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(54) Title: GLUTAMINE ANTAGONISTS FOR USE IN TREATING CEREBRAL EDEMA AND CEREBRAL MALARIA

(57) Abstract: Cerebral malaria is the most deadly complication associated with *P. falciparum* infection, having a fatality rate of 15-25% in African children despite the use of effective antimalarial chemotherapy. The use of a glutamine antagonist, such as 6-diazo-5-oxo-L-norleucine, for treating cerebral edema and restoring blood-brain barrier integrity, such as in patients suffering from cerebral malaria, is described. In an animal model of cerebral malaria, animals administered 6-diazo-5-oxo-L-norleucine at later stages of disease when blood-brain barrier dysfunction, brain swelling and hemorrhaging are evident, were effectively rescued from disease.



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GLUTAMINE ANTAGONISTS FOR USE IN TREATING CEREBRAL EDEMA AND
CEREBRAL MALARIA

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Application No. 62/175,000, filed June 12, 2015, which is herein incorporated by reference in its entirety.

FIELD

10 This disclosure concerns the use of a glutamine antagonist to treat cerebral edema and reverse blood-brain barrier defects in a subject, such as a subject with cerebral malaria.

BACKGROUND

 There are nearly 600 million clinical cases of *Plasmodium falciparum* malaria annually (Snow *et al.*, *Nature* 434, 214-217, 2005). For most individuals living in endemic areas, malaria is
15 uncomplicated and resolves with time. However, in about 1% of cases, almost exclusively among young children, malaria becomes severe and life threatening resulting in 700,000 deaths each year in Africa alone. One of the most deadly complications of *P. falciparum* infection in humans is cerebral malaria (HCM) characterized by the onset of severe neurological signs such as altered consciousness, seizures, and coma (Molyneux *et al.*, *Q J Med* 71, 441-459, 1989). Autopsy and
20 MRI analyses of brains of children with HCM indicate a diffuse symmetrical encephalopathy (Severe falciparum malaria, World Health Organization, Communicable Diseases Cluster, *Trans R Soc Trop Med Hyg* 94, Suppl 1:S1-90, 2000), microhemorrhaging, breakdown of the blood-brain barrier (BBB) (Taylor *et al.*, *Nat Med* 10, 143-145, 2004) and a fatal increase in intracranial pressure resulting from edema (Newton *et al.*, *Lancet* 337, 573-576, 1991; Newton *et al.*, *Arch Dis*
25 *Child* 70, 281-287, 1994; Walker *et al.*, *Trans R Soc Trop Med Hyg* 86, 491-493, 1992; Waller *et al.*, *Trans R Soc Trop Med Hyg* 85, 362-364, 1991; Potchen *et al.*, *Am J Neuroradiol* 33, 1740-1746, 2012; Seydel *et al.*, *N Engl J Med* 372, 1126-1137, 2015). At present, despite effective antimalarial drug treatment, mortality for children presenting with HCM remains at 15-25%. HCM takes a second toll on African children leaving survivors at risk of debilitating neurological defects
30 (Shikani *et al.*, *Am J Pathol* 181, 1484-1492, 2012). Thus, there is an urgent need for the development of effective adjunctive therapies that can be used in conjunction with anti-malarials to treat children with HCM.

SUMMARY

It is disclosed herein that administration of a glutamine antagonist to a subject suffering from cerebral malaria is capable of treating cerebral edema, restoring BBB integrity, inhibiting degranulation of CD8+ T cells in the brain, and promoting survival.

5 Provided herein is a method of treating cerebral edema in a subject by selecting a subject with cerebral edema and administering to the subject a therapeutically effective amount of a glutamine antagonist. In some embodiments, the cerebral edema is caused by cerebral malaria, a traumatic brain injury or a hemorrhagic stroke.

10 Also provided is a method for treating cerebral malaria in a subject by selecting a subject with malaria who has been diagnosed with cerebral malaria (*e.g.*, a subject who is exhibiting at least one neurological sign or symptom of cerebral malaria) and administering to the subject a therapeutically effective amount of a glutamine antagonist.

15 Further provided is a method of inhibiting degranulation of CD8+ T cells in the brain of a subject with malaria by selecting a subject with cerebral malaria and administering to the subject a therapeutically effective amount of a glutamine antagonist.

In particular embodiments of the methods disclosed herein, the glutamine antagonist is 6-diazo-5-oxo-L-norleucine (DON).

Also provided herein are compositions comprising DON and an antimalarial agent.

20 The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

25 **FIGS. 1A-1D: DON treatment reduced the mortality associated with experimental cerebral malaria (ECM).** C57BL/6 mice were infected with *Plasmodium berghei* ANKA (*PbA*) on day 0 and injected intraperitoneally with saline (NoRx) or with DON (1.3 mg/kg) beginning on day 5 post infection (p.i.) at 7 am (DON Rx d5a), on day 5 p.i. at 11 pm (DON Rx d5p), or on day 6 p.i. at 7 am (DON Rx d6a). DON treatment was continued every day or every other day as shown (FIG. 6). (FIG. 1A) Kaplan-Meier survival plots. (FIG. 1B) Clinical scores (Waisberg *et al.*, *PLoS One* 7, e29493, 2012) from 0 (no symptoms) to 10 (moribund) of mice in FIG. 1A. (FIG. 1C) Peripheral blood parasitemia for mice in FIG. 1A. Data for DON Rx d5a were combined from three independent experiments, data for DON Rx d5p were combined from four independent experiments and data for DON Rx d6a were combined from two independent experiments. Shown

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are the mean and standard error of the mean (SEM) for FIG. 1B and FIG. 1C. (FIG. 1C) Mice infected with *PbA* were treated with DON on d5p p.i. or left untreated. On day 6a p.i. mice were transcardially perfused, the brains removed and the fold changes in *PbA* 18s RNA of treated and untreated mice as compared to the brains of untreated mice removed on day 5p p.i. were

5 determined as detailed in Example 1. Each dot represents a mouse with the mean and standard deviation (SD) given. Shown are the results combined from three independent experiments each having three-four mice per group. A Mann-Whitney test showed no significant difference.

FIGS. 2A-2C: DON treatment restored BBB function and reduced brain swelling but did not acutely resolve brain hemorrhages in *PbA*-infected mice. All mice were infected with

10 *PbA* and treated with saline or DON (1.3mg/kg) on day 5p p.i. and the brains removed and analyzed on the days and times indicated. (FIG. 2A) Levels of Evans blue (EB) in the brains were quantified and expressed relative to the levels of EB in the brains of *PbA*-infected, untreated mice on d6a p.i. Each symbol represents one mouse. The data are combined from three independent experiments. The mean and SD are given. (FIG. 2B) Brain water content expressed as the weight

15 of each brain after desiccation divided by the weight before desiccation $\times 100$ is given. Each symbol represents one mouse. Data are combined from two independent experiments. The mean and SD are given. (FIG. 2C) For quantification of brain hemorrhages, mice were perfused with cold phosphate buffered saline (PBS), their brains removed, fixed, sectioned and stained with H&E. Hemorrhages were counted in 10 high power (40x) fields of brain sections and the number of

20 hemorrhages is given. Each symbol represents one mouse. Data are combined from three independent experiments. Mann-Whitney tests were used for comparison of groups (* = p-value <0.05, ** = P-value <0.005), and *** = P-value <0.0005).

FIGS. 3A-3J: DON treatment reduced CD8⁺ T cell degranulation but not the accumulation of immune cell in the brains of *PbA*-infected mice. All mice were either

25 uninfected or infected with *PbA* and treated with DON (1.3 mg/kg) or saline on day 5p p.i. (FIGS. 3A-3E). On day 6a p.i. mice were perfused with cold PBS, the brains and/or spleens removed and single cell suspensions prepared. Cells were analyzed by flow cytometry using the gating strategy show in FIG. 8. Shown are the number of cells per brain for: (FIG. 3A) macrophage/DCs; (FIG. 3B) neutrophils; (FIG. 3C) natural killer (NK) cells; (FIG. 3D) CD 4⁺ T cells; and (FIG. 3E) CD8⁺

30 T cells. Data were combined from three independent experiments. (FIG. 3F) Representative flow cytometry plots of glideosome-associated protein 50 (GAP50) major histocompatibility complex (MHC) class I D^b tetramer binding CD8⁺ T cells in unenriched spleen/lymph node cell populations (left and right panels), and in GAP50-tetramer-binding cells enriched using magnetic beads coated with APC-specific antibodies (middle panel). (FIG. 3G) The number of GAP50-tetramer binding

CD8⁺ T cells in spleen and brains. Data were combined from three independent experiments. (FIG. 3H) Representative flow cytometry plots of CD8⁺ T cells in the spleens of mice that received fluorescently labeled CD107-specific antibodies intravenously one hour before being sacrificed to allow labeling of CD107-expressing cells *in vivo*. The percent of total CD8⁺ T cells that expressed CD107 (FIG. 3I) and the percent of GAP50-tetramer binding CD8⁺ T cells that express CD107 (FIG. 3J) are shown. Data were combined from three independent experiments. Mann-Whitney tests were used for statistical analysis. ns= not significant, * = p-value <0.05, ** = P-value <0.005, and *** = P-value <0.0005.

FIGS. 4A-4F: DON significantly alters citrulline, urea and nitric oxide metabolism in the brain during ECM. Principle components analysis was performed for each of three tissue types collected from mice at day 6a p.i. for the five experimental groups defined by infection, treatment and clinical outcome. Principal components were determined from all detectable metabolites for the (FIG. 4A) brain (438 metabolites), (FIG. 4B) liver (544 metabolites) and (FIG. 4C) serum (563 metabolites). Each symbol represents one tissue sample from one mouse. (FIG. 4D) Venn diagram showing the number of differentially abundant brain metabolites for the two main comparisons: (1) infected, untreated mice vs. uninfected, untreated mice and (2) infected, DON-treated mice with low clinical scores vs. infected, untreated mice. Differential abundance thresholds were an absolute fold-change in abundance of ≥ 1.2 and a false discovery rate of <5% (Welch's t test). Pathways analysis of differentially abundant metabolites for each 2-way comparison demonstrated several significantly overrepresented pathways that are shared between the two comparisons (FIGS. 4E and 4F). Shown are the ratio of overlap of metabolites in the dataset with metabolites in the pathway and the -log (p-value) of the overlap by Fisher's exact test. Metabolites discussed in the present disclosure are shown in bold.

FIGS. 5A-5C: 81 metabolites that were reversed by DON treatment during *PbA* infection. The table lists the 81 metabolites that were differentially abundant in both the *PbA*-infected, untreated vs. uninfected, untreated comparison and the *PbA*-infected, DON treated, low clinical score vs *PbA* infected, untreated comparison. Only metabolites meeting differential abundance thresholds of absolute fold change ≥ 1.2 and a false discovery rate <5% in both comparisons are shown.

FIG. 6: DON treatment schedule. DON treatment (1.3 mg/kg) was initiated on day 5 p.i. at 7 am (d5a), day 5 p.i. at 11 pm (d5p) or day 6 p.i at 7 am (d6a) and continued every day or every other day as shown.

FIG. 7: Late cessation of DON treatment leads to increased parasitemia. C57BL/6 mice were infected with *PbA* on day 0 and given saline (No Rx) or DON (1.3 mg/kg) every other

day beginning at 7 am on day 1 p.i. (DON Rx d1a) and peripheral blood parasitemia was determined by flow cytometry on the days indicated.

FIG. 8: Gating strategy to identify immune cells in the brain and spleens. Single cell suspensions were gated, excluding very small cells/debris, and cell doublets were excluded by side-scatter-width. Using 'aqua' live/dead dye to label dead cells, only living cells were gated. The pan leukocyte marker CD45.2 was used to gate on leukocytes. Cells were gated on CD3+ cells that include CD4+ and CD8+ T cells, NKT and $\gamma\delta$ T cells. CD3+ T cells were gated further on CD8+ and CD4+ cells. The CD3- gate was used to further subset cells into neutrophils (Ly6G+, Ly6C+), macrophages/DC (Ly6G-, Ly6C+) and NK cells (NK1.1+). Leukocytes were counted per spleen and brain preparation on a hemocytometer and the numbers of each cell type was calculated.

FIGS. 9A-9B: The effect of *PbA* infection and DON treatment on the glutaminolysis pathway in the brain, liver and serum. (FIG. 9A) DON blocks the first step of glutaminolysis by inhibiting glutaminase. (FIG. 9B) *PbA* infection and DON treatment of *PbA* infected mice affect the glutaminolysis pathway differently in the brain relative to the liver and serum. Shown are absolute fold change and false discovery rates (FDR) by Welch's t test for the indicated 2-way comparison. Positive fold changes (red shading) represent significantly increased (FDR < 0.05) metabolite abundance in the first group compared to the second group. Negative fold changes (blue shading) represent significantly decreased (FDR < 0.05) metabolite abundance in the first group compared to the second group.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on June 6, 2016, 2.08 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NOs: 1-8 are nucleic acid primer sequences.

SEQ ID NO: 9 is an amino acid sequence of a *PbA* GAP50 peptide.

DETAILED DESCRIPTION

I. Abbreviations

APC	antigen presenting cell
BBB	blood brain barrier

	CM	cerebral malaria
	Ct	cycle threshold
	d5a	post-infection day 5, 7am
	d5p	post-infection day 5, 11pm
5	d6a	post-infection day 6, 7am
	DON	6-diazo-5-oxo-L-norleucine
	EB	Evan's blue
	ECM	experimental cerebral malaria
	FACS	fluorescence activated cell sorting
10	FBS	fetal bovine serum
	FDR	false discovery rate
	GAP50	glideosome-associated protein 50
	H&E	hematoxylin and eosin
	HCM	human cerebral malaria
15	iRBC	infected red blood cell
	MHC	major histocompatibility complex
	NK	natural killer
	PbA	<i>Plasmodium berghei</i> ANKA
	PBS	phosphate buffered saline
20	PE	phycoerythrin
	p.i.	post-infection
	RBC	red blood cell

II. Terms and Methods

25 Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive*
30 *Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

6-diazo-5-oxo-L-norleucine (DON): A glutamine antagonist originally isolated from *Streptomyces* in a sample of Peruvian soil. DON is a non-standard amino acid (an analog of

glutamine). DON is an active site inhibitor of glutaminase, an enzyme responsible for the first step in the glutaminolysis pathway that converts Gln to glutamate plus ammonia (Thangavelu *et al.*, *Sci Rep* 4, 3827, 2014). DON has previously been evaluated as a cancer chemotherapeutic agent.

Acivicin ((alpha S,5S)-alpha-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid): A

glutamate antagonist that is a fermentation product of *Streptomyces*. Acivicin is a glutamine analog that irreversibly inhibits glutamine-dependent amidotransferases involved in nucleotide and amino acid biosynthesis. Acivicin also inhibits gamma-glutamyl transpeptidase and is known to have anti-tumorigenic activity.

Administration: To provide or give a subject an agent, such as a therapeutic agent (*e.g.* a glutamine antagonist), by any effective route. Exemplary routes of administration include, but are not limited to, injection or infusion (such as subcutaneous, intramuscular, intradermal, intraperitoneal, intrathecal, intravenous, intracerebroventricular, intrastriatal, intracranial and into the spinal cord), oral, intraductal, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes.

Antimalarial agent: Any agent that is capable of preventing or treating malaria. Examples of antimalarial agents include, but are not limited to, quinine, chloroquine, quinine sulfate, hydroxychloroquine, mefloquine, quinidine gluconate, doxycycline, tetracycline, clindamycin, mefloquine, atovaquone, artemether/lumefantrine, pyrimethamine/sulfadoxine and artemisinin or an artemisinin derivative (such as artesunate).

Azaserine: A glutamine antagonist. Azaserine is serine derivative diazo compound with antineoplastic and antibiotic properties deriving from its action as a purinergic antagonist and structural similarity to glutamine. Azaserine acts by competitively inhibiting glutamine amidotransferase, a key enzyme responsible for glutamine metabolism.

Blood-brain barrier (BBB): A highly selective permeability barrier that separates the circulating blood from the brain extracellular fluid in the central nervous system. The BBB is formed by brain endothelial cells, which are connected by tight junctions with an extremely high electrical resistivity. The BBB allows the passage of water, some gases, and lipid-soluble molecules by passive diffusion, as well as the selective transport of molecules such as glucose and amino acids that are crucial to neural function. However, the BBB prevents the entry of lipophilic, potential neurotoxins by way of an active transport mechanism mediated by P-glycoprotein. The BBB also protects the brain from bacterial infection.

Cerebral edema: Swelling of the brain caused by excessive accumulation of fluid.

Cerebral malaria: A severe, life-threatening form of malaria. Cerebral malaria is characterized by a number of neurological symptoms (including cerebral edema, disruption of the

BBB, brain hemorrhage, seizures, delirium and unarousable coma), the presence of infected red blood cells (iRBCs) in the peripheral circulation and sequestration of iRBCs on the brain vascular endothelium.

5 **Coma:** A state of unconsciousness in which a person cannot be awakened, fails to respond normally to painful stimuli, light or sound, lacks a normal wake-sleep cycle, and does not initiate voluntary actions.

Degranulation of CD8+ T cells: Refers to the process of releasing cytotoxic molecules, such as perforin and granzymes, from secretory vesicles of a CD8+ T cell.

10 **Delirium:** An acute state of mental confusion characterized by anxiety, disorientation, restlessness, hallucinations, delusions and incoherence of thought and speech. Delirium can be caused by, for example, brain injury, high fever, poisoning, intoxication or shock.

Glutamine antagonist: An inhibitor of biochemical reactions that utilize glutamine. Glutamine antagonists include, but are not limited to, DON, acivicin and azaserine.

Hemorrhage: Bleeding from a ruptured blood vessel in the body.

15 **Hemorrhagic stroke:** At type of stroke (sudden death of brain cells due to a lack of oxygen) that results from an accumulation of blood in or around the brain, such as from a ruptured blood vessel. Hemorrhages in the brain can be caused by a variety of disorders that affect the blood vessels, such as long-term high blood pressure and cerebral aneurysms (a weak or thin spot on a blood vessel wall).

