様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）Stimulating the host innate immune response to fight the early stages of malaria infection

研究課題名（英文）Stimulating the host innate immune response to fight the early stages of malaria infection

研究代表者

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研究成果の概要（和文）：私たちの研究は、免疫応答を誘導する強力なToll-Like Receptor-2（TLR2）アゴニストであるPEG-Pam2Cysが新しい肝臓ステージの抗マラリア薬を構成しうることを示唆する。PEG-Pam2Cysは、マラリア原虫の感染予防と感染後の寄生虫の除去の両方に使用することができる。PEG-Pam2Cysは、通常寄生虫によって誘導されない強い自然免疫応答を刺激するので、この化合物は、一般の薬剤のように寄生虫の耐性を発達させない。マラリアや他の原生動物の寄生虫の対策におけるPEG-Pam2Cysのポテンシャルについて、さらなる研究が必要である。

研究成果の概要（英文）：We investigated a new approach to both protection from and treatment of malaria parasites that involves the direct stimulation of the host innate immune response through the administration of a Toll-Like Receptor-2 (TLR2) agonist. The activity of S-[2,3-bis (palmitoyloxy)propyl] cysteine (Pam2Cys) against the hepatocytic stages, erythrocytic stages and gametocytes of Plasmodium yoelii was investigated. PEG-Pam2Cys significantly reduces the numbers of malaria parasites in the livers of mice following challenge with sporozoites. We also show that treatment can clear parasites from the liver when administered subsequent to the establishment of infection. PEG-Pam2Cys can reduce the numbers of mosquitoes that are infected following blood feeding on gametocytaemic mice. These results suggest that this compound could represent a novel liver stage anti-malarial that can be used both for the clearance of parasites following exposure and for the prevention of the establishment of infection.

研究分野：　原虫学

キーワード：　Malaria　Pam2Cys　vaccine　antimalarial drug

１．研究開始当初の背景

Clinically silent and typically low in number, the liver stages of the malaria parasite, *Plasmodium spp.,* grow and develop in hepatocytes over the course of a number days following the deposition of sporozoites into the skin of the host during an infected mosquito bite. Following their development in the liver, which involves the maturation of single nucleated invasive sporozoites into multi-nucleated schizonts containing thousands of merozoites capable of infecting erythrocytes, malaria parasites enter the blood stream where the symptomatic red-blood cell cycle is initiated. An intervention that effectively targets parasites in the liver has the capacity to prevent the onset of symptomatic malaria, and to break the transmission cycle of the parasite. There is currently only a single licensed drug available, primaquine, which can kill malaria parasites in the liver. Its use, however, is associated with serious side effects in some groups of patients, including those with glucose-6-phosphate dehydrogenase (G6PD) deficiency in which it can cause severe hemolysis, and reports of parasite resistance to the drug are accumulating. New ways of attacking the malaria parasite in the liver are needed.

Liver stage malaria parasites cause no pathology but are recognized and targeted by the innate immune system, although the degree of protection is slight. The recognition of parasites in the liver is relatively poorly understood, but recent evidence suggests that it occurs through the activation of the cytosolic pattern recognition receptor melanoma differentiation-associated gene 5 (Mda5), which in turn triggers mitochondrial antiviral signaling protein (Mavs) stimulation to induce a type I interferon (IFN) response. A role for IFN-γ has also been shown, with parasite killing mostly mediated through the effector functions of liver natural killer T cells (NKT cells). There is also evidence that sporozoites can activate the pattern recognition receptor Toll-like receptor-2 (TLR2) and that this can lead to suppression of the growth of hepatic stage parasites. These findings thus suggest that harnessing and boosting the natural capacity of the innate immune system to kill hepatocytic malaria parasites could form the basis for a valuable intervention against the parasite.

