non-Treg cells was not significantly changed in the colon and spleen of mice maintained with vitamin B9(−) diet (Fig. 5C), which could be explained by the similar concentration of vitamin B9 in the large-intestinal washes and sera of both groups of mice. We also found that Foxp3 and the inhibitory molecules CTLA4 and GITR, which are specifically expressed on Treg cells, were comparable between those mice (Fig. 5D).

Discussion

We have shown that vitamin B9 is crucial for the maintenance of Treg cells. Intriguingly, vitamin B9 was required for the survival of differentiated Treg cells, but was not necessary for the differentiation of naïve T cells into Treg cells. This selective effect of vitamin B9 on Treg cells is opposite to the effect of RA, a vitamin A metabolite, which enhances the differentiation of naïve T cells into Treg cells [11,12,13,14]. RA also induces the expression of gut-homing molecules (e.g., $\alpha4\beta7$ integrin and CCR9) on B and T cells activated by gut dendritic cells [9,10]. Because CCR9 was expressed normally on Treg cells in the LP of mice maintained with vitamin B9(−) diet (data not shown), the deficiency of dietary vitamin B9 did not affect the RA-mediated expression of gut-homing molecules and, predictably, the induction of Treg cells in the small intestine.

Treatment with the vitamin B9 antagonist MTX affected survival of both Treg cells and non-Treg cells, suggesting that the carrier-mediated pathway maintains sufficient amounts of intracellular vitamin B9 for cell survival regardless of the T-cell subset. The indiscriminate effects of MTX could be explained by the ubiquitous expression of the folate carrier [29,34]. As the mechanism of FR4-mediated Treg-cell maintenance, we considered initially that the proliferative activity of Treg cells could require large amounts of vitamin B9 as a source of nucleotides for DNA and RNA. However, the amounts of intracellular vitamin B9 were identical between Treg and non-Treg cells, implying that FR4 specifically recognizes extracellular vitamin B9 for the maintenance of Treg cell survival, consistent with a report that FR4 expressed on Treg cells contributes to their immune function and survival [25]. Additionally, the specific biological functions of vitamin B9 receptors (FR1, FR2, and FR4) have been predicted on the basis of their ~70% amino acid sequence identity, but the expression of each receptor is rigidly restricted, with narrow tissue and cell specificity [35,36]. Because FR1, FR2, and FR4 are glycosyl phosphatidylinositol–anchored proteins [37], adapter molecules may assist FR4 in the maintenance of Treg cell survival. We found that vitamin B9/FR4 was not associated with IL-2–mediated signaling in Treg cells. We will continue to study how FR4-mediated vitamin B9 regulates the survival of Treg cells.

Mammals must obtain vitamin B9 from the diet or from commensal bacteria. The absorption of vitamin B9 from the diet occurs mainly in the small intestine, whereas the uptake of microbial vitamin B9 predominantly occurs in the colon [38]. This explains why depletion of dietary vitamin B9 specifically decreased Treg cells in the small intestine, but not in the colon. It has been proposed that bacterial vitamin B9 absorbed in the colon affects the vitamin B9 status of the host [39,40], which may explain the lack of changes in vitamin B9 in the serum and splenic Treg cells in mice maintained with vitamin B9(−) diet. *Bifidobacterium*, one of the most important genera of commensal bacteria to be used as a probiotic, is well-studied as a vitamin B9 producer [41], and colonic Treg cells are specifically induced by immunological crosstalk with commensal bacteria, especially *Clostridium* clusters IV and XIVa [5]. Although whether *Clostridium* clusters IV and XIVa produce vitamin B9 remains unclear, our current findings suggest that vitamin B9 is an essential survival factor for Treg cells and, in vivo situation, diet vitamin B9 establishes an immunological network in the maintenance of Treg cells specifically in the small intestine.

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Author Contributions

Conceived and designed the experiments: JK. Performed the experiments: JK EH II. Analyzed the data: JK EH II. Wrote the paper: JK HK.

References

1. Kiyono H, Kunisawa J, McGhee JR, Mestecky J (2008) The mucosal immune system. In: Paul WE, ed. Fundamental Immunology. Philadelphia: Lippincott-Raven. pp 983–1030.
2. Littman DR, Rudensky AY (2010) Th17 and regulatory T cells in mediating and restraining inflammation. Cell 140: 845–858.
3. Cebra JJ, Jiang HQ, Boiko NV, Tlaskalva-Hogenova H (2005) The role of mucosal microbiota in the development, maintenance, and pathologies of the mucosal immune system. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, et al., eds. Mucosal Immunology. 3rd ed. San Diego: Academic Press. pp 335–368.
4. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, et al. (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 139: 485–498.
5. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, et al. (2011) Induction of colonic regulatory T cells by indigenous Clostridium species. Science 331: 337–341.
6. Geuking MB, Cahenzli J, Lawson MA, Ng DC, Slack E, et al. (2011) Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity 34: 794–806.
7. Hanson LA, Robertson A, Bjersing J, Herias MV (2005) Undernutrition, Immunodeficiency, and Mucosal Infections. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, et al., eds. Mucosal Immunology. 3rd ed. San Diego: Academic Press. pp 1159–1178.
8. Wintergerst ES, Maggini S, Hornig DH (2007) Contribution of selected vitamins and trace elements to immune function. Ann Nutr Metab 51: 301–323.
9. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, et al. (2004) Retinoic acid imprints gut-homing specificity on T cells. Immunity 21: 527–538.
10. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, et al. (2006) Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 314: 1157–1160.
11. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, et al. (2007) A functionally specialized population of mucosal CD103$^+$ DCs induces Foxp3$^+$ regulatory T cells via a TGF-β and retinoic acid-dependent mechanism. J Exp Med 204: 1757–1764.
12. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, et al. (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 Treg cells via retinoic acid. J Exp Med 204: 1775–1785.
13. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ (2007) All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J Exp Med 204: 1765–1774.
14. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, et al. (2007) Reciprocal Th17 and regulatory T cell differentiation mediated by retinoic acid. Science 317: 256–260.
15. Rivera J, Proia RL, Olivera A (2008) The alliance of sphingosine-1-phosphate and its receptors in immunity. Nat Rev Immunol 8: 753–763.
16. Miller LT, Kerkvliet NI (1990) Effect of vitamin B6 on immunocompetence in the elderly. Ann N Y Acad Sci 587: 49–54.
17. Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, et al. (2005) Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science 309: 1735–1739.
18. Kunisawa J, Kurashima Y, Higuchi M, Gohda M, Ishikawa I, et al. (2007) Sphingosine 1-phosphate dependence in the regulation of lymphocyte trafficking to the gut epithelium. J Exp Med 204: 2335–2348.
19. Iyer R, Tomar SK (2009) Folate: a functional food constituent. J Food Sci 74: R114–122.
20. Stover PJ (2004) Physiology of folate and vitamin B12 in health and disease. Nutr Rev 62: S3–12. discussion S13.
21. Gangjee A, Jain HD, Kurup S (2008) Recent advances in classical and non-classical antifolates as antitumor and antiopportunistic infection agents: Part II. Anticancer Agents Med Chem 8: 205–231.
22. Bourre-Tessier J, Haraoui B (2010) Methotrexate drug interactions in the treatment of rheumatoid arthritis: a systematic review. J Rheumatol 37: 1416–1421.

23. Courtemanche C, Elson-Schwab I, Mashiyama ST, Kerry N, Ames BN (2004) Folate deficiency inhibits the proliferation of primary human $CD8^+$ T lymphocytes in vitro. J Immunol 173: 3186–3192.
24. Troen AM, Mitchell B, Sorensen B, Wener MH, Johnston A, et al. (2006) Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. J Nutr 136: 189–194.
25. Yamaguchi T, Hirota K, Nagahama K, Ohkawa K, Takahashi T, et al. (2007) Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. Immunity 27: 145–159.
26. Gohda M, Kunisawa J, Miura F, Kagiyama Y, Kurashima Y, et al. (2008) Sphingosine 1-phosphate regulates the egress of IgA plasmablasts from Peyer's patches for intestinal IgA responses. J Immunol 180: 5335–5343.
27. Zhao R, Matherly LH, Goldman ID (2009) Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. Expert Rev Mol Med 11: e4.
28. Jansen G, Westerhof GR, Jarmuszewski MJ, Kathmann I, Rijksen G, et al. (1990) Methotrexate transport in variant human CCRF-CEM leukemia cells with elevated levels of the reduced folate carrier. Selective effect on carrier-mediated transport of physiological concentrations of reduced folates. J Biol Chem 265: 18272–18277.
29. Biswal BK, Verma RS (2009) Differential usage of the transport systems for folic acid and methotrexate in normal human T-lymphocytes and leukemic cells. J Biochem 146: 693–703.
30. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, et al. (1998) Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol 10: 1969–1980.
31. Setoguchi R, Hori S, Takahashi T, Sakaguchi S (2005) Homeostatic maintenance of natural $Foxp3^+$ $CD25^+$ $CD4^+$ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J Exp Med 201: 723–735.
32. D'Cruz LM, Klein L (2005) Development and function of agonist-induced $CD25^+Foxp3^+$ regulatory T cells in the absence of interleukin 2 signaling. Nat Immunol 6: 1152–1159.
33. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY (2005) A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat Immunol 6: 1142–1151.
34. Matherly LH, Hou Z, Deng Y (2007) Human reduced folate carrier: translation of basic biology to cancer etiology and therapy. Cancer Metastasis Rev 26: 111–128.
35. Salazar MD, Ratnam M (2007) The folate receptor: what does it promise in tissue-targeted therapeutics? Cancer Metastasis Rev 26: 141–152.
36. Low PS, Kularatne SA (2009) Folate-targeted therapeutic and imaging agents for cancer. Curr Opin Chem Biol 13: 256–262.
37. Jia Z, Zhao R, Tian Y, Huang Z, Tian Z, et al. (2009) A novel splice variant of FR4 predominantly expressed in $CD4^+CD25^+$ regulatory T cells. Immunol Invest 38: 718–729.
38. Said HM, Mohammed ZM (2006) Intestinal absorption of water-soluble vitamins: an update. Curr Opin Gastroenterol 22: 140–146.
39. Aufreiter S, Gregory JF, 3rd, Pfeiffer CM, Fazili Z, Kim YI, et al. (2009) Folate is absorbed across the colon of adults: evidence from cecal infusion of (13)C-labeled [6S]-5-formyltetrahydrofolic acid. Am J Clin Nutr 90: 116–123.
40. Said HM (2011) Intestinal absorption of water-soluble vitamins in health and disease. Biochem J 437: 357–372.
41. Pompei A, Cordisco L, Amaretti A, Zanoni S, Matteuzzi D, et al. (2007) Folate production by bifidobacteria as a potential probiotic property. Appl Environ Microbiol 73: 179–185.

