Chapter

Regulation of T-reg/Th-17 Balance: One Step Closer Towards Immunotherapy Against Malaria Infection

Saikat Mukherjee, Soubhik Ghosh and Arindam Bhattacharyya

Abstract

According to World Malaria Report 2020, the rate of decline in malaria case incidence and deaths caused by malaria has ceased in latter half of the past decade. Though Artemisinin Combination Therapy (ACT) is still the major therapeutic approach globally to treat malaria patients, increased resistance of *Plasmodium* sp. to artemisinin can be looked upon as a major factor responsible for the rate of decline. In the present world, immunotherapeutic approaches are in the limelight to treat several infections, autoimmune disorders, cancers but application of such therapeutic measures in case of malaria are yet not available. Among different immune cells, T-regulatory cells (T-reg) and Th-17 cells and the balance between them, helps in determining the outcome of the immune response in host during both lethal and non-lethal malaria. TGFβ and IL-6 are two major cytokines that play important role in fine tuning the Treg/Th-17 balance by modulating dendritic cell responses, specially by regulating the ratio between myeloid DC and plasmacytoid DC (mDC/pDC). Studies in rodent malaria models have revealed that neutralization of IL-6 by using anti IL-6 monoclonal antibodies *in-vivo* has been found effective in declining the parasitemia, malaria induced deaths and also in reverting back the altered T-reg/Th-17 balance to normal levels. Apart from these, autophagy is one of the major factors which also contributes to regulate the T-reg/Th-17 balance. In malaria infected mice, autophagy induction has been found to normalise the dysregulated T-reg/Th-17 ratio and promote anti-inflammatory Th-2 pathway by supressing pro-inflammatory Th-1 pathway. So, Treg/Th-17 balance and its associated regulators can be important immunotherapeutic targets for malaria prevention in near future.

Keywords: Malaria, drug resistance, immunotherapy, T regulatory cells, Th-17, IL-6, TGFβ, dendritic cells, autophagy

1. Introduction

World Malaria Report 2020 published by World Health Organization estimated 229 million cases of malaria infection around the world in 2019 among which 94%

of the cases were reported from the WHO African region. The number of estimated cases globally in 2019 was 1 million more than that of the previous year. But in the context of last 20 years, the number of the existing malaria cases has declined from 238 million in 2000. Besides, the total number of estimated cases globally, another parameter that has been in the centre of studying the impact of this disease is, malaria case incidence (cases per 1000 population at risk). Malaria case incidence reduced from 80 in 2000 to 57 in 2019 globally but the rate of decline has ceased in the latter half of the past decade. The deaccelerating rate of decline has also been found in case of malaria mortality rate (i.e. deaths per 100000 population at risk). Despite the steady reduction in number of malaria induced deaths in the past two decades, more than 400 thousand malaria deaths have been reported in 2019. Children aged below 5 years account for 67% of the total malaria deaths, which is a major concern [1].

Among various *Plasmodium* strains that can infect human beings, cerebral malaria causing *Plasmodium falciparum* bring about majority of malaria deaths in Africa and parts of Asia. Apart from *Plasmodium falciparum*, another strain, *Plasmodium vivax* also cause malaria deaths in various other parts of the world [2, 3]. Among several available therapeutic and controlling measures, Artemisinin based Combination Therapy (ACT) is being used worldwide and has been of great success in combating this disease [4–6]. But in recent times, the use of ACT got a major setback due to emergence of Artemisinin resistant *Plasmodium* strains [7, 8]. It may be one of the plausible causes behind the diminishing rate of decline in the rate of malaria case incidence and malaria mortality rate since 2015. Researchers worldwide are putting up constant efforts on making ACT more effective and finding other therapeutic strategies to combat this disease in order to eradicate it in near future. Among other therapeutic measures, immunotherapy has been the prime focus of study over the past decade. Nowadays immunotherapy is being used for various infectious diseases and cancer therapy and the success rate of such therapies are quite promising [9–11]. In case of malaria, immunotherapeutic strategies are not yet available for use. This compels researchers worldwide to find various molecules or cells that can be targeted for effective therapeutic measures in malaria infection [12].