20 **Malaria:** Malaria is a parasitic infection of humans and non-human primates by the *Plasmodium* species *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Humans become infected following the bite of an infected anopheline mosquito, the host of the malarial parasite. Malaria occasionally occurs in humans following a blood transfusion or subsequent to needle-sharing. Clinical manifestations of malarial infection may include blackwater fever, cerebral malaria, respiratory failure, 25 hepatic necrosis, and/or occlusion of myocardial capillaries. Additional *Plasmodium* species infect other hosts, such as rodents (*P. berghei*, *P. chabaudi*, *P. vinckei*, and *P. yoelii*), other mammals, birds and reptiles.

Parenteral: Administered outside of the intestine, for example, not via the alimentary tract. Generally, parenteral formulations are those that will be administered through any possible mode 30 except ingestion. This term especially refers to injections, whether administered intravenously, intrathecally, intramuscularly, intraperitoneally, or subcutaneously, and various surface applications including intranasal, intradermal, and topical application, for instance.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers of use are conventional. *Remington's Pharmaceutical Sciences*, by E.W. Martin, Mack Publishing Co.,

Easton, PA, 15th Edition, 1975, describes compositions and formulations suitable for pharmaceutical delivery of the compositions disclosed herein.

In general, the nature of the carrier will depend on the particular mode of administration being employed. In addition to biologically neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Seizure: Uncontrolled electrical activity in the brain, which may produce a physical convulsion, minor physical signs, thought disturbances, or a combination of symptoms. Seizures can be caused by, for example, head injuries, brain tumors, lead poisoning, maldevelopment of the brain, genetic and infectious illnesses, and fevers.

Subject: Living multi-cellular organisms, including vertebrate organisms, a category that includes both human and non-human mammals.

Therapeutically effective amount: A quantity of compound or composition, for instance, a glutamine antagonist (*e.g.* DON), sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to treat cerebral edema and/or restore BBB integrity.

Traumatic brain injury: A form of acquired brain injury that occurs when a sudden trauma causes damage to the brain. TBI can result when the head suddenly and violently hits an object, or when an object pierces the skull and enters brain tissue. Symptoms of a TBI can be mild, moderate, or severe, depending on the extent of the damage to the brain. A person with a mild TBI may remain conscious or may experience a loss of consciousness for a few seconds or minutes. Other symptoms of mild TBI include headache, confusion, lightheadedness, dizziness, blurred vision or tired eyes, ringing in the ears, bad taste in the mouth, fatigue or lethargy, a change in sleep patterns, behavioral or mood changes, and trouble with memory, concentration, attention, or thinking. A person with a moderate or severe TBI may show these same symptoms, but may also have a headache that gets worse or does not go away, repeated vomiting or nausea, convulsions or seizures, an inability to awaken from sleep, dilation of one or both pupils of the eyes, slurred speech, weakness or numbness in the extremities, loss of coordination, and increased confusion, restlessness, or agitation.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. “Comprising A or B” means including A, or B, or A and B. It is further to be

understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below.

- 5 All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

10 **III. Introduction**

Experimental cerebral malaria (ECM) in mice is a widely used model of HCM and provides a valuable tool for elucidating the mechanisms involved in CM pathogenesis and identifying cellular and molecular targets for adjunctive therapy (Grau and Craig, *Future Microbiol* 7, 291-302, 2012). In ECM, 6 to 7 days after infection with *Plasmodium berghei* ANKA (PbA), mice of susceptible strains, such as C57BL/6 develop ataxia, paralysis, seizures, coma and ultimately die (Engwerda *et al.*, *Curr Top Microbiol Immunol* 297, 103-143, 2005). ECM displays key features of HCM, including BBB breakdown, focal hemorrhaging and brain swelling (Nacer *et al.*, *PLoS Pathog* 8, e1002982, 2012; Promeneur *et al.*, *Proc Natl Acad Sci U S A* 110, 1035-1040, 2013; Penet *et al.*, *J Neurosci* 25, 7352-7358, 2005). ECM's pathology also includes sequestration of infected red blood cells (iRBCs) in the brain vasculature (Baptista *et al.*, *Infect Immun* 78, 4033-4039, 2010; McQuillan *et al.*, *Int J Parasitol* 41, 155-163, 2011), a hallmark of HCM (Newton *et al.*, *Lancet* 337, 573-576, 1991). Histological analysis of the brains of children who died of HCM showed that leukocytes, primarily monocytes with phagocytized hemozoin and platelets but also intravascular leukocytes, including CD8⁺ T cells, sequestered in the brain vessels (Clark *et al.*, *Malar J* 2, 6, 2003; Grau *et al.*, *J Infect Dis* 187, 461-466, 2003; Dorovini-Zis *et al.*, *Am J Pathol* 178, 2146-2158, 2011). In ECM, the sequestration of various immune cells including monocytes, NK cells, neutrophils and T cells in the brain vasculature have been implicated in the development of neurological damage (Hansen *et al.*, *J Immunol* 178, 5779-5788, 2007; Belnoue *et al.*, *J Immunol* 169, 6369-6375, 2002). Current evidence indicates that CD8⁺ T cells are the major mediators of death in ECM (Howland *et al.*, *Semin Immunopathol*, 2015) and that antigen-specific CD8⁺ T cells engage parasite antigens cross-presented on MHC class I molecules on brain endothelium resulting in endothelial cell dysfunction by a perforin-dependent mechanism (Nitcheu *et al.*, *J Immunol* 170, 2221-2228, 2003).

A critical role for metabolic reprogramming in regulating immune responses is becoming increasingly appreciated. Upon activation, T cells undergo metabolic reprogramming to meet the increased energetic and biosynthetic demands of growth and effector T cell functions (MacIver *et al.*, *Annu Rev Immunol* 31, 259-283, 2013; O'Sullivan and Pearce, *Trends Immunol* 36, 71-80, 2015; Pollizzi and Powell, *Nat Rev Immunol* 14, 435-446, 2014). Reprogramming involves a shift to aerobic glycolysis and increased glutaminolysis. Activated T cells import large quantities of glutamine (Gln) and increase their expression of glutaminase (Carr *et al.*, *J Immunol* 185, 1037-1044, 2010; Wang *et al.*, *Immunity* 35, 871-882, 2011; Nakaya *et al.*, *Immunity* 40, 692-705, 2014). The studies disclosed herein focus on targeting Gln metabolism for an adjunctive therapy for CM using the Gln analog, 6-diazo-5-oxo-L-norleucine (DON). DON broadly inhibits glutamine metabolism in part by blocking glutamine transport and inhibiting all three isoforms of glutaminase as well as other glutamine-utilizing enzymes such as the amidotransferases and glutamine synthetase (Thangavelu *et al.*, *Sci Rep* 4, 3827, 2014). Consequently, DON has been shown to be a potent inhibitor of T cell proliferation (Wang *et al.*, *Immunity* 35, 871-882, 2011).

DON is an active site inhibitor of glutaminase, an enzyme responsible for the first step in the glutaminolysis pathway that converts Gln to glutamate plus ammonia (Thangavelu *et al.*, *Sci Rep* 4, 3827, 2014). A selective increase in extracellular Glu or ammonia are both neurotoxic and might not be detected by the metabolic analysis of brain tissue. Likewise, the accumulation of Gln in the cytosol of astrocytes, as a result of efforts of the astrocytes to detoxify ammonia, results in astrocyte swelling (Albrecht and Norenberg, *Hepatology* 44, 788-794, 2006) and DON has been shown to prevent ammonia-induced astrocyte swelling *in vitro* (Norenberg *et al.*, *J Bioenerg Biomembr* 36, 303-307, 2004). Furthermore, a recent study by Miranda *et al.* (*Braz J Med Biol Res* 43, 1173-1177, 2010), demonstrated that increased levels of Glu in the cerebral spinal fluid of *PbA*-infected mice is associated with the neurological signs of ECM. Indeed, DON has been shown to reduce excessive Glu release by activated microglia that contribute to neurodegeneration in a number of neurological diseases including ischemic brain injury (Takeuchi *et al.*, *Exp Neurol* 214, 144-146, 2008) and in Japanese encephalitis virus induced encephalitis (Chen *et al.*, *Glia* 60, 487-501, 2012).

The studies disclosed herein show that DON treatment rescues *PbA*-infected mice from ECM at late stages in the disease, at a time when the animals show clinical signs of neurological damage and physical loss of BBB integrity, brain swelling, and hemorrhaging. The ability of DON to reverse disease is concomitant with a decrease in the number and effector function of parasite-specific CD8⁺ T cells in the brains of treated mice. The striking ability of DON treatment to

reverse pathology so late in the disease suggests a fundamental and potentially direct role for Gln metabolism in promoting neuropathology.

IV. Overview of Several Embodiments

5 Provided herein are methods of treating cerebral edema in a subject. In some embodiments, the methods include selecting a subject with cerebral edema and administering to the subject a therapeutically effective amount of a glutamine antagonist. In particular embodiments, the cerebral edema is caused by cerebral malaria, a traumatic brain injury or a hemorrhagic stroke.

10 Also provided herein are methods for treating cerebral malaria in a subject. In some embodiments, the methods include selecting a subject with malaria who is exhibiting at least one neurological sign or symptom of cerebral malaria (or who has been diagnosed with malaria) and administering to the subject a therapeutically effective amount of a glutamine antagonist. In particular embodiments, the at least one neurological sign of cerebral malaria is selected from cerebral edema, loss of blood-brain barrier integrity, brain hemorrhage, infected red blood cells in
15 the brain vasculature, coma, seizure and delirium.

 In some embodiments for treating cerebral malaria, the methods further include administering to the subject an antimalarial agent. Any antimalarial agent effective for the treatment of malaria can be selected. In some examples, the antimalarial agent is quinine, chloroquine, quinine sulfate, hydroxychloroquine, mefloquine, quinidine gluconate, doxycycline,
20 tetracycline, clindamycin, mefloquine, atovaquone, artemether/lumefantrine, pyrimethamine/sulfadoxine, artemisinin or an artemisinin derivative (such as artesunate), or any combination of two or more thereof.

 Further provided are methods of inhibiting degranulation of CD8+ T cells in the brain of a subject with malaria. In some embodiments, the methods include selecting a subject with cerebral
25 malaria and administering to the subject a therapeutically effective amount of a glutamine antagonist.

 The glutamine antagonist can be administered using any suitable route of administration. In some embodiments of the methods disclosed herein, the glutamine antagonist is administered intraperitoneally, intravenously, rectally or orally.

30 Suitable doses and dosing schedules for administration of the glutamate antagonist can be determined by a physician, based on several factors including age, weight, general condition of the subject, the particular condition being treated, the particular glutamate antagonist being used and its mode of administration. In particular examples of the methods disclosed herein, the glutamine

antagonist is administered once a day. In other examples, the glutamine antagonist is administered once every other day.

In some embodiments of the methods, the glutamine antagonist is 6-diazo-5-oxo-L-norleucine, acivicin or azaserine. In specific non-limiting embodiments, the glutamine antagonist is 6-diazo-5-oxo-L-norleucine.

In some instances in which the glutamine antagonist is 6-diazo-5-oxo-L-norleucine, the subject is administered 6-diazo-5-oxo-L-norleucine at a dose of about 0.1 to about 15 mg/kg, about 0.13 mg/kg to about 15 mg/kg, about 0.25 mg/kg to about 15 mg/kg, about 0.5 mg/kg to about 15 mg/kg, about 0.1 to about 10 mg/kg, about 1 to about 10 mg/kg, about 0.1 to about 0.5 mg/kg, about 1 to about 5 mg/kg, about 0.1 to about 2.5 mg/kg, or about 1 to about 2.5 mg/kg. In specific examples, the subject is administered 6-diazo-5-oxo-L-norleucine at a dose of about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1.0 mg/kg mg/kg, about 1.1 mg/kg mg/kg, about 1.2 mg/kg mg/kg, about 1.3 mg/g mg/kg, about 1.4 mg/kg or about 1.5 mg/kg.

In some examples, the 6-diazo-5-oxo-L-norleucine is administered to the subject at a dose of about 1.3 mg/kg once per day. In other examples, the 6-diazo-5-oxo-L-norleucine is administered to the subject at a dose of about 1.3 mg/kg once every other day.

Also provided herein are compositions comprising 6-diazo-5-oxo-L-norleucine and an antimalarial agent. In some embodiments, the antimalarial agent is quinine, chloroquine, quinine sulfate, hydroxychloroquine, mefloquine, quinidine gluconate, doxycycline, tetracycline, clindamycin, mefloquine, atovaquone, artemether/lumefantrine, pyrimethamine/sulfadoxine, artemisinin or an artemisinin derivative (such as artesunate), or any combination of two or more thereof. In some examples, the composition further comprises a pharmaceutically acceptable carrier.

V. Administration of Pharmaceutical Compositions

Glutamine antagonists can be administered, such as for the treatment of cerebral edema, as a composition comprising one or more pharmaceutically acceptable carriers. Such carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions for use in the methods of the present disclosure. In some embodiments herein, the compositions further include an antimalarial agent.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene

glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives can also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Pharmaceutical compositions comprising a glutamine antagonist can be administered orally in liquid, capsule, pill or tablet form, and can include, for example, lactose, microcrystalline cellulose, colloidal silicon dioxide, croscarmellose sodium, magnesium stearate, stearic acid, and other excipients, colorants, pharmacologically compatible carriers, or any combination thereof. Oral formulations of a glutamine antagonist can be taken with a suitable buffer, such as bicarbonate. Alternatively, the glutamine antagonist can be coated with an enteric coating.

The pharmaceutical composition can be presented in unit-dose or multi-dose sealed containers, for example, ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets.

Administration can be accomplished by single or multiple doses. The dose required will vary from subject to subject depending on the species, age, weight, general condition of the subject, the particular condition being treated, the particular glutamate antagonist being used and its mode of administration. An appropriate dose can be determined by one of ordinary skill in the art using only routine experimentation. In some embodiments, the dose of glutamine antagonist (such as DON) is about 0.1 to about 15 mg/kg, about 0.13 mg/kg to about 15 mg/kg, about 0.25 mg/kg to about 15 mg/kg, about 0.5 mg/kg to about 15 mg/kg, about 0.1 to about 10 mg/kg, about 1 to about 10 mg/kg, about 0.1 to about 0.5 mg/kg, about 1 to about 5 mg/kg, about 0.1 to about 2.5 mg/kg, or about 1 to about 2.5 mg/kg. In specific examples, the subject is administered 6-diazo-5-oxo-L-norleucine at a dose of about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1.0 mg/kg, about 1.1 mg/kg, about 1.2 mg/kg, about 1.3 mg/kg, about 1.4 mg/kg or about 1.5 mg/kg.

In some examples disclosed herein, 6-diazo-5-oxo-L-norleucine is administered to a subject at a dose of about 1.3 mg/kg once per day. In other examples, 6-diazo-5-oxo-L-norleucine is administered to a subject at a dose of about 1.3 mg/kg once every other day.

The glutamate antagonist can also be administered in combination with other therapeutic agents, such as antimalarial agents if the subject is infected with *P. falciparum*. Antimalarial agents include, but are not limited to, quinine, chloroquine, quinine sulfate, hydroxychloroquine, mefloquine, quinidine gluconate, doxycycline, tetracycline, clindamycin, mefloquine, atovaquone, 5 artemether/lumefantrine, pyrimethamine/sulfadoxine, artemisinin and artemisinin derivatives (such as artesunate).

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular 10 features or embodiments described.

EXAMPLES

Example 1: Materials and Methods

This example describes the materials and experimental procedures for the studies described 15 in Example 2.

Animal and Malaria Infections

C57BL/6 female mice (7-10 weeks old) were obtained from The Jackson Laboratories. Mice were infected with *PbA* by injecting intraperitoneally 1×10^6 *PbA*-infected RBC obtained 20 from infected C57BL/6 mice. Peripheral blood parasitemia was determined by flow cytometry as described below. Infected mice were monitored for the progression of ECM using a ten point clinical scoring system that rates mice from a score of 0 (no signs) to 10 (moribund) as previously described (Waisberg *et al.*, *PLoS One* 7, e29493, 2012).

6-Diazo-5-oxo-L-norleucine (DON) Treatment

For each DON treatment, mice weighing approximately 25 grams were injected 25 intraperitoneally with 1.3 mg/kg DON (Sigma, Cat#D2141, St. Louis, MO) in 200 μ L PBS.

Quantification of Peripheral Blood Parasitemia by Flow Cytometry

Parasitemia was determined by flow cytometry using a modification of a previously 30 described method (Malleret *et al.*, *Sci Rep* 1, 118, 2011). Briefly, blood was obtained from mouse tail veins, fixed with 0.025% aqueous glutaraldehyde solution, washed with 2 mL PBS, resuspended and stained with the following: the DNA dye Hoechst 33342 (Sigma) (8 μ M), the DNA and RNA dye dihydroethidium (diHET) (10 μ g/mL), the pan C57BL/6 lymphocyte marker APC-conjugated

antibody specific for CD45.2 (BioLegend, San Diego, CA), and the RBC marker APC-Cy7-conjugated antibody specific for Ter119 (BD Pharmingen, San Jose, CA). Cells were analyzed on a BD™ LSRII flow cytometer equipped with UV (325nm), Violet (407nm), Blue (488nm) and Red (633nm) lasers. Data were analyzed using FLOWJO™ software (Tree Star Technologies, Ashland, OR). Infected RBCs (iRBCs) were CD45.2⁻, Ter119⁺, Hoechst⁺ and diHEt⁺. Parasitemia were calculated as the number of iRBCs/total number of RBCs.

