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Characterization of the Gene Expression Patterns in the Murine Liver Following Intramuscular Administration of Baculovirus

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Intramuscular administration of wild-type baculovirus is able to both protect against *Plasmodium* sporozoite challenge and eliminate liver-stage parasites via a Toll-like receptor 9-independent pathway. To investigate its effector mechanism(s), the gene expression profile in the liver of baculovirus-administered mice was characterized by cDNA microarray analysis. The ingenuity pathway analysis gene ontology module revealed that the major gene subsets induced by baculovirus were immune-related signaling, such as interferon signaling. A total of 40 genes commonly upregulated in a Toll-like receptor 9-independent manner were included as possible candidates for parasite elimination. This gene subset consisted of NT5C3, LOC105246895, BTC, APOL9a/b, G3BP3, SLC6A6, USP25, TRIM14, and PSMB8 as the top 10 candidates according to the special unit. These findings provide new insight into effector molecules responsible for liver-stage parasite killing and, possibly, the development of a new baculovirus-mediated prophylactic and therapeutic biopharmaceutical for malaria.

Key words: Liver; Baculovirus; *Plasmodium*; Interferon signaling; cDNA microarray

INTRODUCTION

Malaria is still a significant burden on human health, causing an estimated 228 million cases and 405,000 deaths globally in 2018[1]. The *Plasmodium* parasites invade hepatocytes during the pre-erythrocytic stage and simultaneously stimulate host innate immune responses including the induction of effector molecules. Regarding parasite killing, the production of interferon-γ (IFN-γ), a type II IFN, induced the intracellular generation of nitric oxide, followed by killing of the liver-stage parasites[2]. Unmethylated CpG DNAs such as synthetic oligodeoxynucleotides (ODNs) are known to induce IFN-γ production via a Toll-like receptor (TLR) 9 signaling pathway[3], and stimulation with CpG ODNs in mice provided complete protection from sporozoite infection in an IFN-γ-dependent manner[4]. *Plasmodium* liver-stage infection induces the production of type I IFNs such as IFN-α/β, which were totally independent from TLR signaling[5]. However, this innate response does not eliminate every parasite, which implies that the parasite has evolved resistance strategies. This is an important consideration in the development of new drugs and malaria vaccines because the type I IFN response might reduce the likelihood of transmission of drug-resistant parasites or the escape of attenuated parasites used in vaccination[5]. Liver-stage infection can also induce IFN-γ via CD1d-restricted natural killer (NK) T cells, which are critical to reduce the parasite burden[6]. Thus, the details of host responses that eliminate the liver-stage parasites in terms of direct effector function are still largely unknown, and thus identification of the mechanism remains a top priority to develop not only novel anti-liver-stage drugs but also effective malaria vaccines.

Baculovirus vector (BV), an enveloped DNA virus that infects insects, has been used as an expression vector for protein purification, as well as a transgene expression vector such as a vaccine vector[7]. BV uniquely induces innate immune responses such as the induction of cytokine secretion through TLR9-dependent and -independent pathways when injected into nonhost mammals[8]. Recently, we demonstrated that TLR9 signaling by CpG ODN had protective efficacy against sporozoite challenge, which was consistent with a previous report[4], but had no elimination effect on mature schizonts after liver invasion (the liver-stage parasites). By contrast, BV-mediated induction of the innate immune response conferred both protection against sporozoites and elimination of liver-stage

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parasites[9]. Intramuscular administration of BV in mice markedly induced both IFN-α and IFN-γ in peripheral blood, potentially acting as either direct effector molecules to kill the liver-stage parasites or potent mediators to induce gene expression changes related to the elimination[9]. However, the injection site of BV was distant from the liver as the effector site, and thus the precise mechanism for parasite elimination remains unclear.

The main aim of this study was to identify essential signaling pathways for BV-mediated elimination of parasites using complementary DNA (cDNA) microarray analysis. Previously, gene expression profiling by microarray analysis was conducted for rat brains, human astrocytes, and human neuronal cells in response to BV stimulation, and the results indicated that host antiviral responses were induced in all types of samples as a major reaction[10]. Subsequently, BV was administered into the brain of cynomolgus macaques, nonhuman primates, and the induced genes were mainly associated with innate immunity, such as genes of the RIG-1-like receptor signaling pathway, leading to IFN production[11]. Those studies focused on the gene expression changes in the brain as the local reaction site that was directly injected with BV. In this study, cDNA microarray analysis was conducted for the liver of BV-administered mice, which was distant from the site of injection, to characterize the gene expression patterns. Comprehensive analysis with wild-type (WT) mice and TLR9$^{-/-}$ mice that were administered BV or the TLR9 ligand CpG DNA resulted in identification of a special gene subset that might eliminate the parasites in a TLR9-independent manner.

MATERIALS AND METHODS

Mice and Virus

Female inbred BALB/c (*H-2*^{d}) mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) and were used in the experiment at 7 weeks of age. TLR9-deficient (TLR9$^{-/-}$) mice with a BALB/c background were provided by S. Akira (University of Osaka, Suita, Japan). All animal care and handling procedures were approved by the Animal Care and Ethical Review Committee of Kanazawa University (AP-163700). For animal experiments, all efforts were made to minimize suffering in the animals. Mice were anesthetized with ketamine (100 mg/kg; intramuscularly; Daiichi Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; intramuscularly; Bayer, Tokyo, Japan) when necessary.

