Altered Nucleotide Receptor Expression in a Murine Model of Cerebral Malaria

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In cerebral malaria, the most severe complication of malaria, both neurotransmission mechanisms and energy metabolism are affected. To understand how metabolic changes modify neurotransmission, we examined P2 receptor expression in a murine model of cerebral malaria. Quantitative polymerase chain reaction experiments revealed that parasite deposition was greatest in the cerebellum, compared with other areas of the brain, suggesting a correlation between brain parasitemia and loss of control of movement. Infected mice showed modified patterns of expression of P2 receptor subtype messenger RNA (mRNA), depending on both the specific purinergic receptor and the cerebral region analyzed. Immunohistochemical studies indicated altered levels of protein expression by these receptors in infected brains and, in some cases, a pattern of expression different from that noted in control mice. These differences in both the amount of mRNA and the protein distribution of P2 receptors observed in the different brain sites in infected mice suggest an important role for P2 receptors in either provoking cerebral damage or conferring neuroprotection.

Malaria is still one of the most serious infectious diseases in humans and is the cause of tremendous morbidity, mortality, and economic repercussions in areas of endemicity. The most life-threatening complication of Plasmodium falciparum malaria is cerebral malaria (CM) [1, 2]. This acute neurological condition, which is characterized by seizures, loss of consciousness, and coma, mainly affects children aged 2–6 years in sub-Saharan Africa and adults in Southeast Asia. The mortality rate associated with this complication ranges from 15% to 20%.

The pathology of malaria is associated with the acquired ability of infected red blood cells (IRBCs) to adhere to the endothelial cells of the cerebral microvasculature and other vital organs through specific receptors [3–6]. Other disease events, such as tissue ischemia and immunopathological processes, may occur independently or simultaneously. However, the pathophysiological consequences of CM have not yet been satisfactorily resolved.

As with other neurological pathologies, such as encephalitis, stroke, or neurodegenerative disorders, the cerebral response to initial parasite invasion might be limited to a few common mechanisms that continue to operate even after eradication of IRBCs. Metabolic dysfunction seems to be the main mechanism of the morphological changes observed in CM. Histopathological studies of the brains of patients with CM have revealed a large variety of neurological alterations, including neuronophagia, decreased numbers of glial...
cells, and axonal injury [7, 8], which may result from several coexisting pathogenicity factors, including hypoxia, hypoglycemia, cerebral swelling, hemorrhage, and inflammation. All these and other pathological processes cause cellular damage, which in turn could trigger the release of neurotransmitters, including such nucleotides as adenosine triphosphate (ATP) [9–11].

The pathogenesis of CM has been associated with variations in host gene expression. Thus, genes involved in metabolic energy pathways, inflammatory responses, and neuroprotection/neurotoxicity have been related to resistance or susceptibility to this disease [12–14].

In the extracellular space, nucleotides and their hydrolytic products act through purinergic receptors (ie, P2 receptors), which are classified as ionotropic P2X and metabotropic P2Y receptors coupled to G proteins. To date, 7 different P2X receptors (P2X1–P2X7) and 8 P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y12, and P2Y15) have been cloned in mammals [15–17].

Ionotropic P2X receptors feature 2 transmembrane domains connected by a large extracellular loop and intracellular N and C termini, and they are organized such that they form homor heteromeric functional receptors that contain at least 3 subunits. These receptors mediate rapid and selective permeability to cations [17, 18]. Metabotropic P2Y receptors are composed of 7 transmembrane domains, and, similar to other metabotropic receptors, they have a cytosolic N-terminal and an extracellular C-terminal. These receptors induce slow changes in synaptic transmission that activate different signaling cascades through interactions with G proteins [19, 20]. P2 receptors are widely expressed in the central nervous system (CNS), acting as pre- or postsynaptic receptors, and they are also expressed by glial cells [21–24].

Because high levels of extracellular nucleotides are known to play a role in cell damage repair or induce neurological and neurodegenerative alterations through their specific receptors [10, 25–27], P2 receptor expression in the brain was examined in an experimental murine model of CM in the present study. The results presented in this article indicate that Plasmodium berghei strain ANKA–infected mice with neurological malaria have altered patterns of nucleotide receptor expression in the different brain regions (cerebellum, frontal cortex, olfactory bulb, hippocampus, and thalamus-hypothalamus), as detected by means of quantitative polymerase chain reaction (PCR) and immunohistochemical analysis.

