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Immune Suppressive Properties of Artemisinin Family Drugs

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Abstract

Artemisinin and its derivatives are the first-line antimalarial drugs, and have saved millions of lives across the globe, especially in developing world. The discovery of artemisinin by Youyou Tu was awarded the 2015 Nobel Prize in Physiology or Medicine. In addition to treating malaria, accumulating evidences suggest that artemisinin and its derivatives also possess potent anti-inflammatory and immunoregulatory properties. We recently showed that artesunate, an artemisinin analogue, dramatically ameliorated autoimmune arthritis by selectively diminishing germinal center B cells. Herein, we review the immunosuppressive properties of artemisinin family drugs and the potential underlying mechanisms.

Keywords

artemisinin; germinal center B cells; inflammation; immune regulation; autoimmune diseases; T cells

1. Introduction

Artemisinin was isolated from *Artemisia annua L* in 1972 by Youyou Tu at the Chinese Academy of Traditional Chinese Medicine (reviewed in (Tu, 2011)). By the end of 1975, its unique chemical structure was elucidated, as a sesquiterpene lactone bearing a peroxy group, quite different from all other known antimalarial drugs. Soon afterwards, some artemisinin derivatives were synthesized and proved to possess better bioactivity or solubility, including dihydroartemisinin, artemether, artesunate and arteether. In recent years, by inserting new groups to the parent structure of artemisinin, a series of artemisinin derivatives were synthesized with potent *in vitro* immunosuppressive functions against T cell activation, including SM735, SM905, SM933 and SM934 (L. F. Hou, et al., 2009; Z. Wang, et al.,

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

2007; Z. S. Yang, Wang, Zhou, Zuo, & Li, 2006; Z. S. Yang, et al., 2005). As shown in Figure 1, all of them share core chemical structure, a sesquiterpene lactone containing peroxide bridge, and constitute the artemisinin family drugs (reviewed in (Y. Li, 2012)). Artemisinin family drugs are currently considered the most effective drug in treating cerebral malaria and chloroquine-resistant falciparum malaria (van Hensbroek, et al., 1996; White, 2008). The peroxy group is essential for artemisinin family drugs to exert anti-malarial effects (Olliaro, Haynes, Meunier, & Yuthavong, 2001; Vennerstrom, et al., 2004). Once the red blood cells are infected with Plasmodium, the intracellular energy metabolism system are activated, which results in elevated level of oxidative stress in infected red blood cells. Subsequently, heme or free iron provided by red blood cells breaks the peroxide bridge of artemisinin to form the nucleophilic radical metabolites. These alkylating artemisinin metabolites subsequently act as the free radicals to attack macromolecular bearing electrophilic groups, and finally eliminate the parasites (Robert, Benoit-Vical, Claparols, & Meunier, 2005). It is well established that artemisinin preferentially kills the parasite-infected red blood cells, leaving the healthy red blood cell spared (Golenser, Waknine, Krugliak, Hunt, & Grau, 2006; Mercer, et al., 2007). This distinct pharmacological mechanism endows the artemisinin distinct advantage of efficacy and safety in clinical practice. To date, in addition to anti-malarial functions, artemisinin family drugs have also been reported to have pharmacological actions against viruses, helminthes, fungi, and even a variety of cancer cells (reviewed in (Ho, Peh, Chan, & Wong, 2014)). This review will focus on the anti-inflammatory and immune-regulatory functions of artemisinin family drugs, and discuss the potential application of artemisinin family drugs as novel immune-regulatory agents.

2. Artemisinin family drugs exert immune regulatory functions

2.1. Artemisinin family drugs regulate innate immune cells

In addition to their excellent clinical anti-malarial effects, experimental studies also suggest that artemisinin family drugs possess potent anti-inflammatory properties by regulating both innate and adaptive immunity. Macrophages, representing a key component of the innate immune system, can produce both pro-inflammatory cytokines, such as IL-12/23 P40 and TNF α , and anti-inflammatory cytokines, including IL-10. Most studies investigating the effect of artemisinin analogues on macrophages focused on the cell line RAW264.7, primary peritoneal macrophages, or fibroblast-like synoviocytes (He, et al., 2011; B. Li, et al., 2010; B. Li, et al., 2008; Park, et al., 2012; J. X. Wang, et al., 2009; X. Q. Wang, et al., 2011; Xu, et al., 2007; Z. Yang, et al., 2012). Artesunate was initially reported to reduce significantly the phagocytosis of peritoneal macrophages and the phagocytic index *in vivo* (Lin, Feng, Pan, Zhang, & Xiao, 1995). Artemisinin family drug was also found to inhibit TNF α production from LPS-stimulated peritoneal macrophage by suppressing nuclear translocation of NF- κ B (W. D. Li, Dong, Tu, & Lin, 2006; Y. Li, et al., 2013), which was confirmed by other publications in the last decade ((B. Li, et al., 2010; B. Li, et al., 2008; Park, et al., 2012; Xu, et al., 2007)). In addition to engulf and digest debris, foreign particles, and pathogens through phagocytosis, macrophages are also the critical effector cells downstream of the T cell activation in many autoimmune diseases. For example, in rheumatoid arthritis, upon the stimulation by T cell-derived interferon- γ (IFN- γ),

macrophages and macrophage-like fibroblast cells are activated to produce various mediators including matrix metalloproteinases (MMPs) and nitric oxide (NO) to induce tissue damage, or to secrete IL-12/IL-23 to form the positive feedback loop to further boost the Th1/Th17 responses (Roberts, Dickinson, & Taams, 2015). Besides suppressing pro-inflammatory cytokine production, artemisinin family drugs could also induce the anti-inflammatory cytokine production, such as IL-10. Hou et al reported that SM934, an artemisinin analogue, could increase IL-10 production, whereas decrease IL-12/23p40 production in primary peritoneal macrophages after IFN- γ stimulation *in vitro* or *in vivo* (L. F. Hou, et al., 2012). All these studies suggest that artemisinin family drugs are able to suppress the activation of macrophage and skew the macrophage to be regulatory in autoimmune diseases.