Quantification of Parasite Loads in Brains

Mice were anesthetized and transcardially perfused with cold PBS and brains were removed and immediately frozen in liquid nitrogen. Brains were thawed and 1 ml Qiagen RNEASY™ lysis buffer was added immediately. Brains were homogenized and RNA was extracted from the homogenate using a Qiagen RNEASY™ Mini kit according to manufacturer's instructions. Genomic DNA was digested on a column using RNase-free DNase set (Qiagen, Valencia, CA) and the elimination of genomic DNA was confirmed using no reverse transcriptase controls. cDNA was generated using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). SYBR™ Green PCR master mix (Bio-Rad) was used to determine the relative expression of parasite 18s ribosomal RNA and three mouse housekeeping genes, *hprt*, *gapdh*, and *actb*. The primer sequences were:

Pb 18s – AAGCATTAATAAAGCGAATACATCCTTAC (SEQ ID NO: 1) and
GGAGATTGGTTTTGACGTTTATGTG (SEQ ID NO: 2);
mouse *hprt* – TGCTCGAGATGTGATGAAGG (SEQ ID NO: 3) and
TCCCCTGTTGACTGGTCATT (SEQ ID NO: 4);
mouse *gapdh* – GTGGAGTCATACTGGAACATGTAG (SEQ ID NO: 5) and
AATGGTGAAGGTCGGTGTG (SEQ ID NO: 6); and
mouse *actb* – TCCGGCATGTGCAAAGC (SEQ ID NO: 7) and TCCTTCTGACCCATTCCC
(SEQ ID NO: 8).

The geometrical mean of the cycle threshold (Ct) values of mouse housekeeping genes was first determined to create a normalized base line for the brain to allow a comparison of the delta Ct values of 18s gene amplification. The fold changes in gene expression was then calculated by comparing the delta Ct values of *PbA*-infected mice on day 5 p.i. to that on day 6 p.i. for mice treated with DON on day 5p p.i. or mice left untreated.

Assessment of BBB Integrity

Evans blue (EB; 20 mg/kg) was injected intraorbitally on the specified day and three hours later the mice were anesthetized, perfused with saline and their brains removed and immediately frozen at -80°C for later processing (Kim *et al.*, *Nature* 457, 191-195, 2009). EB was extracted
 5 using N, N-dimethylformamide and quantified using a Varioskan Flash fluorometer (620 nm excitation; 695 nm emission).

Assessment of Brain Swelling

Brains were removed from animals and weighed. Brains were then desiccated at 80°C for
 10 12 hours and weighed again. Percent water content was calculated using the decrease in weight.

Quantification of Brain Hemorrhages

Brain samples were fixed in 10% buffered formalin, embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin (H&E) for ultrastructural examination and
 15 detection of hemorrhages. For quantification of hemorrhages, hemorrhages were counted in 10 microscopic 40× power fields.

Flow Cytometry

For quantification of brain infiltrating leukocytes, mice were anesthetized with
 20 ketamine/xylazine at the specified times and transcardially perfused with cold PBS and the brains and spleens were removed. Brains were dissected, minced and digested with 1 mg/mL collagenase for 30 minutes at 37°C. After passing the tissue through 70 µm nylon mesh, homogenates were placed on a 90-60-40% discontinuous PERCOLL™ gradient, centrifuged for 18 minutes at 1000 × g and the cells at the 40-60% interface containing leukocytes were collected for analysis. Spleens
 25 were mashed using a 70 µm nylon mesh, then red blood cells were lysed. The cells were washed and re-suspended in FACS buffer consisting of PBS and 1% FBS. The following fluorescent dye-conjugated antibodies specific for the following cell surface markers were used for staining: BRILLIAN VIOLET™(BV) 421–NK1.1 (BioLegend), BV605–CD4 (BioLegend), BV785–CD8 (BioLegend), PE–Ly6G (BD Pharmingen), PE–Cy7–CD3 (eBioscience, San Diego, CA), APC–
 30 Ly6C (BD Pharmingen), APC–Cy7–CD45.2 (BD Pharmingen), and LIVE/DEAD™ Fixable AquaDead Cell Stain (Life Technologies, Grand Island, NY). Gating of subsets is depicted in FIG. 6. Cell acquisition data was obtained on a BD™ LSRII flow cytometer. Data was analyzed with FLOWJO™ software (Tree Star Technologies, Ashland, OR).

To quantify GAP50-tetramer binding CD8⁺ T cells (Howland *et al.*, *EMBO Mol Med* 5, 984-999, 2013) in tissues from uninfected mice, GAP50-tetramer-binding CD8⁺ T cells were enriched by magnetic bead separation as described (Haluszczak *et al.*, *J Exp Med* 206, 435-448, 2009). Briefly, a single cell suspension was prepared from spleen and peripheral lymph nodes (axillary, brachial, inguinal, and cervical) and incubated with 0.5 mg GAP-50 tetramer (obtained from the NIH Tetramer Core Facility, Emory University, Atlanta, GA) conjugated to APC for 30 minutes on ice. Cells were washed and incubated for 40 minutes at 4°C with anti-APC beads (Miltenyi Biotec, San Diego, CA). The cells were washed and applied to magnetic columns (Miltenyi). Both the bound GAP50-tetramer+, and unbound cells were incubated for 30 minutes at 4°C with antibodies specific for CD8, CD3, CD44, MHC class II and F4/80 obtained from Biolegend, ebioscience, and Tonbo. Cells were washed, analyzed on a BD LSRFORTRESSA™ flow cytometer (BD Biosciences), and the acquired data analyzed using FLOWJO™ software. To quantify GAP50-tetramer binding CD8⁺ T cells in the tissues of *PbA*-infected mice, brain and spleen were collected and single cell suspensions prepared as described above. Cells were stained for 30 minutes at 4°C with the following fluorescent dye-conjugated antibodies specific for the following cell surface markers: BV785-CD8 (Biolegend); BV421-CD44 (Biolegend); BV605-CD4 (Biolegend); BV711-CD11b (Biolegend); PE-CD107a (Biolegend); PE-CD107b (Biolegend); PerCP-Cy5.5-Thy1.2; Db:GAP50-Bio; APC-streptavidin (Life Technologies); AquaDead Cell Stain (Life Technologies); ALEXA FLUOR™ 700-Ly6C (Biolegend); and APC-Cy7-CD45.2 (BD Pharmingen). Cell acquisition data was obtained on a BD™ LSR II flow cytometer and analyzed with FLOWJO™ software (Tree Star Technologies).

For the detection of *in vivo* degranulation, mice were given PE-conjugated antibodies specific for CD107a (12.5µg) and for CD107b (12.5µg) (Biolegend) by intravenous injection 1 hour prior to sacrificing the mice and removal of their brains and spleens, as previously described (Yuzefpolskiy *et al.*, *Cell Mol Immunol*, 2014).

Metabolic/Metabolomic Profiling

On day 6a p.i., mice from all experimental groups were euthanized to collect brain, liver, and serum. Tissue samples were snap frozen in liquid nitrogen and stored at -80°C until sample preparation. Automated tissue processing and metabolomic profiling by ultrahigh performance liquid chromatography-tandem mass spectroscopy and gas chromatography-mass spectroscopy were performed at Metabolon using multiple quality control standards as described (Shin *et al.*, *Nat Genet* 46, 543-550, 2014). After extraction of raw data, peaks were identified by comparison to known purified standards and recurrent unknown entities present in Metabolon's reference library.

Peak quantification was performed using area-under-the-curve with data normalization to correct for day-to-day variation for studies spanning multiple days. Statistical analyses of metabolomics data were performed in ArrayStudio 5.0 and R 3.1.1 (online at R-project.org). Two-way comparisons between experimental groups were analyzed by Welch's *t* test for each of the three tissue types using log-transformed data in R. To correct for multiple testing, false discovery rates (FDR) were estimated using the q-value method (Storey and Tibshirani, *Proc Natl Acad Sci USA* 100, 9440-9445, 2003), and only metabolites with an FDR <5% were considered significant. Canonical pathways analysis was applied to metabolites meeting a fold-change threshold of > |1.2| and a FDR of < 5% for each two-way comparison and for each tissue using Ingenuity Pathway Analysis (IPA, Qiagen).

Example 2: Targeting Glutamine Metabolism Rescues Mice from Late-Stage Cerebral Malaria

This example describes the finding that in an animal model of cerebral malaria, DON treatment at late stages of disease restores BBB integrity, reduces brain swelling and promotes survival. DON treatment also reduces the number of CD8⁺ effector T cells that degranulate in the brains of infected mice.

DON treatment promotes survival even in late-stage ECM

To determine if blocking glutamine metabolism would inhibit death due to immunopathology in a mouse model of ECM, C57BL/6 mice were infected with *Plasmodium berghei* ANKA (*PbA*) on day 0 and injected intraperitoneally with DON (1.3 mg/kg) or saline, beginning either at 7 am on the morning of day 5p.i. (day 5a p.i.), at 11 pm on day 5 p.i. (day 5p p.i.) or at 7 am on day 6 p.i. (day 6a p.i.). DON treatments were repeated either every day or every other day (FIG. 6). The majority of untreated, *PbA*-infected mice died during day 6 p.i. and all died by day 7 p.i. (FIG. 1A). In contrast, all of the mice treated with DON beginning on day 5a p.i. and 80% of the mice treated on day 5p p.i. survived as followed out to day 12 p.i. Remarkably, nearly 50% of mice treated as late as day 6a p.i. survived.

Mice were evaluated for the development of neurological signs associated with ECM and given clinical scores between 0 (no signs) to 10 (moribund) using previously described criteria (Waisberg *et al.*, *PLoS One* 7, e29493, 2012). Nearly all of the untreated mice that were infected with *PbA* developed neurological signs by day 5p p.i. that in most cases were severe (clinical score >6) by day 6a p.i. (FIG. 1B). Treatment with DON beginning on day 5a p.i. prevented the development of neurological symptoms in all *PbA*-infected mice (FIG. 1B). Treatment of mice

with DON beginning on day 5p p.i., a point at which most mice had clinical scores of two, not only prevented the worsening of clinical signs but promoted the rapid resolution of symptoms (FIG. 1B). Remarkably, treatment of mice on day 6a p.i., when many mice had already developed clinical scores of five, blocked the progression of the disease and rapidly resolved the symptoms in half the mice (FIG. 1B). Thus, DON was able to reverse disease even when the mice were already displaying signs of neurologic damage.

In both DON-treated and untreated *PbA*-infected mice, parasitemias increased similarly with time, reaching peak levels of approximately 8-12% between d5 and d6 p.i. (FIG. 1C). Nearly all untreated mice died by day 7 p.i., with peripheral parasitemia of 10-12%. In the DON-treated mice that survived beyond day 6 p.i., the peripheral parasitemias decreased beginning on day 7 p.i., reaching low levels by day 8 p.i. However, during the critical period on day 6 p.i. during which untreated mice die and DON treated mice survive, the parasite loads were indistinguishable in the brains of the mice treated on day 5p p.i. and untreated mice (FIG. 1D), suggesting that inhibition of parasite growth by DON is not the primary mechanism promoting survival. Consistent with the ability of DON to inhibit parasite replication, once neurologic symptoms were resolved, continued DON treatment suppressed parasitemia to under 5% as followed out to day 17 p.i. and upon stopping DON treatment, parasitemia increased to a mean of 40% by day 21 p.i. (FIG. 7).

DON treatment restores BBB integrity and reduces brain swelling but does not have an immediate effect on brain hemorrhaging

Compromise of the BBB, brain swelling and hemorrhaging are major components of the neuropathology observed in ECM. Thus, the effect of DON treatment on the integrity of the BBB was assessed by measuring the leakage of the dye Evans blue (EB) into the brains (Yen *et al.*, *PLoS One* 8, e68595, 2013; Nag, *Methods Mol Med* 89, 133-144, 2003). Mice were injected retroorbitally with EB, sacrificed three hours later and their brains removed and EB quantified. By both visual inspection and by EB quantification, the brains of *PbA*-infected, untreated mice showed significant leakage of EB into their brains on day 5p p.i. that increased further on day 6a p.i. (FIG. 2A). Remarkably, in the brains of *PbA*-infected DON-treated mice, EB leakage was significantly less on day 6a p.i. as compared to untreated mice and decreased significantly further by day 7a p.i. (FIG. 2A). The effect of DON treatment on the water content of the brain as a measure of cerebral edema was determined by weighing the brains before and after desiccation. As compared to uninfected mice, the brains of *PbA*-infected mice had a significantly greater water content measured on both day 5p and day 6a p.i. (FIG. 2B). Treating mice with DON on day 5p p.i. significantly reduced the water content of the brains measured on day 6a p.i. Thus, not only could

DON arrest the disease process, but it also promoted resolution even when treatment was first initiated at a time when significant BBB dysfunction and brain swelling were already manifest.

To quantify brain hemorrhages, brain sections were stained with hematoxylin and eosin (H&E). The brains of *PbA*-infected, untreated mice developed petechial hemorrhages throughout the brain by day 5p p.i. that increased in number by day 6a p.i. (FIG. 2C). Treatment of mice with DON beginning on day 5p p.i. had no significant effect on the number of hemorrhages that developed. However, by day 15 p.i. the hemorrhages were no longer evident (FIG. 2C). Thus, DON treated mice survived local hemorrhaging in the brain when the overall integrity of the BBB was restored and brain swelling was reduced.

DON treatment reduces the number of CD8⁺ effector T cells that degranulate in the brains of infected mice

CD8⁺ T cells have been shown to play a major role in promoting death in ECM (Howland *et al.*, *Semin Immunopathol*, 2015). Glutamine metabolism is critical for the differentiation, proliferation and function of effector T cells. Thus, studies were conducted to determine the effect of DON treatment on T cells in the brains of infected mice. DON was administered to *PbA*-infected mice on day 5p p.i. and on day 6a p.i. mice were terminally anesthetized and transcardially perfused with cold PBS. The brains were collected and single cell suspension were prepared and analyzed by flow cytometry. First, immune cell infiltration of both the treated and untreated mice was examined. As compared to uninfected mice, the brains of infected mice showed large increases in all immune cell types analyzed including CD8⁺ and CD4⁺ T cells, neutrophils, macrophages and NK cells. Interestingly, in spite of its ability to reverse disease, DON treatment had no effect on the absolute numbers of immune cell subsets in the brains of infected mice (FIGS. 3A-3E).

Next, the effect of DON on the expansion and function of parasite-specific CD8⁺ T cells was examined. The accumulation of *PbA*-specific CD8⁺ T cells in the brains and spleens was quantified using a peptide-MHC class I tetramer composed of the *PbA* glideosome-associated protein 50 (GAP50) peptide, SQLLNAKYL (SEQ ID NO: 9), bound to MHC class I D^b (referred to as a GAP50-tetramer) that identifies approximately 5% of splenic CD8⁺ T cells in *PbA*-infected mice (Howland *et al.*, *EMBO Mol Med* 5, 984-999, 2013). It was verified that in the spleens of uninfected mice the frequency of GAP50-tetramer-binding CD8⁺ T cells was less than 0.1% (FIG. 3F, left panel). Enrichment for GAP50-tetramer-binding cells in the spleen and lymph nodes of uninfected mice by GAP50-tetramer-bound magnetic bead purification showed approximately 2,700 GAP50-tetramer-binding CD8⁺ T cells, the majority of which were resting, CD44⁺ cells

(FIG. 3F, middle panel). In contrast, in *PbA*-infected mice the frequency of GAP50-tetramer-binding T cells in the spleen was approximately 3% and these were CD44⁺ effector T cells (FIG. 3F, right panel). DON treatment of *PbA*-infected mice resulted in a small decrease in the number of GAP50-tetramer-binding CD8⁺ T cells in the spleens but had little effect on the number of GAP50-tetramer binding T cells in the brains (FIG. 3G). These observations suggest that the ability of DON to reverse neuropathology at this late stage in the disease process is not dependent on its ability to inhibit the expansion of *Pb*-specific CD8⁺ T cells.

Having observed that DON was not inhibiting expansion of parasite-specific CD8⁺ T cells, a study was conducted to determine the effect of DON on CD8⁺ T cell effector function. The development of ECM has been shown to be dependent on degranulation and perforin release of CD8⁺ T cells that accumulate in the brains of *PbA*-infected mice (Howland *et al.*, *Semin Immunopathol*, 2015). Degranulation of CD8⁺ T cells results in the expression of CD107 on their plasma membranes. To assess the ability of DON to inhibit CD8⁺ T cell degranulation in the brains of infected mice, fluorescently-labeled CD107-specific monoclonal antibodies were administered intravenously to mice one hour before tissues were removed for analysis to allow *in vivo* labeling of CD107⁺ cells. Shown are representative flow cytometry plots showing the percent of CD44⁺, CD8⁺ T cells that express CD107 in the spleens of uninfected mice, *PbA*-infected mice and *PbA*-infected mice treated with DON (FIG. 3H). DON treatment of *PbA*-infected mice resulted in significant decreases in the percent of CD8⁺ T cells that were CD107⁺ in both the spleens and brains (FIG. 3I). The percent of GAP50-tetramer-binding CD8⁺ T cells that were CD107⁺ also decreased significantly in the brains of *PbA*-infected mice (FIG. 3J). Thus, while the late-stage treatment of DON does not block the expansion of the *PbA*-specific CD8⁺ T cells, it does seem to block their effector function as measured by degranulation.

DON treatment reverses *PbA*-induced metabolic changes in the brain

The ability of DON to rapidly reverse ECM at such a late stage of disease led to studies to determine whether in addition to inhibiting immune-mediated pathology, DON might also be affecting pathology induced by altered brain metabolism. To investigate this possibility, metabolites in the brain, liver and serum were profiled from five groups of mice that differed in their infection, treatment and/or clinical status (Table 1). Principal component analysis of all detected metabolites revealed distinct clustering of samples according to infection status in all tissues (FIGS. 4A-4C). Notably, it was observed that infected, DON-treated mice with low clinical scores (<6) clustered with uninfected mice for metabolites in the brain (FIG. 4A). In contrast, when the effect of DON on systemic metabolism was examined, as determined by serum

metabolites or the effect of DON on liver metabolism, this correlation did not hold (FIGS. 4B-4C) suggesting that DON treatment in ECM results in specific effects on brain metabolites.