METHODS

All experiments were conducted at the Universidad Complutense de Madrid, in accordance with the guidelines of the International Council for Laboratory Animal Science.

Mice, Parasites, Infection, and Disease Assessment

The animals used in the study were 5-week-old male C57BL/6 mice obtained from Harlan Ibérica.

Experimental infection was achieved using mouse blood that contained an infective dose of 10⁷ P. berghei strain ANKA IRBCs preserved in liquid nitrogen until the day of infection.

Sixteen C57BL/6 mice were infected by intraperitoneal inoculation of 10⁷ IRBCs, and 10 uninfected mice were used as control mice. Progression of infection was monitored daily by looking for clinical symptoms of CM, including hemiplegia or paraplegia, head deviation, a tendency to roll over on stimulation, ataxia, and convulsions. Individual peripheral blood parasitemia was determined by Giemsa staining that was followed by counting done with the use of a microscope.

Once infected, mice showing acute clinical symptoms of CM were killed by cervical dislocation and decapitated and then had the olfactory bulb, frontal cortex, hippocampus, thalamus-hypothalamus, and cerebellum removed. Twelve of the 16 infected mice were used for DNA and RNA studies, whereas the other 4 mice were used for the immunohistochemical procedures.

Quantification of the Level of Cerebral Infection by Means of Quantitative PCR

Extraction of parasite DNA from the peripheral blood of infected mice. P. berghei strain ANKA genomic DNA was extracted from peripheral IRBCs by means of the NuncPrep protocol of chemical isolation of DNA from whole blood in the ABI Prism 6100 Nucleic Acid Prep Station (Applied Biosystems), in accordance with the manufacturer’s instructions. A total of 5 μL of each DNA sample was used for amplification.

Extraction of DNA from mouse brain regions. Genomic DNA from the olfactory bulb, hippocampus, frontal cortex, thalamus-hypothalamus, and cerebellum of control mice and mice with CM was isolated using NuncPrep chemistry isolation of genomic DNA in the ABI Prism 6100 Nucleic Acid Prep Station, in accordance with the manufacturer’s protocol. Animal tissue samples were previously treated with 20 mg/mL proteinase K (Sigma) at 65°C for 1 h. A total of 5 μL of each sample was used for amplification.

Quantitative PCR. Oligonucleotide primers for the P. berghei strain ANKA 18S ribosomal gene subunit were designed to quantify parasite genomic DNA levels of P. berghei strain ANKA (GenBank accession number M14599). For quantification of host DNA in the DNA samples, the mouse β-actin gene was chosen (GenBank accession number NC_000071). The specific pairs of primers used to identify the P. berghei 18S ribosomal RNA and mouse β-actin were as follows: for P. berghei 18S, 5'-GGAGATTGGTTTTGACGTTTATGTG-3' (forward) and 5'-AAGCATTAAATAAACGGAATACATCCTTTAG-3' (reverse) and, for β-actin, 5'-ACGGCCAGGTCACTACATATTG-3'.
(forward) and 5′-ACTATGGCCTCAGGAGTTTTGTCA-3′ (reverse). Primers were designed in our laboratory by use of Primer Express software (Applied Biosystems). Parasite and host DNA quantification was assessed using the double-stranded DNA-binding dye SYBR green I. Reactions to amplify the *P. berghei* strain ANKA 18S and mouse *β*-actin genes were conducted on the same DNA sample in separate tubes. In both cases, the 20-μL PCR mixture contained 1× Fast SYBR Green Master Mix (Applied Biosystems), 300 nmol/L each specific primer, and 5 μL of DNA template. The amplification conditions were enzyme activation at 95°C for 20 s and 40 cycles at 95°C for 10 s and at 60°C for 30 s. Samples were always run in triplicate. Amplification, data acquisition, and data analysis were performed using the ABI 7700 Prism Sequence Detector system (Applied Biosystems). Quantification involved experimental determination of the cycle threshold (Ct) of each gene. After amplification of the *P. berghei* strain ANKA 18S and mouse *β*-actin sequences, the melting curves of the PCR products were acquired by stepwise increases in temperature from 60°C to 95°C for 35 min.