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Review

Immunological Function of Sphingosine 1-Phosphate in the Intestine

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Abstract: It has been shown that dietary materials are involved in immune regulation in the intestine. Lipids mediate immune regulation through a complex metabolic network that produces many kinds of lipid mediators. Sphingosine-1-phosphate (S1P) is a lipid mediator that controls cell trafficking and activation. In this review, we focus on the immunological functions of S1P in the regulation of intestinal immune responses such as immunoglobulin A production and unique T cell trafficking, and its role in the development of intestinal immune diseases such as food allergies and intestinal inflammation, and also discuss the relationship between dietary materials and S1P metabolism.

Keywords: intestinal immunity; lipid; IgA antibody; intraepithelial T lymphocytes; food allergy

1. Introduction

It is generally accepted that dietary components are involved in immune regulation. The intestinal immune system, especially, seems to be directly affected by the digestion and absorption of dietary materials. Intestinal tissues are primary sites for infection by many pathogenic microorganisms, and commensal bacteria are abundant. Thus, the intestinal immune system has to create harmonious immunological condition, and the disruption of the intestinal immune homeostasis leads to the development of allergic, inflammatory, and infectious diseases [1,2].

Dietary lipids seem to be the dietary materials most involved in the regulation of intestinal immune responses after the conversion into lipid mediators [3]. Among various lipid mediators, sphingosine-1-phosphate (S1P) is a biologically active sphingolipid that regulates cell trafficking and activation [4,5]. S1P is abundantly present in the blood and lymph, which is originated from the cell membranes from sphingomyelin and is produced mainly by platelets, erythrocytes, and endothelial cells [6]. It is degraded by S1P lyase in the lymphoid tissues [7]. This metabolic pathway establishes an S1P gradient between the blood/lymph and lymphoid tissues and mediates cell trafficking.

The S1P gradient is recognized by cells expressing S1P receptors, and these cells migrate toward high concentrations of S1P. Of the five types of S1P receptor, type-1 S1P receptors (S1P1) are preferentially expressed by lymphocytes, and they determine lymphocyte emigration from and retention in the lymphoid tissues [8]. S1P1 is highly expressed in naive lymphocytes, including single-positive thymocytes expressing either CD4 or CD8, and expression is decreased upon lymphocyte activation. S1P1 expression recovers once the activated lymphocytes are fully differentiated and this recovery leads to their emigration from the lymphoid tissues into the blood circulation [4,5]. Studies indicate that the trafficking of macrophages, dendritic cells, and natural killer cells is mediated by S1P2, S1P3, and S1P5, respectively [9–11].

Recent studies have revealed additional functions of S1P in immune regulation that are independent of cell trafficking [4]. For example, differentiation of T cells is regulated by S1P1-mediated signaling [12–14]. It has also been demonstrated that a S1P2-mediated pathway is involved in the activation of mast cells [15] and macrophages [16], and that S1P3 are involved in dendritic cell endocytosis [10]. These findings together suggested that the S1P plays critical role in the activation and differentiation of immunocompetent cells involved in the both innate and acquired phases of immune responses in addition to their function of cell trafficking.

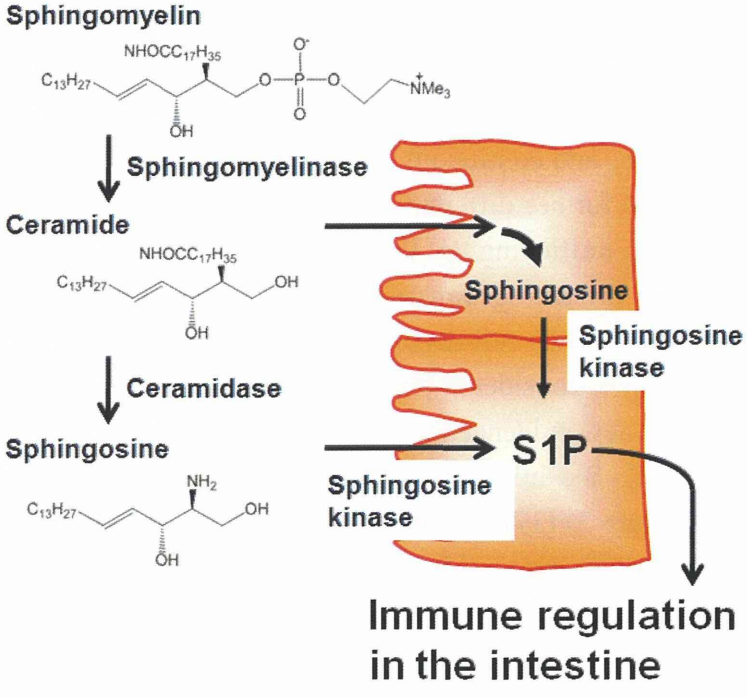
These biological and immunological functions show that S1P is involved in the maintenance of immunosurveillance as well as the development of immune diseases. In this review, we discuss the relationship between dietary materials (e.g., lipids, vitamin, and colorant) and S1P metabolism and describe the immunological functions of S1P, such as regulation of immunoglobulin A (IgA) production and intraepithelial T-lymphocyte trafficking, and its role in the development of intestinal immune diseases such as food allergy and intestinal inflammation.

2. Relationship Between S1P and Dietary Lipids

Several lines of evidence demonstrate that intestinal tissues contain higher levels of sphingolipids, including S1P, than other tissues [17]. There is no evidence of intestinal uptake of sphingolipids from

the blood, and germfree rats have comparable levels of sphingolipids in the intestine to conventional specific pathogen-free (SPF) rats [18]. Therefore, it is plausible that a source of sphingolipids in the intestine could be daily consumed diet. Adult humans ingest around 0.3 to 0.4 g sphingolipids per day, especially sphingomyelin from meat, milk, egg, and fish [19]. Dietary sphingomyelin is not directly absorbed, but is first degraded into ceramide and sphingosine [20,21] by alkaline sphingomyelinase and ceramidase, respectively, which are expressed on the apical membranes of epithelial cells [22,23]. Because epithelial cells express several key enzymes (e.g., sphingosine kinase) in the production of S1P from ceramide and sphingosine [23,24], it is possible that epithelial cells obtain ceramide and sphingosine from dietary sphingomyelin to produce S1P (Figure 1), thereby regulating intestinal immune responses and the associated intestinal immune diseases. Consistent with this, several studies showed that the incidence and severity of intestinal inflammation was changed by the uptake of dietary sphingomyelin [25,26] and the enzymatic activity of sphingomyelinase [27] and sphingosine kinase [28]. In addition, it was reported that dietary cholesterol inhibits the intestinal absorption of sphingolipids [29], implicating that cholesterol-rich Western diets may affect the availability of S1P precursors and consequently interfere with S1P-mediated intestinal immunity.

Figure 1. Dietary sphingolipids in epithelial-cell S1P production. Dietary sphingomyelin is degraded into ceramide and subsequently sphingosine by alkaline sphingomyelinase and ceramidase, respectively, which are expressed on the apical membranes of epithelial cells. In the epithelial cells, absorbed ceramide is metabolized into sphingosine. Together with absorbed sphingosine, sphingosine kinase metabolizes sphingosine into S1P, which then participates in immune regulation in the intestine.



3. Regulation of S1P Metabolism by Dietary Materials

In addition to dietary lipids, other dietary materials are also involved in the regulation of S1P metabolism. For instance, S1P lyase, a key enzyme to degrade S1P and thus keep optimal S1P low

concentration, requires vitamin B6 as a co-factor [7]. Thus, administration of vitamin B6 antagonist impaired S1P lyase activity, which consequently led to the defect of lymphocyte trafficking caused by inappropriate S1P gradient [7]. Similar effect was noted in 2-acetyl-4-tetrahydroxybutylimidazole (THI), a component of caramel food colorant III used in food products. THI inhibits S1P lyase and thus, like treatment with vitamin B6 antagonist, prevents normal lymphocyte trafficking [7]. These findings led to the use of THI for the treatment of immune diseases [30–32].