Preparation of BES-GL3, a recombinant BV-expressing luciferase, has been described previously[9,12].

Microarray Analysis

BALB/c (WT or TLR9$^{-/-}$) mice were intramuscularly injected with 10^8 pfu of BES-GL3 or 50 μg of CpG ODN 1826. Six hours later, the livers of the treated mice were surgically removed. Total RNA was isolated from the homogenates of the livers using an RNeasy Mini kit (Qiagen) along with on-column DNase I treatment (RNase-free DNase kit; Qiagen K.K.) as described previously[9]. The quality of the extracted RNA was analyzed using a Bioanalyzer 2100 (Agilent Technologies Inc.), and the RNA integrity number (https://www.chem-agilent.com/pdf/5989-1165EN.pdf) values were all confirmed to be above 9.0. RNA expression was profiled using the Affymetrix GeneChip® Mouse Gene 2.0 ST Array. cDNA synthesis of transcripts and biotin labeling of the cDNA were performed using the WT Plus reagent kit (Affymetrix, Santa Clara, CA, USA) in accordance with the manufacturer's protocol. The labeled and fragmented DNA was hybridized onto the microarray for 16 h in the GeneChip Hybridization oven 640 at 45°C while being rotated at 60 rpm. The hybridized samples were washed and stained using the Affymetrix fluidics station 450. After staining, the microarrays were immediately scanned using an Affymetrix GeneArray Scanner 3000 7G Plus.

GeneSpring and Ingenuity Pathway Analysis (IPA)

The obtained hybridization intensity data were imported into the GeneSpring GX 14.9 software (Agilent Technologies Inc.). The gene expression data were normalized to the robust multiarray average (RMA), and expression ratios were compared between genes from treated mice (BV or CpG administered) and control mice [phosphate-buffered saline (PBS) administered] for scatter plots, Venn diagrams, and hierarchical clustering analysis.

Genes with expression fold changes of more than 2 or less than 1/2 were considered significant, and they were further analyzed using IPA software. Canonical pathways and biological functions were automatically predicted based on the IPA's reference database and other ingenuity-supported third-party databases restricted to mouse tissues and cell lines. These sets of pathways allowed us to establish connections among genes according to both experimentally observed and predicted relationships with high confidence.

RESULTS

Profiling of Gene Expression Patterns in Murine Livers After Intramuscular Administration of BV in a TLR9-Independent Manner

Our previous study clearly demonstrated that BV-mediated elimination of liver-stage parasites was highly dependent on type I IFN signaling rather than TLR9 signaling[9]. However, type I IFN signaling induces more than 300 IFN-stimulated genes[13], which made it difficult to identify essential genes for parasite elimination. Therefore, we performed experiments to identify TLR9-dependent gene expression changes and then excluded them from the whole gene subset induced by BV. To achieve this,

BV was intramuscularly administered to both WT and TLR9$^{-/-}$ knockout (KO) BALB/c mice. CpG ODN 1826 (CpG), a murine TLR9 ligand, and PBS were also administered to WT mice as controls. After 6 h, the whole livers were dissected from four groups of mice (n = 2) including WT mice with BV (WT-BV), TLR9$^{-/-}$ mice with BV (KO-BV), WT mice with CpG (WT-CpG), and PBS-administered control mice. The gene expression patterns were examined using an Affimetrix GeneChip Mouse Gene 2.0 ST Array that covered a total of 35,240 RefSeq transcripts. The resulting microarray indicates that a total of 812, 690, and 1,204 gene probes in WT-BV, KO-BV, and WT-CpG, respectively, showed a >2-fold difference compared with mock samples. Among the three groups, the numbers of upregulated genes were 562, 372, and 842 in WT-BV, KO-BV, and WT-CpG, respectively, and the numbers of downregulated genes were 250, 318, and 362, respectively, compared with the mock samples.