**Mouse Nucleotide Receptor Expression Assays**

Total RNA was isolated from the olfactory bulb, hippocampus, frontal cortex, thalamus-hypothalamus, and cerebellum of control mice and mice with CM by use of an ABI Prism 6100 Nucleic Acid Prep Station, in accordance with the manufacturer’s instructions (Applied Biosystems). Reverse-transcription
Figure 2. A, Quantification of peripheral blood parasitemia. B, Quantification of the levels of infection in the olfactory bulb, hippocampus, frontal cortex, cerebellum, and thalamus-hypothalamus of mice with cerebral malaria (CM) by use of oligonucleotides directed toward Plasmodium berghei strain ANKA 18S and mouse β-actin genes. Data are the mean value ± standard error of 10 determinations made for different animals. IRBCs, infected red blood cells.

(RT) reactions were performed using the High-Capacity cDNA Archive Kit (Applied Biosystems), which is recommended for RNA extracted as indicated above. RT buffer to RNA was used in a 1:1 proportion.

Nucleotide receptor mRNA levels were determined by quantitative RT-PCR. Relative abundances of mouse P2 receptors were assessed using the 5′ fluorogenic nuclease assay (TaqMan) of the ABI 7700 Prism Sequence Detector system. In the PCRs, a commercial mixture of mouse-specific primers and probes for P2XR1–P2XR4, P2XR7, P2Y<sub>1</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, P2Y<sub>13</sub>, and the housekeeping β-actin genes (Assays-on-Demand Gene Expression products and TaqMan 3′ Minor Groove Binder probes [FAM dye labeled]) was used, with each mixture prepared as a TaqMan PCR Master Mix without AmpErase UNG (uracil-N-glicosilasa) (all from Applied Biosystems). The specificity of the primers and probes used was verified by BLAST (Basic Local Alignment Search Tool) analysis comparing the mouse genes selected with the P. berghei genome, and no significant similarity was found. The β-actin gene served as an endogenous control to check for any slight variation in the initial concentration, the total RNA quality, and the conversion efficiency of the RT reaction. PCR reactions were conducted with an initial incubation at 95°C for 10 min for polymerase activation, followed by 40 cycles (melting at 95°C for 15 s and annealing and extension at 60°C for 1 min), by use of an ABI Prism 7700 Sequence Detection System. The relative amount of RNA was calculated using the comparative Ct method (ie, 2−ΔΔCt method) [28], once experiments for validation of efficiency were performed. These validation experiments involved constructing standard plots for P2 receptors and β-actin amplified by quantitative PCR. These experiments revealed the similar efficiencies of both pairs of primers that were close to a value of 2. This is the fold increase in input complementary DNA required to decrease the cycle number by 1.

Immunohistochemical Analysis

Control mice and infected mice were killed, and whole brains were removed and fixed overnight at 4°C in 4% paraformaldehyde in phosphate buffer saline (wt/vol). Paraffin-embedded samples were cut into 4-μm sections, deparaffinated, and stained with hematoxylin-eosin. These sections were also used for the complete histopathological study that was performed for each animal.

Immunohistochemical analysis (using the streptavidin-biotin–complex peroxidase method) was performed as described elsewhere [29]. Sections were preincubated with normal pig serum diluted 1:30 (Dakocytomation). Incubations with the
Figure 3. Relative patterns of expression of messenger RNA (mRNA) of P2XR1 (1), P2XR2 (2), P2XR3 (3), P2XR4 (4), P2XR7 (5), P2Y1 (6), P2Y2 (7), P2Y12 (8), and P2Y13 (9) nucleotide receptors in the olfactory bulb, hippocampus, frontal cortex, thalamus-hypothalamus, and cerebellum of control mice. P2 receptor mRNA values were normalized to those of mouse β-actin gene oligonucleotides. Data are expressed as the mean value ± standard error of 10 determinations made for different animals.

Statistical Analysis

Data are presented as the mean value ± standard error. Statistical analysis was performed using Student’s t test. Differences between mean values were considered to be significant at $P < .05$.