Table 1: Infection and treatment groups for metabolomics studies

Group description	Number of Samples		
	Brain	Liver	Serum
Uninfected, untreated	5	5	5
Uninfected, DON treated	5	5	5
<i>PbA</i> -infected, DON treated, low clinical scores (<6) on day 6 post-infection	10	10	10
<i>PbA</i> -infected, DON treatment, high clinical scores (≥ 6) on day 6 post-infection	6	6	6
<i>PbA</i> -infected, untreated	8	7	7

5

Since DON blocks the initial step in cellular glutaminolysis by inhibiting glutaminase (Thangavelu *et al.*, *Sci Rep* 4, 3827, 2014) (FIG. 9), the metabolites of glutaminolysis were examined. It was observed that *PbA* infection alone affects these metabolites differently in the brain compared to the liver and serum (FIG. 9B). Notably, *PbA* infection increased Gln in the brain but decreased Gln in the serum. In *PbA*-infected mice with low clinical scores, treatment with DON significantly increased Gln in both the liver and the serum, as expected, but modestly decreased Gln in the brains relative to infected, untreated mice. DON treatment of infected mice that have the good clinical outcomes have Gln levels that are closer to the uninfected mice, which are lower than the infected, untreated mice. The same is true of Glu, except that the levels are higher than the infected, untreated mice.

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Of the 225 brain metabolites that changed significantly in infected versus uninfected mice, 81 overlapped with the 89 significantly affected metabolites identified by comparing infected, DON-treated mice with low clinical scores to infected, untreated mice (FIG. 4D). Pathways significantly affected by *PbA* infection involved citrulline metabolism, the urea cycle or nitric oxide (NO) biosynthesis (aspartate, citrulline, ornithine, and urea) and were similar to pathways affected by DON treatment of *PbA*-infected mice (FIGS. 4E-4F). Strikingly, all 81 overlapping metabolites were metabolites that were reversed by DON treatment (FIGS. 5A-5C). Unsupervised hierarchical clustering analysis on these 81 brain metabolites accurately grouped the samples by infection, treatment, and clinical status, with 9 out of 10 *PbA*-infected, DON treated mice with low

20

clinical scores (<6) clustering together and demonstrating remarkable metabolic similarities to uninfected mice. DON appears to reverse brain metabolism associated with disease acting through its immunomodulatory effects as well as potentially directly on brain metabolism. While these data do not identify the specific metabolites promoting disease, they do identify a set of metabolites which are specifically associated with ECM and are selectively reversed by treatment with DON.

Summary of Results

HCM is a deadly complication of *P. falciparum* malaria despite treatment with effective anti-malarial drugs. That is, even in the setting of effectively suppressing the replication of *P. falciparum*, once signs of neurologic disease have commenced, there is no effective adjunctive treatment for HCM and overall mortality remains high. As such, the identification of a treatment which can arrest and reverse disease in the late stages is urgently needed. It is disclosed herein that the Gln analog, DON, is an effective therapy for ECM even when treatment is first initiated after infected animals show neurological signs of disease. This clinical response was accompanied by the ability of DON to inhibit pathology as measured by decreases in BBB dysfunction, brain swelling and degranulation of parasite-specific CD8⁺ T cells that accumulated in the brain. Furthermore, DON is able to reverse metabolic changes associated with the disease state.

Previously, DON has been shown to have anti-parasitic activity, albeit weak both *in vitro* and *in vivo* (Plaimas *et al.*, *Infect Genet Evol* 20, 389-395, 2013; Queen *et al.*, *Antimicrob Agents Chemother* 34, 1393-1398, 1990). The findings disclosed herein indicate that the anti-parasitic activity of DON is not playing a major role in its ability to arrest and reverse ECM. Indeed, DON had little effect on the parasite load in the brains of *PbA*-infected mice during the critical period of time when the BBB was restored and brain swelling decreased in DON-treated mice. Under continual treatment with DON, *PbA*-infected mice were able to control parasitemia, keeping it well under 2%. However, when DON treatment was stopped the parasitemias rose rapidly and mice died of severe anemia.

On the other hand, CD8⁺ effector T cells that release perforin have been shown to play a critical role in promoting the pathogenesis which constitutes ECM (Howland *et al.*, *Semin Immunopathol*, 2015). Recently, it was observed that the mTOR inhibitor rapamycin protected infected mice from death due to ECM when administered within the first 4 days of infection (Gordon *et al.*, *mBio* 6, e00725-00715, 2015). This protection was associated with a marked decrease in CD8⁺ T cells in the brain and it was hypothesized that rapamycin prevents the development of ECM in part by inhibiting the generation of CD8⁺ effector cells. CD8⁺ T cell activation results in a dramatic shift from oxidative metabolism to aerobic glycolysis and

glutaminolysis necessary for T cell expansion and effector function (MacIver *et al.*, *Annu Rev Immunol* 31, 259-283, 2013; O'Sullivan and Pearce, *Trends Immunol* 36, 71-80, 2015; Pollizzi and Powell, *Nat Rev Immunol* 14, 435-446, 2014). Both T cell cytokine production and proliferation are blocked by restricting the availability of extracellular Gln (Carr *et al.*, *J Immunol* 185, 1037-1044, 2010) or by treating T cells with DON (Wang *et al.*, *Immunity* 35, 871-882, 2011).

Interestingly, unlike the previous studies employing rapamycin, DON treatment of *PbA*-infected mice did not prevent or decrease the accumulation of CD8⁺ T cells in the brain. Rather, the number of CD8⁺ T cells that degranulated, was decreased following DON treatment. These observations indicate that DON is not mediating its protective effect by preventing proliferation and generation of CD8⁺ effector cells, but rather by blocking CD8⁺ T cell effector function. This may account in part for the remarkably fast kinetics by which DON mediates its protective effect. Along these lines, unlike DON, rapamycin treatment was not effective when administered later in the disease. It is the ability of DON to promote survival at such a late stage of the disease that distinguishes the present findings from all other attempts to treat and reverse ECM.

While the studies disclosed herein were motivated by the ability of DON to inhibit immune function, the effect of DON treatment on infected brain metabolism was also investigated by interrogating the brain for known metabolites. Over 81 metabolic changes in the brains of the infected mice compared to uninfected mice were observed and these changes were reversed by DON. Such findings do not unequivocally reveal the mechanism by which DON is mediating its effects. The complexity of the fluctuations in Gln and Glu levels in the brain during infection and upon treatment most likely reflect the fact that DON not only inhibits glutaminase activity but also Gln transport and other glutamine-utilizing enzymes (Thangavelu *et al.*, *Sci Rep* 4, 3827, 2014). However, these findings provide a metabolic profile, a biomarker for diseased brains in ECM.

In summary, the studies disclosed herein demonstrate for the first time the ability of pharmacologic intervention to reverse disease and promote survival in the late stages of ECM. Once neurologic symptoms are manifested, there is a high mortality rate in HCM. Therefore, the findings disclosed herein have relevant and immediate clinical implications. Furthermore, these studies reveal a selective metabolic signature for ECM and the subsequent reversal of diseases.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

1. A method of treating cerebral edema in a subject, comprising:

selecting a subject with cerebral edema; and

5 administering to the subject a therapeutically effective amount of a glutamine antagonist, thereby treating cerebral edema in the subject.

2. The method of claim 1, wherein the cerebral edema is caused by cerebral malaria, a traumatic brain injury or a hemorrhagic stroke.

3. A method of treating cerebral malaria in a subject, comprising:

selecting a subject with malaria who is exhibiting at least one neurological sign or symptom of cerebral malaria; and

15 administering to the subject a therapeutically effective amount of a glutamine antagonist, thereby treating cerebral malaria in the subject.

4. The method of claim 3, wherein the at least one neurological sign of cerebral malaria is selected from cerebral edema, loss of blood-brain barrier integrity, brain hemorrhage, infected red blood cells in the brain vasculature, coma, seizure and delirium.

5. The method of claim 3 or claim 4, comprising selecting a subject who has been diagnosed with cerebral malaria.

6. The method of any one of claims 3-5, further comprising administering to the subject an antimalarial agent.

7. The method of claim 6, wherein the antimalarial agent is selected from quinine, chloroquine, quinine sulfate, hydroxychloroquine, mefloquine, quinidine gluconate, doxycycline, tetracycline, clindamycin, mefloquine, atovaquone, artemether/lumefantrine, pyrimethamine/sulfadoxine, artemisinin and artesunate.

8. A method of inhibiting degranulation of CD8⁺ T cells in the brain of a subject with malaria, comprising:

selecting a subject with cerebral malaria; and

administering to the subject a therapeutically effective amount of a glutamine antagonist, thereby inhibiting degranulation of CD8+ T cells in the brain of the subject.

5 9. The method of any one of claims 1-8, wherein the glutamine antagonist is administered intraperitoneally or intravenously.

 10. The method of any one of claims 1-9, wherein the glutamine antagonist is administered once a day.

10 11. The method of any one of claims 1-9, wherein the glutamine antagonist is administered once every other day.

 12. The method of any one of claims 1-11, wherein the glutamine antagonist is 6-diazo-5-oxo-L-norleucine, acivicin or azaserine.
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 13. The method of claim 12, wherein the glutamine antagonist is 6-diazo-5-oxo-L-norleucine.

 14. The method of claim 13, wherein the 6-diazo-5-oxo-L-norleucine is administered to
20 the subject at a dose of about 0.1 to about 5 mg/kg.

 15. The method of claim 13, wherein the 6-diazo-5-oxo-L-norleucine is administered to the subject at a dose of about 1.3 mg/kg once per day.

25 16. The method of claim 13, wherein the 6-diazo-5-oxo-L-norleucine is administered to the subject at a dose of about 1.3 mg/kg once every other day.

 17. A composition comprising 6-diazo-5-oxo-L-norleucine and an antimalarial agent for use in treating cerebral malaria.
30

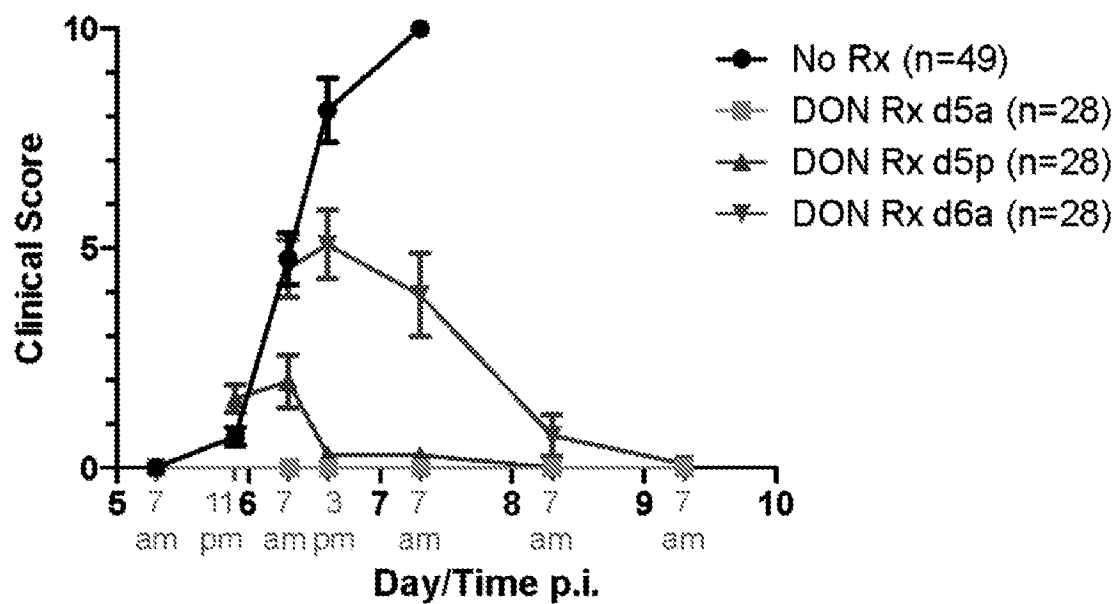
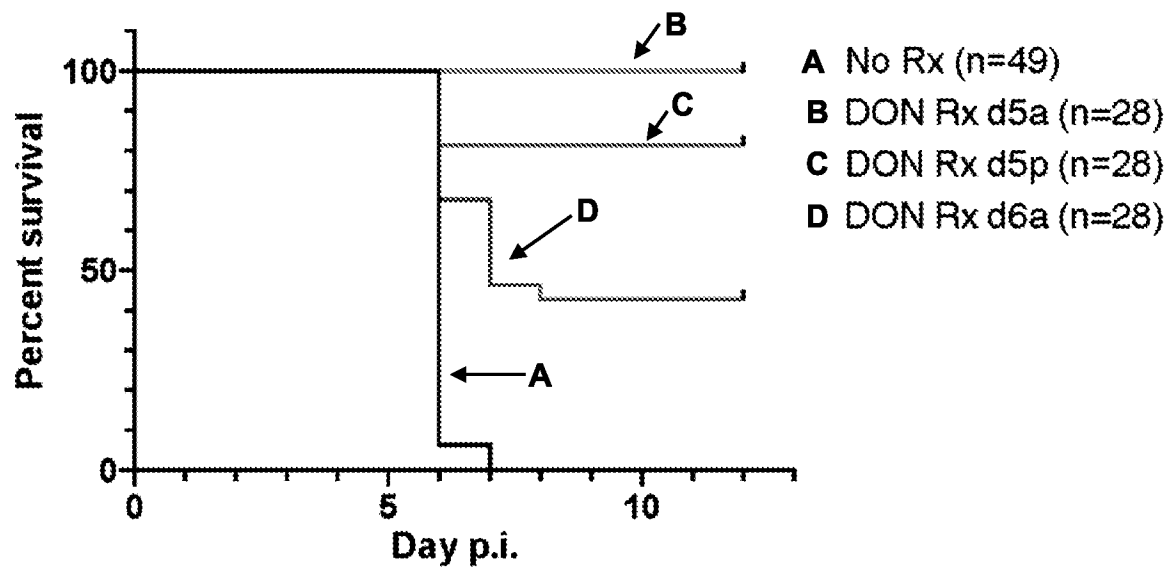
FIG. 1A**FIG. 1B**

FIG. 1C

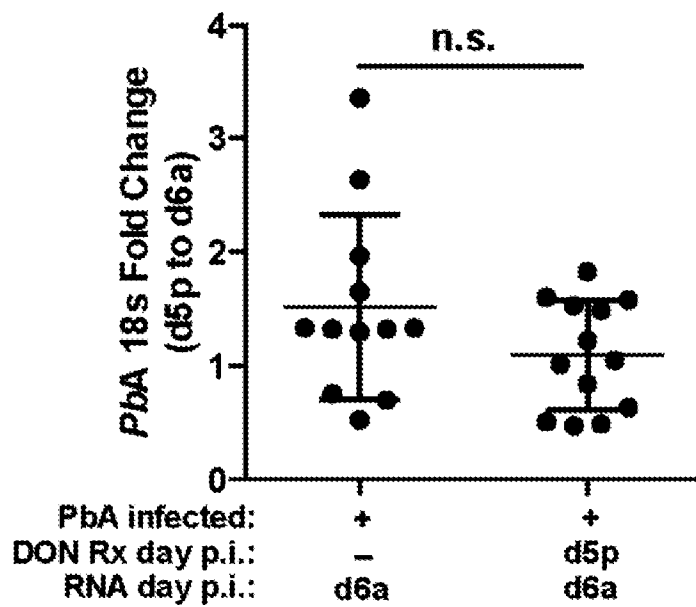
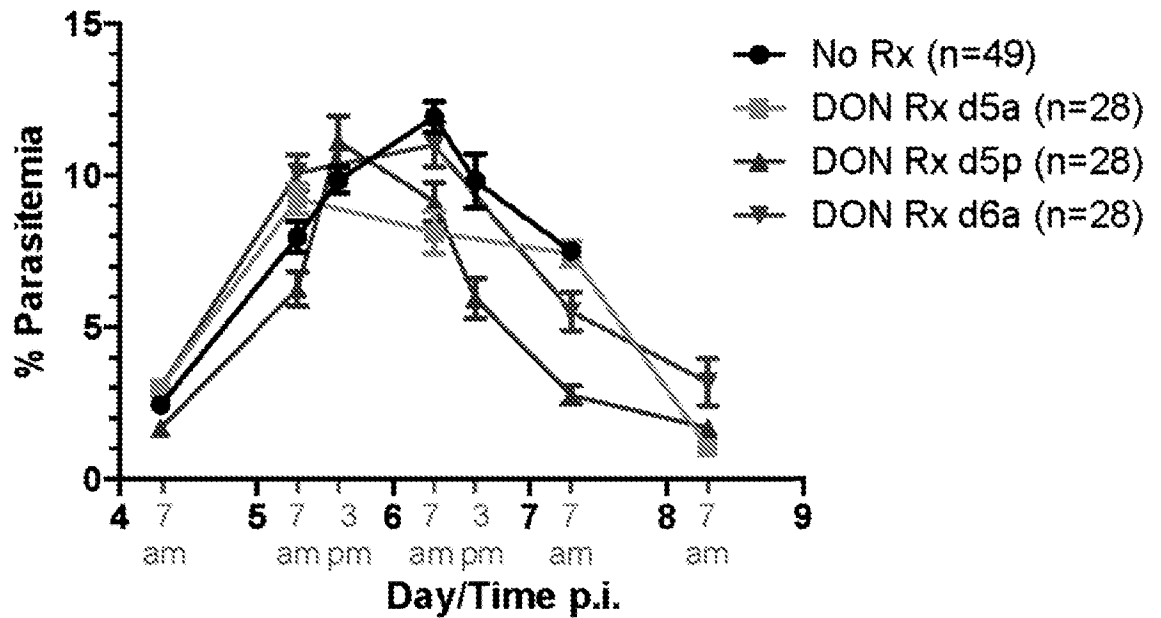