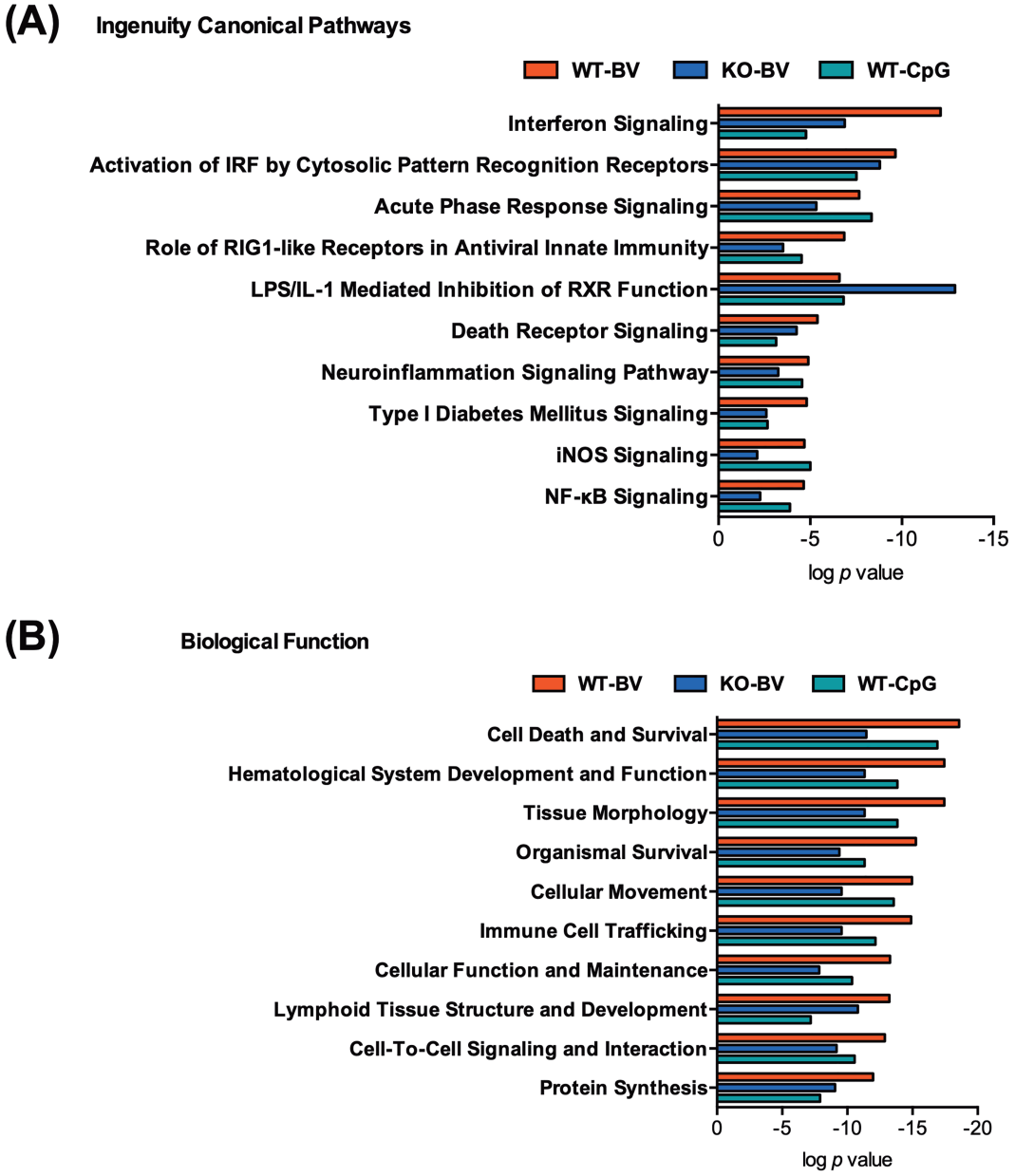

Figure 1. Microarray analysis of gene expression patterns in the liver after intramuscular administration of baculovirus vector (BV). Upregulated genes of wild-type (WT)-BV, knockout (KO)-BV, and WT-CpG when compared with the mock control (fold changes ≥ 2) were analyzed by ingenuity pathway analysis to identify their involvement in ingenuity canonical pathways (A) and their biological functions (B). In (A), only z-score-positive pathways are shown.

Table 1. Immune-Related Gene Expression in WT-BV Liver Samples According to Ingenuity Canonical Pathway Analysis in Comparison With KO-BV and WT-CpG Samples (Fold Changes[a])

	WT-BV	KO-BV	WT-CpG
Interferon signaling			
BAK	2.01	1.87	1.40
ISG15 (GIP2)	9.98	17.79	4.12
IFI35	4.24	4.01	2.79
IRF1	2.80	1.80	2.37
IRF9	3.47	2.45	3.00
JAK1	2.05	1.25	1.67
OAS1	8.28	0.86	5.96
PSMB8	2.16	3.75	1.35
RELA	2.55	2.46	1.71
SOCS1	2.92	2.17	2.07
STAT1	11.05	10.17	5.72
STAT2	3.65	2.19	2.87
TAP1	8.74	8.21	4.08
Activation of IRF by cytosolic pattern recognition receptors			
ADAR1	4.54	2.79	2.30
c-JUN (AP-1)	5.40	4.06	3.14
DAI	16.85	12.12	11.33
IRF7	9.13	10.74	4.51
LGP2	10.85	7.59	7.01
MDA5	7.81	5.57	3.85
p300/CBP	2.09	0.79	1.88
RIG-1	5.70	2.71	3.80
RIPK1	2.44	1.30	2.30
TBK1 (NAK)	3.27	2.97	1.92
Other genes duplicated above: IRF9, ISG15, RELA, STAT1, STAT2			
Acute-phase response			
FN1	2.03	1.16	2.26
GP130	2.34	1.37	2.31
IL-1	3.33	1.24	4.21
IL-6R	2.34	1.41	2.49
IL-1R/TLR	7.55	8.18	9.76
LBP	4.52	3.17	4.92
MEK1	2.87	2.43	1.34
MEKK1	2.07	1.39	1.92
MyD88	4.85	4.31	4.05
OSMR	8.60	2.47	7.91
SAA	61.64	56.26	102.73
SERPINA3	3.09	2.39	30.18
SERPINE1	6.11	2.26	9.12
SOCS3	4.73	4.61	3.00
STAT3	3.33	2.04	2.82
TNFR	2.70	2.42	2.51
Other genes duplicated above: c-JUN, RELA, RIPK1			
Role of RIG1-like receptors in antiviral innate immunity			
Caspase 8/10	2.61	1.86	1.95
TRIM25	2.49	1.70	1.45
Other genes duplicated above: IRF7, LGP2, MDA5, RELA, RIG-1, RIPK1, TBK1			
iNOS signaling			
CD14	4.55	2.36	8.93
Other genes duplicated above: c-JUN, IRF1, JAK1, LBP, MyD88, p300/CBP, RELA, STAT1			

(*continued*)

Table 1. (Continued)

	WT-BV	KO-BV	WT-CpG
NF-κB signaling			
Growth factor	3.89	2.92	4.19
HDAC1/2	2.08	1.39	1.12
PKR	6.93	5.12	2.59
TIRAP	2.58	1.76	2.14
Other genes duplicated above: Caspase 8/10, IL-1, IL-1R/TLR, MEKK1, MyD88, p300/CBP, RELA, RIPK1, TBK1, TNFR			

[a]Fold-changes (>2) in upregulated genes in WT-BV are shown.