The lipopeptide S-[2,3-bis(palmitoyloxy) propyl] cysteine (Pam_2Cys), a synthetic analogue of the lipid component of macrophage activating lipopeptide-2 (MALP2) is a potent Toll-like Receptor 2 (TLR2) agonist, which has been shown to confer rapid protection against the influenza A virus and secondary bacterial infections. This protection is conferred through stimulation of the innate immune system, and specifically through the induction of macrophages and neutrophils to secrete inflammatory cytokines including interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α) and IFN-γ. Given the ability of PEG-Pam_2Cys to invoke a response in this manner and the fact that liver stage malaria parasites are susceptible to innate immune responses involving the IFN-γ pathway, we hypothesized that PEG-Pam_2Cys could work as both an immunotherapeutic to clear liver stage parasites, and also as an immunoprophylactic to prevent the establishment of parasites in the liver following sporozoite challenge. Here, we provide the results of experiments that show that administration of PEG-Pam_2Cys several hours prior to challenge with sporozoites of the rodent malaria *Plasmodium yoelii*, significantly and dramatically reduces the numbers of parasites that are able to grow in the livers of mice. Furthermore, the administration of PEG-Pam_2Cys 24 hours after infection with sporozoites also provides significant protection against the development of parasites in the liver.

２．研究の目的

Liver stage malaria parasites are recognized and targeted by the innate immune system, although the degree of protection is slight. Harnessing and boosting the natural capacity of the innate immune system to kill malaria parasites would provide a valuable weapon against the parasite. The lipopeptide S-[2,3-bis(palmitoyloxy)propyl] cysteine (Pam_2Cys), is a potent Toll-like receptor 2 agonist that induces a strong innate immune response.

We hypothesized that Pam_2Cys could work both to prevent the establishment of parasites in the liver and the removal of parasites after they infect the liver, as well as affecting other stages of the parasite's life cycle.

During the course of this project, we

investigated the following topics:

① The ability of Pam_2Cys to prevent liver-stage malaria parasite infection
② The ability of Pam_2Cys to remove established liver-stage malaria parasites
③ The effect of Pam_2Cys on erythrocytic parasites
④ The effect of Pam_2Cys on the progression of experimental cerebral malaria
⑤ The effect of Pam_2Cys on the ability of gametocytes to cause infection in mosquitoes
⑥ The immunological mechanisms underlying the effect of Pam_2Cys

３．研究の方法

(1) *Action of Pam_2Cys against liver stage parasites*

The rodent malaria parasites *Plasmodium yoelii yoelii* (strain 17X1.1pp) and *P. berghei* ANKA were used in these experiments. To measure the protective effect of Pam_2Cys against liver stage parasites, sporozoites were intravenously inoculated into mice which had been previously treated with Pam_2Cys. Alternatively, sporozoite-inoculated mice were treated with Pam_2Cys 24 hours following challenge, in order to assess the effect of the compound to clear parasites from the liver. Parasite growth in the liver was measured by qPCR 42 hours after sporozoite challenge.

(2) *Action of Pam_2Cys against transmission stages, erythrocytic stages and experimental cerebral malaria*

Mice infected with *P. yoelii* parasites were treated with Pam_2Cys or control saline, and 24 hours later, *Anopheles stephensi* mosquitoes were allowed to feed on them. The development of oocysts on the midgut of these mosquitoes was quantified 10 days later by microscopy. The effect of Pam_2Cys on the development of erythrocytic stages was measured by the inoculation of the compound into mice prior to, or during a blood stage infection of *P. yoelii*. Similarly, the effect of Pam_2Cys on the progression of experimental cerebral malaria was measured in mice infected with *P. berghei* ANAKA.

(3) *The immunological mechanisms underlying the effect of Pam_2Cys*

Blood cytokine levels (IFN-γ, TNF-α, IL-6 or IL-10) were measured by ELISA, and NKT cell numbers in the liver were quantified by FACS analyses.