RESULTS

Monitoring Progression of CM in Mice Infected with P. berghei Strain ANKA

The infection process was assessed daily by recording clinical symptoms of CM and quantifying peripheral blood parasitemia.
Figure 4. The n-fold changes (log scale) in nucleotide receptor expression observed in mice with cerebral malaria (CM) relative to noninfected control (C) mice. Levels of P2X1–P2X4, P2X7, and P2Y1, P2Y2, P2Y12, and P2Y13 nucleotide receptor expression were examined in the olfactory bulb, hippocampus, frontal cortex, thalamus-hypothalamus, and cerebellum of C mice and mice with CM. Data are the mean value ± standard error of 10 determinations made for different animals. The n-fold changes were calculated by expressing the quantity of messenger RNA (mRNA) for each P2 receptor present in each mouse with CM vs the quantity of mRNA for the same P2 receptor present in one of the C mice. To analyze the variation in P2 receptor abundance in C mice, n-fold changes for each individual C mouse were obtained as for the mice with CM, as indicated in the figure. Statistical analysis of data was performed using Student’s t test. *P < .05; **P < .01; ***P < .001.

in individual mice. Most infected animals demonstrated the first clinical symptoms on days 4–5 after infection. These symptoms were weakness, elevation of the pelvis, rolling over on receiving a stimulus, and slight shaking. As infection progressed, these symptoms appeared in a greater number of mice and worsened up to days 8–10 after infection, at which time the mice demonstrated full symptoms of CM and were killed.

The peripheral blood samples that were obtained from mice with CM on the day that they were killed revealed the presence of anemia (figure 1A). Anemia was also detected in brain tissue samples (figure 1B).

Histologic examination of the brains of control mice and mice with CM revealed vascular congestion, generalized hemorrhage, malaria pigmentation, vascular infiltrates, and ischemic changes, especially in the olfactory bulb and frontal cortex of mice with CM (figure 1C).
DNA equivalent in a milligram of cerebral tissue, 5 independent DNA extractions from 3 to 25 mg of uninfected frozen murine cerebellum, hippocampus, olfactory bulb, frontal cortex, and thalamus-hypothalamus tissue were performed using the Puregene DNA Purification Kit (Gentra Systems), because this commercial kit provides higher DNA yields. Based on this estimate, the final results were expressed as the number of IRBCs per milligram of tissue from each host brain region (figure 2B). The highest IRBC levels were detected in samples from the cerebellum, and the lowest levels were detected in samples from the hippocampus region.

Patterns of P2 Receptor Expression

Purinergic receptor expression was assessed by quantitative RT-PCR and immunohistochemical assays of mouse brain tissue.

Quantitative RT-PCR. The results of these experiments revealed a particular P2 receptor distribution pattern, depending on the cerebral area of control mice that was examined (figure 3). The thalamus-hypothalamus region showed greater P2 expression than did the remaining regions examined. The subtypes P2XR4, P2XR7, and P2Y12 were the most abundant subtypes in all regions, but their expression was especially high in the cerebellum.

Mice infected with *P. berghei* strain ANKA exhibited a modified nucleotide receptor gene expression pattern, compared with that noted in control mice, in terms of both the amounts of given P2 receptors and the different cerebral region in which these appeared (figure 4). Thus, although in several areas of the brain some receptor subtypes were significantly down-regulated, such as *P2XR2* (*P* < .016, for the thalamus-hypothalamus; *P* < .033, for the cortex), *P2XR3* (*P* < .018, for the thalamus-hypothalamus), *P2XR4* (*P* < .039, for the thalamus-hypothalamus), *P2XR7* (*P* < .010, for the olfactory bulb), *P2Y1* (*P* < .008, for the thalamus-hypothalamus), and *P2Y12* (*P* < .021, for the cortex), other receptors subtypes were significantly up-regulated, such as *P2RX1* (*P* < .016, for the hippocampus; *P* < .009, for the olfactory bulb), *P2Y2* (*P* < .001, for the hippocampus; *P* < .003 for the cortex; and *P* < .001, for the thalamus-hypothalamus), and *P2Y12* (*P* < .014, for the cerebellum), and *P2Y13* (*P* < .001, for the cerebellum). Most alterations were observed in the thalamus-hypothalamus region, because the expression of most of the P2 receptors in this region was down-regulated.