FIG. 1D

FIG. 2A

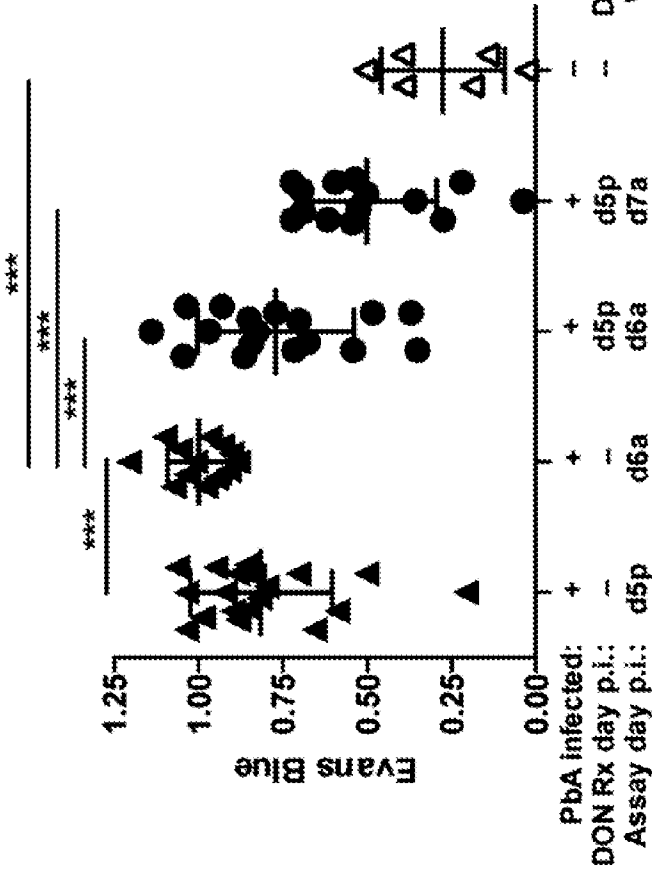


FIG. 2B

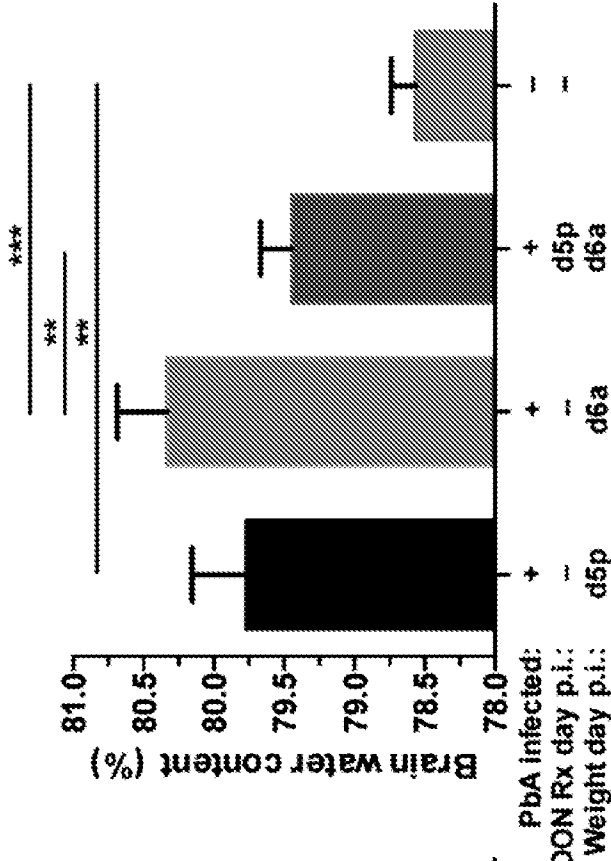
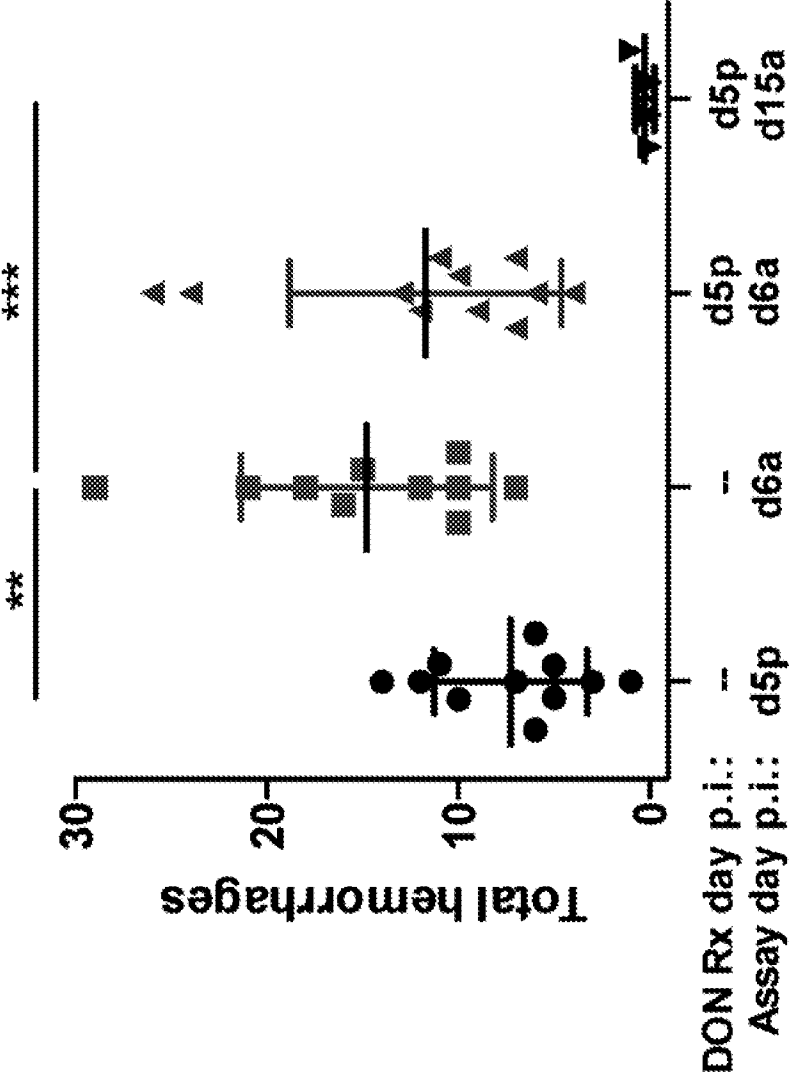


FIG. 2C



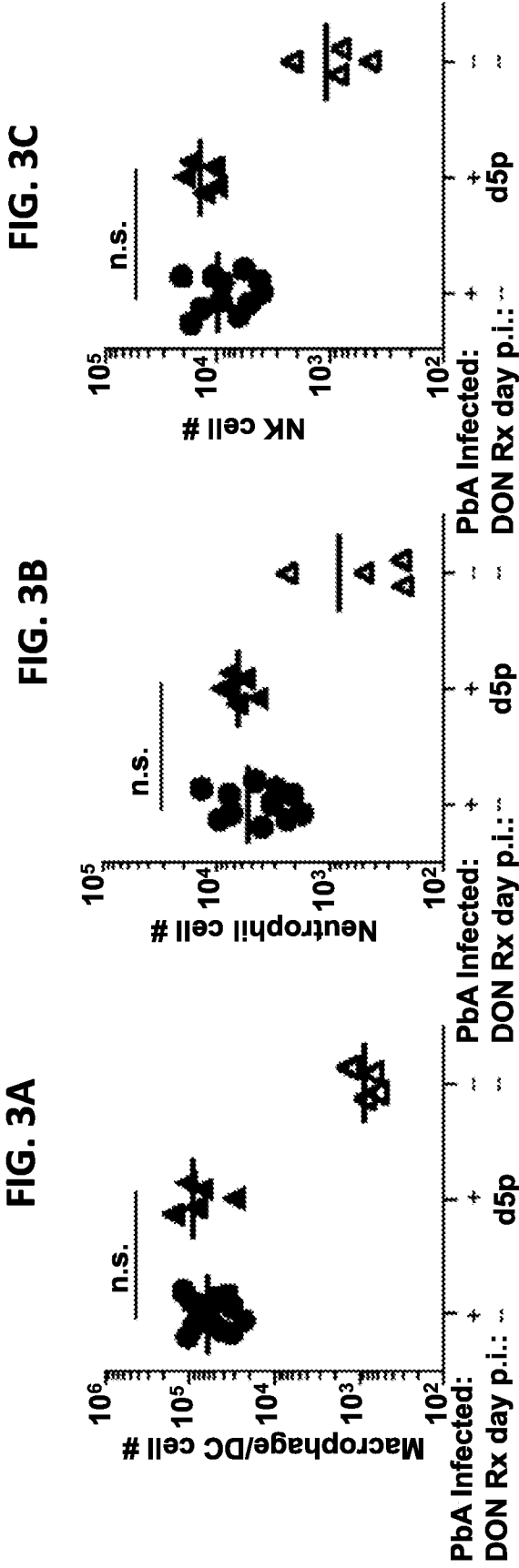


FIG. 3D

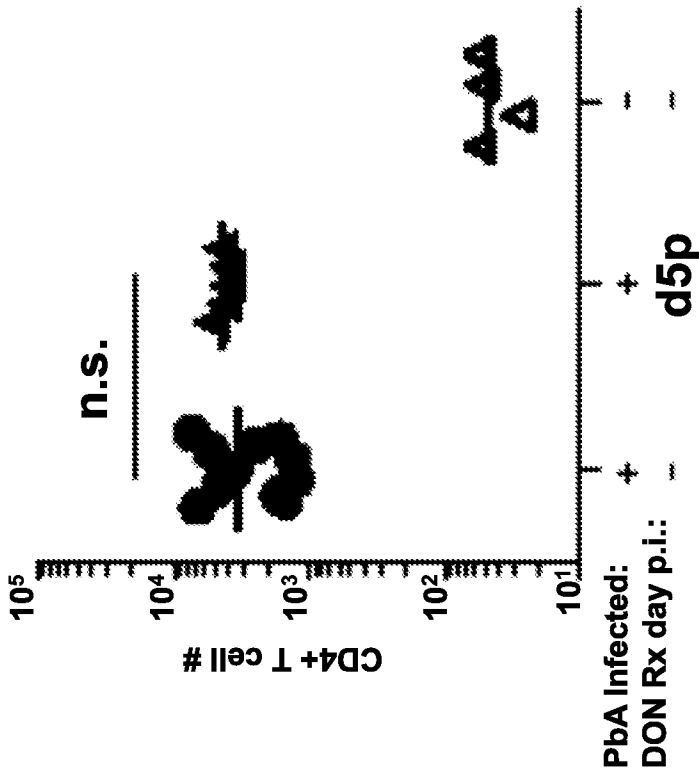


FIG. 3E

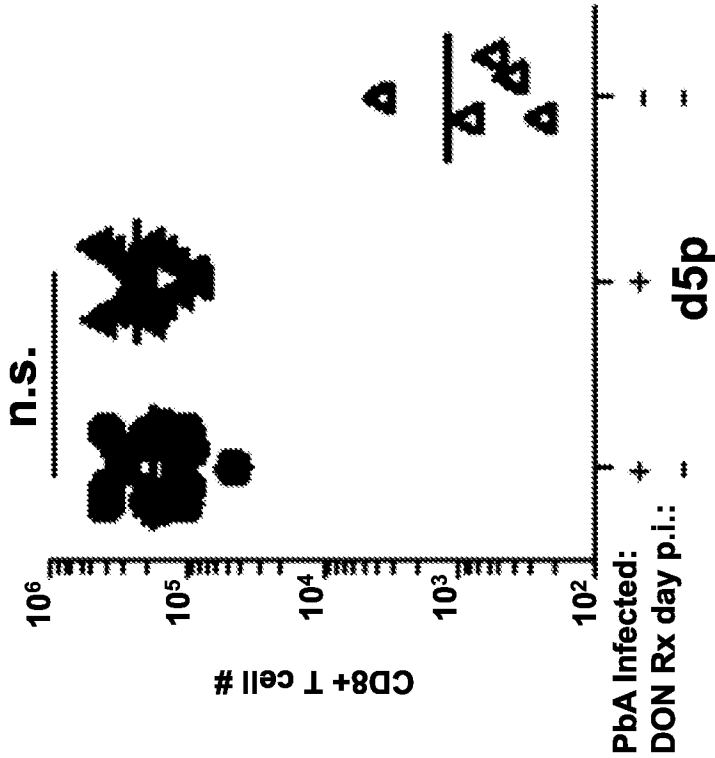
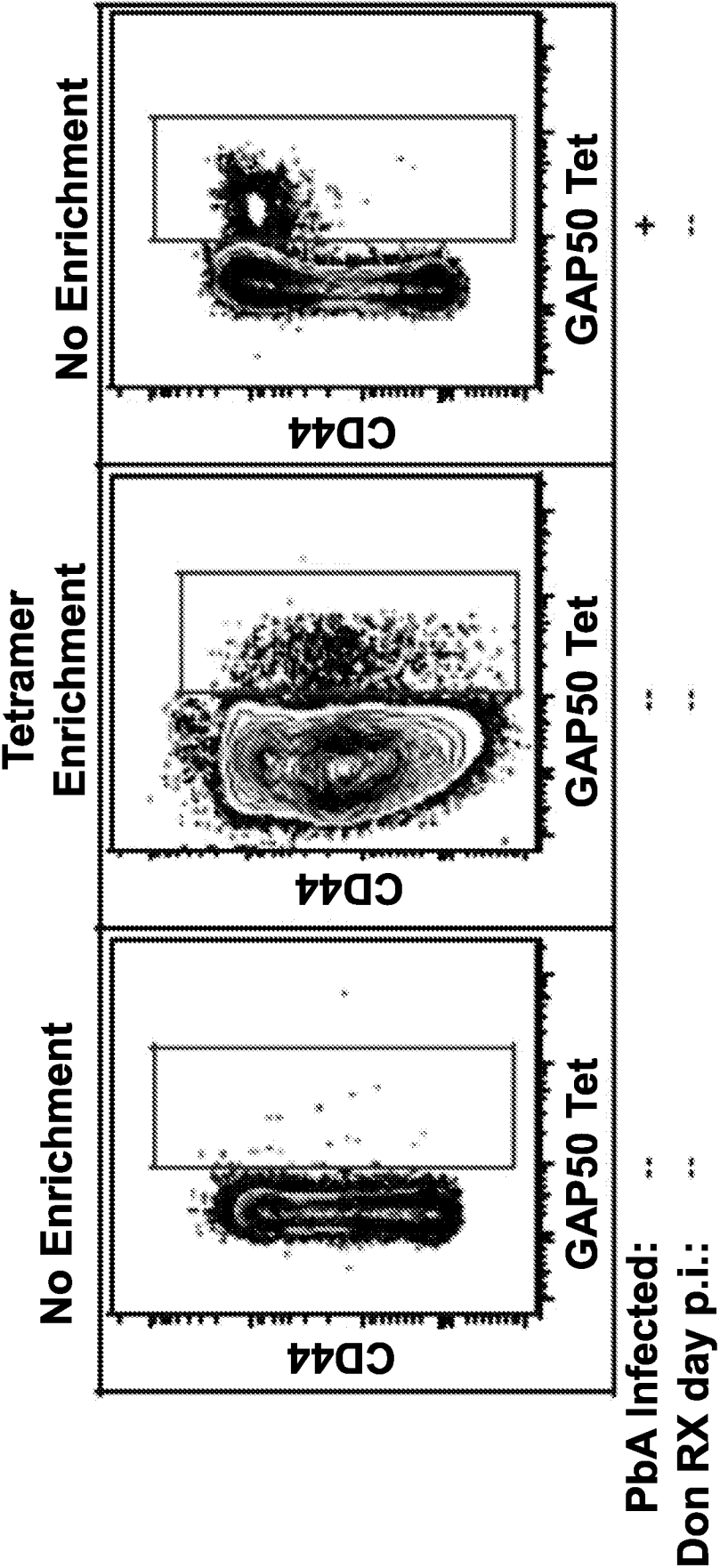


