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エイズ対策研究事業

免疫不全に伴う脳内潜伏トキソプラズマ原虫再活性化の事前予想と再活性化原発局所における宿主遺伝子発現レベルの網羅的解析

平成20年度　総括・分担研究報告書

主任研究者　高島　康弘

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厚生労働科学研究費補助金（エイズ対策　研究事業）

総括研究報告書

主任研究者　　高島　康弘　岐阜大学　応用生物科学部　准教授

研究要旨　免疫不全に伴う潜伏中のトキソプラズマ原虫再活性化は致死性の脳炎を引き起こし、AIDS 患者の死亡原因の上位を占めている。しかしながら現在のところ再活性化の直接的引き金となる現象が把握できていない。本研究は脳内局所環境における原虫再活性化の直接的引き金となる分子を同定することを研究課題としている。本研究では H18 年度において新技術を確立し、本原虫の再活性化原発部位の同定をはじめて可能とした。この系を用いた実験により H19 年度には本原虫の潜伏様式について新たな考え方を提唱した。すなわち、spontaneous な活性化によってシストから脱出した虫体を宿主免疫系が速やかに排除することによる潜伏の維持という従来の考え方に変って、潜伏中の虫体に対する免疫反応が潜伏型虫体のステージ変換そのものを阻んでいるという解釈である。言い換えれば、潜伏中の原虫に対する免疫反応が宿主細胞の内部環境を「原虫の潜伏を維持できる状態」にしているということである。これを踏まえ、H20 年には本原虫の潜伏を許す宿主細胞内中の環境を同定することを目的とする。

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A. 研究目的

現在 AIDS 患者においては、末梢血 CD4 陽性細胞数が一定以下になった場合に日和見感染症のリスクが高いと判断されて予防的投薬が開始されている。しかし CD 4 陽性 T 細胞数は個々の日和見感染症病原体の活動レベルとは直接関係のない値である。したがって CD 4 陽性 T 細胞数を指標とすると日和見感染症発症リスクを見逃したり、それほど危険がない時期にも投薬を行ってしまったりする可能性が高い。日和見感染発症にいたる直接的な宿主側要因を同定しこれをモニターできるようになれば、ピンポイントに再活性化時期を予測することが可能となり、真にハイリスクな時期のみの短期間投薬が可能となろう。AIDS にともなう日和見感染症には、潜伏感染からの再活性化によって発症にいたるものが少なくない。そこで本研究ではこのような発症メカニズムをとる病原体のひとつであるトキソプラズマ脳炎をモデルとして「再活性化原発部位での遺伝子発現変化の網羅的解析」をおこない、本原虫の脳内再活性化の直接的な宿主側要因候補を見出すことで「潜伏した日和見感染症の再活性化時期の予想」「再活性化の阻止」という新しい対処法へ向けて有用な知見を提供することを当初の目的とした。本研究では H18 年度において新技術を確立し、本原虫の再活性化原発部位の同定をはじめて可能とした。この系を用いた実験により H19 年度には本原虫の潜伏様式について新たな考え方を提唱した。すなわち、spontaneous な活性化によってシストから脱出した虫体を宿主免疫系が速やかに排除することによる潜伏の維持という従来の考え方に変って、潜伏中の虫体に対する免疫反応が潜伏型虫体のステージ変換そのものを阻んでいるという解釈である。言い換えれば、潜伏中の原虫に対する免疫反応が宿主細胞の内部環境を「原虫の潜伏を維持できる状態」にしているということである。

これを踏まえ、H20 年には本原虫の潜伏を許す宿主細胞内中の環境を同定することを目的とする。

B. 研究方法

モデル抗原（アルブミン）をステージ特異的に発現する原虫の作成：トキソプラズマ原虫タキゾイト期（活性化期）に特異的に活性を有する SAG1 プロモーター支配下、ブラディゾイト期（潜伏期）に特異的に活性を有する BAG 1 プロモーター支配下に卵白アルブミン遺伝子をつないだ発現ユニットを構築しそれぞれを PLK 株に組み込んだ。この際、卵白アルブミン遺伝子のシグナル領域に改変を加えて発現された分子が虫体表面にとどまるようにする。えられた

組み換え原虫をそれぞれPLK/S-OVA、PLK/B-OVAとする。これらをマウスに感染させ、OVAに対する免疫を誘導するか否か確認する。この作業により「潜伏中の虫体表面の抗原は免疫系に認識されない」という従来の概念の正否を問う。

PLK/DUAL株の解析：H19年度に作成したPLK/DUAL株（潜伏期に緑色、活性期に赤色蛍光を発する）を免疫染色し、蛍光色とステージ特異的分子（SAG1およびBAG1）の発現様式が一致していることを確認する。

宿主細胞P38αMAPKの関与：P38αMAPK欠損マウス胎児から樹立した繊維芽細胞（KOP）とそのリバータント（RKOP）にPLK/DUAL株を感染させ、細胞外からの潜伏誘導刺激がない状態で培養する。原虫の発する蛍光色を経時的に観察することで宿主細胞P38αMAPK情報伝達系の欠如が原虫のステージ転換に影響を及ぼすか否か調べる。

原虫の潜伏を許す宿主細胞内微小環境をつくりだす遺伝子の網羅的解析：Gene Trap Mutagenesis法によってCHO細胞の遺伝子をランダムに破壊したライブラリを作成し、そこにPLK/DUAL株を感染。外部からの潜伏誘導刺激がないままでも原虫が潜伏してしまう細胞クローンが存在するか否か確認。存在した場合は当該クローンにおいて破壊されている遺伝子を同定する。

（倫理面への配慮）

岐阜大学または日本大学の動物実験委員会の指針に従い承認のもとですべての動物実験を実施。

C. 研究結果

モデル抗原（アルブミン）をステージ特異的に発現する原虫の作成：

5クローンのPLK/S-OVAおよび7クローンのPLK/B-OVAを得た。

PLK/DUAL株の解析：

抗BAG-1抗体を作成した。PLK/DUAL株を市販の抗SAG1抗体および抗BAG1抗体で染色した。緑色蛍光を強く発する虫体ほど抗BAG1抗体に強く反応しており、蛍光色の変化とステージ特異的分子の発現が厳密に一致していることを確認した。