４．研究成果

Our major findings may be summarised as follows:

(1) *PEG-Pam_2Cys reduces the liver parasite burden when administered prior to sporozoite challenge* (**Figure 1**)

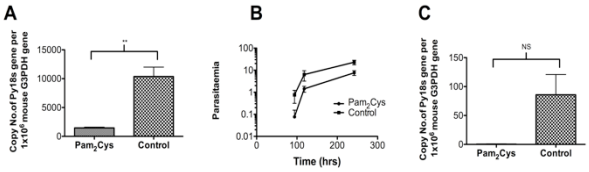

Figure 1. Liver parasite burden of mice inoculated intravenously (iv) with 10,000 sporozoites of *Plasmodium yoelii* 17x1.1pp (**Panel A**), or exposed to the bites of 5 female *Anopheles stephensi* mosquitoes fed two weeks prior on mice infected with gametocytes of *P. yoelii* (**Panel C**). Livers were removed 42 hours post-inoculation, homogenised in Isogen solution, total RNA extracted and converted to cDNA by reverse transcriptase PCR. Groups were treated 6 hours prior to sporozoite inoculation with 10nM inocula of Pam_2Cys or saline control. Parasite liver burden was measured by qPCR quantification of *P. yoelii 18s* gene copy number with reference to the copy number of mouse *g3pdh* gene measured in the same sample. ** $P < 0.001$, Student's two-tailed t-test, $t = 5.394$, $df = 8$. $n = 5$ mice per group (**Panel A**), and 3 mice in the Pam_2Cys and 4 in the control group (**Panel C**). (**Panel B**), Parasitaemia following challenge with 10,000 sporozoites of *P. yoelii* 17x1.1pp intravenously, following intravenous inoculation of 10 nM PEG-Pam_2Cys or saline control 6 hours earlier. $n = 4$ mice per group. Two-way ANOVA repeated measures mixed effects model; PEG-Pam_2Cys accounts for 5.84% of the total variance (after adjusting for matching). $F = 5.27$. $DFn = 1$ $DFd = 6$, $P = 0.0615$. If PEG-Pam_2Cys has no effect overall, there is a 6.2% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is therefore considered not quite significant. Data is representative of two repeat experiments.

(2) *PEG-Pam$_2$Cys reduces liver parasite burden when administered after sporozoite infection* (**Figure 2**).

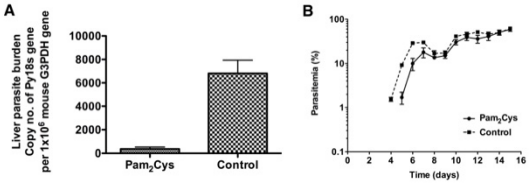

Figure 2. Panel A: Liver parasite burden (LPB) of mice inoculated intravenously with 15,000 *Plasmodium yoelii* 17X 1.1pp sporozoites 42 hours post-inoculation measured from genomic DNA extracted from a 0.5cm^3 piece of liver tissue. Mice were inoculated with sporozoites 24 hours prior to intravenous administration of 7.5nM PEG-Pam$_2$Cys inoculation or with PBS (control). LPB was measured by quantitative PCR of *P. yoelii* 18s gene copy number with reference to the copy number of mouse *g3pdh* gene measured in the same sample. Error bars indicate the standard error of the mean (SEM), n = 4 mice per group. ** P value < 0.05, Student's two-tailed t-test, t = 5.72, df = 6. **Panel B:** Parasitaemia of mice first inoculated intravenously with 500 sporozoites of *P. yoelii* 17X1.1pp intravenously then inoculated with 7.5nM PEG-Pam$_2$Cys 24 hours later and the animals then monitored for 16 days. From Day 0 to Day 4 no parasitaemia was observed. Error bars indicate the standard error of the mean (SEM), n = 5 mice per group. On day 12 one mouse from the control group died.

(3) *PEG-Pam$_2$Cys reduces the percentage of mosquitoes infected with malaria parasites, and the oocyst burden of those infected, following mosquito feeding on infected mice treated 24 hours previously* (**Figure 3**).