4. S1P Regulates Innate and Acquired Phases of Intestinal IgA Responses

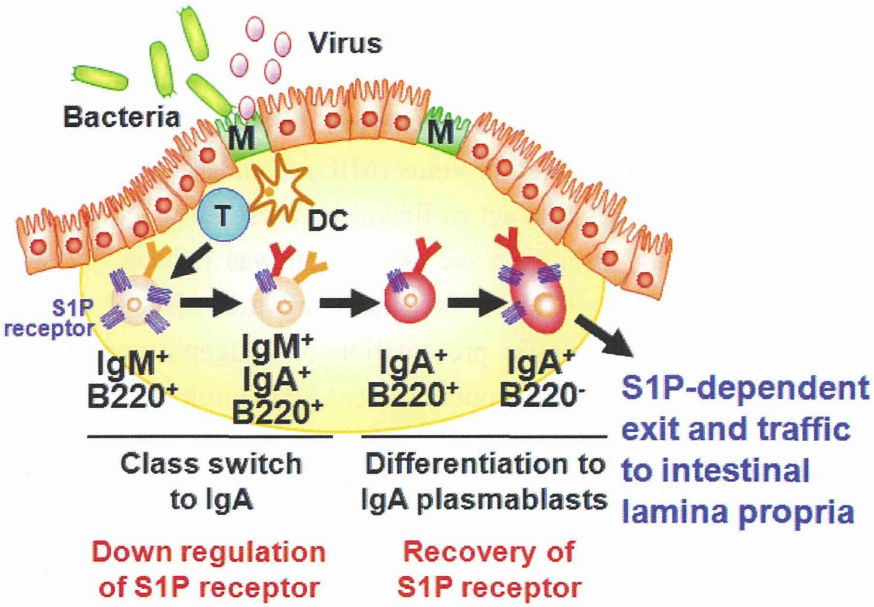
IgA is the most frequently observed antibody isotype in the intestinal compartments and provides the first line of defense against pathogenic microorganisms invading through mucosal tissues. Therefore, the induction of appropriate IgA responses is a logical strategy for the development of oral vaccines [33]. Since IgA antibody is one of the major arms of the mucosal immune system in the digestive tract, which covers a large surface area, the intestinal IgA is originated from several induction sites including Peyer's patches (PPs), isolated lymphoid follicles, and the peritoneal cavity [34].

A well characterized gut-associated lymphoid tissue (GALT) is PPs. PPs act as induction sites for the initiation of IgA responses against T-cell-dependent antigens [35]. PPs are covered with a specialized epithelium known as follicle associated epithelium (FAE) containing antigen-sampling M cells, which are responsible for the uptake and transport of antigens from the intestinal lumen to antigen-presenting cells such as dendritic cells (DCs) (Figure 2) [36]. Then, DCs capture antigens from the M cells, process and present them to T cells. It has been shown that the formation of PP DC-T cell clusters provide both cellular and molecular environment for the generation of IgA committed B cells in PPs [34]. In this pathway, some of the activated T cells differentiate into follicular helper T cells to help the antibody class switching of B cells in the germinal centers [34]. Because of the unique cytokine environment (e.g., TGF-β, IL-4, and IL-21) and continuous stimulation by commensal bacteria in the intestine, PPs have been shown to equip with efficient molecular and cellular environment for the spontaneous and continuous B cell class switching from IgM to IgA [34,35]. After class switching to IgA, B cells further differentiate into IgA plasmablasts and then migrate out from the PPs for their subsequent trafficking to the intestinal lamina propria, where they terminally differentiate into plasma cells producing dimeric (or polymeric) forms of IgA. This process mainly contributes to the development of T cell-dependent antigen-specific immune responses. Thus, the PP-mediated induction pathway is considered to be a major arm of the acquired IgA response [34].

Our investigation provided new evidence that S1P regulated the B cell trafficking in the PPs for the intestinal IgA production [37]. We initially found that S1P1 expression in B cells changes during differentiation in the PPs (Figure 2) [37]. High levels of S1P1 expression were detected in IgM^+ naive B cells, and expression was down-regulated when B cells started class switching to IgA. The low expressions of S1P1 allowed newly class-switched IgA^+ B cells to retain in the PPs for the sufficient differentiation into the IgA^+ plasmablasts. S1P1 expression was restored on the IgA^+ plasmablasts, resulting in their emigration from the PPs. Mice treated with FTY720, an immunosuppressant inducing S1P1 downregulation [38], show selective accumulation of IgA^+ plasmablasts in the PPs, leading to the disturbance of continuous delivery of IgA committed B cells from the PPs to the lamina propria of intestine. Consequently, the decrease of same population in the intestinal lamina propria was noted,

which associated with the reduction of intestinal antigen-specific IgA responses against orally immunized protein antigen [37].

Figure 2. Sequential changes in S1P1 expression during B-cell differentiation in Peyer's patches. Dendritic cells (DC) take the antigens transported by M cells from intestinal lumen and present them to T cells for their activation. Through the interaction with T cells and DCs, IgM^+ naive B cells show class-switch from IgM to IgA. During this process, S1P1 is expressed at high levels in IgM^+ naive B cells and downregulated on B cells class-switching from IgM to IgA and subsequently recovered on IgA^+ $B220^-$ plasmablasts, resulting in their emigration from the Peyer's patches and traffic into the intestinal lamina propria.



In the IgA production pathway in the gut, peritoneal B cells are an additional source of intestinal IgA [39]. A number of peritoneal B cells belong to a unique B-cell subset, termed as B1 cells, which produces antibodies against T-cell-independent antigens such as lipids and polysaccharides. Because these T-cell-independent antigens are conserved in various microorganisms, B1-cell-derived antibodies undiscriminatingly react to commensal and pathogenic bacteria and prevent their attachment and invasion into the host. This reaction is opposite to antibody responses against protein antigen mediated by PP B cells, which show rigid specificity against microorganisms. Therefore, it has been considered that B1-cell-derived IgA is categorized as to be innate-type antibodies that recognize a wide range of microorganisms in the intestine [39].

Trafficking of peritoneal B1 cells into the intestine requires S1P-mediated signaling [40]. Like B cells in the PPs, peritoneal B1 cells identically expressed S1P1. Thus, trafficking of peritoneal B cells into the intestine and consequent production of intestinal IgA are diminished by treatment with FTY720, mainly because of the inhibition of B1 cell emigration from the parathymic lymph nodes, which drain to the peritoneal cavity [40]. This impaired trafficking in FTY720-treated mice was associated with the decreased IgA responses against phosphorylcholine (a T-cell-independent antigen) induced by oral immunization with heat-killed *Streptococcal pneumoniae* [40].

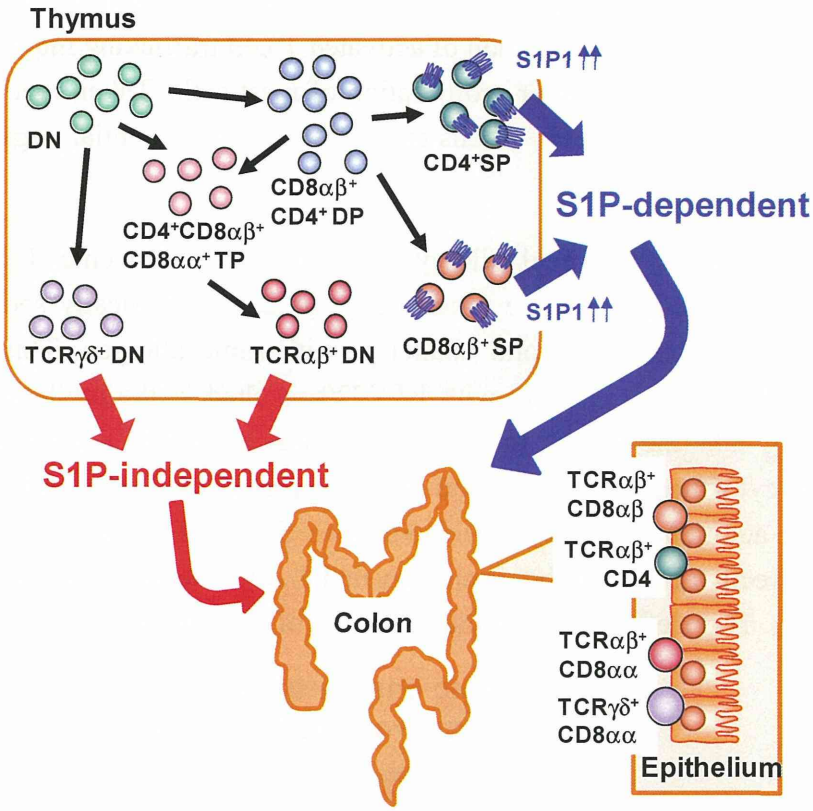
We also found that S1P-mediated regulation of peritoneal B-cell trafficking requires crosstalk with stromal cells in the peritoneal cavity [41]. This interaction mediated by adhesion molecules (e.g., ICAM-1 and VCAM-1) on stromal cells and the expression is regulated by NFκB-inducing kinase (NIK). Therefore, NIK-mutant aly/aly mice show decreased sensitivity to FTY720 in the regulation of peritoneal B-cell trafficking due to the impaired expression of adhesion molecules although peritoneal B1 cells in aly/aly mice expressed comparable levels of S1P1.