宿主細胞P38αMAPKの関与：

PLK/DUAL株をP38αMAPK欠損株であるKOP細胞に感染させたところ、外部からの潜伏誘導刺激をくわえなくても、感染後2−3日で緑色蛍光を発し始めた。このような現象はP38αMAPK遺伝子を導入したリバータント（RKOP）では見られなかった。

原虫の潜伏を許す宿主細胞内微小環境をつくりだす遺伝子の網羅的解析：

ライブラリにPLK/DUALを感染させたところ、外部からの潜伏誘導刺激なしで緑色蛍光を発する虫体がみられた。

D. 考察

当初計画では潜伏維持中にみられるspontaneousな再活性化像（発症に至らない）の周辺と免疫抑制群に見られる再活性化原発部位（発症にいたると思われる）周辺の比較を予定していたが、潜伏維持中のマウスからは再活性化像が得られなかった。一方で、免疫抑制群からは再活性化像が得られた。この結果から「spontaneousな活性化」という概念に疑義が生じ、「spontaneousな活性化によってシストから脱出した虫体を宿主免疫系が速やかに排除することによる潜伏の維持」という従来の考え方だけで潜伏現象を説明するのは無理があると考えられた。すなわち、潜伏中の虫体に対する免疫反応が潜伏型虫体のステージ変換そのものを阻んでいるため、免疫正常群では再活性化した赤色原虫が見られないと考えるほうが実験結果を解釈しやすいということである。本研究計画にある発現解析においては（従来計画にあったspontaneousな再活性化後の虫体に対する免疫反応だけでなく）潜伏型原虫に対する微弱な免疫反応をも考慮にいれて実施すべきであると考えられた。

E. 結論

本年度の成果として、P38αMAPKが不活化した状態の細胞内では、細胞外からの特定の刺激がなくともトキソプラズマ原虫は潜伏するということが明らかになった。今後は、P38αMAPK情報伝達系の下流に位置する分子群をちゅうしんにかいせきをすすめ、「原虫の潜伏を許す細胞内微小環境」の詳細を解き明かす必要がある。また同様のことが生体内でも起こっているかどうか確認することでトキソプラズマ脳炎の制御に益する知見が得られるものと思われる。

F. 健康危険情報

特になし。

G 研究発表

1）Unno A., Suzuki K., Batanova T., Cha S. Y, Jang H. K., Kitoh K., **Takashima Y.*** 2009. Visualization of *Toxoplasma gondii* stage conversion by expression of stage-specific dual fluorescent proteins. Parasitology. *In press*

2）Unno A, Suzuki K., Xuan X., Nishikawa Y., Kitoh K., Takashima Y. 2008. Dissemination of extracellular and intracellular *Toxoplasma gondii* tachyzoites in the blood flow. Parasitol. Int. 57(4): 515-8.

3）Ota H., Takashima Y., Matsumoto Y., Hayashi Y., Matsumoto Y. 2008. Pretreatment of macrophages with the combination of IFN-gamma and IL-12 induces resistance to Leishmania major at the early phase of infection. J Vet Med Sci. 70(6): 589-93.

H. 知的財産権の出願・登録情報

なし。

厚生労働科学研究費補助金（エイズ対策　研究事業）
分担研究報告書

分担研究者　　鈴木　和彦　日本大学　生物資源科学部　助手
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研究要旨　　主任研究者の作成する蛍光蛋白発現原虫を解析し、マウス組織内および培養細胞内で原虫のステージと蛍光蛋白の発現が同調していることを確認する。とりわけ2色の蛍光を発するPLK/DUALについて、マウス体内における原虫の形態を詳細に観察し、各ステージにある原虫の形態学的特徴と一致するか否か検証する。

A. 研究目的

免疫不全に伴う潜伏中のトキソプラズマ原虫再活性化は致死性の脳炎を引き起こし、AIDS 患者の死亡原因の上位を占めている。しかしながら現在のところ、再活性化の引き金は「免疫力の低下」というきわめて曖昧な概念で捉えられているに過ぎず、再活性化の直接的引き金となる現象が把握できていない。このため、再活性化時期の予想・再活性化の阻止といった分野では基礎的研究さえ開始できない状態である。本研究は脳内局所環境における原虫再活性化の直接的引き金となる分子を同定することを研究課題としており、これを通じて「トキソプラズマ原虫の再活性化時期の予想」「再活性化の阻止」という新しい対処法へ向けての研究の端緒として必要不可欠な知見を提供することが研究期間全体を通じた目的である。

本研究班ではステージ特異的に蛍光を発する組換え原虫を用いて原虫の脳内再活性化を迅速に捉えることを計画している。平成20年度の1年間においては、高島らが作成した組換え原虫 PLK/DUAL がマウス組織内および培養細胞内で原虫のステージと蛍光蛋白の発現が同調していること、とりわけ2色の蛍光を発する PLK/DUAL について、マウス体内における原虫の形態を詳細に観察し、各ステージにある原虫の形態学的特長と一致するか否か検証することを目的とする。

B. 研究方法

1．テクノビット包埋

PLK/DUAL に感染したマウスを頸椎脱臼の後、脳、肝臓、脾臓、肺を採取した。採取した臓器は4％パラホルムアルデヒド(PFA)に入れて 4℃で一晩固定した。固定後の臓器を 6.8%シュクロース液（溶媒は PBS）に入れて 4℃でさらに一晩おいた後、アセトンに5分間漬けて脱水した。脱水後の臓器を再度新しいアセトンに1時間つけた後、テクノビット樹脂（ヘレウス・クルツァー社）にて説明書にしたがって包埋した。

2．原虫の形態学的研究

薄切切片ならびに臓器の圧潰標本を共焦点レーザー顕微鏡で観察した。

（倫理面への配慮）

本研究班の主任研究者は岐阜大学動物実験委員会の指針に従い、その承認のもとですべての動物実験を実施している。動物由来の材料は主任研究者が上記の指針に従って準備したのみを使用する。