In malaria different stages of the parasitic life cycle can trigger both the innate and adaptive immune response within the host. It is quite difficult to study whether the immune cells play protective or pathogenic or dual roles, especially in human [13]. Still, long-term research reveals specific roles of antibodies and B cells in protection of the host body against the malaria parasite. Besides, several other immune cells like inflammatory cytokines (TNF α, TGFβ, IFN-γ etc.), different subsets of T cells (T-helper cells and Cytotoxic T cells), NK cells and Macrophages also play their part in protection or pathogenesis or both depending on the type of malaria parasite and the stage of life cycle they are in [14]. During life cycle of *Plasmodium* sp. within the host, several major organs and the immune environment within those organs show changes due to presence of parasite factors. Spleen, being a major lymphoid organ and the main blood filtration unit, harbours most of these immune cells [15, 16]. In presence of *Plasmodium* sp. in host body, the immune environment changes rapidly in a day specific manner post infection. Investigation of the changes and regulatory mechanisms within splenic compartment during infections in humans is difficult for several reasons. Most of the study is restricted to observations of clinical symptoms and analysis of tissue sections that are available only after post-mortem. So, there is always lack of enough samples available to investigate the changes and their associated mechanisms in spleen and other lymphoid organs properly [17, 18]. To overcome this, researchers worldwide have focused on studying the major changes in

rodent models of malaria. Murine malaria models are very much in use for their ready availability. Various rodent specific parasite strains like *Plasmodium berghei* ANKA, *Plasmodium yoelii*, *Plasmodium chabaudi* are constantly used in laboratories and they almost resemble different parameters (i.e. anaemia, body temperature changes, loss of weight, and occasional death) shown by human during malaria infection. Apart from these basic parameters, several immune parameters like changes in T helper cell and Cytotoxic T cell percentages in lymphoid organs, activities of B cell, concentration of antibodies, disruption of blood brain barrier and migration of immune cells in the brain during cerebral malaria infection also show resemblance to that of human malaria infections. *Plasmodium berghei* ANKA and *Plasmodium chabaudi* infections show similar symptoms, immunological changes as discussed with that of *Plasmodium falciparum* infection in human which might be due to similarities in infective strategies. Both these rodent and human strains can disrupt the blood brain barrier in a similar manner and immune cells (majorly T cells) infiltrate in the brain which can be lethal to the respective hosts. Another rodent specific non-lethal strain *Plasmodium yoelii* has similar effect on the host immune system to that of *Plasmodium vivax* infection in humans [19]. Working with these rodent strains of *Plasmodium* sp. has been found effective in inferring how the immune system is being regulated during malaria and the elaborated regulatory mechanisms that controls the inflammatory balance that occurs. The balance between pro-inflammatory and regulatory immune responses determines the outcome of malaria infection [20]. The balance is maintained by various cytokines, chemokines, several immune cells (macrophages, dendritic cells) and processes like autophagy. The role of CD4+ T helper cells and CD8+ cytotoxic T cells has been found important in regulating the immune response during malaria infection using both rodent models and human samples. The focus has now been shifted to find out the exact role of different subsets of CD4+ T helper cells and how the balance between them defines the outcome of malaria infection. Among these subsets, Th1/Th2 balance and the cytokines regulating this balance has been found crucial for monitoring the immune homeostasis [21, 22]. But recently, balance between two other subsets of T helper cells was found to be important in regulation of immune responses in various infections, autoimmunity and also cancer immunology. These are termed as T regulatory cells that regulates immune-tolerance by secretion of IL-10 and Th17 cells which inflicts inflammatory responses by secreting IL-17, IL-22, IL-23. Naïve CD4+ T cells differentiate into T-regulatory cells (T-reg) in presence of TGFβ and into Th-17 in presence of TGFβ and IL-6. Majority of functions executed by these cells are regulated by their major transcription factors FOXP3 and RORγT for T-reg and Th17 cells respectively [23–25]. As discussed, the differentiation of Treg and Th17 cells is reciprocally regulated by shared and different cytokines and recent studies even show the plasticity of these cells which states that each subset can convert itself to the other one under different inflammatory stimuli [26–28]. These stimuli modulates the cytokine environment of the host and also changes the homeostatic balance between pro-inflammatory and anti-inflammatory cytokines that culminates into Treg/Th17 disbalance. So, T-reg/Th17 balance and regulation of factors that influence this balance has been found to be pivotal in several viral, bacterial and parasitic infections. In case of several autoimmune disorders like rheumatoid arthritis (RA), psoriasis, inflammatory bowel disease (IBD), multiple sclerosis (MS), Th-17 is the major role player and the T-reg/Th-17 balance skews towards pro-inflammatory Th-17 mediated response. Therapeutic approaches which target Th-17 cells and its functional transcription factor RORγT has been successful in reverting the T-reg/Th-17 cell ratio to normal levels [29]. Monoclonal antibodies designed against the human IL-6R, and drugs like sarilumab and tocilizumab