FIG. 3F



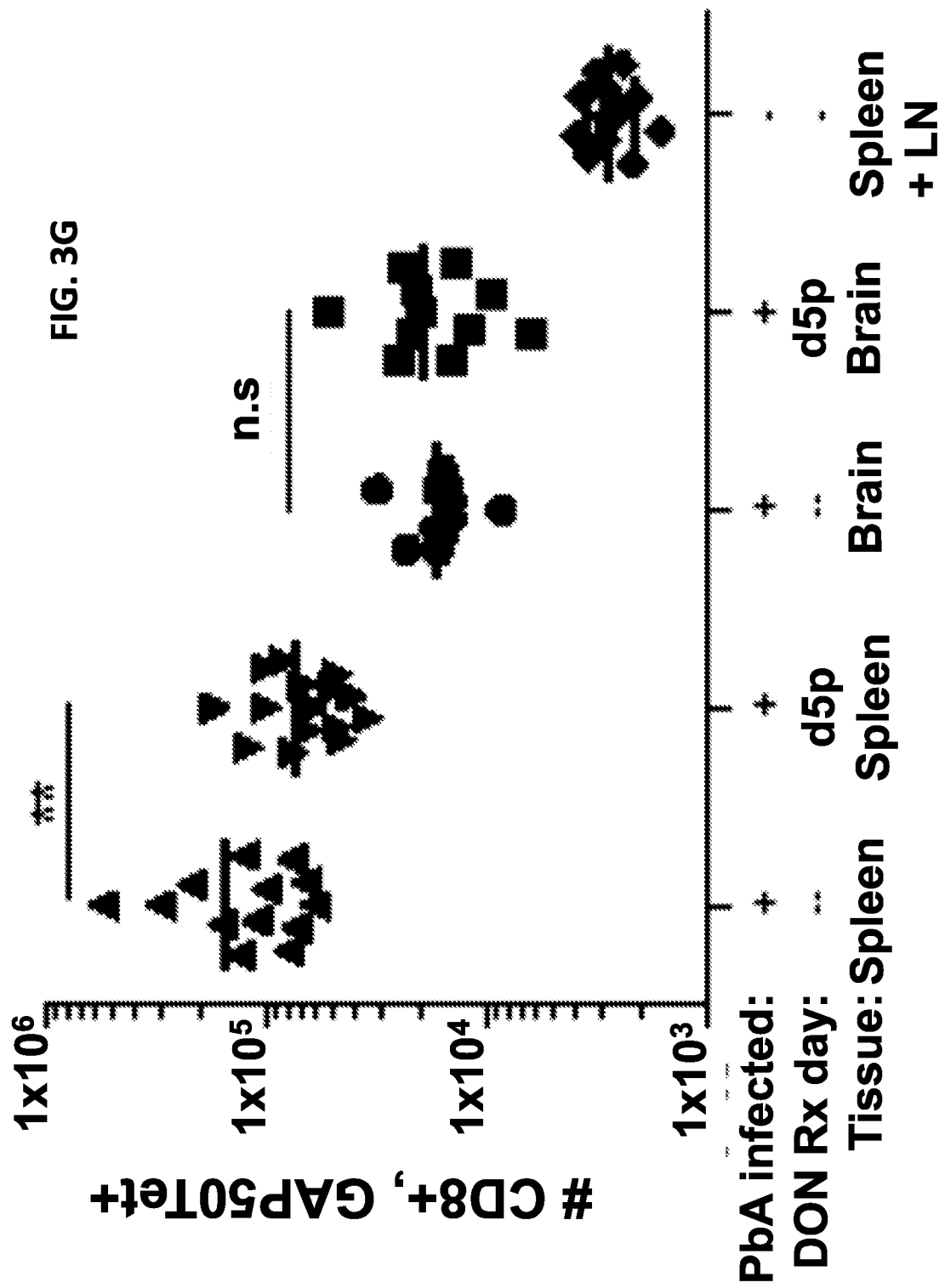


FIG. 3H

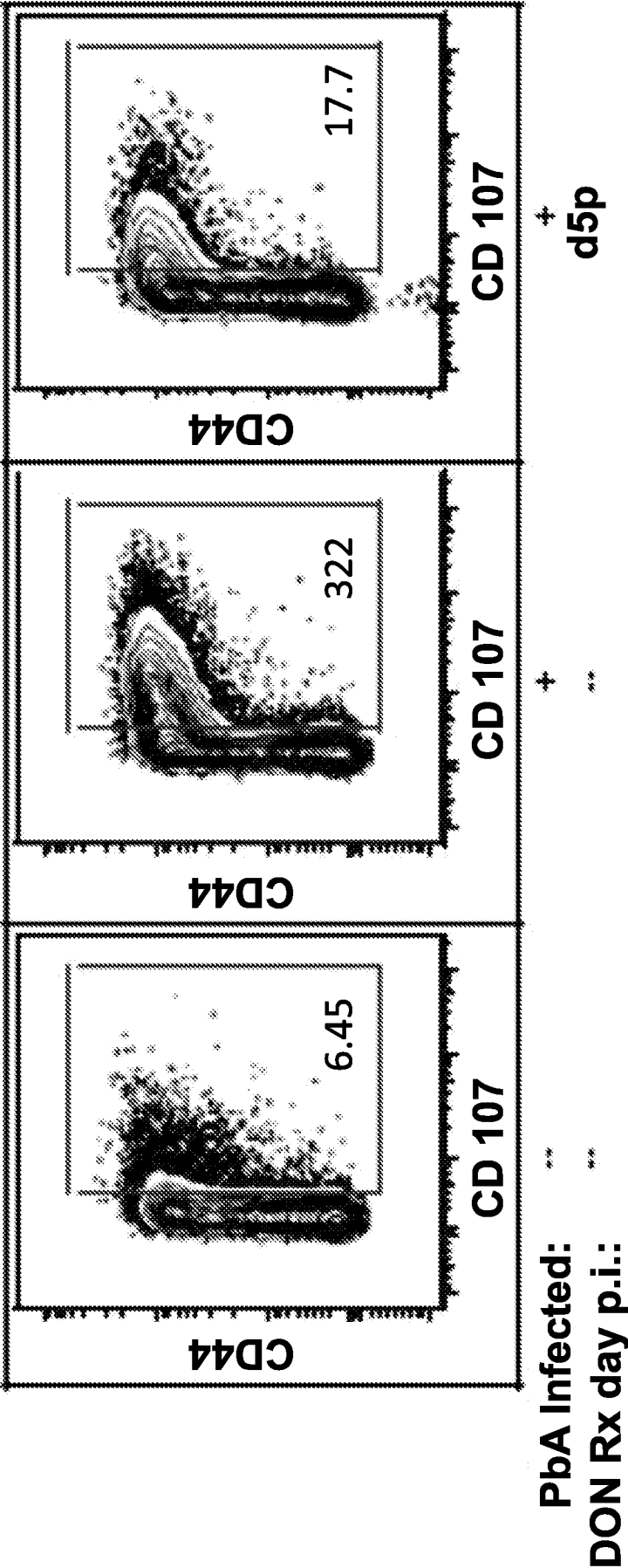


FIG. 3I

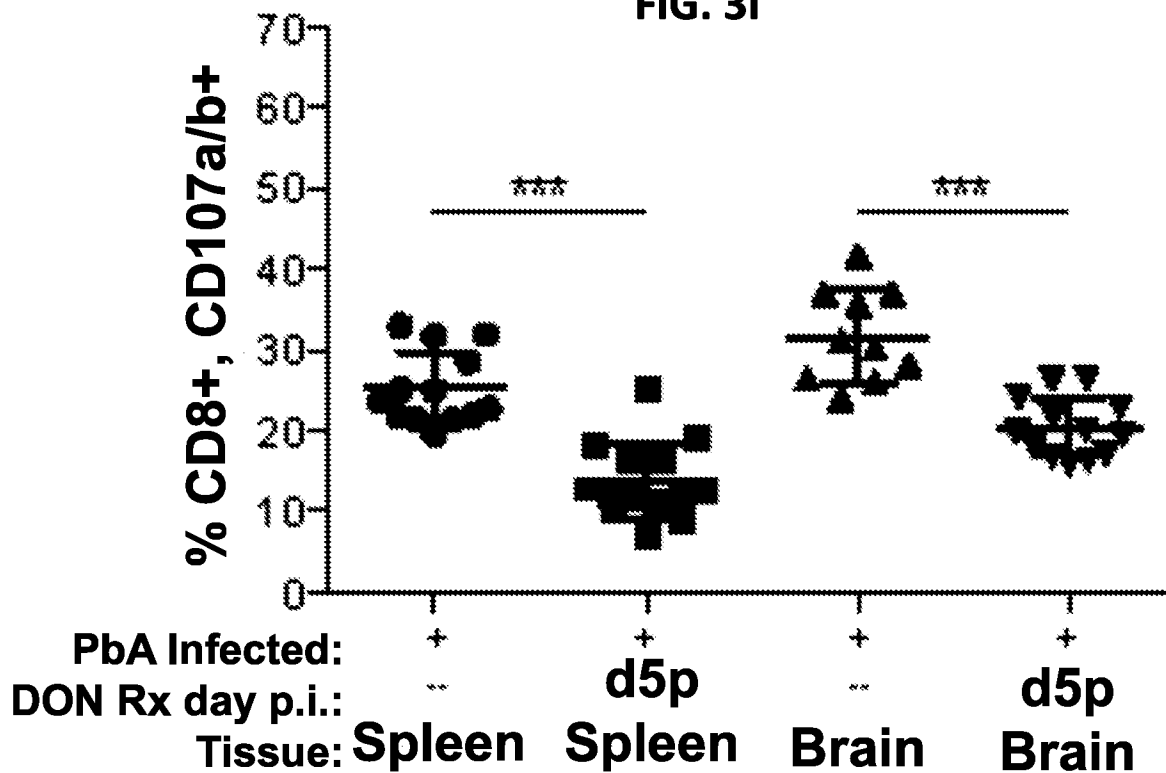


FIG. 3J

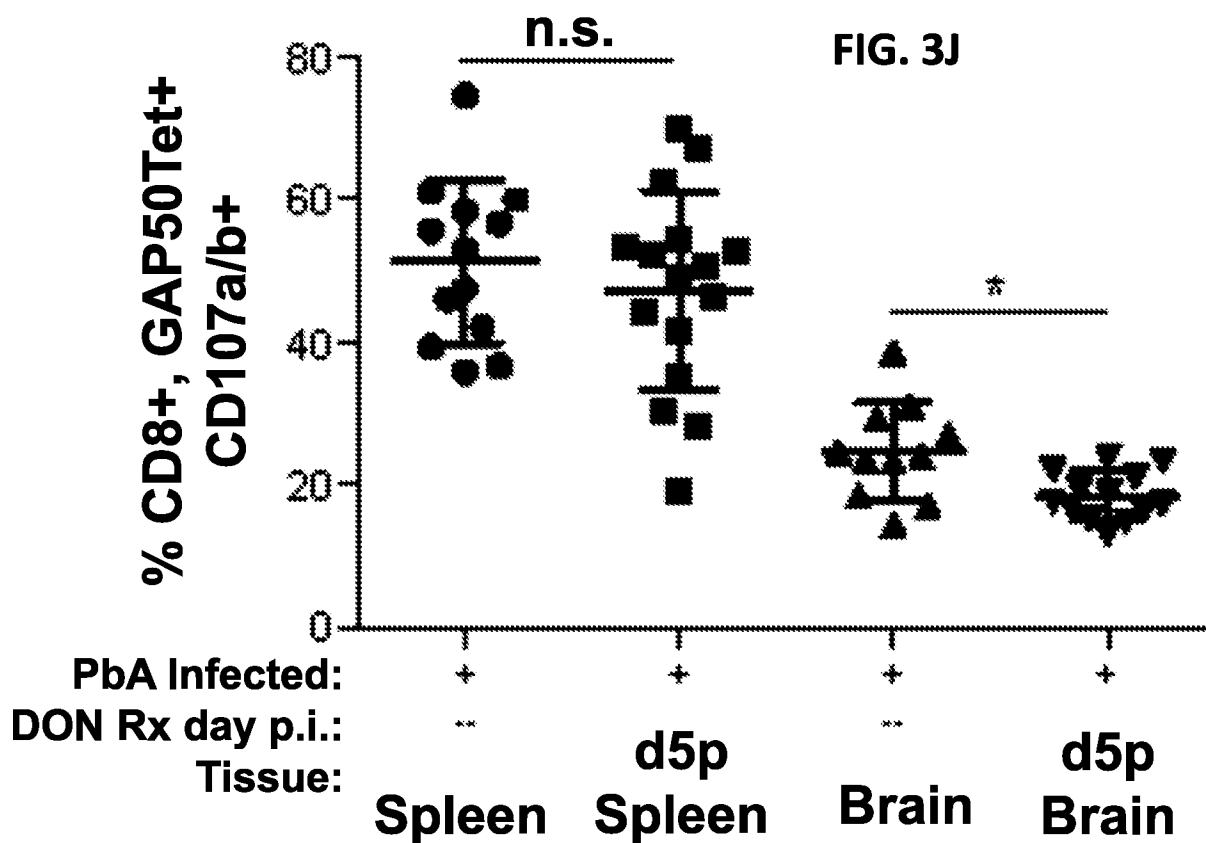


FIG. 4A

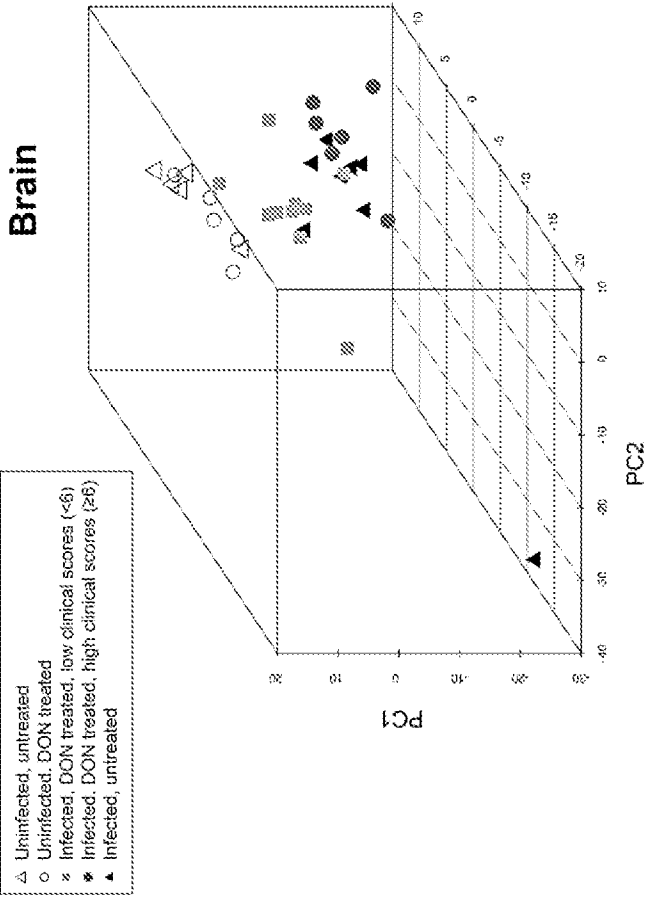


FIG. 4B

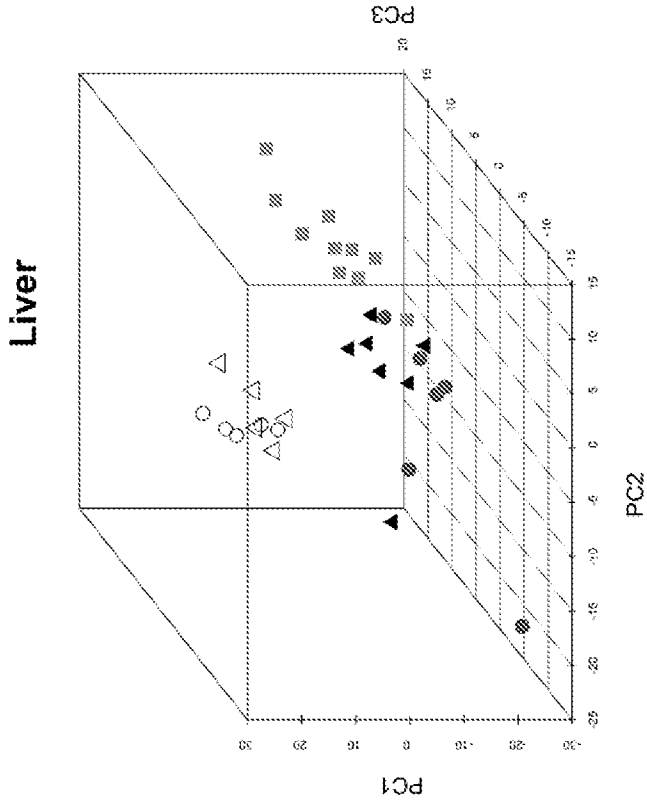


FIG. 4C

Serum

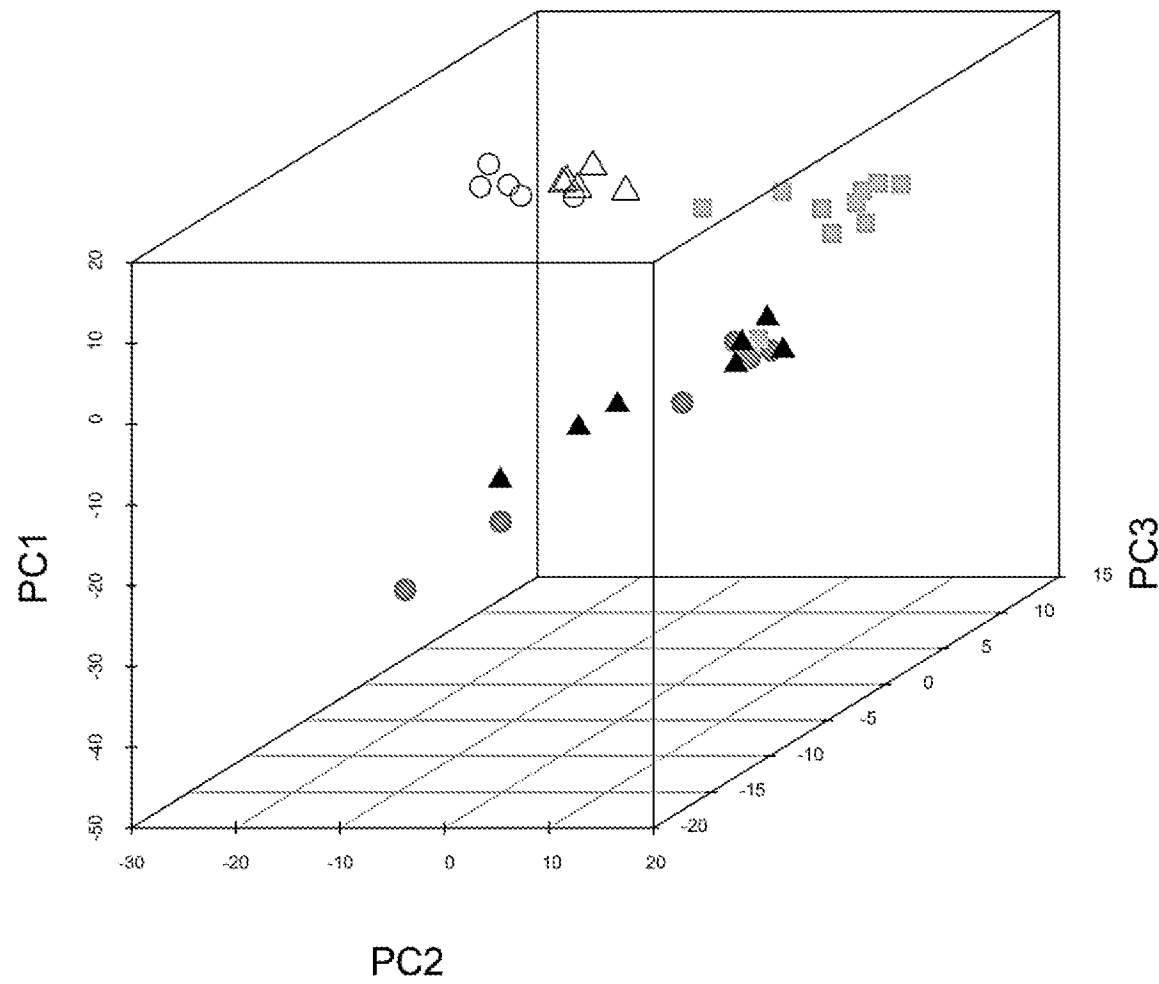


FIG. 4D

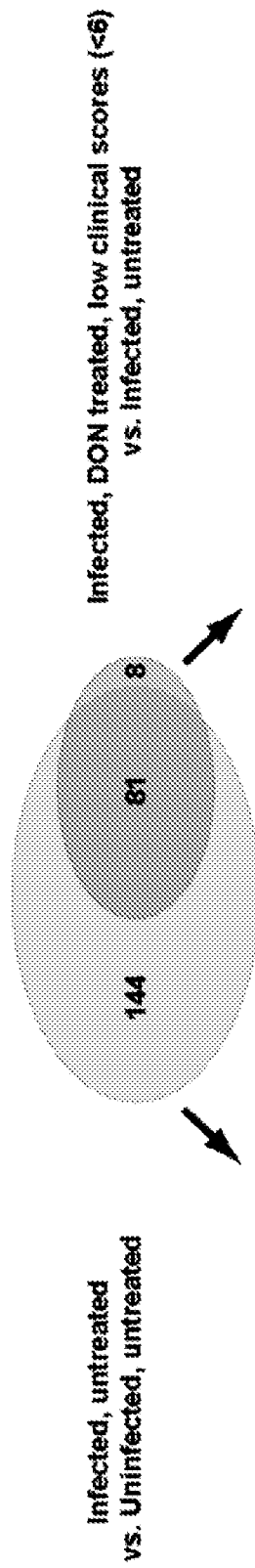


FIG. 4E

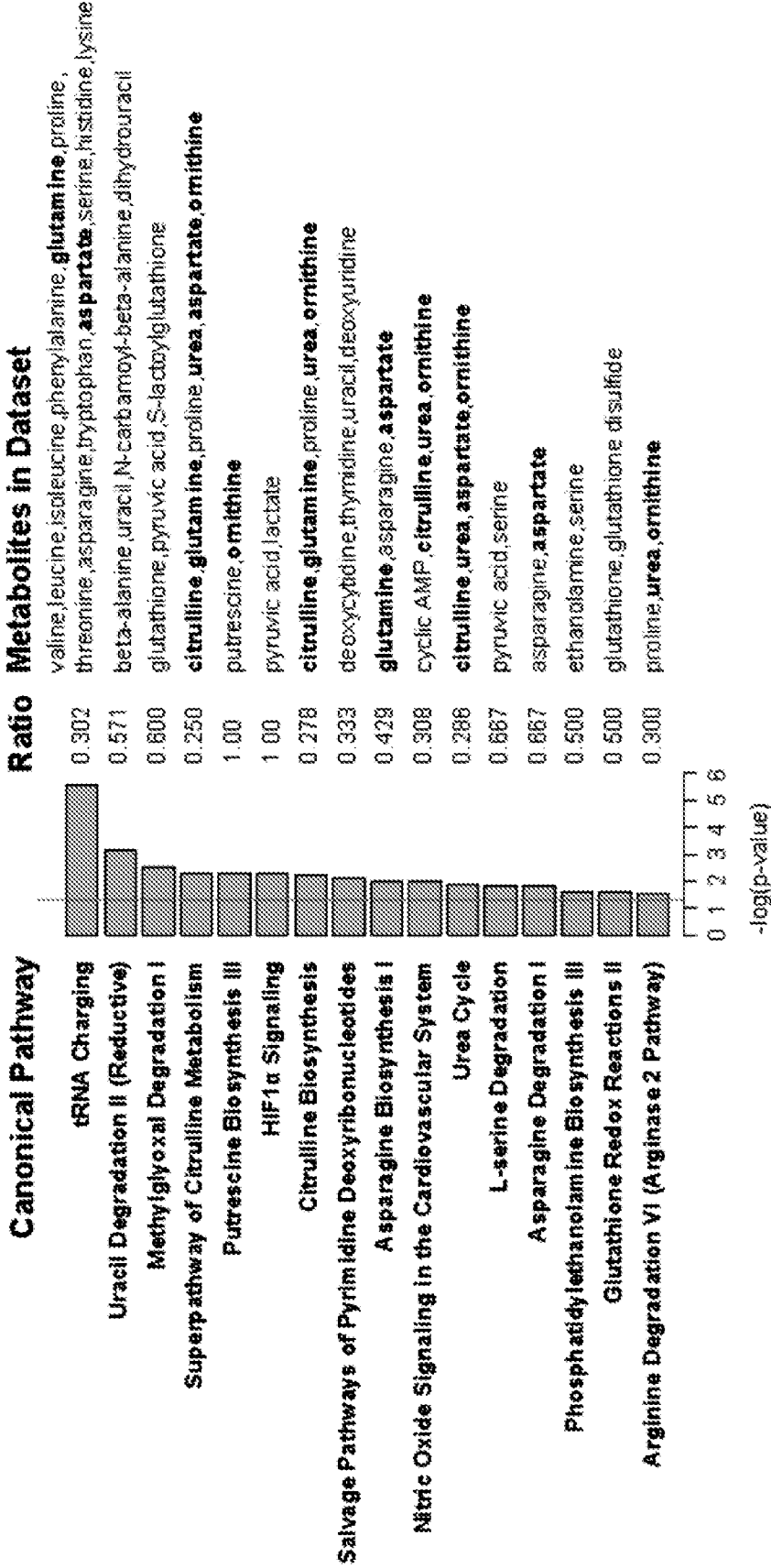
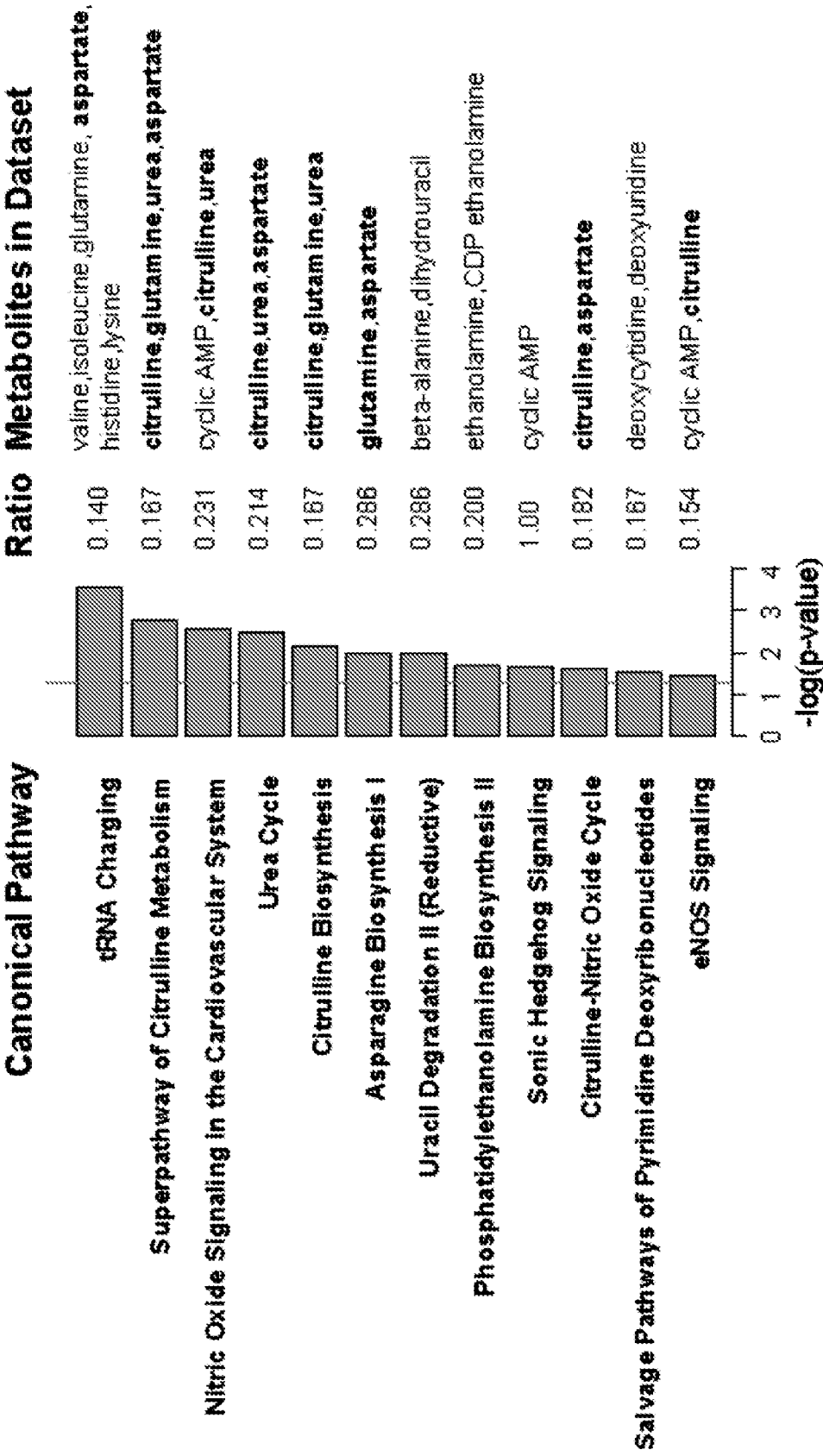


FIG. 4F



The 81 metabolites which were reversed by DON treatment during *PbA* infection.

FIG. 5A

Metabolite	Main Pathway	Sub Pathway	Mass	Pubchem ID	HMDB ID	Comparison groups (Welch's t tests)			
						PbA infected, untreated vs uninfected, untreated	Fold Change	False Discovery Rate	PbA infected, DON treated, low clinical score (<6) vs PbA infected, untreated
1-arachidonoylglycerophosphoethanolamine	Lipid	Lysolipid	500.27826	42607465	HMDB11517	1.56	0.0176	0.0036	0.0006
1-linoleoylglycerophosphoethanolamine	Lipid	Lysolipid	476.27826	52925130	HMDB11507	2.87	0.00066561	0.0006	0.0006
1-oleoylglycerophosphoethanolamine	Lipid	Lysolipid	478.28391	9547071	HMDB11506	2.35	0.0007	0.0031	0.0031
1-oleoylglycerophosphoinositol	Lipid	Lysolipid	597.30453			1.57	0.0421	0.0005	0.0005
1-oleoylglycerophosphoserine	Lipid	Lysolipid	522.28374	9547099		2.58	0.0002	0.0005	0.0005
1-oleoylplasmalogenethanolamine	Lipid	Lysolipid	462.29899			2.58	0.0002	0.0032	0.0032
1-palmitoylglycerophosphoethanolamine	Lipid	Lysolipid	452.27826	9547069	HMDB11503	1.90	0.0004	0.0006	0.0006
1-palmitoylglycerophosphoinositol	Lipid	Lysolipid	571.28888		HMDB11505	1.51	0.0317	0.0006	0.0006
1-palmitoylglycerophosphoserine	Lipid	Lysolipid	496.26809	9547100		2.99	0.0188	0.0127	0.0127
1-palmitoylplasmalogenethanolamine	Lipid	Lysolipid	436.28334			2.30	0.0003	0.0021	0.0021
1-stearoylglycerophosphoethanolamine	Lipid	Lysolipid	480.30956	9547068	HMDB111130	2.48	0.0005	0.0027	0.0027
1-stearoylglycerophosphoglycerol	Lipid	Lysolipid	511.30414			2.94	0.0012	0.0022	0.0022
1-stearoylglycerophosphoinositol	Lipid	Lysolipid	599.32018		HMDB11506	1.95	0.0013	0.0005	0.0005
1-stearoylglycerophosphoserine	Lipid	Lysolipid	524.29939	9547101		2.28	0.0004	0.0005	0.0005
1-stearoylplasmalogenethanolamine	Lipid	Lysolipid	464.31464			2.36	0.0024	0.0009	0.0009
2-aminobutyrate	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	130	439691	HMDB00650	18.07	3.4872E-06	0.0129	0.0129
2-hydroxy-3-methylvalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	131.07135	164623	HMDB000317	7.56	0.0000878	0.0031	0.0031
2-hydroxybutyrate (AHB)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	103.04006	440864	HMDB000008	12.68	0.000008827	0.0168	0.0168
2-methylbutyrylcamitine (C5)	Amino Acid	Leucine, Isoleucine and Valine Metabolism	246.16999	6426901	HMDB000378	5.17	0.0001	0.048	0.048
2-stearoylglycerophosphoethanolamine	Lipid	Lysolipid	480.30956			2.42	0.0047	0.001	0.001
2'-deoxycytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	226.08333	13711	HMDB000014	2.01	0.001	0.0113	0.0113
2'-deoxyuridine	Nucleotide	Pyrimidine Metabolism, Uracil containing	111.01997	13712	HMDB000012	1.68	0.0006	0.0109	0.0109
3-hydroxybutyrate (BHBA)	Lipid	Ketone Bodies	103.04007	441	HMDB000357	3.76	0.000012987	0.0002	0.0002
3-hydroxydecanoate	Lipid	Fatty Acid, Monohydroxy	187.13396	26612	HMDB002203	2.78	0.0005	0.0049	0.0049
3-hydroxyisobutyrate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	103.04007	87	HMDB000336	3.47	0.00007885	0.0021	0.0021