C. 研究結果

高島らの作成した組み換え原虫、PLK/DUAL は厳密に「活性型であるタキゾイト期に赤色、潜伏型であるブラディゾイト期に緑色の蛍光蛋白を発現している」ことが明らかになった。また、マウス体内におけるそれぞれのステージの原虫はその構造学的特長において野生型のトキソプラズマ原虫と差がなかった。

D　結論

高島らの作成した組み換え原虫 PLK/DUAL の発する蛍光を原虫ステージの指標として利用することは In vitro でも In vivo でも適切なモデルであると考えられる。

F.　健康危険情報

特になし。

G　研究発表

1）Unno A., Suzuki K., Batanova T., Cha S. Y, Jang H. K., Kitoh K., **Takashima Y.*** 2009. Visualization of *Toxoplasma gondii* stage conversion by expression of stage-specific dual fluorescent proteins. Parasitology. *In press*

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H.　知的財産権の出願・登録情報

なし。

研究成果による特許権等の知的財産権の出願・登録状況

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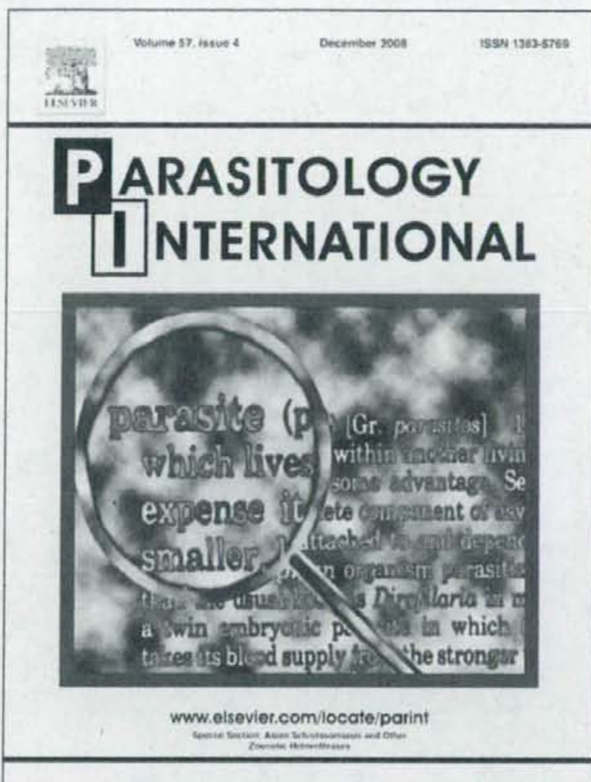

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Short communication

Dissemination of extracellular and intracellular *Toxoplasma gondii* tachyzoites in the blood flow

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ABSTRACT

Toxoplasma gondii is an intracellular parasite. It has been thought that *T. gondii* can disseminate throughout the body by circulation of tachyzoite-infected leukocytes (intracellular parasite) in the blood flow. However, a small number of parasites exist as free extracellular tachyzoites in the blood flow (extracellular parasite). It is still controversial whether the extracellular parasites in the blood flow disseminate into the peripheral tissues. In this study, we evaluated the dissemination efficiency of the extracellular and intracellular parasites in the blood flow using GFP-expressing transgenic parasite (PLK/GFP) and DsRed Express-expressing transgenic parasite (PLK/RED). When PLK/GFP and PLK/RED tachyzoites were injected, as intracellular and extracellular forms respectively, at the same time into the tail vein of a mouse, many disseminated green fluorescent PLK/GFP tachyzoites were observed in the lung, the spleen, the liver and the brain. However, only a few red fluorescent PLK/RED tachyzoites were detected in these organs. When PLK/GFP and PLK/RED tachyzoites were injected in the opposite manner, that is, as extracellular and intracellular forms respectively, the majority of tachyzoites in these tissues were PLK/RED tachyzoites. Collectively, these results indicate that intracellular tachyzoites mainly disseminate throughout the body and that extracellular tachyzoites hardly contribute to parasite dissemination.

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Toxoplasma gondii is an apicomplexan parasite that causes congenital infection and abortion as well as fatal infection in immune-compromised individuals. Infection generally occurs from oral ingestion of oocysts or tissue cysts. After oral ingestion, *T. gondii* initially crosses the intestinal epithelium and invades the general circulation. It was reported that *T. gondii* organisms circulate in the blood only 1 h after infection [1,2]. In the blood flow, the majority of parasites exist intracellularly, inside infected leukocytes [1–3] (Fig. 1A). The sera of several hosts, including humans, seem to have a lethal effect on extracellular tachyzoites in the absence of specific antibodies [4,5]. To escape from the lethal effect of serum, it might be necessary for *T. gondii* to infect leukocytes and be separated from serum. Recently, it was reported that infected CD11b+ leukocytes transport intracellular *T. gondii* into the brain extravascular space [3]. In this study, *T. gondii* DNA was not detected in the brain after intravenous injection of 10 or 50 extracellular tachyzoites [3]. It is suspected that trafficking of leukocytes contribute to dissemination of intracellular parasites in a 'Trojan horse' mechanism. However, despite these reports, a small number of extracellular parasites were observed in the blood flow [3] (Fig. 1A). It is also reported that *T. gondii* tachyzoites are resistant to human complement in the absence of specific antibodies [6,7]. An *in vivo* study using IgM−/− mice also showed that natural IgM does not function to limit parasite dissemination in the absence of specific antibodies against *T. gondii* [8]. These reports suggest the possibility that extracellular parasites survive in the blood flow and contribute to the dissemination to the peripheral tissues. The contribution of extracellular parasites to *T. gondii* dissemination to the peripheral tissues is controversial. In this study, we investigated disseminations of extracellular and intracellular tachyzoites in the blood flow. All experiments using animals were performed in accordance with the Gifu University Animal Care and Use Committee guidelines.

To compare the abilities of the intracellular and extracellular tachyzoites in the blood flow to disseminate into the peripheral tissues, we designed an experiment using PLK strain (Type II) [9] derived transgenic *T. gondii* tachyzoites stably expressing green and red fluorescence, PLK/GFP and PLK/RED, respectively [10–12]. In this experiment, PLK/GFP and PLK/RED tachyzoites are directly injected into the blood flow of one mouse in intracellular and extracellular forms, at the same time. Then, disseminations of both parasites were chased *in vivo* by detection of green and red fluorescent parasite in mouse tissues.