can reduce Th-17 cells and increase T-reg cells that helps in amelioration of RA in humans [30]. In malaria, the T-reg cells has been found to help the malaria parasite to evade the immune response [31]. Apart from T-regs, Th-17 cells have been also known to play an important role in blood brain barrier disruption, which is a prime reason behind deaths due to cerebral malaria. This article summarizes the recent advancements on understanding Treg/Th-17 balance with respect to malaria [32].

2. Differential role of T-regulatory cells and Th-17 cells in malaria

During malaria, failure in development of an effective pro-inflammatory and anti-inflammatory balance has been found to contribute towards unrestricted replication of parasite and severe immunopathology [31, 33]. Several subsets of T cells (Th-1, Th-2, NKT cells) are involved in controlling the lethal and non-lethal malaria infection [34]. T-reg cells have been primarily found to control the immune evading mechanism of the *Plasmodium* sp. in both mouse and human [35]. A number of other studies have also reported that T-regs may play an important part in facilitating parasite clearance and enhance parasite burden [36, 37]. However, in a separate study, depletion of Foxp3+ T-regs failed to provide protection against experimental cerebral malaria (ECM), which questions the actual role of T-regs in lethal and non-lethal malaria [38]. Augmented generation of Th-17 cells and quick death due to high inflammation in several organs in adult healthy mice upon ablation of T-reg cells, point towards a counter regulatory pathway that might control the pathogenic Th-17 pathway [39]. Th-17 cell itself and cytokines associated with its differentiation from naïve CD4+ T cells has been found to play a role in blood brain barrier (BBB) disruption and cooperate with each other to allow migration of T cells into the brain [40]. As BBB disruption is a salient feature of lethal cerebral malaria, Th-17 pathway and its probable counter regulatory pathway controlled by T-regulatory cells is thought to be important in depicting the probable outcome of the immune response elicited by the host against the malaria parasite. In malaria, the balance between pro-inflammatory and anti-inflammatory factors was found to be important when we reported differential expressions of anti-inflammatory TGFβ and pro-inflammatory TNFα and their role in regulation of splenocyte apoptosis [41]. Keeping the outcome of evaluation of TGFβ and TNFα in context to splenocyte apoptosis and shared requirements of TGFβ during differentiation of T-regs and Th-17 cells, we checked whether the balance between anti-inflammatory T regulatory cells and pro-inflammatory Th-17 cells (T-reg/Th-17) is important in malaria immunology in both spleen and brain. T regulatory cells were found to increase in spleen of non-lethal *P. yoelii* infection at 8 days post infection (dpi) in a day specific manner but in case of lethal *P. berghei* ANKA infection, it decreased with an increase in the infection and the percentage of T-regs in spleen was lowest at 8 dpi. Not only Tregs but the transcription factors, specially FOXP3 also showed similar trend in spleen of lethal and non-lethal malaria infection. In contrast to the T-regulatory cells, Th-17 cells increased significantly at 8 dpi in lethal *P. berghei* ANKA infection but decreased optimally at 8 dpi after an initial surge at 2 dpi. The major transcription factor of Th-17 cells shows the similar trend in both lethal and non-lethal malaria infection as does Th-17 cells [42]. Not only in spleen but also in cerebral cortex and cerebellum of the *P. berghei* ANKA infected mice, differential expression of FOXP3 and RORγT has been found to be critical in regulating the glial cell mediated neuroinflammation and neuronal cell death [43]. So, the contrasting behaviour shown