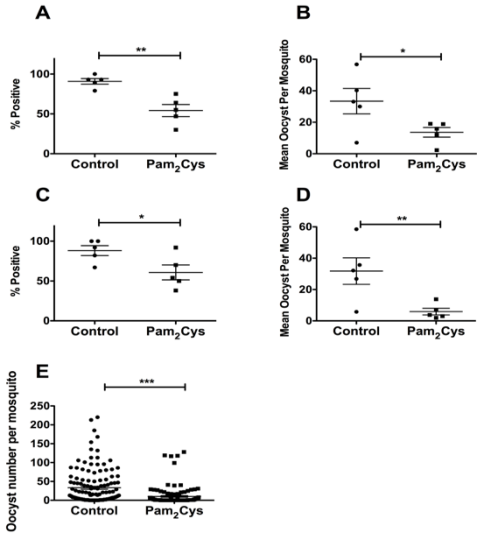

Figure 3. Percentage of *Anopheles stephensi* mosquitoes with at least one oocyst (**A** and **C**), and the mean number of oocysts per mosquito (**B** and **D**) for mosquitoes fed on gametocytaemic CBA/n (n = 5) (**A** and **B**) or BALB/c (n = 5) (**C** and **D**) mice. Mice were infected with 1x10^6 *Plasmodium yoelii yoelii* parasitized red blood cells and treated with 10 nM PEG-Pam$_2$Cys 24 hours prior to mosquito feeding, which occurred on Day 3 post-parasite challenge. Groups of 20-30 mosquitoes were allowed to feed on individual mice. Oocysts were counted on dissected mosquito midguts 10 days post mosquito feeding. Data are representative of two independent experiments for each mouse genotype. **Panel E** shows the oocyst burden of individual mosquitoes fed on mice treated with PEG-Pam$_2$Cys or saline solution in the two separate experiments represented in panels A-D (total of 261 mosquitoes fed on 10 individual mice). **A**, ** P < 0.01, Student's one-tailed t-test, t = 4.369, df = 8 n = 5 mice per group; **B**, * P < 0.05, Student's one-tailed t-test, t = 2.292, df = 8 n = 5 mice per group; **C**, * P < 0.05, Student's one-tailed t-test, t = 2.440, df = 8 n = 5 mice per group; **D**, ** P < 0.01, Student's one-tailed t-test, t = 2.965, df = 8 n = 5 mice per group; **E**, *** P < 0.0001, Student's one-tailed t-test, t = 5.086, df = 259. Error bars indicate the standard error of the means.

(4) *PEG-Pam$_2$Cys inoculation during an ongoing blood stage malaria parasite infection can increase parasitaemia, and does not protect against experimental cerebral malaria*

(5) *PEG-Pam$_2$Cys inoculation results in increased blood levels of IFN-γ, TNF-α and IL-6 but not IL-10 after 6 hours*

(6) *PEG-Pam$_2$Cys inoculation results in an increase in the numbers of Natural Killer (NK) T-cells in the livers of mice.*

IFN-γ has been shown to be involved in innate immune system mediated killing of liver stage parasites through the recruitment and activation of NKT cells. We investigated whether the increase in IFN-γ observed following treatment with PEG-Pam$_2$Cys is associated with an increase in liver NKT cells. Groups of CBA/n mice (n = 4) were inoculated with 10nM PEG-Pam$_2$Cys and 72 hours later, the numbers of NKT cells in liver homogenates were quantified by flow cytometry. Our results show that mice inoculated with PEG-Pam$_2$Cys exhibited significantly higher numbers of NKT cells in their livers than those

inoculated with saline.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　13 件）

① Moriyasu T, Nakamura R, Deloer S, Senba M, Kubo M, Inoue M, Culleton R, Hamano S, Schistosoma mansoni infection suppresses the growth of Plasmodium yoelii parasites in the liver and reduces gametocyte infectivity to mosquitoes, PLOS Neglected Tropical Diseases, 査読有, Vol. 12, No. 1, 2018, e0006197, DOI:10.1371/journal.pntd.0006197

② Alvarenga DA, Culleton R, Pina-Costa Ad, Rodrigues D, Junior CB, Silva S, Nunes AJ, J Souza jr JCd, Hirano Z, Moreira ZB, Pissinatti A, Abreu F, Areas AL, Lourenco-de-Oliveira R, Zalis MG, Brasil P, Ferreira-da-Cruz MdF, Daniel-Ribeiro CT, Alves de Brito CF, An assay for the identification of Plasmodium simium infection for diagnosis of zoonotic malaria in the Brazilian Atlantic Forest, Scientific Reports, 査読有, Vol. 8, 2018, DOI:10.1038/s41598-017-18216-x