The tachyzoites of PLK/GFP and PLK/RED were passaged in Vero cells maintained in RPMI-1640 medium supplemented with 7.5% fetal

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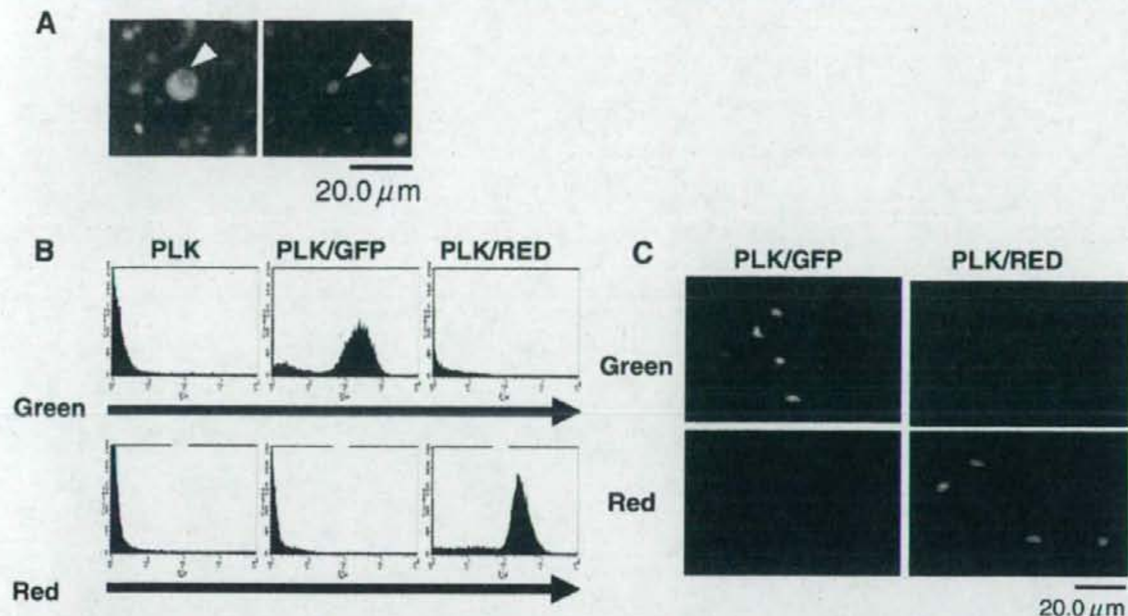

Fig. 1. Fluorescence of PLK/GFP and PLK/RED tachyzoites. (A) Intracellular and extracellular tachyzoites. To facilitate the confirmation of intracellular and extracellular tachyzoites in the blood flow, GFP-expressing transgenic mice (C57BL/6-Tg(CAG-EGFP) C14-Y01-FM131 Osb) [13,14] was i.p. infected with 10^6 red fluorescent *T. gondii* (PLK/RED) tachyzoites. The peripheral blood samples were observed 4 days p.i. Left and right panels show PLK/RED tachyzoite in a leukocyte expressing GFP (intracellular tachyzoite) and PLK/RED tachyzoite outside leukocytes (extracellular tachyzoite), respectively. (B) Fluorescence intensity of PLK, PLK/GFP and PLK/RED tachyzoites (left, center and right, respectively). Upper panels show green fluorescence detected by the FL-1 channel; lower panels show red fluorescence detected by the FL-2 channel. (C) Extracellular tachyzoites of PLK/GFP (left) and PLK/RED (right) after the purification procedure. Scale bar = 20.0 µm.

calf serum (FCS) and 20 µg/ml gentamicin and incubated at 37 °C in a 5% CO_2 incubator. To confirm whether PLK/GFP and PLK/RED are distinguished by fluorescence, these transgenic parasites were observed using flow cytometry and fluorescent microscope. Flow cytometry was performed on a FACSCalibur system (BD Biosciences Pharmingen, San Diego, CA) using the single tachyzoite suspension. As shown in Fig. 1B, green fluorescence from PLK/GFP was detected in the green fluorescent (FL-1) channel (upper-center panel). Leakage into the red fluorescent (FL-2) channel, which may interfere with clear distinction of PLK/GFP from PLK/RED, was not detected (Fig. 1B, lower-center panel). Similarly, PLK/RED showed red fluorescence detected in the FL-2 channel, but did not show leakage into the FL-1 channel (Fig. 1B, upper-right and lower-right). In the case of observations using a fluorescent microscope, slight leakage of fluorescence from PLK/GFP into the red channel was detected (Fig. 1C). However, the weak red fluorescence from PLK/GFP did not interfere with the ability to distinguish PLK/GFP and PLK/RED, because PLK/RED showed much brighter red fluorescence than PLK/GFP, and green fluorescence from PLK/RED was under the detectable level (Fig. 1C). These results indicate that PLK/GFP and PLK/RED tachyzoites can be distinguished from each other based on their fluorescence. Therefore we prepared the intracellular and extracellular tachyzoites using PLK/GFP and PLK/RED.

Extracellular tachyzoites were released from infected Vero cells by rapid extrusion through a 27-gauge needle, 3 times. After centrifugations at 2000 rpm for 10 min at room temperature, the pellet was re-suspended in 2 ml of phosphate-buffered saline (PBS). Cell debris was removed by filtration through a filter with a pore size of 5 µm (Millipore, Bedford, MA). The purified extracellular tachyzoites were suspended in PBS at a concentration of 2×10^4 tachyzoites/ml. To estimate the damage to parasites caused by the purification procedure, the numbers of tachyzoites conserving infectivity among 1000 obtained tachyzoites were measured as follows. The obtained tachyzoites were re-suspended in RPMI-1640 medium supplemented with 7.5% FCS and 20 µg/ml gentamicin. Extracellular tachyzoites were added to wells of the 24-well plates (10^3 tachyzoites/300 µl RPMI medium/well) and plates were incubated for 8 h at 37 °C in a 5% CO_2 incubator. After incubation for 8 h, Vero cells were washed twice with 300 µl of RPMI medium and the media were substituted by Eagle medium containing 0.5% methylcellulose supplemented with 7.5% FCS, 2 mM L-glutamine and 0.15% $NaHCO_3$ (pH 7.4). Within 16 h of the medium replacement, the numbers of PLK/GFP and PLK/RED tachyzoite clusters were counted. Each cluster contained from one to four parasites (data not shown). The experiments were independently repeated six times and the 95% confidence intervals [CIs] of the population means for the numbers of infectious tachyzoites were estimated. The numbers of infectious PLK/GFP and PLK/RED tachyzoites were 744.5 ± 38.1 (95%CI: 649.8 to 839.2) and 679.0 ± 145.6 (95%CI: 526.2 to 831.8), respectively.