by these two cells and their transcription factors highlights the importance of T-reg/Th-17 balance and their regulators in malaria.

3. Role of cytokines (TGFβ and IL-6) in regulation of T-reg/Th-17 balance in malaria

TGFβ and IL-6 are cytokines that play major roles in the regulation of innate and adaptive immune responses in different viral (viz. influenza A, Respiratory Syncytical virus etc.), bacterial (viz. *Streptococcus, Mycobacterium* etc), parasitic (viz. *Leishmania*, *Trypanosoma*, *Toxoplasma* etc.) infections, cancers and auto-immune disorders [44–47]. In malaria, IL-6 is found in circulation of patients infected with *Plasmodium vivax* and *Plasmodium falciparum* and it plays a major role in host response [48–50]. There are reports stating that decreased IL-6 levels upon treatment with anti-malarial compounds is associated with decreased parasitaemia [51–53]. However, several reports raise question on actual involvement of IL-6 in the pathogenesis of cerebral malaria [54–56]. In case of TGFβ, we have found that low concentration of TGFβ was found to be pro-inflammatory where high concentration of TGFβ have anti-inflammatory effects [41]. So, as factors responsible for disease outcome in malaria, both of these cytokines and their regulatory effect on T-reg/Th-17 balance seem to be important. We neutralized TGFβ and IL-6 by administration of neutralizing antibodies *in-vivo* at specific concentration. Parasitaemia was highest in TGFβ neutralized group than any other groups whereas parasitaemia was lowest in IL-6 neutralized group. This has been supported by the results of survival percentages of mice, where TGFβ neutralized group showed lowest survival percentage and IL-6 neutralized group showed the highest survival percentage of mice. Thus, it is quite evident that TGFβ and IL-6 directly affects the outcome of the immune response elicited by the host in malaria. Focusing on the effect of these two cytokines on the T-reg/Th-17 balance, it is found that neutralization of TGFβ results in significant induction of Th-17 cells at 8 dpi than control and infected ones. Whereas neutralization of IL-6 causes reduction in percentage and number of Th-17 cells than *Plasmodium berghei* ANKA infected group. Analysis of percentage and number of T regulatory cells in spleen show the reverse phenomenon to that of Th-17 cells upon neutralization of TGFβ and IL-6. Thus T-reg/Th-17 balance, which is skewed towards Th-17 in *Plasmodium berghei* ANKA infection is dependent on fine tuning maintained by TGFβ and IL-6. IL-6 neutralization reverts the dysregulated T-reg/Th-17 balance to homeostatic levels by inhibiting Th17 induction, but neutralization of TGFβ has opposing effect and causes the balance to skew more towards Th17. These changes in T-reg/Th17 balance by regulatory effects of TGFβ and IL-6 is mainly maintained by expression of STAT3 and STAT5, which are the major signalling molecules that take part in the signalling mechanism of these two cytokines [57]. Neutralization of TGFβ and IL-6 not only have its impact on splenic T-reg/Th-17, but also in that of cerebral cortex and cerebellum. In Anti-IL-6 treated *Plasmodium berghei* ANKA infected mice, glial cell mediated neuroinflammation is reduced whereas the anti-TGFβ treated mice upon infection show similar level of neuroinflammation as that of only infected mice. Consistent to that, astrocyte and microglia activation levels show similar changes in IL-6 and TGFβ neutralized groups. Regarding T-reg/Th-17, the major transcription factor of T-reg cells, FOXP3 expression was significantly higher in Anti-IL-6 treated infected group and significantly lower in Anti-TGFβ treated infected mice. The expression of IL-17, a major cytokine secreted by Th-17 cells, show the opposite result to that of FOXP3 in both the groups than the only