③ Brasil P, Zalis MG, de Pina-Costa A, Siqueira AM, Bianco Junior C, Silva S, Areas ALL, Pelajo-Machado M, de Alvarenga DAM, Santelli ACFdS, Albuquerque HG, Cravo P, de Abreu FVS, Peterka CL, Zanini GM, Suarez-Mutis MC, Pissinatti A, Lourenco-de-Oliveira R, Brito CFAd, Ferreira-da-Cruz MdF, Culleton R, Daniel-Ribeiro CT, Outbreak of human malaria caused by Plasmodium simium in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation, The Lancet Global Health, 査読有, Vol. 5, 2017, e1038-46, DOI:10.1016/S2214-109X(17)30333-9

④ Lu F, Zhang M, Culleton RL, Xu S, Tang J, Zhou H, Zhu G, Gu Y, Zhang C, Liu Y, Wang W, Cao Y, Li J, He X, Cao J, Gao Q, Return of chloroquine sensitivity to Africa? Surveillance of African Plasmodium falciparum chloroquine resistance through malaria imported to China, Parasites & Vectors, 査読有, Vol. 10, 2017, p. 355, DOI:10.1186/s13071-017-2298-y

⑤ Lu F, Culleton R, Cao J, Artemisinin-Resistant Plasmodium falciparum in Africa, New England Journal of Medicine, 査読有, Vol. 377, 2017, pp. 305-306, DOI:10.1056/nejmc1705789

⑥ Abkallo H, Martinelli A, Inoue M, Ramaprasad A, Xangsayarath P, Gitaka J, Tang J, Yahata K, Zoungrana A, Mitaka H, Hunt P, Carter R, Kaneko O, Mustonen V, Illingworth CJR, Pain A, Culleton R, Rapid identification of genes controlling virulence and immunity in malaria parasites, PLOS Pathogens, 査読有, Vol. 13, 2017, p. e1006447, DOI:10.1371/journal.ppat.1006447

⑦ Maeno Y, Quang NT, Culleton R, Kawai S, Masuda G, Hori K, Nakazawa S, Marchand RP, Detection of the Plasmodium falciparum Kelch-13 gene P553L mutation in sporozoites isolated from mosquito salivary glands in South-Central Vietnam, Parasites & Vectors, 査読有, Vol. 10, 2017, p. 308, DOI:10.1186/s13071-017-2247-9

⑧ Lu F, Culleton R, Zhang M, Ramaprasad A, von Seidlein L, Zhou H, Zhu G, Tang J, Liu Y, Wang W, Cao Y, Xu S, Gu Y, Li J, Zhang C, Gao Q, Menard D, Pain A, Yang H, Zhang Q & Cao J, Emergence of Indigenous Artemisinin Resistant Plasmodium falciparum in Africa, New England Journal of Medicine, 査読有, Vol. 376, 2017, pp. 991-993, DOI:10.1056/NEJMc1612765

⑨ Maeno Y, Culleton R, Quang NT, Kawai S, Marchand RP, Nakazawa S, Plasmodium knowlesi and human malaria parasites in Khan Phu, Vietnam: Gametocyte production in humans and frequent co-infection of mosquitoes, Parasitology, 査読有, Vol. 144, 2017, pp. 527-535, DOI:10.1017/S0031182016002110

⑩ Mulenge FM, Hunja CW, Magiri E, Culleton R, Kaneko A, Aman RA, Genetic Diversity And Population Structure Of Plasmodium Falciparum In Lake Victoria Islands, A Region Of Intense Transmission, American Journal of Tropical Medicine & Hygiene, 査読有, Vol. 95, 2016, pp. 1077-1085, DOI:10.4269/ajtmh.16-0383

⑪ Martinelli A & Culleton R, Non-human Primate Malaria Parasites: Out of the forest and into the laboratory, Parasitology, 査読有, Vol. 145, 2016, pp. 41-54, DOI:10.1017/S0031182016001335