Immunohistochemical analysis. Brain slices from control mice showed the ubiquitous and heterogeneous distribution of P2 receptors in the different cerebral areas. Some P2 subtypes were expressed at the somatic and axodendritic levels, such as *P2X1*, *P2X3*, *P2X4*, *P2X7*, *P2Y1*, and *P2Y13* (figure 5). All these receptor subtypes were detected in all regions analyzed, but the intensities were higher in certain areas. Thus, the *P2X1* receptor

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Figure 5. Immunohistochemical expression profiles of P2 receptors in slices of brain tissue obtained from control mice. CA2, CA2 hippocampal region; Cb, cerebellum; Cx, prefrontal cortex; DG, dentate gyrus; OB, olfactory bulb. Scale bar, 25 μm.

Although anemia was observed in blood samples obtained on the day that the mice were killed, levels of peripheral blood parasitemia were <10% (figure 2A).

Quantification of Parasites in Brain Samples from Infected Mice

The tissue parasite load was expressed as the number of parasites detected per milligram of host tissue. To measure parasites in host tissue, the *P. berghei* 18S and host β-actin genes were amplified, and DNA was quantified by interpolating experimental Ct values for the 2 genes on 2 independent standard plots of a log known number of either parasites or milligrams of host tissue against amplification Ct values, respectively (figure A2 in the Appendix, which appears only in the electronic version of the Journal).

In experimentally infected mice, 10-fold diluted tissue templates were analyzed for both *P. berghei* strain ANKA 18S and mouse β-actin genes. The amount of host tissue and the number of IRBCs present in each experimental sample were quantified by interpolation of the corresponding Ct values in the standard curves (figure A2 in the Appendix, which appears only in the electronic version of the Journal). To estimate the host DNA equivalent in a milligram of cerebral tissue, 5 independent DNA extractions from 3 to 25 mg of uninfected frozen murine cerebellum, hippocampus, olfactory bulb, frontal cortex, and thalamus-hypothalamus tissue were performed using the Puregene DNA Purification Kit (Gentra Systems), because this commercial kit provides higher DNA yields. Based on this estimate, the final results were expressed as the number of IRBCs per milligram of tissue from each host brain region (figure 2B). The highest IRBC levels were detected in samples from the cerebellum, and the lowest levels were detected in samples from the hippocampus region.
was mostly expressed in the olfactory bulb. P2X3 and P2X4 were most abundant in the hippocampus, cerebellum, and olfactory bulb, with P2X3 showing lower levels of expression and a mostly somatic distribution. P2X7 was expressed mostly in the hippocampus, prefrontal cortex, and thalamus-hypothalamus. P2Y1 immunostaining was most intense in the olfactory bulb, cerebellum, and thalamus-hypothalamus, and P2Y13 was expressed mainly in the cerebellum, prefrontal cortex, and thalamus-hypothalamus. Some of the P2 receptors, such as P2X2, P2Y2, and P2Y12, had a somatic distribution and were similarly expressed in all brain regions (figure 5).

For brain slices from mice with CM, results of immunostaining for P2 receptor expression differed slightly from results for brain slices from control mice. The results obtained are summarized in figure 6. Compared with control mice, mice with CM had higher levels of expression of the subtypes P2X1, P2Y2, and P2Y13, with this last subtype showing the least expression. For the remaining P2 receptor subtypes, immunostaining revealed lower intensities in infected mice than control mice. Some of the purinergic receptors showed a change in their distribution pattern in infected mice. This was true of the P2X2, P2X4, and P2Y13 subtypes, which showed a somatic distribution in the brains of control mice and a more diffuse expression pattern affecting axodendritic fibers in the infected mice.

**DISCUSSION**

C57BL/6 mice experimentally infected with *P. berghei* strain ANKA have been described as a valid model of CM for the assessment of disease progression, parasite deposition, and neurological dysfunction [30–32].

The relative abundance and distribution of purinergic P2 receptors observed in the different brain regions of mice with CM allowed us to assess anomalies in purinergic neurotransmission in this disease state. The present study is, to our knowledge, the first report of abnormal nucleotide receptor expression in the CNS induced by a systemic infectious disease.

The presence of most purinergic receptors in healthy subjects has been described [20, 33] in the different brain areas examined in this study at the RNA, protein, and/or functional level. We detected broad heterogeneous distribution of these subtypes in the different areas of the brain, along with preferential expression and somatic or axodendritic distribution of each P2 receptor, depending on the area of the brain. This specific expression of purinergic subtypes indicates their important and specific functions in the different regions of the CNS [23, 33].