Plasmodium berghei ANKA infected ones [43]. But the actual percentages of the T-reg and Th-17 in cerebral cortex and cerebellum and their changes upon neutralization of these two cytokines is not yet investigated. Though there are few reports that cerebral malaria development is independent of IL-17 [58], several other reports shows that significant amount of IL-17 is found in circulation of malaria infected mice and human patients [59–61]. Genetic variants of IL-17 and its receptor IL-17RA increase the risk of malaria as investigated in African population [62]. Protective role of IL-17 during malaria pathogenesis has been found by working with IL-17RA deficient mice, in which IL-17 doesn't function in a proper way. These IL-17RA deficient mice show increased parasitemia, earlier onset of malaria, increased mortality during acute stage than the wild type mice [63]. So, it can be summarised that IL-17 itself and IL-17 expressing CD4+ T helper cells (Th17 cells) is of pivotal importance during malaria but the actual outcome of the immune response against the malaria parasite is dependent on the Treg/Th-17 balance, which is maintained majorly by TGFβ and IL-6.

4. Role of plasmacytoid dendritic cells (pDC) and myeloid dendritic cells (mDC) in regulation of Treg/Th-17 balance in malaria

Dendritic cells (DC), a professional antigen presenting cell, function as a bridge between innate and adaptive immune responses. In various infections, including malaria, different subsets of dendritic cells and co-stimulatory molecules (CD40, CD80, CD86, MHC-II etc.) expressed by them show significant changes which indicates that dendritic cells play a major role in the regulation of T cell differentiation and function [64]. Among different subsets, plasmacytoid DC (pDC), specially the tolerogenic pDCs induces and regulates the function of T regulatory cells [65]. Myeloid DC (mDC), on the other hand mainly secretes factors which are important for differentiation of Th-17 cells from naïve CD4+ T cells in several inflammatory disorders. Regulation of mDC function by several microRNA or other factors has its effect on Th-17 induction and function [66, 67]. In malaria, it has already been reported that mDC/pDC ratio has an impact on host immune response against *Plasmodium* sp. and disease pathogenesis [68, 69]. Analysis of splenic mDC/pDC ratio in *Plasmodium berghei* ANKA infection has shown that the ratio is increased significantly and the result is consistent with Th-17 mediated response against the murine cerebral malaria. This increased mDC/pDC ratio has been shown to revert back to homeostatic levels upon neutralization of IL-6, which also has its impact on Th-17 cells and functions in controlling the disease progression as discussed earlier [57]. Thus mDC/pDC ratio may be crucial in serving as a mediator that regulates the T-reg/Th-17 ratio in malaria. However, further investigation is still required to actually find out how exactly mDC/pDC ratio regulates the T-reg/Th-17 balance and how it influences the outcome of the immune response against malaria parasite.