To prepare intracellular tachyzoites, female 6–8 week-old C57BL/6J mice (purchased from Oriental Yeast Co., Ltd., Tokyo, Japan) were i.p. infected with 10^6 PLK/GFP or PLK/RED tachyzoites. Four days after the injection, the blood was collected and erythrocytes were lysed using NH_4Cl solution (0.145 M NH_4Cl, 17 mM Tris–HCl pH 7.65). After centrifugations at 1500 rpm for 5 min at room temperature to pellet leukocytes, pellets were washed twice with 10 ml of PBS and re-suspended in PBS. The obtained peripheral leukocytes were used as intracellular tachyzoite samples. The proportions of infected leukocytes were determined by observing more than 200 leukocytes using a fluorescent microscope. 1.48% and 2.91% of leukocytes were infected with PLK/GFP and PLK/RED, respectively. Although the majority of infected cells harbored a single parasite, a small number of cells containing more than two tachyzoites were also detected (data not

shown). The leukocyte suspensions were diluted in PBS to adjust the concentration of tachyzoites to 1×10^4 tachyzoites/ml. In this study, in order to facilitate the collection of enough number of infected leukocytes (intracellular tachyzoites), mice were infected i.p. with huge number of *T. gondii* tachyzoites (10^6 tachyzoites). It is not the route of natural infection of *T. gondii*. Therefore, from the i.p. inoculation to the appearance of parasites in the blood flow, dissemination route of the inoculated parasites is different from that of naturally infected ones. However, the difference would not have an impact on conclusions of this study because the subject of this study is phenomenon after the appearance of parasites in the blood flow.

Equal volumes (1:1) of the suspension of leukocytes infected with PLK/GFP and the suspension of extracellular PLK/RED tachyzoites were mixed. 200 µl of the mixed suspension was injected into the tail veins of each mouse (containing 1000 intracellular PLK/GFP and 2000 extracellular PLK/RED). A mixed suspension of extracellular PLK/GFP and leukocytes infected with PLK/RED was also prepared and injected in the same manner. Considering the survival rate of extracellular tachyzoites after the purification procedure, it appeared that approximately 1400 extracellular tachyzoites conserved their infection ability among the injected 2000 tachyzoites. Therefore, in this experimental design, approximately 1400 (little more than 1000) extracellular and 1000 intracellular infectious tachyzoites invaded the blood flow. Nine days after the injection, a huge number of disseminated PLK/RED tachyzoites were detected in the brain, the spleen, the liver and the lungs of mice infected with extracellular PLK/GFP and intracellular PLK/RED (Fig. 2A and C). However, only a few PLK/GFP tachyzoites were also detected in these organs (Fig. 2A and C). When PLK/GFP and PLK/RED tachyzoites were injected in the opposite combination, that is, intracellular PLK/GFP and extracellular PLK/RED, a huge number of PLK/GFP tachyzoites disseminated into these organs (Fig. 2B and C). PLK/RED tachyzoite was not detected in these organs, except for two PLK/RED tachyzoites detected in the lungs (Fig. 2C). The several preliminary experiments also showed the same tendency (data not shown). It is impossible to eliminate the possibility that the proliferation speeds of the two transgenic parasites, PLK/GFP and PLK/RED, are slightly different, and that this affected the experiment results. However, such an effect of proliferation speed would not have an impact on the conclusion of the experiments, because the massive majority of disseminated parasites in the tissues were intracellular tachyzoites, in both combinations of injection (extracellular PLK/GFP and intracellular PLK/RED; extracellular PLK/RED and intracellular PLK/GFP) (Fig. 2). These results indicate that intracellular tachyzoites mainly disseminate throughout the body and that extracellular tachyzoites hardly contribute to dissemination.

In our experiments, only a few parasites that had been administered into the blood flow as an extracellular form were detected in tissues (Fig. 2). These data suggest that the majority of extracellular tachyzoites could not arrive at the peripheral tissue. When we injected 10^6 extracellular PLK/RED tachyzoites into the tail vein of GFP-expressing transgenic mouse (C57BL/6-Tg(CAG-EGFP) C14-Y01-FM131 Osb) [13,14] and collected the peripheral blood after 1 h, the number

