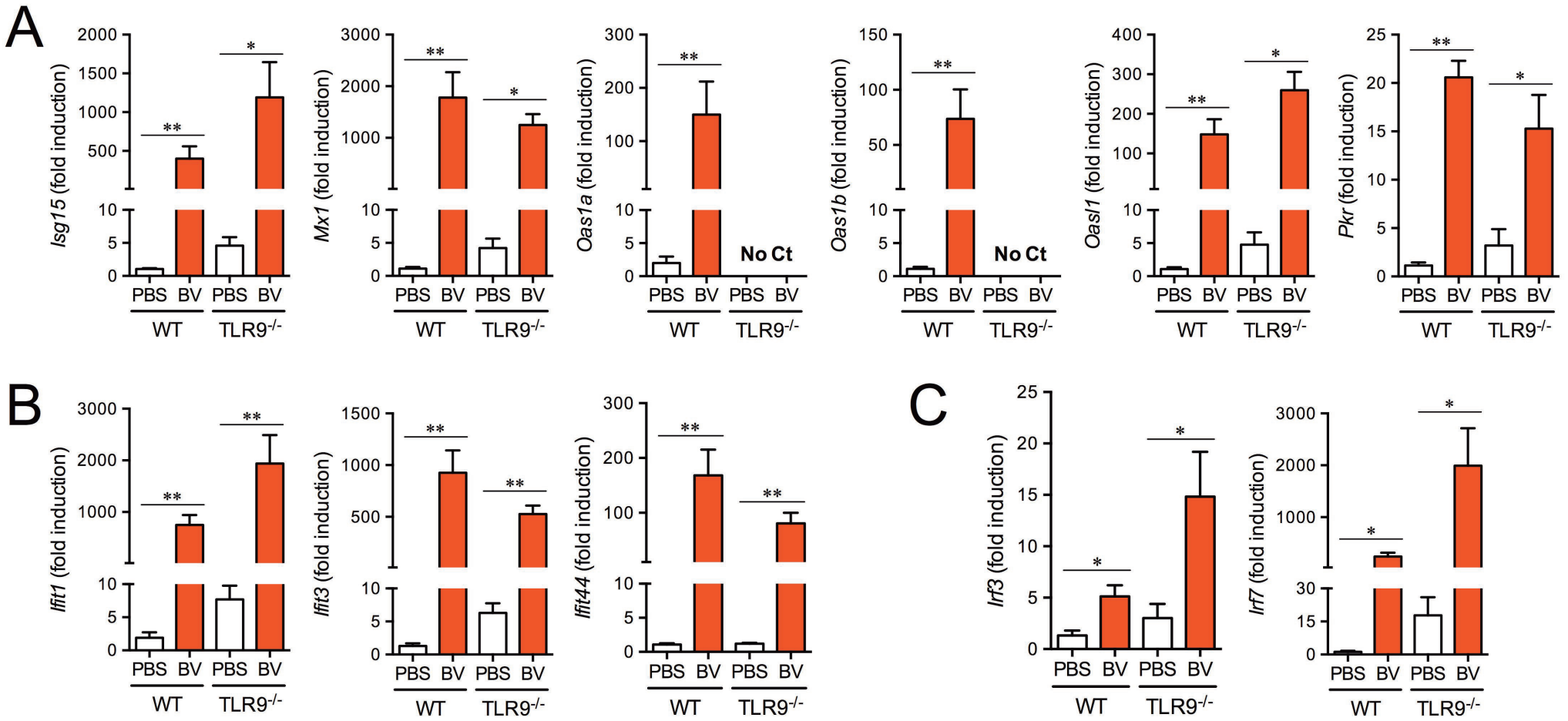

A
Time post Pb-Luc spz infection
24 h
72 h
BV i.m.
PBS
6000
5000
4000
3000
2000
100000
80000
60000
40000
20000


B
Time post Pb-Luc spz infection
42 h
72 h
PBS
BV i.m.
50000
40000
30000
20000
10000
100000
80000
60000
40000
20000


C
% Parasitaemia
PBS
CpG 24hr
10^8 BV 42hr
Primaquine
*
**
****
Day post infection


D
% Parasitaemia
PBS
10^4 BV 24hr
10^6 BV 24hr
**
Day post infection


A
IFN-α (pg/ml)
10000
8000
6000
4000
2000
200
100
0
****
****
PBS
BV
CpG
PBS
BV
WT
TLR9-/-
B
IFN-γ (pg/ml)
3000
2000
1000
200
100
0
****
**
PBS
BV
CpG
PBS
BV
WT
TLR9-/-
C
IL-12 p40 (ng/ml)
30
20
10
1.0
0.5
0.0
****
PBS
BV
CpG
PBS
BV
WT
TLR9-/-
D
Proportion not reaching 1% parasitaemia
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day post infection
PBS
BV sera
BV sera + anti-IFN-α
BV sera + anti-IFN-γ
***
*
E
Proportion not reaching 1% parasitaemia
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day post infection
PBS
BV sera
IFN-α
IFN-γ
**
**


A
Isg15 (fold induction)
Mx1 (fold induction)
Oas1a (fold induction)
Oas1b (fold induction)
Oasl1 (fold induction)
Pkr (fold induction)
No Ct
No Ct
B
Ifit1 (fold induction)
Ifit3 (fold induction)
Ifit44 (fold induction)
C
Irf3 (fold induction)
Irf7 (fold induction)
PBS BV PBS BV
WT TLR9-/-