5. Distinct S1P Dependency of Trafficking of Intraepithelial T-Lymphocytes in the Gut

Large numbers of lymphocytes are also present in the intestinal epithelium and called as intraepithelial lymphocytes (IELs) [42]. IELs are mostly T cells, but unlike in conventional T cells observed in the systemic compartments (e.g., spleen) which predominantly express the αβ T-cell receptor (αβTCR), in the IEL subset there is an abundance of T cells expressing the γδ T cell receptor (γδTCR) in addition to $\alpha\beta TCR^+$ T cells [42]. αβTCR recognizes peptide antigen presented via major histocompatibility complex (MHC) molecules, whereas γδTCR recognizes non-classical MHC molecules such as MHC class I chain-related proteins (MIC) A and B (MICA/B) in human and Rae-1 in mouse [43]. Unlike MHC molecules that act as ligand by presenting peptide antigen, non-classical MHC molecules act as a ligand by itself and the expression was induced by stress (e.g., infection, tumors, or chemical treatment) [44]. Thus, it is considered that αβTCR is involved in acquired immunity through the activation by specific presentation of antigenic peptides, whereas γδTCR is involved in innate immunity by the ligation of non-classical MHC molecules [42]. A distinctive pattern of CD8 expression has also been noted in IELs. Conventional $\alpha\beta TCR^+$ T cells express CD8 as a heterodimer of α and β (CD8αβ). In contrast, some IELs uniquely express CD8 as a homodimer (CD8αα) [42]. A previous study identified a unique precursor of CD8αα IELs in the thymus [45]. In the thymus, $CD4^-$ $CD8^-$ double-negative thymocytes differentiate into $CD4^+$ $CD8^+$ double-positive thymocytes and then further differentiate into single-positive thymocytes expressing either CD4 or CD8. $CD8\alpha\beta^+$ IELs are derived mainly from $CD8^+$ single-positive thymocytes expressing αβTCR. $CD8\alpha\alpha^+$ IELs, however, originate from double-negative thymocytes expressing either αβTCR or γδTCR that have themselves differentiated from unique $CD4^+$ $CD8\alpha\alpha^+$ $CD8\alpha\beta^+$ triple-positive thymocytes (Figure 3) [45].

S1P has been involved in the regulation of cell trafficking of different subsets of IELs originated from thymus. We found that each type of IEL shows a different dependency on S1P in its trafficking from the thymus to the intestine, especially in the colon (Figure 3) [46]. When mice were treated with FTY720, decreased numbers of $CD8\alpha\beta^+$ IELs were observed. In contrast, the numbers of $CD8\alpha\alpha^+$ IELs were barely affected. These data suggest that, in the colonic epithelium, $CD8\alpha\beta^+$ IELs are S1P dependent and $CD8\alpha\alpha^+$ IELs are S1P independent. Consistent with this finding, $CD8^+$ single-positive thymocytes—the precursors of $CD8\alpha\beta^+$ IELs—express high levels of S1P1 [8], whereas no S1P1 expression has been noted on double-negative thymocytes, the precursors of $CD8\alpha\alpha^+$ IELs [46]. These findings suggest that S1P1 expression was different in different subsets of thymic precursors of IELs and provide versatile immunological pathways in the intestine.

Figure 3. Distinct dependency on S1P in T-cell trafficking into the colonic epithelium. In the thymus, $CD4^-$ $CD8^-$ double-negative (DN) thymocytes differentiate into $CD4^+$ $CD8^+$ double-positive (DP) thymocytes and then into single-positive (SP) thymocytes expressing either CD4 or CD8 and αβTCR. These SP thymocytes express high levels of S1P1 and migrate out from the thymus and into the colon in an S1P-dependent manner. DN thymocytes express TCRαβ or TCRγδ. DN thymocytes expressing TCRαβ are derived from $CD4^+$ $CD8αα^+$ $CD8αβ^+$ triple-positive (TP) thymocytes differentiated from DN or DP thymocytes. Little or no S1P1 expression is noted in the DN thymocytes expressing TCRαβ or TCRγδ, so traffic to the colonic epithelium proceeds in an S1P-independent manner.

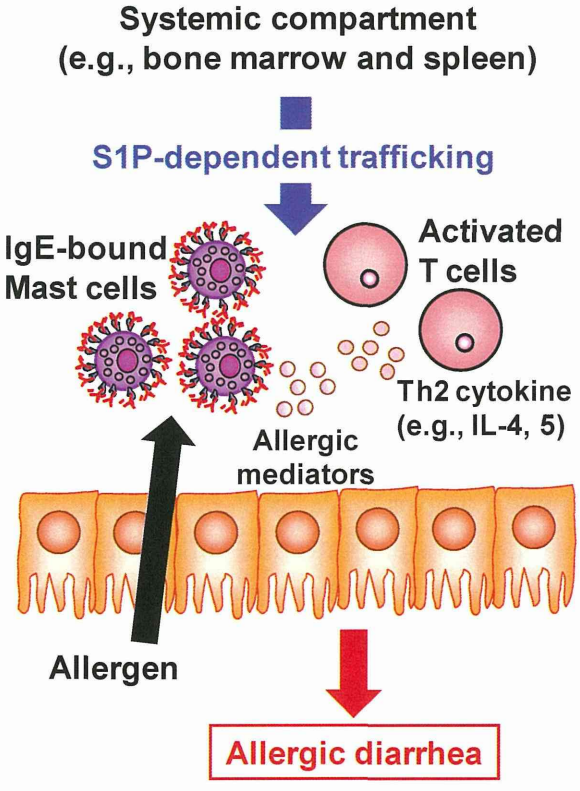


6. S1P-Mediated Regulation in the Development of Intestinal Immune Diseases

Accumulating evidence has revealed the pivotal role of S1P in the development of inflammatory diseases such as autoimmune type 1 diabetes, rheumatoid arthritis, and multiple sclerosis [5]. FTY720 prevents the egress of autoreactive lymphocytes from the lymph nodes into the peripheral circulation and subsequent across the blood–brain barrier into the central nerve system and thus has recently been approved as an oral therapy for multiple sclerosis [47]. In addition to being involved in these immune diseases at the systemic immune compartments, S1P is involved in the development of intestinal immune diseases including food allergies and intestinal inflammation [5]. The number of patients with food allergies has increased not only in children but also in adults; the development of effective preventive and therapeutic strategies for food allergies is therefore required to improve patients' quality of life. Using the ovalbumin-induced murine food-allergy model developed by our group [48], we examined the molecular and cellular mechanisms underlying the development of food allergies and found that, in allergic mice, activated T cells migrate into the colon, where they produced high

amounts of Th2 cytokines such as IL-4 and IL-5 [48]. We demonstrated that the trafficking of pathogenic T cells from the systemic compartments into the colon was mediated by S1P (Figure 4) [49]. Indeed, activated T cells in the colon of allergic mice expressed S1P1 and their infiltration into the colon and subsequent production of Th2 cytokines (e.g., IL-4 and IL-5) were inhibited by the treatment with FTY720 [49]. In addition, the infiltration of mast cells, effector cells in the development of food allergy, into the colon was also prevented in the FYT720-treated mice [49]. As a mechanism of FTY720-mediated inhibition of mast cell infiltration, it was likely that FTY720 directly and indirectly prevented the mast cell infiltration into the colon. Direct effect of FTY720 was predicted by results that mast cells expressed S1P1 and their *in vitro* migration was inhibited by FTY720 [49]. Indirect effect is mediated by activated T cells producing Th2 cytokines which enhanced the proliferation and recruitment of mast cells [50]. Thus, inhibition of activated T cell trafficking into the colon by FTY720 resulted in the reduced recruitment and/or proliferation of mast cells. Taken together, involvement of S1P in the trafficking of both pathogenic T cells and mast cells is a potential target for prevention and treatment of food allergies.

Figure 4. S1P mediates intestinal allergy by regulating pathogenic T and mast cell infiltration into the colon. In murine food allergy model, systemically sensitized T cells migrate into the colon upon the oral challenge with same allergen. This trafficking is mediated by S1P and thus treatment with FTY720 resulted in the inhibition of activated T cell trafficking into the colon. In the colon, these activated T cells produced high amounts of Th2 cytokines such as IL-4 and IL-5 for promotion of mast cell recruitment and proliferation. In addition, mast cell itself expresses S1P1. Therefore, FTY720 treatment directly and indirectly (Th2 cytokine from activated T cells) decreases the numbers of mast cells in the colon. These effects lead to the inhibition of allergic diarrhea.



Similarly, several lines of evidence have demonstrated that the FTY720 treatment prevents the development of intestinal inflammation [51–53]. For example, in a spontaneous colitis model in interleukin-10-deficient mice, administration of FTY720 suppressed the infiltration of pathogenic T cells producing interferon-γ [51]. Infiltration of the colon by pathogenic T cells was also inhibited by treatment with FTY720 in both a dextran sulfate sodium (DSS)-induced colitis model and a T-cell transfer model in mice [52,53]. Although S1P regulates the activation of several inflammatory cells via modulation of the signaling of certain innate receptors such as toll-like receptors, TNF receptor, and protease-activated receptor 1, and S1P itself is produced by activated inflammatory cells [4], collectively these findings suggest that S1P–S1P1 axis participates mainly in the development of intestinal immune diseases at the stage of pathogenic cell trafficking into the colon.

7. Conclusion

It is clear from past and current studies that S1P plays an important role in the regulation of the immune system of the gut in both healthy and disease states. In general, S1P is derived from sphingomyelin and is produced mainly by platelets, erythrocytes, and endothelial cells in the body. However, in the intestine, it is likely that epithelial cells contribute most to the production of S1P. Most importantly, S1P produced by epithelial cells seems to originate from dietary sphingolipids, especially sphingomyelin. Thus, elucidation of the complex networks established by dietary lipids will create a new era in nutrition-based mucosal immunology and should provide a new strategy against intestinal immune diseases.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Kiyono, H.; Kunisawa, J.; McGhee, J.R.; Mestecky, J. The Mucosal Immune System. In *Fundamental Immunology*; Paul, W.E., Ed.; Lippincott-Raven: Philadelphia, PA, USA, 2008; Volume 6, pp. 983–1030.
2. Maslowski, K.M.; Mackay, C.R. Diet, gut microbiota and immune responses. *Nat. Immunol.* **2011**, *12*, 5–9.