The IPA gene ontology module reviewed the top 10 significantly affected ingenuity canonical pathways of WT-BV, along with KO-BV and WT-CpG (Fig. 1A). The pathways consisted mainly of immune-related signaling such as "IFN signaling," "activation of IRF," "acute phase response," "role of RIG1-like receptors," "LPS/IL-1 mediated inhibition of RXR function," "iNOS signaling," and "NF-κB signaling" (Fig. 1A). Details of their fold changes are provided in Table 1. In general, the association with each signaling pathway in KO-BV was decreased compared with WT-BV, while the association with WT-CpG was relatively high, indicating the contribution of TLR9 signaling following BV administration (Fig. 1A).

In biological function analysis, the following categories were identified for WT-BV: "cell death and survival," "hematological system development," "tissue morphology," "organismal survival," "cellular movement," "immune cell trafficking," "cellular function and maintenance," "lymphoid tissue structure and development," "cell-to-cell signaling and interaction," and "protein synthesis" (Fig. 1B). These functions were mainly related to tissue mortality and development, and cellular movement and the contributions in KO-BV were less than those in WT-BV and WT-CpG.

The Cluster of Effector Molecule Candidates "Intersection" That Was Independent of the TLR9 Signaling Pathway

Because the elimination of liver-stage parasites was independent from TLR9 signaling[9], the common upregulated genes in both WT-BV and KO-BV but not WT-CpG were categorized. The 40 specific genes identified from the microarray data were hierarchically clustered, and the cluster was designated "Intersection" (Fig. 2A and B). The genes of the Intersection cluster are listed in Table 2 according to the specific unit related to the TLR9-independent pathway. The fold changes in expression were high for both WT-BV and KO-BV, but low for WT-CpG. The Intersection cluster included NT5C3, LOC105246895, BTC, APOL9a/b, G3BP3, SLC6A6, USP25, TRIM14, and PSMB8 as the top 10 candidates. The significance of these genes and possible effector functions against *Plasmodium* are described in more detail in the Discussion section.

DISCUSSION

In this study, intramuscular administration with BV induced gene expression changes in the livers of mice. Our previous study showed that (i) BV is able to eliminate

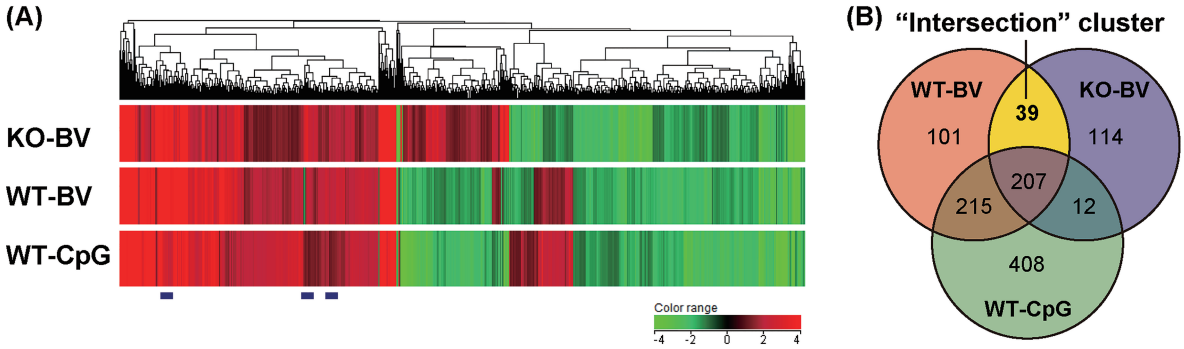

Figure 2. "Intersection" cluster induced by BV in a Toll-like receptor (TLR) 9-independent manner. (A) Hierarchical clustering analysis of the gene expression patterns in the liver from WT-BV, KO-BV, and WT-CpG mice compared with mock control mice. Gene sets that are indicated by blue bars represent the "Intersection" cluster, which may be BV specific but independent from TLR9 signaling. (B) Numbers of upregulated genes in WT-BV, KO-BV, and WT-CpG, and their overlaid clusters are shown. The Intersection cluster (yellow) consists of genes that were upregulated in both WT-BV and KO-BV but not WT-CpG.