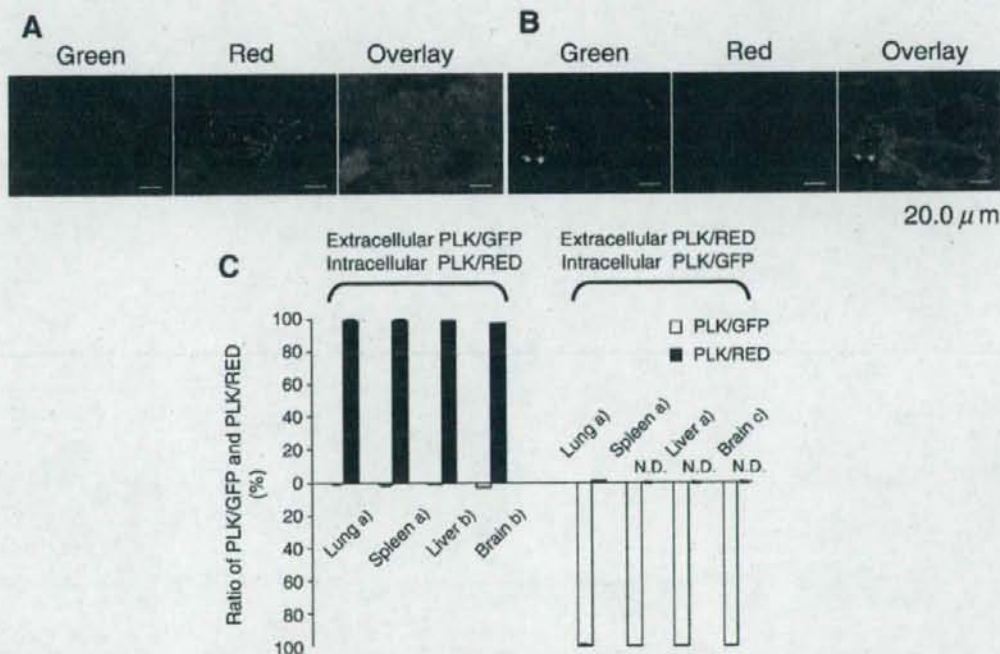

Fig. 2. Dissemination of extracellular and intracellular tachyzoites *in vivo*. (A) Half of each lung of mice infected with extracellular PLK/GFP and intracellular PLK/RED tachyzoites was fixed in 4% paraformaldehyde and embedded in plastic resin (Technovit 8100, Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Thin sections of the embedded organs were directly observed using a fluorescent microscope. Results shown are representative of three mice in the group. Scale bar = 20.0 µm. (B) The lung of mouse infected with extracellular PLK/RED and intracellular PLK/GFP tachyzoites was observed as described above. Scale bar = 20.0 µm. (C) Ratios of PLK/GFP and PLK/RED tachyzoites in the lung, the spleen, the liver and the brain of mice infected with extracellular PLK/GFP and intracellular PLK/RED tachyzoites (left), and with extracellular PLK/RED and intracellular PLK/GFP tachyzoites (right). The remaining half of lungs and other organs were chopped up into small pieces, crushed onto glass slides and observed using a fluorescent microscope. The numbers of detected PLK/GFP and PLK/RED tachyzoites were determined. Except for some organ samples in which only a few tachyzoites were detected (shown as superior letter 'b' and 'c'), in total, more than 200 (the lung) or 100 (the brain, the liver and the spleen) tachyzoites were observed in each organ. Open and solid bars show levels of PLK/GFP and PLK/RED, respectively. N.D. means non-detection. a) Values are means ± SD (n = 3). b) Means of data from 2 mice are shown because only a few tachyzoites (all detected tachyzoites were PLK/RED) were detected in the other mouse, in spite of more than 200 fields of view being observed (×400). c) Data of one mouse are shown because only a few tachyzoites (all detected tachyzoites were PLK/GFP) were detected in the other two mice, in spite of more than 200 fields of view being observed (×400).

of extracellular tachyzoites (red tachyzoites outside green leukocyte), in the blood sample was under detectable level (data not shown). It appears that extracellular tachyzoites were immediately eliminated by innate immunity after the direct exposure to serum.

Recently, it was reported that infected CD11c- and CD11b-expressing leukocytes pass through the blood–brain barrier and transport intracellular *T. gondii* into the brain [3]. In this report, extracellular tachyzoites inoculated into mice via the tail vein were unable to pass through the blood–brain barrier and invade the brain [3]. By contrast, we detected a few PLK/GFP tachyzoites, which had been inoculated via the tail vein as the extracellular form, in the brain (Fig. 2C). It is impossible to eliminate the possibility that extracellular tachyzoites are able to pass through the brain–blood barrier at a low efficiency, and that the low level invasion of extracellular tachyzoites into the brain was missed in previous studies. However, it is likely that the inoculated extracellular PLK/GFP tachyzoites infected leukocytes in other organs (for example, the spleen, liver and lymph nodes) or in the blood vessel and then passed through the blood–brain barrier as an intracellular form, because we detected PLK/GFP tachyzoites not only in the brain, but also in the liver and the spleen on the 9th day after inoculation (Fig. 2). As shown in Fig. 2C, although greatly outnumbered, extracellular tachyzoites were also able to disseminate. Previously, it was also suggested that the gliding motility of extracellular parasites was involved in their crossing of biological barriers [15,16]. In spite of the killing effect of serum [4,5], extracellular tachyzoites may survive short-term exposure to serum. Taken together, these findings suggest that infected leukocytes transport tachyzoites into the peripheral tissues and that the infected leukocytes migrate into parenchyma as a "Trojan horse"horse", and/or transported intracellular tachyzoites egress from leukocytes in the microvasculature of peripheral tissues and invade into parenchyma as extracellular forms during a short period of time. Regardless, transportation by infected leukocytes seems to be important for dissemination of *T. gondii*. To examine whether extracellular tachyzoites can migrate into leukocytes in the blood flow, 10^6 extracellular PLK/RED tachyzoites were injected into the tail veins of GFP-expressing transgenic mice [13,14], and peripheral leukocytes were observed after 1 h. However, green cells harboring red PLK/RED tachyzoites, like that shown in Fig. 1A, were not observed (data not shown). This result indicates that invasion of extracellular parasites into leukocytes does not occur, or occurs at low frequency in the blood flow. Infection of tachyzoites into leukocytes may mainly occur not in the blood flow, but in the lymphoid organs.

Our study demonstrates that intracellular tachyzoites in the blood flow are the major contributing factor to dissemination of *T. gondii* from the general circulation into the peripheral tissues. This indicates that to understand the mechanism by which *T. gondii* is disseminated into the peripheral tissues, further study of the behaviors of leukocytes in the blood flow harboring *T. gondii* is necessary.