3-hydroxyaurate	Lipid	Fatty Acid, Monohydroxy	215.16526	94216	HMDB000387	3.32	0.0002	0.0146	0.0146
3-hydroxyoctanoate	Lipid	Fatty Acid, Monohydroxy	159.10266	26613	HMDB001954	2.68	0.0001	0.0014	0.0014
3-indoxyl sulfate	Amino Acid	Tryptophan Metabolism	212.0023	10258	HMDB000622	3.53	0.0042	0.0224	0.0224

FIG. 5B

3-methylglutaryl carnitine	Amino Acid	Lysine Metabolism	290.15982	128145	HMDB000552	20.85	0.000003694	-1.52	0.0295
4-hydroxyphenylpyruvate	Amino Acid	Phenylalanine and Tyrosine Metabolism	179.03498	979	HMDB000707	-3.13	0.000027222	1.96	0.0047
5-dodecanoate (12:1n7)	Lipid	Medium Chain Fatty Acid	197.1547	5312378	HMDB000529	2.14	0.0023	-1.89	0.014
5,6-dihydrouracil	Nucleotide	Pyrimidine Metabolism, Uracil containing	115.05021	649	HMDB000075	-1.33	0.0322	1.93	0.0031
adenosine 3',5'-cyclic monophosphate (cAMP)	Nucleotide	Purine Metabolism, Adenine containing	328.04524	6076	HMDB000058	-2.94	3.4872E-06	1.76	0.0428
allantoin	Nucleotide	Purine Metabolism, (Hypoxanthine)inosine containing	157.03671	204	HMDB000462	8.34	9.2603E-06	-2.04	0.0209
ascorbate (Vitamin C)	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	332.1		HMDB000044	-2.44	0.0017	2.02	0.0302
aspartate	Amino Acid	Alanine and Aspartate Metabolism	134.04479	5960	HMDB000191	-1.43	0.0095	1.44	0.0064
beta-alanine	Nucleotide	Pyrimidine Metabolism, Uracil containing	174	239	HMDB000056	-1.45	0.0086	1.33	0.0473
beta-hydroxyisovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	117.05572	69362	HMDB000754	3.27	0.0001	-1.96	0.0047
betaine	Amino Acid	Glycine, Serine and Threonine Metabolism	118.08626	247	HMDB000043	5.38	5.4137E-06	-2.00	0.0031
butyrylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	232.16434	439829	HMDB002013	5.08	0.000055261	-1.85	0.0473
choline	Lipid	Phospholipid Metabolism	104.10899	306	HMDB000097	-1.49	0.0011	1.28	0.0048
citrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	176.10297	9750	HMDB000094	2.11	0.0004	-1.96	0.0031
corticosterone	Lipid	Steroid	347.22169	5753	HMDB001547	4.52	0.0017	-2.00	0.0031
cytidine 5'-diphosphocholine	Lipid	Phospholipid Metabolism	533.10853	13804	HMDB001413	-1.75	0.0045	1.71	0.0224
cytidine 5'-diphosphoethanolamine	Lipid	Phospholipid Metabolism	445.0531	123727	HMDB001864	-1.27	0.025	1.33	0.0334
ethanolamine	Lipid	Phospholipid Metabolism	174.1	700	HMDB000149	-1.69	0.0032	1.57	0.0141
ethylmalonate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	131.03498	11756	HMDB000622	2.44	0.0001	-1.67	0.0109
gamma-glutamylglutamate	Peptide	Gamma-glutamyl Amino Acid	277.10303	92865	HMDB111737	-1.61	0.0009	1.50	0.0098
glutamate	Amino Acid	Glutamate Metabolism	148.06044	611	HMDB000148	-1.49	0.0004	1.29	0.0205
glutamine	Amino Acid	Glutamate Metabolism	147.07642	5961	HMDB000641	1.42	0.0000878	-1.25	0.0051
glycerophosphorylcholine (GPC)	Lipid	Phospholipid Metabolism	258.1101	71920	HMDB000086	-6.88	9.4428E-06	2.10	0.0109
heptanoate (7:0)	Lipid	Medium Chain Fatty Acid	129.0921	8094	HMDB000666	-3.70	7.8856E-07	1.96	0.013
hexanoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	260.18564	6426853	HMDB000705	12.56	0.0007	-3.86	0.02
histidine	Amino Acid	Histidine Metabolism	154.0622	6274	HMDB000177	2.14	0.000071544	-1.39	0.0295
hydroxybutyrylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	248.14925	53481617	HMDB13127	10.86	0.000003694	-3.57	0.0008
imidazole propionate	Amino Acid	Histidine Metabolism	141.06586	70630	HMDB002271	3.13	0.0361	-4.76	0.0189