3. Margioris, A.N. Fatty acids and postprandial inflammation. *Curr. Opin. Clin. Nutr. Metab. Care* **2009**, *12*, 129–137.
4. Chi, H. Sphingosine-1-phosphate and immune regulation: Trafficking and beyond. *Trends Pharmacol. Sci.* **2011**, *32*, 16–24.
5. Rivera, J.; Proia, R.L.; Olivera, A. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat. Rev. Immunol.* **2008**, *8*, 753–763.
6. Pappu, R.; Schwab, S.R.; Cornelissen, I.; Pereira, J.P.; Regard, J.B.; Xu, Y.; Camerer, E.; Zheng, Y.W.; Huang, Y.; Cyster, J.G.; *et al.* Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* **2007**, *316*, 295–298.
7. Schwab, S.R.; Pereira, J.P.; Matloubian, M.; Xu, Y.; Huang, Y.; Cyster, J.G. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science* **2005**, *309*, 1735–1739.
8. Matloubian, M.; Lo, C.G.; Cinamon, G.; Lesneski, M.J.; Xu, Y.; Brinkmann, V.; Allende, M.L.; Proia, R.L.; Cyster, J.G. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **2004**, *427*, 355–360.
9. Michaud, J.; Im, D.S.; Hla, T. Inhibitory role of sphingosine 1-phosphate receptor 2 in macrophage recruitment during inflammation. *J. Immunol.* **2010**, *184*, 1475–1483.
10. Maeda, Y.; Matsuyuki, H.; Shimano, K.; Kataoka, H.; Sugahara, K.; Chiba, K. Migration of CD4 T cells and dendritic cells toward sphingosine 1-phosphate (S1P) is mediated by different receptor subtypes: S1P regulates the functions of murine mature dendritic cells via S1P receptor type 3. *J. Immunol.* **2007**, *178*, 3437–3446.
11. Walzer, T.; Chiossone, L.; Chaix, J.; Calver, A.; Carozzo, C.; Garrigue-Antar, L.; Jacques, Y.; Baratin, M.; Tomasello, E.; Vivier, E. Natural killer cell trafficking *in vivo* requires a dedicated sphingosine 1-phosphate receptor. *Nat. Immunol.* **2007**, *8*, 1337–1344.
12. Liu, G.; Yang, K.; Burns, S.; Shrestha, S.; Chi, H. The S1P1–mTOR axis directs the reciprocal differentiation of Th1 and Treg cells. *Nat. Immunol.* **2010**, *11*, 1047–1056.
13. Wang, W.; Huang, M.C.; Goetzl, E.J. Type 1 sphingosine 1-phosphate G protein-coupled receptor (S1P1) mediation of enhanced IL-4 generation by CD4 T cells from S1P1 transgenic mice. *J. Immunol.* **2007**, *178*, 4885–4890.
14. Huang, M.C.; Watson, S.R.; Liao, J.J.; Goetzl, E.J. Th17 augmentation in OTII TCR plus T cell-selective type 1 sphingosine 1-phosphate receptor double transgenic mice. *J. Immunol.* **2007**, *178*, 6806–6813.
15. Oskeritzian, C.A.; Price, M.M.; Hait, N.C.; Kapitonov, D.; Falanga, Y.T.; Morales, J.K.; Ryan, J.J.; Milstien, S.; Spiegel, S. Essential roles of sphingosine-1-phosphate receptor 2 in human mast cell activation, anaphylaxis, and pulmonary edema. *J. Exp. Med.* **2010**, *207*, 465–474.
16. Hughes, J.E.; Srinivasan, S.; Lynch, K.R.; Proia, R.L.; Ferdek, P.; Hedrick, C.C. Sphingosine-1-phosphate induces an antiinflammatory phenotype in macrophages. *Circ. Res.* **2008**, *102*, 950–958.
17. Bouhours, D.; Bouhours, J.F. Developmental changes of monohexosylceramide and free ceramide in the large intestine of the rat. *J. Biochem.* **1985**, *98*, 1359–1366.

18. Gustafsson, B.E.; Karlsson, K.A.; Larson, G.; Midtvedt, T.; Stromberg, N.; Teneberg, S.; Thurin, J. Glycosphingolipid patterns of the gastrointestinal tract and feces of germ-free and conventional rats. *J. Biol. Chem.* **1986**, *261*, 15294–15300.
19. Vesper, H.; Schmelz, E.M.; Nikolova-Karakashian, M.N.; Dillehay, D.L.; Lynch, D.V.; Merrill, A.H., Jr. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.* **1999**, *129*, 1239–1250.
20. Nilsson, A. Metabolism of sphingomyelin in the intestinal tract of the rat. *Biochim. Biophys. Acta* **1968**, *164*, 575–584.
21. Schmelz, E.M.; Crall, K.J.; Larocque, R.; Dillehay, D.L.; Merrill, A.H., Jr. Uptake and metabolism of sphingolipids in isolated intestinal loops of mice. *J. Nutr.* **1994**, *124*, 702–712.
22. Nilsson, A.; Duan, R.D. Alkaline sphingomyelinases and ceramidases of the gastrointestinal tract. *Chem. Phys. Lipids* **1999**, *102*, 97–105.
23. Tani, M.; Ito, M.; Igarashi, Y. Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. *Cell. Signal.* **2007**, *19*, 229–237.
24. Duan, R.D.; Nilsson, A. Metabolism of sphingolipids in the gut and its relation to inflammation and cancer development. *Prog. Lipid Res.* **2009**, *48*, 62–72.
25. Mazzei, J.C.; Zhou, H.; Brayfield, B.P.; Hontecillas, R.; Bassaganya-Riera, J.; Schmelz, E.M. Suppression of intestinal inflammation and inflammation-driven colon cancer in mice by dietary sphingomyelin: Importance of peroxisome proliferator-activated receptor gamma expression. *J. Nutr. Biochem.* **2011**, *22*, 1160–1171.
26. Fischbeck, A.; Leucht, K.; Frey-Wagner, I.; Bentz, S.; Pesch, T.; Kellermeier, S.; Krebs, M.; Fried, M.; Rogler, G.; Hausmann, M.; *et al.* Sphingomyelin induces cathepsin D-mediated apoptosis in intestinal epithelial cells and increases inflammation in DSS colitis. *Gut* **2011**, *60*, 55–65.
27. Sjoqvist, U.; Hertervig, E.; Nilsson, A.; Duan, R.D.; Ost, A.; Tribukait, B.; Lofberg, R. Chronic colitis is associated with a reduction of mucosal alkaline sphingomyelinase activity. *Inflamm. Bowel Dis.* **2002**, *8*, 258–263.
28. Maines, L.W.; Fitzpatrick, L.R.; French, K.J.; Zhuang, Y.; Xia, Z.; Keller, S.N.; Upson, J.J.; Smith, C.D. Suppression of ulcerative colitis in mice by orally available inhibitors of sphingosine kinase. *Dig. Dis. Sci.* **2008**, *53*, 997–1012.
29. Nyberg, L.; Duan, R.D.; Nilsson, A. A mutual inhibitory effect on absorption of sphingomyelin and cholesterol. *J. Nutr. Biochem.* **2000**, *11*, 244–249.
30. Allende, M.L.; Bektas, M.; Lee, B.G.; Bonifacino, E.; Kang, J.; Tuymetova, G.; Chen, W.; Saba, J.D.; Proia, R.L. Sphingosine-1-phosphate lyase deficiency produces a pro-inflammatory response while impairing neutrophil trafficking. *J. Biol. Chem.* **2011**, *286*, 7348–7358.
31. Bagdanoff, J.T.; Donoviel, M.S.; Nouraldeen, A.; Tarver, J.; Fu, Q.; Carlsen, M.; Jessop, T.C.; Zhang, H.; Hazelwood, J.; Nguyen, H.; *et al.* Inhibition of sphingosine-1-phosphate lyase for the treatment of autoimmune disorders. *J. Med. Chem.* **2009**, *52*, 3941–3953.
32. Bandhuvula, P.; Honbo, N.; Wang, G.Y.; Jin, Z.Q.; Fyrst, H.; Zhang, M.; Borowsky, A.D.; Dillard, L.; Karliner, J.S.; Saba, J.D. S1p lyase: A novel therapeutic target for ischemia-reperfusion injury of the heart. *Am. J. Physiol. Heart Circ. Physiol.* **2011**, *300*, H1753–H1761.