Table 2. The Genes of the Cluster of Effector Molecule Candidates "Intersection"

Gene Symbol	Gene Name Description	Fold Change			Specific Unit
		WT-BV	KO-BV	WT-CpG	
NT5C3	5′-Nucleotidase, cytosolic III	4.77	2.22	1.13	3.09
LOC105246895|Gm11772	Unknown	3.84	4.26	1.40	2.89
BTC	Betacellulin, an epidermal growth factor	2.71	2.76	1.00	2.74
APOL9a	Apolipoprotein L9a	3.97	5.29	1.70	2.72
APOL9b	Apolipoprotein L9b	2.66	3.24	1.19	2.48
G3BP2	GTPase activating protein binding protein 2	5.13	4.13	1.93	2.40
SLC6A6	Solute carrier family 6, a neurotransmitter transporter	3.51	2.58	1.33	2.29
USP25	Ubiquitin-specific processing protease 25	3.49	2.46	1.34	2.22
TRIM14	Tripartite motif-containing 14	2.31	2.07	1.00	2.19
PSMB8	Proteasome subunit, beta type 8	2.16	3.75	1.35	2.19
PCGF5	Polycomb group RING finger protein 5	2.22	2.32	1.06	2.14
PARP10|PLEC	Poly(ADP-ribose) polymerases 10	3.41	3.48	1.67	2.06
DBNL	Drebrin-like protein	2.03	2.55	1.14	2.01
MAP2K1	MEK1	2.87	2.43	1.34	1.98
TDRD7	Tudor domain-containing 7	3.15	2.94	1.55	1.96
TNFRSF1a	TNF receptor superfamily member 1a	2.03	2.42	1.14	1.95
BST2	Tetherin; CD317	2.26	3.54	1.52	1.91
EXT1	Exostosin-1, a glycosyltransferse	2.88	2.45	1.40	1.90
TOR1AIP1	Torsin 1A interaction protein 1	4.58	2.10	1.81	1.85
ZC3HAV1	Zinc finger protein	3.26	2.61	1.60	1.83
B4GALT1	β(1,4)-galactosyltransferase	3.07	2.83	1.65	1.79
BAG3	BCL2-associated athanogene 3	3.14	3.20	1.84	1.72
H2-T24	MHC class I	3.38	2.32	1.68	1.70
CMTR1	Cap Methyltransferase 1	2.71	2.12	1.45	1.67
TBK1	TANK-binding kinase 1	3.27	2.97	1.92	1.63
LY6e	Lymphocyte antigen 6 family member E	2.02	2.35	1.40	1.56
NUB1	The ubiquitin-like protein NEDD8-interacting protein	3.29	2.19	1.78	1.54
FAS	Death receptor; CD95	2.77	2.51	1.79	1.47
RELA	NF-κB p65, a transcription factor	2.55	2.46	1.71	1.46
HIP1R	Huntingtin-interacting protein 1 related	2.71	2.49	1.78	1.46
Gm20412	Unknown	2.89	2.28	1.77	1.46
SH3BP2	SH3-domain binding protein 2	2.24	2.16	1.54	1.43
GSDMD	Gasdermin D	2.61	2.75	1.88	1.43
STX18	Syntaxin 18, a SNARE protein	2.57	2.83	1.95	1.38
USB1	U6 snRNA phosphodiesterase 1	2.40	2.12	1.65	1.37
CPSF2	Cleavage and polyadenylation specificity factor 2	2.60	2.08	1.71	1.37
VPS37c	Vacuolar protein sorting-associated protein 37C	2.19	2.46	1.77	1.31
ARF4	ADP-ribosylation factor 4, a small G-protein	2.40	2.24	1.83	1.27
GRN	Granulin	2.54	2.07	1.86	1.24
GNL3	Guanine nucleotide binding protein-like 3; Nucleostemin	2.07	2.19	1.79	1.19

Abbreviations: GTPase, guanosine triphosphatase; RING, Really interesting new gene; ADP, adenosine diphosphate; MEK1, mitogen-activated protein kinase and extracellular signal-regulated kinase kinase 1; TNF, tumor necrosis factor; BCL2, B-cell lymphoma 2; MHC, major histocompatibility complex; TANK, Traf family member-associated NF-κB activator; NEDD8, neural precursor cell expressed developmentally downregulated 8; NF-κB, nuclear factor-kappa B; SH3, Src homology 3; SNARE, soluble NSF attachment protein receptor. Specific units that were independent from TLR9 were calculated by the following formula: [(WT-BV + KO-BV)/2] ÷ WT-CpG.

liver-stage parasites by intramuscular administration, (ii) TLR9 signaling via CpG ODN stimulation has only limited efficacy for the elimination of parasites, and (iii) BV-mediated elimination is mainly dependent on type I IFN signaling[9]. To investigate which effector molecules are responsible for the elimination of liver-stage parasites, the gene expression changes in WT and TLR9$^{-/-}$ mice in the presence or absence of BV or CpG ODN were examined. Our data indicated that the major gene subsets induced by BV were related to IFN signaling, and this,

along with secondary signaling, may contribute to the elimination of liver-stage parasites.