CM can be described as an encephalopathy involving severe neurological defects [34], which could be the outcome of different pathological processes [8]. These abnormalities induce
generalized cell damage affecting neurons, endothelial cells, erythrocytes, and glial cells, resulting in the release of ATP. Increased nucleotide levels in the extracellular microenvironment can act through purinergic P2 receptors and can mediate either cerebral damage or a neuroprotective effect. ATP and other nucleotides modulate the inflammatory processes induced by microglial cells that contribute to maintaining CNS integrity and/or to the development of pathologic processes, such as glutamate toxicity [25]. Altered P2 receptor expression has been observed in some of these pathologies [35].

C57BL/6 mice infected with P. berghei strain ANKA showed some of the typical symptoms of CM, including reduced body weight and abnormal sensory responses. These symptoms appeared 4–7 days after infection, and most of the mice developed motor alterations over the following days. However, the mice with CM did not demonstrate elevated levels of IRBCs in peripheral blood, compared with murine models of noncerebral malaria [31]. This set of symptoms and the accompanying level of infection have been described elsewhere [36]. However, high recruitment of IRBCs was detected in the different areas of the brain, and the cerebellum was found to be the most parasitized area. This finding is consistent with the symptoms of CM, because mice with this condition have ataxia and altered movements, indicating cerebellar damage.

Mice with CM in the present study displayed signs of anemia that also affected the brain, as described elsewhere [37]. Several factors could lead to anemia, including the hemolysis of infected erythrocytes, spleen removal of noninfected red blood cells, or the destruction of infected and noninfected erythrocytes by the immune system [37, 38]. An additional possible factor causing anemia could be reduced erythrocyte recycling resulting from the production of proinflammatory cytokines or noneffective maturation of erythrocytes [37]. Although the mechanism underlying the development of anemia is not fully understood, early down-regulation of erythropoietic genes in CM has been described elsewhere [12].

We observed severe hemorrhaging in the brains of infected mice, mainly in the olfactory bulb and cerebral cortex regions. This finding could be the consequence of the cerebral edema that occurs during the initial stages of infection [39].

Compared with those in control mice, the P2 receptor transcripts in the different brain areas of infected mice revealed the modified relative abundance of some of these receptors. We observed that the thalamus–hypothalamus was the region that showed the most intensely altered expression of purinergic receptors and that the cerebellum was the least affected region.

The P2Y2 subtype was found to be most altered in infected mice, because levels of expression were elevated in most of the regions analyzed, mainly the olfactory bulb, prefrontal cortex, and hippocampus. Because inflammation is among the most important physiopathological processes of CM [40], the increased level of this receptor could be a consequence of the inflammatory response. In effect, levels of the P2Y2 receptor subtype are known to be increased by inflammation–mediating molecules [41]. By regulating such genes as Bel-2 and Bel-xl, P2Y2 receptors can also promote cell survival [42].

Coactivation of P2Y1 and P2Y12 receptors mediates platelet aggregation [43–45]. The reduced levels of P2Y1 and/or P2Y12 subtype transcripts that were observed in the olfactory bulb, prefrontal cortex, and thalamus–hypothalamus could point to a possible role for these receptors in the cerebral hemorrhages observed in the brains of mice with CM. Thus, their reduced expression could modify platelet aggregation and thus cause hemorrhage. Platelets have been attributed a role in the genesis of CM through the regulation of homeostasis and inflammation [46]. In contrast, the elevated P2Y12 receptor expression that was noted in the cerebellum could modulate platelet activation and induce the release of prothrombotic agents, including adenosine diphosphate. In turn, high concentrations of adenosine diphosphate could also act through P2Y12 receptors, the levels of which were augmented in the cerebellum, promoting neuron survival by inhibition of glycogen synthase kinase 3 [47].

The genetic expression patterns of the purinergic receptors observed in the present study indicate highly variable levels of expression, depending on the cerebral area and whether the mouse was infected or not. Although experimental animal models cannot exactly reproduce human disease, they show some similarities to human CM [31]. The results obtained point to an important role of P2 receptors in the pathogenesis of CM and prompt further research into the possibility of their use as pharmacological targets for the treatment of this infectious disease in humans.

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**References**