33. Kunisawa, J.; McGhee, J.; Kiyono, H. Mucosal S-IgA Enhancement: Development of Safe and Effective Mucosal Adjuvants and Mucosal Antigen Delivery Vehicles. In *Mucosal Immune Defense: Immunoglobulin A*; Kaetzel, C., Ed.; Kluwer Academic/Plenum Publishers: New York, NY, USA, 2007; pp. 346–389.
34. Fagarasan, S.; Kawamoto, S.; Kanagawa, O.; Suzuki, K. Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. *Annu. Rev. Immunol.* **2010**, *28*, 243–273.
35. Kunisawa, J.; Nochi, T.; Kiyono, H. Immunological commonalities and distinctions between airway and digestive immunity. *Trends Immunol.* **2008**, *29*, 505–513.
36. Neutra, M.R.; Mantis, N.J.; Kraehenbuhl, J.P. Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat. Immunol.* **2001**, *2*, 1004–1009.
37. Gohda, M.; Kunisawa, J.; Miura, F.; Kagiyama, Y.; Kurashima, Y.; Higuchi, M.; Ishikawa, I.; Ogahara, I.; Kiyono, H. Sphingosine 1-phosphate regulates the egress of IgA plasmablasts from Peyer's patches for intestinal IgA responses. *J. Immunol.* **2008**, *180*, 5335–5343.
38. Graler, M.H.; Goetzl, E.J. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J.* **2004**, *18*, 551–553.
39. Kunisawa, J.; Kiyono, H. A marvel of mucosal T cells and secretory antibodies for the creation of first lines of defense. *Cell Mol. Life Sci.* **2005**, *62*, 1308–1321.
40. Kunisawa, J.; Kurashima, Y.; Gohda, M.; Higuchi, M.; Ishikawa, I.; Miura, F.; Ogahara, I.; Kiyono, H. Sphingosine 1-phosphate regulates peritoneal B-cell trafficking for subsequent intestinal IgA production. *Blood* **2007**, *109*, 3749–3756.
41. Kunisawa, J.; Gohda, M.; Kurashima, Y.; Ishikawa, I.; Higuchi, M.; Kiyono, H. Sphingosine 1-phosphate-dependent trafficking of peritoneal B cells requires functional NFκB-inducing kinase in stromal cells. *Blood* **2008**, *111*, 4646–4652.
42. Kunisawa, J.; Takahashi, I.; Kiyono, H. Intraepithelial lymphocytes: Their shared and divergent immunological behaviors in the small and large intestine. *Immunol. Rev.* **2007**, *215*, 136–153.
43. Vivier, E.; Tomasello, E.; Paul, P. Lymphocyte activation via NKG2D: Towards a new paradigm in immune recognition? *Curr. Opin. Immunol.* **2002**, *14*, 306–311.
44. Natarajan, K.; Dimasi, N.; Wang, J.; Mariuzza, R.A.; Margulies, D.H. Structure and function of natural killer cell receptors: Multiple molecular solutions to self, nonself discrimination. *Annu. Rev. Immunol.* **2002**, *20*, 853–885.
45. Gangadharan, D.; Lambolez, F.; Attinger, A.; Wang-Zhu, Y.; Sullivan, B.A.; Cheroutre, H. Identification of pre- and postselection TCRαβ$^+$ intraepithelial lymphocyte precursors in the thymus. *Immunity* **2006**, *25*, 631–641.
46. Kunisawa, J.; Kurashima, Y.; Higuchi, M.; Gohda, M.; Ishikawa, I.; Ogahara, I.; Kim, N.; Shimizu, M.; Kiyono, H. Sphingosine 1-phosphate dependence in the regulation of lymphocyte trafficking to the gut epithelium. *J. Exp. Med.* **2007**, *204*, 2335–2348.
47. Sharma, S.; Mathur, A.G.; Pradhan, S.; Singh, D.B.; Gupta, S. Fingolimod (FTY720): First approved oral therapy for multiple sclerosis. *J. Pharmacol. Pharmacother.* **2011**, *2*, 49–51.
48. Kweon, M.N.; Yamamoto, M.; Kajiki, M.; Takahashi, I.; Kiyono, H. Systemically derived large intestinal CD4$^+$ Th2 cells play a central role in STAT6-mediated allergic diarrhea. *J. Clin. Invest.* **2000**, *106*, 199–206.

49. Kurashima, Y.; Kunisawa, J.; Higuchi, M.; Gohda, M.; Ishikawa, I.; Takayama, N.; Shimizu, M.; Kiyono, H. Sphingosine 1-phosphate-mediated trafficking of pathogenic Th2 and mast cells for the control of food allergy. *J. Immunol.* **2007**, *179*, 1577–1585.
50. Gurish, M.F.; Boyce, J.A. Mast cell growth, differentiation, and death. *Clin. Rev. Allergy Immunol.* **2002**, *22*, 107–118.
51. Mizushima, T.; Ito, T.; Kishi, D.; Kai, Y.; Tamagawa, H.; Nezu, R.; Kiyono, H.; Matsuda, H. Therapeutic effects of a new lymphocyte homing reagent FTY720 in interleukin-10 gene-deficient mice with colitis. *Inflamm. Bowel Dis.* **2004**, *10*, 182–192.
52. Fujii, R.; Kanai, T.; Nemoto, Y.; Makita, S.; Oshima, S.; Okamoto, R.; Tsuchiya, K.; Totsuka, T.; Watanabe, M. FTY720 suppresses $CD4^{+}$ $CD44^{high}$ $CD62L^{-}$ effector memory T cell-mediated colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2006**, *291*, G267–G274.
53. Deguchi, Y.; Andoh, A.; Yagi, Y.; Bamba, S.; Inatomi, O.; Tsujikawa, T.; Fujiyama, Y. The S1P receptor modulator FTY720 prevents the development of experimental colitis in mice. *Oncol. Rep.* **2006**, *16*, 699–703.

MALARIA JOURNAL

METHODOLOGY Open Access

A simple and inexpensive haemozoin-based colorimetric method to evaluate anti-malarial drug activity

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Abstract

Background: The spread of drug resistance in malaria parasites and the limited number of effective drugs for treatment indicates the need for new anti-malarial compounds. Current assays evaluating drugs against *Plasmodium falciparum* require expensive materials and equipment, thus limiting the search for new drugs, particularly in developing countries. This study describes an inexpensive procedure that is based on the advantage of a positive correlation between the haemozoin level of infected erythrocytes and parasite load.

Methods: The relationship between parasitaemia and the haemozoin level of infected erythrocytes was investigated after converting haemozoin into monomeric haem. The 50% inhibitory concentration (IC_{50}) values of chloroquine, quinine, artemisinin, quinidine and clotrimazole against *P. falciparum* K1 and 9A strains were determined using the novel assay method.

Results: The haemozoin of parasites was extracted and converted into monomeric haem, allowing the use of a colorimeter to efficiently and rapidly measure the growth of the parasites. There was a strong and direct linear relationship between the absorbance of haem converted from haemozoin and the percentage of the parasite ($R^2 = 0.9929$). Furthermore, the IC_{50} values of drugs were within the range of the values previously reported.

Conclusion: The haemozoin-based colorimetric assay can be considered as an alternative, simple, robust, inexpensive and convenient method, making it applicable in developing countries.

Keywords: Anti-malarial, Assay, Haemozoin, Malaria

Background

Malaria is more than just a problem for tropical countries, it also is a major global public health concern. Annually, there are approximately 300 million new malaria infections and millions of deaths worldwide due to malaria [1,2]. Because a vaccine for malaria is not available, chemotherapy is the main treatment. However, the rapid spread of resistance to current quinoline anti-malarials has made malaria a major global and important problem. In addition, artemisinin, from a Chinese herb (*Qinghaosu*) that has been used in the treatment of fevers for more than a thousand years, is now considered an essential component of artemisinin-based combination therapy against drug-resistant malaria [3,4]. However, the malaria parasites have recently been found to be resistant to artemisinin [5-7]. The alarming spread of drug resistance and the limited number of effective drugs for treatment indicates how important it is to find new anti-malarial compounds.

For decades, the anti-malarial activity of a drug has been measured *in vitro* by quantifying the uptake of radioactive substrates by a parasite as a measure of growth and viability in the presence of the test drug [8,9]. Although several *in vitro* methods exist, the ^{3}H-hypoxanthine method [8] is a popular test for novel anti-malarial drugs, but it is labelled with radiation that presents a potential risk to safety, and it relies on

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relatively expensive radio-isotopes and includes multi-step procedures that become increasingly problematic and impractical when the incidence of testing is increased. The other methods, including the PicoGreen [10,11] and the SYBR Green I [12,13] methods, are also considered to be expensive approaches regarding equipment and chemicals. In addition, there are other methods that are based on enzymatic reaction and/or antibodies that specifically detect the presence of histidine-rich protein II or parasite lactate dehydrogenase [14-16]. However, these methods involve multiple complex steps that are too expensive for developing countries, which makes them ill suited for screening potential anti-plasmodial drugs.

During the development and proliferation stage in host erythrocytes, the malaria parasites degrade haemoglobin for use as a major source of amino acids. This is accompanied by the release of free haem. With haem as a prosthetic group of haemoglobin, the iron is in the ferrous state, but free haem loses one electron and assumes the ferric state. This ferric haem could be oxidatively active and toxic to both the host cells and malaria parasites, and can even cause parasite death. Moreover, due to the absence of haem oxygenase, the parasite is unable to cleave haem into an open-chain free haem, which is necessary for cellular excretion [17]. To protect itself, it is necessary for the parasite to convert haem to non-toxic metabolites. Principally, the parasite detoxifies free haem via neutralization with histidine-rich protein 2 [18,19] and degradation with reduced glutathione [20,21], but mostly with crystallization into haemozoin, which is a water-insoluble malarial pigment produced in the food vacuole [19,22]. Therefore, a simple and inexpensive *in vitro* assay was developed based on the colorimetric quantification of haemozoin in infected red blood cells to evaluate the anti-malarial activity of drugs.