Our microarray study identified the Intersection cluster that included possible effector molecules for the elimination of the liver-stage parasites that were independent of TLR9 signaling. The top 10 genes according to the special unit are detailed in Table 2 and discussed below. **[Gene 1]** 5′-nucleotidase cytosolic III (NT5C3) mainly catalyzes the dephosphorylation of pyrimidine nucleoside monophosphates, which play a critical role in nucleotide pool balance and in the metabolism of nucleoside analogues such as gemcitabine and cytosine arabinoside[14]. The blood-stage *Plasmodium falciparum* parasites rely mostly on pyrimidine nucleotides synthesized through the de novo biosynthetic pathway[15]. Recently, Antonova-Koch et al. identified subsets of chemical compounds that strikingly inhibited the growth of liver-stage *P. berghei* by high-throughput technology and found that they contained a high proportion of mitochondrial inhibitors (43%)[16]. The mitochondrion is critical for pyrimidine biosynthesis, a pathway that is essential for cell replication[17]. Collectively, this kind of nucleotidase may also contribute to control nucleotide metabolism in the liver-stage parasites. **[Gene 2]** LOC105246895 is an uncharacterized gene. **[Gene 3]** Betacellulin (BTC) is a member of the epidermal growth factor (EGF) family that is expressed in several tissues such as the kidney and liver[18], and not only stimulates EGF receptor tyrosine phosphorylation but also activates erbB-4, a member of the *erbB* gene family[19]. MEK1, which is listed in Table 2, is also implicated in downstream signaling of EGF receptors[20]. A previous study showed that travelers of Canada were more susceptible to severe malaria than people residing in endemic areas and produced less EGF in plasma than the residents, suggesting that EGF signaling is involved in *Plasmodium* elimination[15]. **[Genes 4 and 5]** Apolipoprotein L9 (APOL9) homologs such as APOL9a and APOL9b were strongly expressed in mouse liver and had antiviral activity against neurotropic Theiler's murine encephalomyelitis virus, which might be caused by interaction with cellular prohibitin 1 and prohibitin 2[21]. Innate immunity against *Trypanosoma brucei brucei* involved activation of APOL1, an APOL family member, and the lack of this protein enhanced the patient's infectivity to *Trypanosoma evansi*, because APOL1 was the lytic factor in normal human serum[22,23]. Additionally, APOL9 has been reported to be a key protein involved in autophagy[24], and thus, this protein is likely to induce lysis of the liver-stage parasites. **[Gene 6]** GTPase activating protein binding protein 2 (G3BP2) and G3BP1, together with RNA, are components of stress granules, which function to protect RNAs from harmful conditions[25–27]. Recently, Hanson et al. demonstrated that infection by liver-stage parasites did not induce stress granule formation in the host cells, and therefore the parasites evaded host cell sensing by stress inducible signaling[28]. Conversely, the successful induction of G3BP2 by BV might contribute to parasite killing, as similarly demonstrated for poliovirus[29]. **[Gene 7]** The gene *slc6a6* (taut gene) encodes the taurine transporter[30]. It was previously shown that taut$^{-/-}$ mice lose their ability to self-heal a blood-stage infection with *Plasmodium chabaudi*[31]. The livers of taut$^{-/-}$ mice showed a higher frequency of hepatocyte apoptosis and activation of the CD95 death receptor (Fas)[32], which is listed in Table 2. Based on this evidence, we hypothesize that taurine metabolism in the liver may control not only liver injury but also parasite growth. **[Gene 8]** Ubiquitin-specific processing protease 25 (USP25) is a member of the deubiquitinating enzymes that cleave ubiquitin or ubiquitin-like proteins from pro-proteins or target proteins[33,34]. USP25 was able to cleave ubiquitin chains from RIG-I, tumor necrosis factor receptor-associated factor (TRAF) 2, TRAF3, and TRAF6 that were induced via type I IFN signaling by Sendai virus or vesicular stomatitis virus[35,36], suggesting that USP25 could be a negative regulator of inflammation after BV administration. **[Gene 9]** Tripartite motif-containing 14 (TRIM14) localizes to mitochondria and facilitates RIG-I-like receptor-mediated IFN regulatory factor (IRF) 3 and nuclear factor-κB (NF-κB) activation[37]. TRIM14 mediates cell proliferation, clone formation, cell cycle procession, migration, and invasion in vitro and promotes tumor growth in vivo; it also activates the AKT pathway[38]. Therefore, TRIM14 might induce changes in several genes that belong to bio-functional pathways (Fig. 1B). **[Gene 10]** The gene encoding proteasome subunit beta type 8 (*psmb8*) encodes the IFN-γ inducible subunit (b5i/LMP7) of the immunoproteasome, which degrades ubiquitin-tagged cytoplasmic proteins into peptides that are especially suited for presentation by major histocompatibility complex (MHC) class I molecules to CD8$^+$ cytotoxic T cells[39]. The downregulation of PSMB8 expression leads to suppression of MHC class I molecule surface expression[40]. H2-24, an MHC class I molecule, was also listed in the Intersection cluster (Table 2) and may play a role in enhancing the acquired immune response against sporozoite-infected hepatocytes. In summary, we hypothesized that parasite killing in the liver by BV administration might be partly mediated by the inhibition of pyrimidine biosynthesis required for parasite growth and the acceleration of lysis of the parasites by several signaling pathways, which were mediated by EGF receptors, autophagy, stress granules, or a taurine transporter in combination with Fas.

Although IFN signaling is essential to reduce the parasite burden in the liver, the majority of genes in the Intersection cluster, with the exception of MEK1,

PSMB8, TBK1, and RELA (NF-κB p65), were not listed in the main pathways related to immune signaling (Tables 1 and 2). This discrepancy supports the idea that secondary signaling pathways may contribute to parasite elimination. In addition to direct activation of hepatocytes, BV-mediated induction of IFN might activate circulating blood cells such as NK cells. Type I IFN enhances the production of IFN-γ by NK cells and promotes NK cell-mediated cytotoxicity[41,42]. Type I IFN also promotes NK cell expansion during viral infection and protects against cell death via fratricide[43]. *Plasmodium* infection increases the number of NK cells in the liver, and these cells can restrict the development of liver-stage parasites but not blood-stage parasites[44]. Thus, NK cells could contribute to parasite elimination through the activation by BV-induced IFN-α. Although BV administration also induced IFN-γ secretion, its involvement in parasite elimination was partial (Fig. 2)[9]. Based on this, BV-induced IFN-α could not only induce gene expression changes in the liver but also activate NK cells to secrete IFN-γ and other unknown effectors to eliminate the liver-stage parasites. This hypothesis needs to be clarified in future studies involving an NK cell depletion assay.