5. Role of autophagy in the regulation of T-reg/Th17 balance in malaria infection

Autophagy is a well-known process which plays a beneficial role against infectious disease not only by degrading pathogens but also by activating host immune system. Autophagy plays an important role in multiple aspects of immune system like cytokine balance, modulation of immune cells, innate and adaptive immunity and antigen presentation [70]. In our study we have found increased expression of

all five major markers of autophagy pathway viz. BECLIN1, ATG3, ATG5, ATG7, p62 with the progression of disease and the expressions were highest at 8 dpi *Plasmodium berghei* ANKA infection. An increase in the expression of LC3B has also been found. Simultaneously, the ratio of LC3B:LC3A increased at 8 dpi *Plasmodium berghei* ANKA infection which indicates the conversion of LC3A to LC3B and an upregulation of autophagic flux [71]. It has been reported that pDC harbours live *Plasmodium* parasite which have the ability to cause malaria symptoms when transferred to naïve mice [72]. Rapamycin (known autophagy inducer) treatment reduces the plasmodium load in splenic pDC. Autophagic induction increases the expression of CD205 and MHC I on pDC which stimulates antigen processing and antigen presentation respectively as compared to non-treated PbA infected group. Relative downregulation of proinflammatory cytokines like IL-6 and TNFα and positive induction of anti-inflammatory cytokines like IL10 was observed in autophagy induced mice. A tilt towards low Treg/Th-17 and high mDC/pDC ratio have been observed during malaria infection which induce Th1 pathway mediated immune regulation and poor prognosis for host. But autophagy induction can shift the Treg/Th17 balance towards increased T-reg population along with increased pDC population which can alter the mDC/pDC ratio, suppress the proinflammatory response and promote Th2 pathway [73]. Autophagic regulation of splenic red pulp macrophages show similar results in context to Treg/Th-17 balance [74]. Upregulation of proinflammatory cytokines production and alteration of Treg/Th-17 balance towards increased population of Th17 is a major cause for poor prognosis of malaria. Autophagy induction can revert the imbalance and help in betterment of host immune response.

6. Conclusion and future perspectives

Despite of continuous efforts towards invention of a proper and effective vaccines for malaria prevention, very few of them have their impact on reducing the number of malaria cases and malaria induced mortality. ACT still is the major therapeutic strategy in combating this disease, although emergence of Artemisinin resistance has been a major worry for the effectiveness of ACT during treatment of malaria patients. Immunotherapeutic strategies have been quite promising in several inflammatory disorders, cancers, autoimmune disorders and other infections. In case of malaria, although immunomodulation is very effective in murine studies, causing declination of parasitemia and increasing the survival percentages, application of those immunotherapeutic strategies in human is still awaiting. The balance between two T helper cell subsets i.e. T regulatory cells and Th-17 cells has been found to be important in both lethal and non-lethal malaria and factors which regulate this balance seems to play a pivotal role in disease manifestation. Studies using murine models has been quite effective in determining the factors and how they influence the disease outcome by regulating the Treg/Th-17 balance. Among those factors, TGFβ and IL-6 directly regulate the percentage of cells, expression of their characteristic transcription factors and functional cytokines secreted by Treg and Th-17 cells. Neutralization of IL-6 has direct effect on parasitaemia and survival percentages of mice infected with *Plasmodium* sp. It also reverses the dysregulated Treg/Th-17 ratio to optimal levels and can be a target for future therapeutic interventions against malaria infection. mDC/pDC ratio also play the role of a regulator and as a bridge to control Treg/Th-17 ratio. IL-6 neutralization can also bring the altered mDC/pDC ratio to normal levels. Apart from these, autophagic regulation of dendritic cells and macrophages in the spleen has its effect on Treg/Th-17 balance. Though, use of T-regulatory cells and drugs that directly

regulate the altered ratio is regarded as a potentially attractive therapeutic strategy in autoimmune disorders, application of these approaches in malaria and other parasitic infections needs more attention and caution. Further investigations are still required to achieve the goal of a malaria free world.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Saikat Mukherjee, Soubhik Ghosh and Arindam Bhattacharyya*
Immunology Laboratory, Department of Zoology, University of Calcutta, Kolkata, West Bengal, India

*Address all correspondence to: arindam19@yahoo.com

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