⑫ Ansari HA, Templeton JT, Subudhi AK, Ramaprasad A, Tang J, Lu J, Naeem R, Oguike MC, Benavente ED, Clark TG,

Sutherland CJ, Barnwell JW, Culleton R, Cao J, Pain A, Genome-scale comparison of expanded gene families in Plasmodium ovale wallikeri and Plasmodium ovale curtisi with Plasmodium malariae and with other Plasmodium species, International Journal for Parasitology, 査読有, Vol. 46, 2016, pp. 685-696, DOI: 10.1016/j.ijpara.2016.05.009

⑬ Mita T, Culleton R, Takahashi N, M Nakamura, Tsukahara T, Hunja CW, Win ZZ, Htike WW, Marma AS, Dysoley L, Ndounga M, Dzodzomenyo M, Akhwale WS, Kobayashi J, Uemura H, Kaneko A, Hombhanje F, Ferreira MU, Bjorkman A, Endo H, Ohashi J, Little Polymorphism at the K13 Propeller Locus in Worldwide Plasmodium falciparum Populations Prior to the Introduction of Artemisinin Combination Therapies, Antimicrobial Agents and Chemotherapy, 査読有, Vol. 60, 2016, pp. 3340-3347, DOI: 10.1128/AAC.02370-15

〔学会発表〕(計 6件)

① Culleton R, Phenotypic, Genotypic, and Transcriptomic Characterisation of the Plasmodium vinckei clade of Rodent Malaria Parasites, 第16回あわじしま感染症・免疫フォーラム, 2017

② Culleton R, Genetic analysis of Plasmodium malariae and Plasmodium ovale from asymptomatic adolescents in South-East Nigeria, 第86回日本寄生虫学会, 2017

③ Culleton R, Genetic analysis of Plasmodium malariae and Plasmodium ovale from asymptomatic adolescents in South-East Nigeria, 13th Nagasaki - Singapore Medical Symposium / Leading Program International Symposium, 2017

④ Culleton R, Malaria: A Deadly Zoonosis, Japan-Brazil Malaria Research Workshop - fostering new partnership, 2017

⑤ Culleton R, Genome-scale comparison of expanded gene families in Plasmodium ovale wallikeri and Plasmodium ovale curtisi with Plasmodium malariae and with other Plasmodium species, 第15回あわじしま感染症・免疫フォーラム, 201

⑥ Culleton R, Pam_2Cys as a novel immunochemoprophylactic and immunochemotherapeutic in malaria, The 4th Meeting of the Consortium for the Control of Zoonoses, 2016

〔図書〕(計 0件)

〔産業財産権〕

○出願状況 (計 1件)
名称:Oligonucleotides, set of nucleotides, assay for detection of Plasmodium simium, kit for diagnosis and discrimination of infection by Plasmodium simium and probe
発明者:DE BRITO, Cristiana Ferreira Alves, ALVARENGA, D. A. M.,DANIEL-RIBEIRO, C. T., PINA-COSTA, A, BRASIL, P., Lourenco-de-Oliveira R, CULLETON, R, HIRANO, Z. M. B., SOUZA-JUNIOR, J. C.
権利者:DE BRITO, Cristiana Ferreira Alves, ALVARENGA, D. A. M.,DANIEL-RIBEIRO, C. T., PINA-COSTA, A, BRASIL, P., Lourenco-de-Oliveira R, CULLETON, R, HIRANO, Z. M. B., SOUZA-JUNIOR, J. C.
種類:特許
番号:BR1021080061879
取得年月日:平成29年3月27日
国内外の別:外国

○取得状況 (計 1件)
名称:RAPID IDENTIFICATION OF GENES CONTROLLING VIRULENCE AND IMMUNITY IN MALARIA PARASITES
発明者:ARNAB, Pain, CULLETON, Richard, ILLINGWORTH, Christopher J.R.
権利者:ARNAB, Pain, CULLETON, Richard, ILLINGWORTH, Christopher J.R.
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番号:62/504,798
取得年月日:平成30年5月11日
国内外の別:外国

〔その他〕
ホームページ等
www.tm.nagasakiu.ac.jp/malariaunit/Culleton_Lab/Home.html

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