The identification of effector molecules from hosts capable of killing liver-stage parasites is key for developing both pre-erythrocytic vaccines and antihypnozoite drugs. The present study not only showed the potential of BV as a new agent against liver-stage parasites but also offers insight into understanding the mechanism of parasite killing in the liver stage. To further determine the critical effector molecules, it would be necessary to validate whether the candidate genes are upregulated using different methods [such as reverse transcription polymerase chain reaction (RT-PCR)] in a follow-up study, and develop an in vitro sporozoite invasion assay using transgenic hepatocytes, in which the individual gene candidates are silenced by siRNA or overexpressed by an expression vector.

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REFERENCES

1. World malaria report 2019. The World Health Organization 2019. https://www.who.int/publications/i/item/9789241565721
2. Mellouk S, Green SJ, Nacy CA, Hoffman SL. IFN-gamma inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. J Immunol. 1991;146(11):3971–6.
3. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K and others. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408(6813):740–5.
4. Gramzinski RA, Doolan DL, Sedegah M, Davis HL, Krieg AM, Hoffman SL. Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice. Infect Immun. 2001;69(3):1643–9.
5. Liehl P, Zuzarte-Luis V, Chan J, Zillinger T, Baptista F, Carapau D, Konert M, Hanson KK, Carret C, Lassnig C and others. Host-cell sensors for *Plasmodium* activate innate immunity against liver-stage infection. Nat Med. 2014;20(1):47–53.
6. Miller JL, Sack BK, Baldwin M, Vaughan AM, Kappe SH. Interferon-mediated innate immune responses against malaria parasite liver stages. Cell Rep. 2014;7(2):436–47.
7. Lin SY, Chung YC, Hu YC. Update on baculovirus as an expression and/or delivery vehicle for vaccine antigens. Expert Rev Vaccines 2014;13(12):1501–21.
8. Abe T, Matsuura Y. Host innate immune responses induced by baculovirus in mammals. Curr Gene Ther. 2010;10(3):226–31.
9. Emran TB, Iyori M, Ono Y, Amelia F, Yusuf Y, Islam A, Alam A, Tamura M, Ogawa R, Matsuoka H, et al. Baculovirus-induced fast-acting innate immunity kills liver-stage *Plasmodium*. J Immunol. 2018;201(8):2441–51.
10. Boulaire J, Zhao Y, Wang S. Gene expression profiling to define host response to baculoviral transduction in the brain. J Neurochem. 2009;109(5):1203–14.
11. Balasundaram G, Kwang TW, Wang S. cDNA microarray assays to evaluate immune responses following intracranial injection of baculoviral vectors in non-human primates. J Neurochem. 2017;140(2):320–33.
12. Iyori M, Yamamoto DS, Sakaguchi M, Mizutani M, Ogata S, Nishiura H, Tamura T, Matsuoka H, Yoshida S. DAF-shielded baculovirus-vectored vaccine enhances protection against malaria sporozoite challenge in mice. Malar J. 2017;16(1):390.
13. Borden EC, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR, Stark GR. Interferons at age 50: Past, current and future impact on biomedicine. Nat Rev Drug Discov. 2007;6(12):975–90.
14. Aksoy P, Zhu MJ, Kalari KR, Moon I, Pelleymounter LL, Eckloff BW, Wieben ED, Yee VC, Weinshilboum RM, Wang L. Cytosolic 5′-nucleotidase III (NT5C3): Gene sequence variation and functional genomics. Pharmacogenet Genomics 2009;19(8):567–76.
15. Krungkrai SR, Krungkrai J. Insights into the pyrimidine biosynthetic pathway of human malaria parasite *Plasmodium falciparum* as chemotherapeutic target. Asian Pac J Trop Med. 2016;9(6):525–34.
16. Antonova-Koch Y, Meister S, Abraham M, Luth MR, Ottilie S, Lukens AK, Sakata-Kato T, Vanaerschot M, Owen E, Jado Rodriguez JC and others. Open-source discovery