FIG. 5C

isobutyrylcarnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	232.15434	168379	HMDB00736	7.99	0.000042116	-2.33	0.0288
isoleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	132.10191	6306	HMDB00172	1.59	0.0015	-1.35	0.0428
isovalerylcarnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	246.16999	6426851	HMDB00688	11.45	0.000017712	-2.56	0.0109
kynurenine	Amino Acid	Tryptophan Metabolism	209.09207	161166	HMDB00684	32.79	2.3284E-07	-2.04	0.0097
linoleate (18:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	279.23295	5280450	HMDB00673	1.95	0.0006	-1.45	0.0064
linoleylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	424.34214	6450015	HMDB00646	140.92	0.000054612	-6.67	0.0408
lysine	Amino Acid	Lysine Metabolism	147.11281	5962	HMDB00182	1.84	0.0002	-1.37	0.0223
myristoleate (14:1n5)	Lipid	Long Chain Fatty Acid	225.186	5281119	HMDB02000	1.87	0.001	-1.54	0.0249
N-acetylglutamine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	172.09791	70912	HMDB11766	6.25	0.0002	-3.45	0.0034
N-acetylphenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism	208.09632	74839	HMDB00512	20.58	0.000003694	-3.70	0.0022
N-acetyltyrosine	Amino Acid	Phenylalanine and Tyrosine Metabolism	222.07718	68310	HMDB00866	1.71	0.0203	-2.13	0.016
N-delta-acetylornithine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	175.10772	9920500		2.87	0.0023	-2.04	0.0473
N1-Methyl-2-pyridone-5-carboxamide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	153.06586	69698	HMDB04193	3.56	0.0025	-25.00	7.7062E-06
orotidine	Nucleotide	Pyrimidine Metabolism, Orotate containing	287.05209	92751	HMDB00788	-1.52	0.0086	1.52	0.036
palmitoyl-linoleoyl-glycerophosphocholine	Lipid	Lysolipid	802.56049			2.18	5.1316E-06	-1.33	0.0098
pipecolate	Amino Acid	Lysine Metabolism	130.08626	849	HMDB00070	32.00	0.000003694	-2.33	0.014
ribonate	Carbohydrate	Pentose Metabolism	165.04046	5460677	HMDB00867	-4.35	0.0002	1.85	0.0097
S-methylcysteine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	119.01613	24417	HMDB02108	4.34	0.0071	-2.70	0.0473
tartronate (hydroxymalonate)	Xenobiotics	Bacterial/Fungal	118.99859	45	HMDB35227	-2.50	9.4428E-06	1.68	0.0034
tauro-beta-muricholate	Lipid	Primary Bile Acid Metabolism	514.28439	168408	HMDB00932	25.51	0.0009	-11.11	0.0077
taurocholate	Lipid	Primary Bile Acid Metabolism	514.28439	6675	HMDB00036	9.56	0.0004	-5.88	0.0031
tiglyl carnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	244.15434	22833596	HMDB02366	88.06	2.3284E-07	-2.78	0.0349
urea	Amino Acid	Urea cycle; Arginine and Proline Metabolism	121.07201	1176	HMDB00629	1.89	0.0067	-1.89	0.0212
valerate	Lipid	Short Chain Fatty Acid	101.0608	7991	HMDB00889	-3.85	7.8856E-07	2.05	0.0109
valine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	118.08626	6287	HMDB00883	2.42	0.0003	-1.72	0.0171

FIG. 6

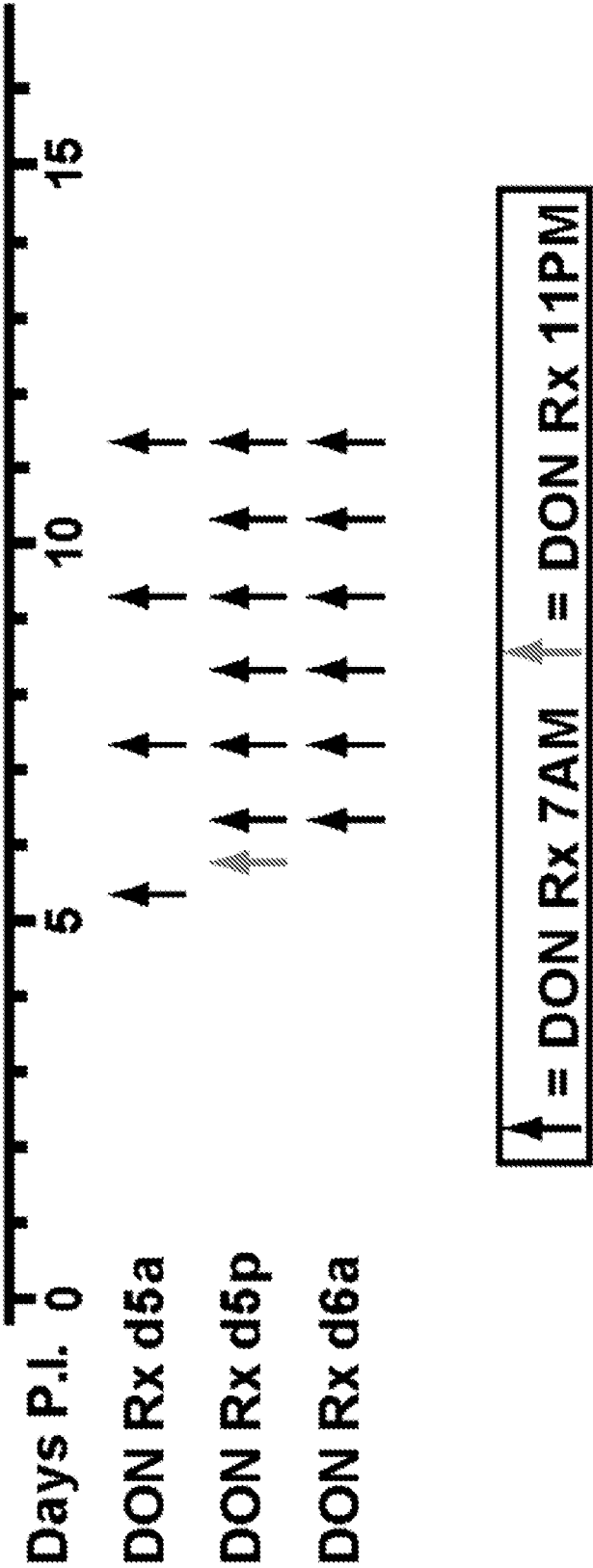
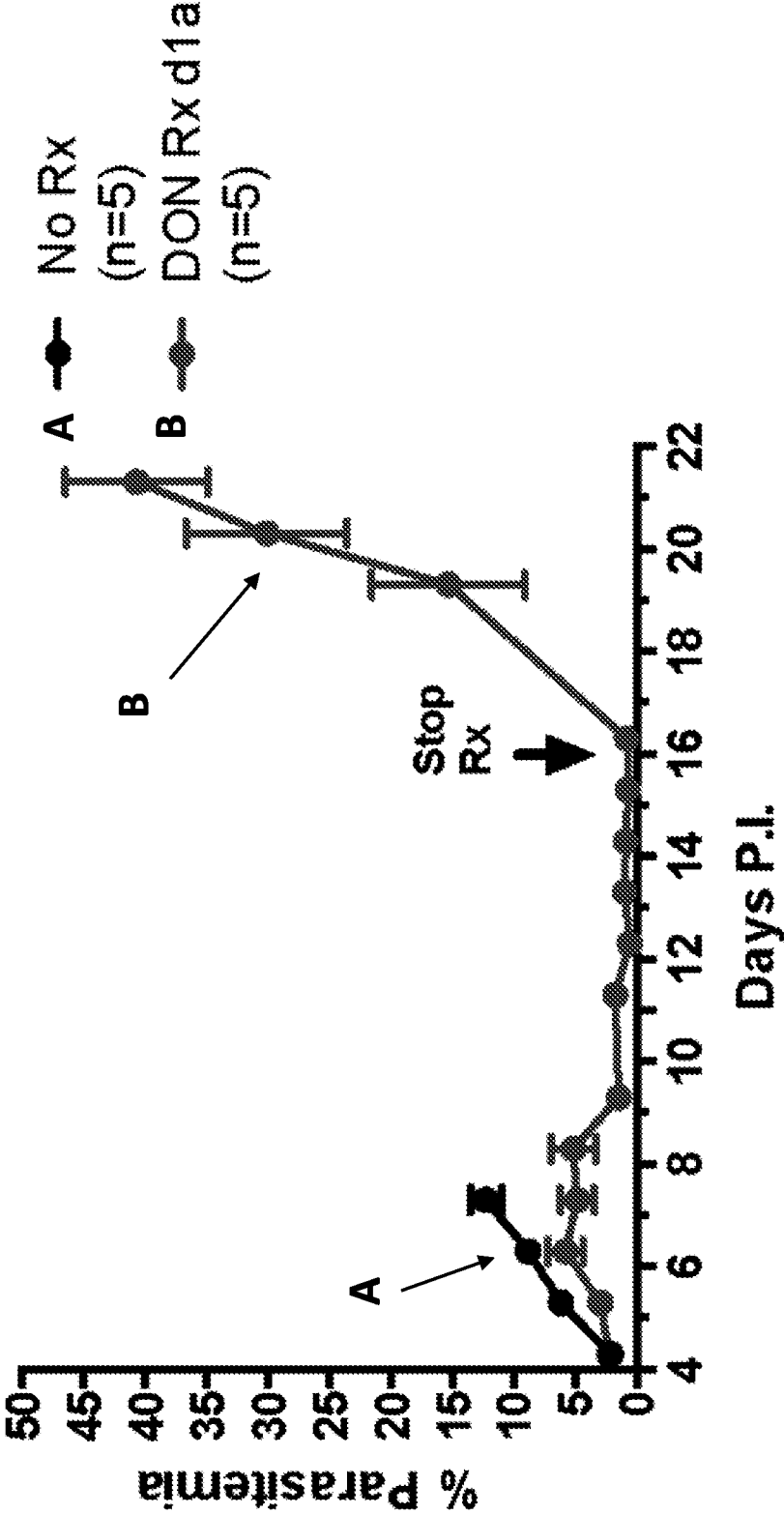


FIG. 7



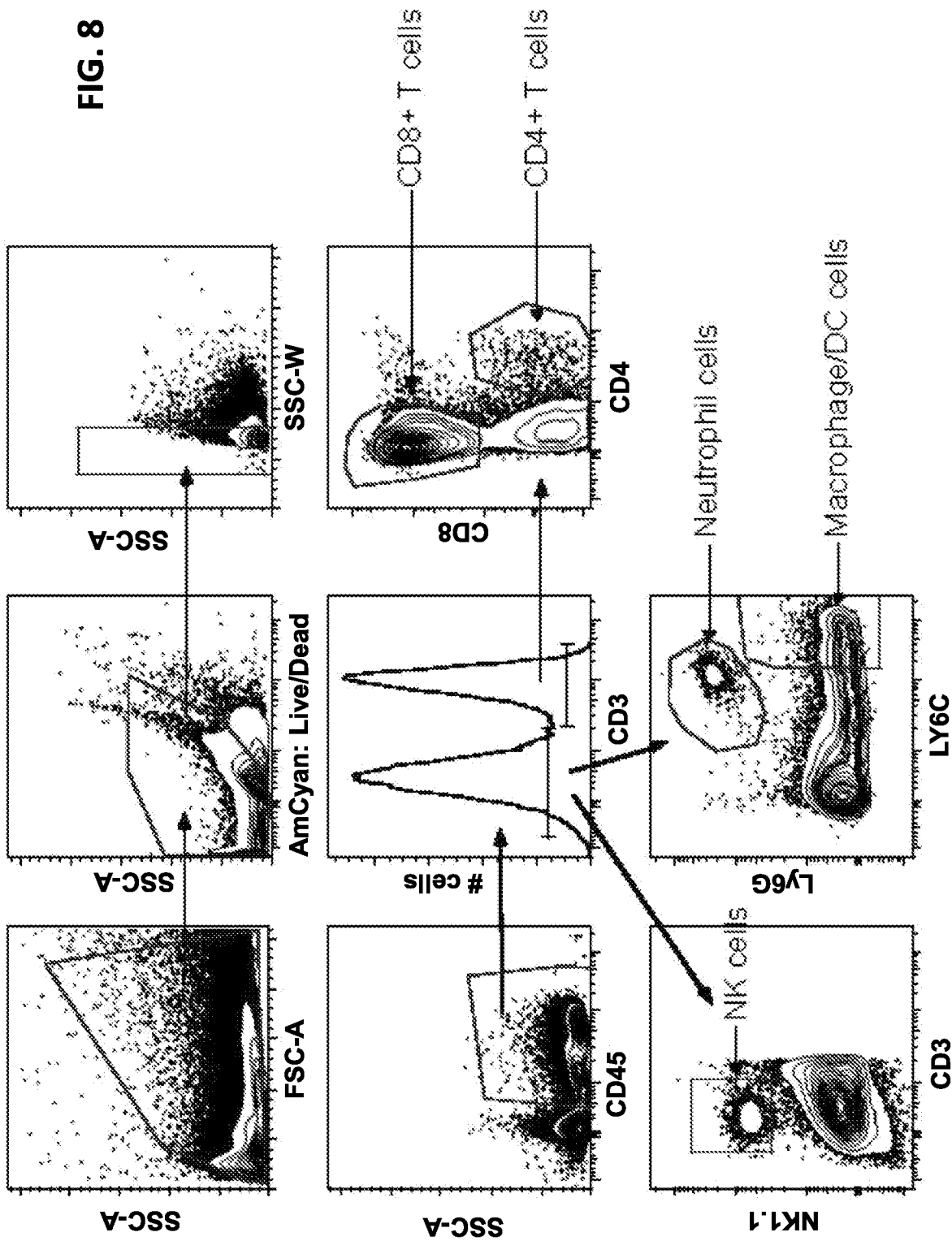


FIG. 9A

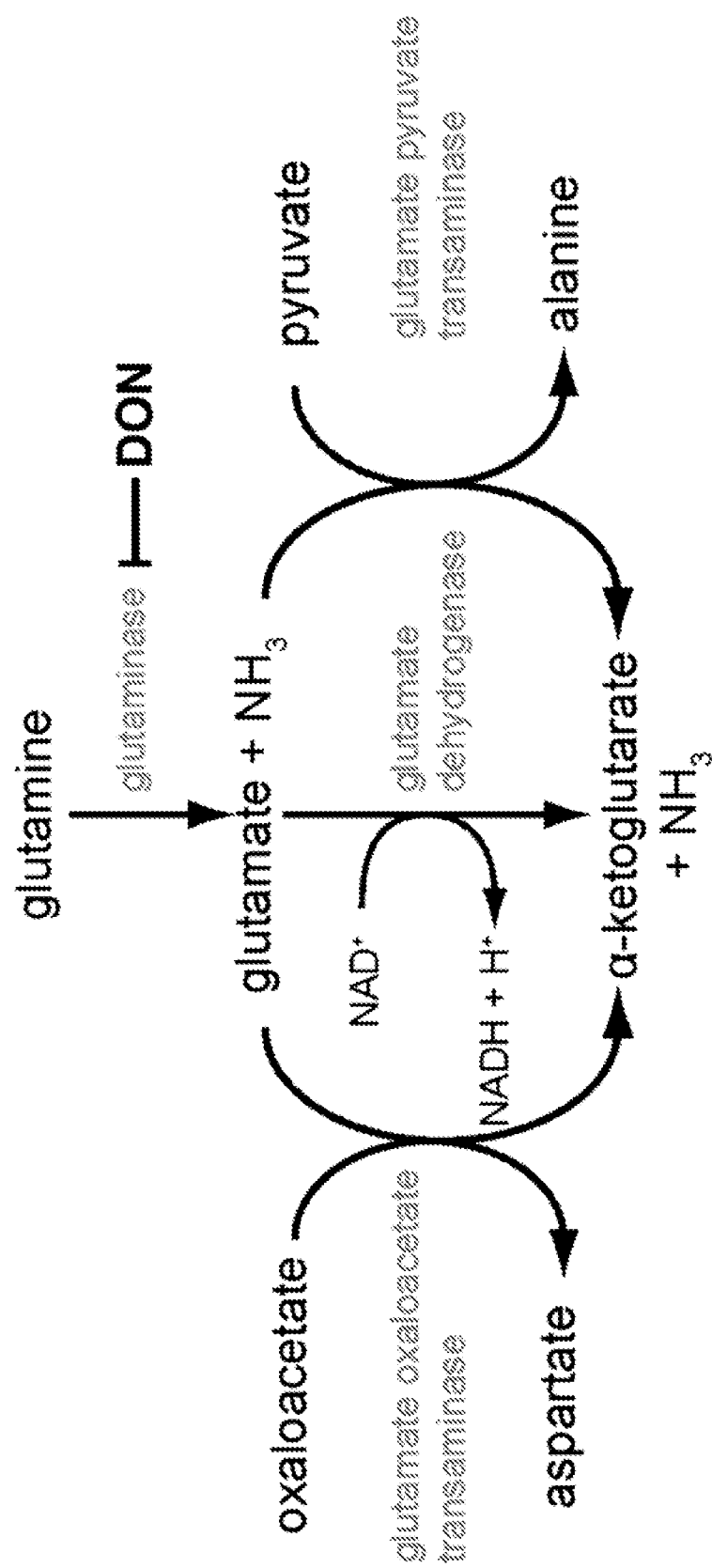


FIG. 9B

Metabolite	Infected, untreated vs Uninfected, untreated						DON treated, low clinical scores vs. Infected, untreated					
	Brain		Liver		Serum		Brain		Liver		Serum	
	Fold change	FDR	Fold change	FDR	Fold change	FDR	Fold change	FDR	Fold change	FDR	Fold change	FDR
glutamine	1.42	0.0001	1.07	0.18	-1.49	0.0053	-1.25	0.0051	1.49	0.0005	1.81	0.0043
glutamate	-1.49	0.0004	1.26	0.028	1.54	0.0028	1.29	0.021	1.37	0.0019	-1.05	0.37
ammonia	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected
pyruvate	-1.89	0.0076	-1.27	0.028	-1.72	0.0010	1.00	0.67	-1.03	0.38	2.25	0.0160
alanine	1.05	0.3376	-1.37	0.025	-1.82	0.0088	-1.01	0.67	1.58	0.0008	2.76	0.0043
oxaloacetate	not detected	not detected	-1.28	0.24	not detected	not detected	not detected	not detected	1.27	0.27	not detected	not detected
aspartate	-1.43	0.0095	1.14	0.047	2.13	0.0025	1.44	0.0064	1.33	0.0022	1.00	0.44
α -ketoglutarate	-1.11	0.12	1.03	0.24	-5.88	4.8E-06	1.02	0.56	-1.02	0.35	2.03	0.0043

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/036996

A. CLASSIFICATION OF SUBJECT MATTER		
INV. A61K45/06	A61K31/00	A61K31/198 A61K31/22 A61K31/42
ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, BIOSIS, EMBASE, SCISEARCH, CHEM ABS Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 19 August 2016		Date of mailing of the international search report 26/08/2016
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Cielen, Elsie

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International application No

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