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Visualization of Toxoplasma gondii stage conversion by expression of stage-specific dual fluorescent proteins

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1 **Visualization of *Toxoplasma gondii* stage conversion by expression of stage-specific**
2 **dual fluorescent proteins**
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21 Running title: Visualization of *Toxoplasma gondii* stage conversion

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22 **SUMMARY**

23 To recognize the stage conversion of *Toxoplasma gondii* between tachyzoite and
24 bradyzoite in live host cells, a transgenic *T. gondii* line, which expressed stage-specific
25 red and green fluorescence, was constructed. *T. gondii* PLK strain tachyzoites were
26 stably transformed with genes encoding red fluorescent protein (DsRed Express) and
27 green fluorescent protein (GFP) under the control of tachyzoite-specific SAG1 and
28 bradyzoite-specific BAG1 promoters, respectively. The resulting transgenic parasite
29 was designated PLK/DUAL. When PLK/DUAL was cultured under in pH 7.0
30 media, the PLK/DUAL zoites expressed red fluorescence, but no detectable levels of
31 green fluorescence were observed. The PLK/DUAL zoites reacted with anti-SAG1
32 antibody, but not anti-BAG1 antiserum. When PLK/DUAL was cultured under high
33 pH conditions, or in the presence of the p38 MAPK inhibitor SB202190, a small
34 number of zoites expressed green fluorescence and were BAG1 positive. C57BL/6J
35 mice were infected with PLK/DUAL tachyzoites. During the acute and reactivating
36 phase, zoites expressed red fluorescence. However, green fluorescence was not
37 detectable. By contrast, latent cysts expressed green fluorescence. The stage-specific
38 dual fluorescence of PLK/DUAL facilitates identification of the parasitic stage in
39 live cells, with the advantage that fixation or immunostaining is not required.

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41 **Key words**

42 *Toxoplasma gondii*, stage conversion, dual fluorescent proteins

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6 46 **Introduction**

47 *Toxoplasma gondii* is an intracellular coccidian with a complex life cycle, belonging to the phylum Apicomplexa. Although *T. gondii* infects a wide range of intermediate hosts, such as mammal and bird species, as well as humans, the sexual cycle of this parasite occurs only in the feline intestinal epithelium, thereby yielding sporozoite-containing oocysts in the feces (Innes, 1997; Montoya and Liesenfeld, 2004). In the body of the intermediate host, two asexual forms, namely acute lytic tachyzoites and latent bradyzoite tissue cysts, are found (Lyons *et al.* 2002). Infection of intermediate hosts with *T. gondii* occurs by ingestion of either tissue cysts (bradyzoites) or oocysts (sporozoites). After infection, bradyzoites and sporozoites rapidly differentiate into tachyzoites and disseminate throughout the body; this is referred to as an acute infection. Although the majority of tachyzoites are usually eliminated by the host's immune response, a small percentage of tachyzoites survive and differentiate into bradyzoites, primarily in the brain and muscle. The bradyzoites persist for the life of the host, and are essentially dormant and harmless (Carruthers and Suzuki 2007).

62 Stage conversion between tachyzoites and bradyzoites is a sporadic phenomenon, rather than occurring en masse (Ferreira *et al.* 2008). The co-existence of tachyzoites and bradyzoites in one vacuole was demonstrated in an *in vitro* experimental study (Bohne *et al.* 1993). Although such a vacuole, containing both tachyzoites and bradyzoites, has not yet been observed in animal tissue, latent cysts containing both SAG1-positive and -negative zoites have been observed in the mouse brain (Silva *et al.* 1998; Bohne *et al.* 1998). The co-existence of latent cysts and tachyzoites in the brain has also been shown in animal and human studies (Ferguson *et al.* 1989; Takashima *et al.* 2008). Thus, *T. gondii* stage conversion seems to be a gradual and progressive event.

72 Time-lapse analysis is a powerful tool for studying gradually progressing events. In the field of virology, for example, simultaneous tracking of capsid, tegument, and envelope proteins of herpes simplex virus-1 characterized how virus particles are produced, providing the first evidence that these three proteins are assembled in the trans-Golgi network of the host cell (Sugimoto *et al.* 2008). *T. gondii* stage conversion is also a gradually progressing event, like the assembly of a virus particle. However, to further understand this phenomenon, the behavior of stage-converting zoites needs to be characterized over time. To date, *T. gondii* stage conversion and the detection of stage-specific antigens has been demonstrated utilizing immunohistochemistry (Ferguson. 2004; Lüder *et al.* 1999; Gross *et al.* 1995; Bohne *et al.* 1994; Bohne *et al.*

1993). The necessity of a fixation procedure for immunohistochemistry makes it impossible to observe living parasites during stage conversion. To observe temporal changes in gene-expression patterns in stage-converting *T. gondii* zoites, Lyons, R. et al. (2001) harvested mRNA from cell cultures infected with stage-converting zoites once every 24 hr. They clearly described the stage conversion process in a mass of parasites in terms of the changes in stage-specific gene expression. However, even with this method, it is impossible to fully characterize the mechanism underlying the stage conversion of an individual parasite.

To identify the mechanism underlying the stage conversion of an individual parasite, it is necessary to ascertain the stage the observed parasite is in without killing it. That is, it is necessary to utilize live-cell imaging. Fluorescent proteins have proven to be powerful tools for live-cell imaging (Day and Schaufele 2008). In the present study, a transgenic *T. gondii* line expressing stage-specific red and green fluorescent proteins (DsRed Express and GFP, respectively) was constructed. This transgenic parasite allows for live imaging of the stage conversion in *T. gondii*.

Materials and Methods

Mice

Female, 6-8-week-old, C57BL/6J and BALB/c mice were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The C57BL/6J mice were infected with parasites and their organs excised after euthanasia by cervical dislocation. BALB/c mice were used for the production of polyclonal antibody. Experiments were performed in accordance with the Gifu University Animal Care and Use Committee guidelines.

Cells and Parasites

The African green monkey-kidney cell line Vero was cultured in RPMI1640 medium supplemented with 7.5% FCS at 37°C in a 5% CO_2 incubator. *T. gondii* tachyzoites of the following strains were propagated for routine use in Vero cells: *T. gondii* P(LK) HXGPRT-strain (Roos *et al.* 1994; Pfefferkorn and Borotz 1994), GFP-expressing stable transgenic *T. gondii* (PLK/GFP) (Nishikawa *et al.* 2008; Zhang *et al.* 2007), DsRed Express-expressing stable transgenic *T. gondii* (PLK/RED) (Takashima *et al.* 2008), and the dual fluorescent protein-expressing stable transgenic *T. gondii* (PLK/DUAL), engineered as described below. The PLK/GFP line expresses GFP during the tachyzoite and bradyzoite stages (Nishikawa *et al.* 2008; Zhang *et al.* 2007). PLK/RED expresses DsRed Express only during the tachyzoite stage (Takashima *et al.* 2008). PLK/Dual expresses DsRed Express and GFP as described below.