of chemical leads for next-generation chemoprotective antimalarials. Science 2018;362(6419).
17. Phillips MA, Goldberg DE. Toward a chemical vaccine for malaria. Science 2018;362(6419):1112–3.
18. Sasada R, Ono Y, Taniyama Y, Shing Y, Folkman J, Igarashi K. Cloning and expression of cDNA encoding human betacellulin, a new member of the EGF family. Biochem Biophys Res Commun. 1993;190(3):1173–9.
19. Riese DJ, 2nd, Bermingham Y, van Raaij TM, Buckley S, Plowman GD, Stern DF. Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-beta. Oncogene 1996;12(2):345–53.
20. Saito T, Okada S, Ohshima K, Yamada E, Sato M, Uehara Y, Shimizu H, Pessin JE, Mori M. Differential activation of epidermal growth factor (EGF) receptor downstream signaling pathways by betacellulin and EGF. Endocrinology 2004;145(9):4232–43.
21. Kreit M, Vertommen D, Gillet L, Michiels T. The interferon-inducible mouse apolipoprotein l9 and prohibitins cooperate to restrict Theiler's virus replication. PLoS One 2015;10(7):e0133190.
22. Pays E, Vanhollebeke B, Vanhamme L, Paturiaux-Hanocq F, Nolan DP, Perez-Morga D. The trypanolytic factor of human serum. Nat Rev Microbiol. 2006;4(6):477–86.
23. Vanhollebeke B, Truc P, Poelvoorde P, Pays A, Joshi PP, Katti R, Jannin JG, Pays E. Human *Trypanosoma evansi* infection linked to a lack of apolipoprotein L-I. N Engl J Med. 2006;355(26):2752–6.
24. Arvind TA, Rangarajan PN. Mouse Apolipoprotein L9 is a phosphatidylethanolamine-binding protein. Biochem Biophys Res Commun. 2016;479(4):636–42.
25. Kedersha N, Ivanov P, Anderson P. Stress granules and cell signaling: More than just a passing phase? Trends Biochem Sci. 2013;38(10):494–506.
26. Buchan JR, Parker R. Eukaryotic stress granules: The ins and outs of translation. Mol Cell 2009;36(6):932–41.
27. Kobayashi T, Winslow S, Sunesson L, Hellman U, Larsson C. PKCalpha binds G3BP2 and regulates stress granule formation following cellular stress. PLoS One 2012;7(4):e35820.
28. Hanson KK, Mair GR. Stress granules and *Plasmodium* liver stage infection. Biol Open 2014;3(1):103–7.
29. Piotrowska J, Hansen SJ, Park N, Jamka K, Sarnow P, Gustin KE. Stable formation of compositionally unique stress granules in virus-infected cells. J Virol. 2010;84(7):3654–65.
30. Jhiang SM, Fithian L, Smanik P, McGill J, Tong Q, Mazzaferri EL. Cloning of the human taurine transporter and characterization of taurine uptake in thyroid cells. FEBS Lett. 1993;318(2):139–44.
31. Delic D, Warskulat U, Borsch E, Al-Qahtani S, Al-Quraishi S, Haussinger D, Wunderlich F. Loss of ability to self-heal malaria upon taurine transporter deletion. Infect Immun. 2010;78(4):1642–9.
32. Warskulat U, Borsch E, Reinehr R, Heller-Stilb B, Monnighoff I, Buchczyk D, Donner M, Flogel U, Kappert G, Soboll S, et al. Chronic liver disease is triggered by taurine transporter knockout in the mouse. FASEB J. 2006;20(3):574–6.
33. Reyes-Turcu FE, Ventii KH, Wilkinson KD. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem. 2009;78:363–97.
34. Valero R, Marfany G, Gonzalez-Angulo O, Gonzalez-Gonzalez G, Puelles L, Gonzalez-Duarte R. USP25, a novel gene encoding a deubiquitinating enzyme, is located in the gene-poor region 21q11.2. Genomics 1999;62(3):395–405.
35. Zhong H, Wang D, Fang L, Zhang H, Luo R, Shang M, Ouyang C, Ouyang H, Chen H, Xiao S. Ubiquitin-specific proteases 25 negatively regulates virus-induced type I interferon signaling. PLoS One 2013;8(11):e80976.
36. Lin D, Zhang M, Zhang MX, Ren Y, Jin J, Zhao Q, Pan Z, Wu M, Shu HB, Dong C and others. Induction of USP25 by viral infection promotes innate antiviral responses by mediating the stabilization of TRAF3 and TRAF6. Proc Natl Acad Sci USA 2015;112(36):11324–9.
37. Zhou Z, Jia X, Xue Q, Dou Z, Ma Y, Zhao Z, Jiang Z, He B, Jin Q, Wang J. TRIM14 is a mitochondrial adaptor that facilitates retinoic acid-inducible gene-I-like receptor-mediated innate immune response. Proc Natl Acad Sci USA 2014;111(2):E245–54.
38. Xu G, Guo Y, Xu D, Wang Y, Shen Y, Wang F, Lv Y, Song F, Jiang D, Zhang Y and others. TRIM14 regulates cell proliferation and invasion in osteosarcoma via promotion of the AKT signaling pathway. Sci Rep. 2017;7:42411.
39. Basler M, Kirk CJ, Groettrup M. The immunoproteasome in antigen processing and other immunological functions. Curr Opin Immunol. 2013;25(1):74–80.
40. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. Immunol Today 2000;21(9):455–64.
41. Hunter CA, Gabriel KE, Radzanowski T, Neyer LE, Remington JS. Type I interferons enhance production of IFN-gamma by NK cells. Immunol Lett. 1997;59(1):1–5.
42. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: Function and regulation by innate cytokines. Annu Rev Immunol. 1999;17:189–220.
43. Madera S, Rapp M, Firth MA, Beilke JN, Lanier LL, Sun JC. Type I IFN promotes NK cell expansion during viral infection by protecting NK cells against fratricide. J Exp Med. 2016;213(2):225–33.
44. Roland J, Soulard V, Sellier C, Drapier AM, Di Santo JP, Cazenave PA, Pied S. NK cell responses to *Plasmodium* infection and control of intrahepatic parasite development. J Immunol. 2006;177(2):1229–39.