Methods

Materials

Chloroquine diphosphate, quinine sulphate, primaquine, clotrimazole, haemin chloride (haem), RPMI 1640 medium, hypoxanthine, and gentamycin were purchased from Sigma Aldrich Chemical Company (Tokyo, Japan). Albumax II (Gibco), and the other chemicals used in the present study were of a high grade. *Plasmodium falciparum* K1 (chloroquine resistant) and 3D7-9A (chloroquine susceptible) strains [23] were provided from Dr Osamu Kaneko and Dr Shusuke Nakazawa, respectively, from the Institute of Tropical Medicine, Nagasaki University, Japan.

Plasmodium cultivation

Plasmodium falciparum K1 and 9A strains were maintained *in vitro* with continuous culture according to a previously described method with a slight modification [24]. The culture medium consisted of RPMI 1640 supplemented with 0.025 mg/ml gentamicin, 0.01 mM hypoxanthine, 23.8 mM $NaHCO_3$, 11 mM glucose and 0.5% albumax II, and adjusted to a pH of 7.3 to 7.4. The parasite was cultured and maintained in a tissue culture flask with complete culture medium containing 5% human erythrocytes. The parasite density was maintained at about 1.5% parasitaemia under an atmosphere of an AnaeroPack sachet (Mitsubishi Gas Chemical Company Inc, Tokyo, Japan) to create 20% CO_2 and remove O_2 (<0.1%) 37°C [25]. Every two days, infected erythrocytes were transferred into fresh medium containing 5% human erythrocyte. The level of parasitaemia was determined by light microscopy on a Giemsa-stained thin blood smear, and parasitized erythrocytes were diluted when parasitaemia was higher than 5% in erythrocytes contained at 5% in culture medium, in order to lower parasitaemia and allow continuous growth. Parasite culture was diluted with fresh uninfected erythrocytes and culture medium to achieve a starting parasitaemia of 2% and a haematocrit of 5%. This final parasite culture was immediately used for anti-malarial assay.

The relationship between parasitaemia and haemozoin level

A culture of the *P. falciparum* K1 strain was serially diluted with uninfected erythrocytes in complete medium to yield a haematocrit of 5% and parasitaemia ranging from 0 to 10%. The serial culture containing 200 μl was prepared independently in triplicate in microtubes, followed by the addition of 800 μl of 2.5% sodium dodecyl sulphate in 0.1 M sodium bicarbonate pH 8.8, then the samples were mixed at room temperature for 15 min. After centrifugation at 13,000 rpm for 10 min, the supernatant was removed. The pellet was washed twice with 800 μl of 2.5% sodium dodecyl sulphate in 0.1 M sodium bicarbonate (pH 8.8), then 200 μl of 5% sodium dodecyl sulphate was added to 50 mM NaOH to convert the haemozoin into haem. After incubation at room temperature for 30 min, the sample (200 μl) was transferred to a 96-well microplate and scanned at 405/750 nm ($A_{405\ nm}$ minus $A_{750\ nm}$) using an IMark microplate reader (Bio-Rad). After the background absorbance of haemozoin was purified of uninfected erythrocytes (5% haematocrit) then subtracted, the amount of haemozoin in the infected erythrocytes was presented as the absorbance at 405/750 nm and then plotted against parasitaemia.

Evaluating the anti-malarial activity of drugs using the haemozoin-based spectrophotometric method

The *P. falciparum* K1 and 9A strains were used to evaluate the anti-malarial activity of quinine, chloroquine,

pyrimethamine, artemisinin and clotrimazole by using the haemozoin-based spectrophotometric method. Stocks of drugs were prepared in dimethyl sulphoxide or phosphate buffer saline (for chloroquine) and were then serially diluted with complete culture medium. To each well of a microplate, 10 μl of serially diluted drug solution was added into 200 μl of final asynchronous parasite culture. Dimethyl sulphoxide or phosphate buffer saline were also tested by adding a similar amount to control wells. The microplates were cultured 72 hr under the conditions described above. The haemozoin of infected erythrocytes was extracted, purified, and quantified, as described above. The 50% inhibitory concentration (IC_{50}) value was calculated by non-linear fitting of the absorbance at 405/750 nm against the logarithm of the drug concentration using the GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Sigmoidal doses with variable slope models were used with the following equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(\{\log IC_{50} - x\} \times Hill\ slope)}}$$

where y is the absorbance at 405/750 nm; x is the logarithm of the drug concentration, min is the absorbance at 405/750 nm measured at time zero (starting point of assay), and max is the maximal absorbance of a particular drug. The Hill slope is the steepness of the curve. The logarithm of the concentration at zero was defined at 2 log lower than the lowest concentration of a particular drug.

Results and discussion

Relationship between the absorbance of the haem content of haemozoin and parasitaemia

The relationship between the haemozoin amount in a parasitized erythrocyte and parasitaemia was revealed by measuring the absorbance of the haem content of the haemozoin obtained from the parasitized erythrocytes after degradation to monomer haem. As shown in Figure 1, the absorption of converted haem showed a direct and linear correlation with the level of parasitaemia. Low and unsynchronized parasitaemia (about 1%) could be detected using this haemozoin-based colorimetric method. The results indicate that this novel assay of parasites is applicable for monitoring parasite growth and for screening new antimalarial compounds.

Haemozoin-based colorimetric assay to determine the IC_{50} values

The IC_{50} values of some anti-malarial drugs were determined with a dose-response experiment using a haemozoin-based colorimetric assay. The result showed that increasing the concentration of anti-malarial drugs resulted in a decreased absorbance at 405/750 nm (Figure 2). The data was best fitted by a typical sigmoidal dose-response model with a variable slope (four parameters) that agreed well with previous reports [26,27].

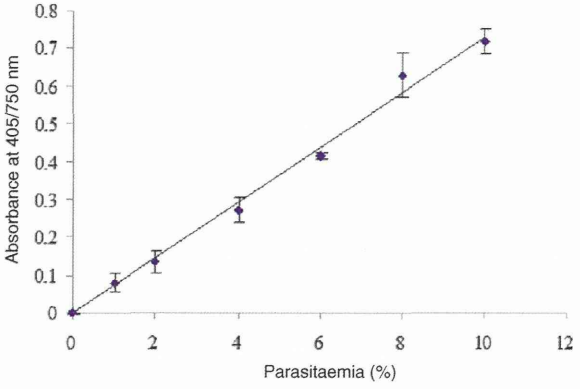

Figure 1 The linear relationship between the haemozoin level of a parasite and parasitaemia. Haemozoin concentration of infected erythrocytes is presented by the absorbance at 405/750 nm of monomeric haem after conversion from haemozoin using an NaOH solution. Absorption values (means ± standard errors of triplicate wells) are plotted against parasitaemia. A well correlated, linear relationship (R^2 = 0.9929) is strong evidence of the sensitivity of the method.

Table 1 summarizes the results of the assay to determine IC_{50} using the haemozoin-based colorimetric method. The *P. falciparum* K1 strain was primarily observed for quinine, chloroquine, clotrimazole, pyrimethamine and artemisinin, with IC_{50} values of 0.258,

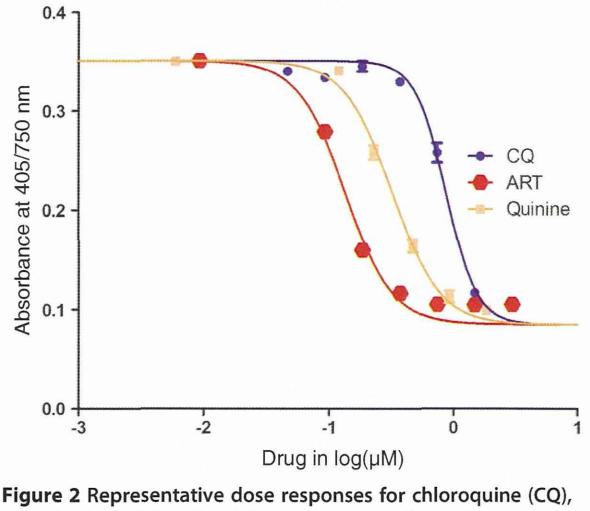

Figure 2 Representative dose responses for chloroquine (CQ), artemisinin (ART) and quinine against the *Plasmodium falciparum* K1 strain. Parasite growth after incubation of parasitized erythrocytes for 72 hr with a drug was measured using a haemozoin-based colorimetric method. The symbols and error bars are the average absorption values at 405/750 nm and standard deviations in triplicate, respectively. The sigmoidal dose response model with a variable slope is the best fit to the data.

Table 1 *In vitro* anti-malarial activities of drugs against chloroquine-susceptible (9A) and -resistant (K1) strains of *Plasmodium falciparum*

Drugs	Mean IC_{50} and 95% CI (μM)	
	P. falciparum K1 strain	*P. falciparum* 9A strain
Quinine	0.258 (0.242 - 0.275)	0.398 (0.307 - 0.516)
Chloroquine	0.873 (0.824 - 0.926)	0.132 (0.082 - 0.211)
Clotrimazole	0.805 (0.689 - 0.939)	1.67 (1.18 – 2.37)
Pyrimethamine	23.03 (18.36 - 28.90)	Not done
Artemisinin	0.139 (0.124 - 0.155)	0.483 (0.376 - 0.622)

Parasitized red blood cells were incubated with different concentrations of drugs for 72 hr and parasite growth was evaluated using the haemozoin-based colorimetric method. The IC_{50} and its 95% confidence interval (95% CI) were calculated from the concentration-response curve of the haemozoin level *vs* the log concentration of a drug.