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8 119 **Construction of the transfer vector**
9 120 The promoter region of the bradyzoite-specific antigen 1 (BAG1) gene was PCR
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11 121 amplified from the plasmid Bag/cat (kindly provided by Dr. Bohne, Georg-August
12 122 University) using the following primer set:
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14 123 5'-AAGCTTCTTCCAGTTGCCCGGCTCTGGGTACCTTCT-3' and
15 124 5'-GGATCCCTTTTTTGAATATCATACGGGACCTGGGCT-3'.
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17 125 The amplified fragment was cloned into the pT7Blue T-Vector (Novagen, Darmstadt,
18 126 Germany), and the resulting plasmids were designated as pT7/BAGp. The BAG1
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20 127 gene promoter was obtained from pT7/BAGp by digestion with *Bam*HI and *Hind* III,
21 128 followed by ligation with the *Bam*HI- and *Hind* III-digested plasmid pUC/GRA
22
23 129 (Takashima *et al.* 2008). The resulting plasmid was designated pUC/BAG-GRA. The
24 130 gene for enhanced GFP was obtained from the plasmid, pCX-EGFP (Niwa *et al.*
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26 131 1991) (kindly provided from Dr. J. Miyazaki, Osaka University). The obtained GFP
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28 132 gene was blunt-ended with *Klenow* fragments and inserted into the blunt-ended *Bam*
29 133 HI site of the plasmid pUC/BAG-GRA. The resulting plasmid was designated
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31 134 pBAG-GFP. GFP expression units, under the control of the BAG1 promoter, and
32 135 DsRed Express (Clontech, Palo Alto, CA), under the control of the major surface
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34 136 antigen 1 (SAG1) promoter, were obtained from the plasmids pBAG-GFP and
35 137 pSAG-RED (Takashima *et al.* 2008). The obtained GFP and DsRed Express
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37 138 expression units were inserted into the *Hind* III and *Eco* RI sites of the plasmid
38 139 pminiHXGPRT (kindly provided by Dr. Roos, Stanford University) (Roos *et al.* 1994;
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40 140 Pfefferkorn *et al.* 1994), respectively, as shown in Fig. 1A. The resulting plasmid was
41 141 designated pminiHXGPRT/DUAL.
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45 143 **Parasite transfection and selection**
46 144 A total of 100 μg of the plasmid pminiHXGPRT/DUAL was electroporated into
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48 145 P(LK) HXGPRT–tachyzoites as described previously (Soldati and Boothroyd 1993),
49 146 and the transfected parasites were then added to Vero cell cultures. Forty-eight hours
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51 147 after electroporation, medium containing 25 μg/ml mycophenolic acid and 40 μg/ml
52 148 xanthine was added to the Vero cell culture. Stable transgenics were selected for in the
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54 149 presence of mycophenolic acid and xanthine for 10 days. Following selection,
55 150 cloning of red fluorescent tachyzoites was performed, and the resulting clone was
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57 151 designated PLK/DUAL.
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60 153 ***In vitro* stage conversion**

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6 154 Stage conversion of tachyzoites into bradyzoites was induced by high pH or the p38
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8 155 MAPK inhibitor SB202190, as previously described (Soete *et al.* 1993, Radke *et al.*
9 156 2006) with minor modifications. For induction of stage conversion by high pH, Vero
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11 157 cells that were infected with free PLK/DUAL or PLK/RED tachyzoites were
12 158 cultured in D-MEM medium (pH 8.1), supplemented with 2.0% FCS and 20 μg/ml
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14 159 gentamicin, at 37°C in a humidified incubator for four or seven days. For induction
15 160 of stage conversion by the p38 MAPK inhibitor SB202190, Vero cells were
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17 161 pre-incubated in D-MEM medium containing 1.0% FCS and 8 nM SB202190 (In
18 162 Solution™ SB202190, Calbiochem, U.S.A) for 3 hr prior to tachyzoite infection, and
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20 163 washed with D-MEM medium containing 1.0% FCS. Following incubation with
21 164 PLK/DUAL tachyzoites suspended in D-MEM medium containing 1.0% FCS for 4
22
23 165 hr, supernatant was removed and fresh medium containing 1.0% FCS and 8 nM
24 166 SB202190 was added. The infected Vero cell cultures were incubated at 37°C in a 5%
25
26 167 CO_2 incubator for 6 days.
27
28 168
29 169 **Flow cytometry**
30
31 170 Flow cytometry was performed on a FACSCalibur (BD Biosciences PharMingen,
32 171 San Diego, CA) to detect green and red fluorescence in purified free tachyzoites from
33
34 172 Vero cells, as described previously (Unno *et al.* 2008). GFP and DsRed Express
35 173 expression were detected using Fl-1 and Fl-2, respectively.
36
37 174
38 175 ***In vivo* stage conversion**
39
40 176 To observe parasites during the acute phase, C57BL/6J mice were infected
41 177 intraperitoneally with 1×10^6 PLK/DUAL tachyzoites. The brains of the infected
42
43 178 mice were excised 6 days later, and fixed and embedded in plastic resin (Technovit
44 179 8100, Heraeus Kulzer, Wehrheim, Germany). Thin brain sections were directly
45
46 180 observed using fluorescent microscopy, as previously described (Takashima *et al.*
47 181 2008). To prepare latently infected mice, C57BL/6J mice were intraperitoneally
48
49 182 infected with 20-1000 PLK/DUAL or PLK/GFP tachyzoites and observed for 6
50
51 183 weeks. Mice that survived more than 6 weeks without symptoms were labeled as
52 184 latently infected mice and used in subsequent experiments. To observe parasites
53
54 185 during the latent phase, the brains of latently infected mice were excised and chopped
55 186 into small pieces. The brain pieces were then crushed onto glass slides and observed
56
57 187 under fluorescence microscopy and confocal laser microscopy. Red fluorescence
58 188 from the crushed samples was detected by short (1/3.0 sec) and long (1.5 sec) time
59
60 189 exposure. Green fluorescence from the crushed samples was detected by just