0.873, 0.805, 23.03, and 0.139 μM, respectively. For the *P. falciparum* 9A strain, pyrimethamine was not evaluated, and the IC_{50} values were 0.398, 0.132, 1.67 and 0.483 μM in succession for quinine, chloroquine, clotrimazole and artemisinin, respectively. The IC_{50} values for quinine, chloroquine, clotrimazole, and pyrimethamine were in a range that was similar to those observed in previous reports [28,29]. On the other hand, the IC_{50} values for artemisinine were higher than previous reports, probably due to asynchronous cultures, ring stage-specific target of artemisinine, accumulation of released hemozoin in the continuous cultures, or several rounds of continuous cultures and cloning of parasite strains in our laboratories. Therefore, further studies are required to compare the novel assay with recent developed methods to validate the accuracy in the screening new antimalarial compounds [30]. Another limitation of the novel method is that it is not easily adaptable for a high throughput screening of anti-malarial drug candidates, which is under-developed using 96-well filter plates [31].

In recent years, the number of laboratories, diagnosis centres and research institutes has risen in developing countries. However, most of them lack the modern equipment and expensive chemicals to apply new methods for screening anti-malarial candidates. In addition, some methods have potential risks of toxicity, so it is prudent to wear disposable gloves at all times when proceeding. Another obstacle is that many laboratories lack the facilities to treat toxic contamination before the toxin is discarded in the environment. The novel anti-malarial assay is safe, non-expensive and easy to apply in laboratories.

Conclusions

The standard curve obtained in this study was strongly linear between the absorbance of monomeric haem converted from haemozoin and the percentage of parasitaemia. The IC_{50} values of chloroquine and quinine obtained from the haemozoin-based colorimetric method are similar to other methods. Even though this report describes specific conditions, the current experiment has introduced an assay that is adaptable to a wide range of conditions. The results also show that using this method has several advantages over using current methods. First, the method is fast and is based on a simple technique that uses a microplate reader, which is available in most laboratories. Second, the assay is based on inexpensive chemicals with no requirement of cold storage. Last but not least, the assay of the inhibition of *P. falciparum* growth using a haemozoin-based colorimetric method is feasible, reproducible, non-toxic, and more convenient than other assays, which makes it particularly useful for developing countries in the screening of novel anti-plasmodials, as a useful high throughput screening method for anti-malarial drug candidates.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NTH and KK developed the idea for the project. TTM and NTH conceived and designed the experiments. TTM, NTH and MNS carried out the laboratory work. TTM, NTH, DTXT, KH, and KK analysed and interpreted the data. TTM, NTH, MNS, KH and KK contributed reagents/materials/analysis tools. . TTM, NTH, DTXT and KK wrote the paper. All authors had full access to all data in the study, read and approved the manuscript.

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References

1. Breman JG, Alilio MS, Mills A: **Conquering the intolerable burden of malaria: what's new, what's needed: a summary.** *AmJTrop Med Hyg* 2004, **71**:1–15.
2. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR: **Epidemiology of drug-resistant malaria.** *Lancet Infect Dis* 2002, **2**:209–218.
3. Adjuik M, Babiker A, Garner P, Olliaro P, Taylor W, White N: **Artesunate combinations for treatment of malaria: meta-analysis.** *Lancet* 2004, **363**:9–17.
4. WHO: *Guidelines for the treatment of malaria.* Geneva, Switzerland: World Health Organization; 2006.
5. Muller O, Sie A, Meissner P, Schirmer RH, Kouyate B: **Artemisinin resistance on the Thai-Cambodian border.** *Lancet* 2009, **374**:1419.
6. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ: **Artemisinin resistance in *Plasmodium falciparum* malaria.** *N Engl J Med* 2009, **361**:455–467.

7. Noedl H, Se Y, Sriwichai S, Schaecher K, Teja-Isavadharm P, Smith B, Rutvisuttinunt W, Bethell D, Surasri S, Fukuda MM, Socheat D, Chan Thap L: **Artemisinin resistance in Cambodia: a clinical trial designed to address an emerging problem in Southeast Asia.** *Clin Infect Dis* 2010, **51**:e82–e89.
8. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD: **Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique.** *Antimicrob Agents Chemother* 1979, **16**:710–718.
9. Elabbadi N, Ancelin ML, Vial HJ: **Use of radioactive ethanolamine incorporation into phospholipids to assess *in vitro* antimalarial activity by the semiautomated microdilution technique.** *Antimicrob Agents Chemother* 1992, **36**:50–55.
10. Corbett Y, Herrera L, Gonzalez J, Cubilla L, Capson TL, Coley PD, Kursar TA, Romero LI, Ortega-Barria E: **A novel DNA-based microfluorimetric method to evaluate antimalarial drug activity.** *AmJTrop Med Hyg* 2004, **70**:119–124.
11. Quashie NB, de Koning HP, Ranford-Cartwright LC: **An improved and highly sensitive microfluorimetric method for assessing susceptibility of *Plasmodium falciparum* to antimalarial drugs *in vitro*.** *Malar J* 2006, **5**:95.
12. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M: **Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening.** *Antimicrob Agents Chemother* 2004, **48**:1803–1806.
13. Vossen MG, Pferschy S, Chiba P, Noedl H: **The SYBR Green I malaria drug sensitivity assay: performance in low parasitemia samples.** *AmJTrop Med Hyg* 2010, **82**:398–401.
14. Noedl H, Wernsdorfer WH, Miller RS, Wongsrichanalai C: **Histidine-rich protein II: a novel approach to malaria drug sensitivity testing.** *Antimicrob Agents Chemother* 2002, **46**:1658–1664.
15. Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL, Hinrichs DJ: **Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity.** *AmJTrop Med Hyg* 1993, **48**:739–741.
16. Druilhe P, Moreno A, Blanc C, Brasseur PH, Jacquier P: **A colorimetric *in vitro* drug sensitivity assay for *Plasmodium falciparum* based on a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay.** *AmJTrop Med Hyg* 2001, **64**:233–241.
17. Eckman JR, Modler S, Eaton JW, Berger E, Engel RR: **Host heme catabolism in drug-sensitive and drug-resistant malaria.** *J Lab Clin Med* 1977, **90**:767–770.
18. Huy NT, Serada S, Trang DT, Takano R, Kondo Y, Kanaori K, Tajima K, Hara S, Kamei K: **Neutralization of toxic heme by *Plasmodium falciparum* histidine-rich protein 2.** *J Biochem* 2003, **133**:693–698.
19. Sullivan DJ Jr, Gluzman IY, Goldberg DE: **Plasmodium hemozoin formation mediated by histidine-rich proteins.** *Science* 1996, **271**:219–222.
20. Atamna H, Ginsburg H: **Heme degradation in the presence of glutathione. A proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobinopathic red blood cells.** *J Biol Chem* 1995, **270**:24876–24883.
21. Huy NT, Kamei K, Yamamoto T, Kondo Y, Kanaori K, Takano R, Tajima K, Hara S: **Clotrimazole binds to heme and enhances heme-dependent hemolysis: proposed antimalarial mechanism of clotrimazole.** *J Biol Chem* 2002, **277**:4152–4158.
22. Francis SE, Sullivan DJ Jr, Goldberg DE: **Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*.** *Annu Rev Microbiol* 1997, **51**:97–123.
23. Maeno Y, Nakazawa S, le Dao D, Yamamoto N, Giang ND, Van Hanh T, le Thuan K, Taniguchi K: **A dried blood sample on filter paper is suitable for detecting *Plasmodium falciparum* gametocytes by reverse transcription polymerase chain reaction.** *Acta Trop* 2008, **107**:121–127.
24. Trager W, Jensen JB: **Human malaria parasites in continuous culture.** *Science* 1976, **193**:673–675.
25. Takeuchi Y, Yamakawa N, Kaetsu M, Fujiwara K, Okada J: **Experiences with AnaeroPack systems.** *J Jpn Assoc Anaerob Infect Res* 1992, **22**:106–112.
26. Shuaibu MN, Wuyep PA, Yanagi T, Hirayama K, Tanaka T, Kouno I: **The use of microfluorometric method for activity-guided isolation of antiplasmodial compound from plant extracts.** *Parasitol Res* 2008, **102**:1119–1127.
27. Bhattacharya A, Mishra LC, Bhasin VK: ***In vitro* activity of artemisinin in combination with clotrimazole or heat-treated amphotericin B against *Plasmodium falciparum*.** *AmJTrop Med Hyg* 2008, **78**:721–728.
28. Karl S, Wong RP, St Pierre TG, Davis TM: **A comparative study of a flow-cytometry-based assessment of *in vitro* Plasmodium falciparum drug sensitivity.** *Malar J* 2009, **8**:294.
29. Kurosawa Y, Dorn A, Kitsuji-Shirane M, Shimada H, Satoh T, Matile H, Hofheinz W, Masciadri R, Kansy M, Ridley RG: **Hematin polymerization assay as a high-throughput screen for identification of new antimalarial pharmacophores.** *Antimicrob Agents Chemother* 2000, **44**:2638–2644.
30. Franke-Fayard B, Djokovic D, Dooren MW, Ramesar J, Waters AP, Falade MO, Kranendonk M, Martinelli A, Cravo P, Janse CJ: **Simple and sensitive antimalarial drug screening *in vitro* and in vivo using transgenic luciferase expressing *Plasmodium berghei* parasites.** *Int J Parasitol* 2008, **38**:1651–1662.
31. Baniecki ML, Wirth DF, Clardy J: **High-throughput *Plasmodium falciparum* growth assay for malaria drug discovery.** *Antimicrob Agents Chemother* 2007, **51**:716–723.

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