2.2. Artemisinin family drugs regulate adaptive immune cells

T and B lymphocytes play pivotal roles in adaptive immune responses to drive cellular and humoral immunity. Upon T cell receptor (TCR) engagement with antigens presented by MHC moleculars, T cells are activated and secrete growth factor IL-2 and express its high-affinity receptor IL-2R α chain (CD25); subsequently, by autocrine/paracrine proliferative loop, IL-2 induces clonal expansion and promotes survival of activated T cells; finally, after successful clearance of pathogen or antigen, activated T cells undergo apoptosis to maintain immune homeostasis (Alberola-Ila, Takaki, Kerner, & Perlmutter, 1997; Lea, et al., 2003). Artemisinin family drugs can suppress T cell activation both *in vitro* and *in vivo*. Artemether was reported to suppress T cell proliferation and IL-2 production in response to TCR engagement or mitogens *in vitro* (J. X. Wang, et al., 2007). Interestingly, among all the artemisinin family drugs and derivatives, SM934 has unique properties. On one hand, similar to artemether and SM905, SM934 inhibits T cell proliferation stimulated by anti-CD3, concanavalin A, and alloantigens (mixed lymphocyte reaction). On the other hand, unlike artemether and SM905, SM934 does not affect IL-2 production from activated T cells (L. F. Hou, et al., 2009). Although IL-2 is important to induce effector T cell proliferation, IL-2 is also pivotal for generating regulatory T cells (Treg), and deficiency of IL-2 or CD25 leads to severe systematic autoimmune diseases in mice (reviewed in (Nelson, 2004)). Clinical practices demonstrated that inhibiting T cell proliferation, rather than IL-2 production, may increase the proportion of Treg subset. For example, rapamycin, which does not inhibit IL-2 production, is used in transplantation and leads to Treg predominance (Baan, et al., 2005; Coenen, Koenen, van Rijssen, Hilbrands, & Joosten, 2006). SM934 was well characterized in regulating the balance of effector T and regulatory T cells. SM934 suppresses the differentiation and accumulation of Th1 and Th17 cells, whereas induces the differentiation and expansion of Treg cells, which was demonstrated by both *in vitro* T cell differentiation system and *in vivo* in autoimmune disease models (L. F. Hou, et al., 2012; L. F. Hou, et al., 2011; X. Li, et al., 2013). Similar to SM934, dihydroartemisinin and artesunate were also able to regulate the balance of effector T cells and regulatory T cells (L. Hou, Block, & Huang, 2014; T. Li, et al., 2013; Zhao, et al., 2012). The mechanism for artemisinin family drug to enhance Treg while suppress Th1 and Th17 differentiation is still elusive. Zhao et al reported that dihydroartemisinin works by attenuating the mTOR/Akt signaling pathway (Zhao, et al., 2012). However, in a recent study, Khor et al. showed that artemisinin and other 14 novel Treg cell enhancers appear to work independently of mTOR

(Khor, et al., 2015). Nevertheless, accumulating evidence clearly show that most of artemisinin family drugs have potent immunosuppressive effects against T cell activation, enhance the Treg differentiation *in vitro*, and increase the peripheral Treg numbers *in vivo*.

B lymphocytes are central players in the adaptive immune response and are often the major pathogenic driver in many autoimmune diseases through secreting autoantibodies. In early 1990s, Tawfik et al. and Lin et al. reported that artemisinin family drugs were able to suppress humoral response against sheep red blood cells, but not the delayed-type hypersensitivity (Lin, et al., 1995; Tawfik, Bishop, Ayalp, & el-Feraly, 1990), suggesting artemisinin family drugs may preferentially affect antibody production upon antigen immunization. Later, Youyou Tu et al. reported that dihydroartemisinin treatment decreased the anti-dsDNA autoantibodies in lupus-prone BXSB mice (Dong, Li, & Tu, 2003). Subsequent reports showed that artemisinin family drugs were able to inhibit anti-collagen antibody production in collagen-induced arthritis (CIA) model (Mirshafiey, et al., 2006), anti-nuclear and anti-dsDNA antibodies in lupus-prone MRL/lpr mice and NZB/NZW mice (L. F. Hou, et al., 2012; L. F. Hou, et al., 2011; Jin, et al., 2009). Collectively, artemisinin family drugs are established to suppress autoantibodies in various autoimmune disease models. However, the direct action of artemisinin family drugs on B cells was not well studied. Because of the prominent inhibitory effects of artemisinin family drugs on T cell proliferation and activation, the reduction in antibody levels were always interpreted as the secondary effects on T cell inhibition. Recently, we investigated the effect of artesunate on K/BxN mouse model of rheumatoid arthritis, in which disease mechanisms have been well characterized (L. Hou, et al., 2014; Kouskoff, et al., 1996; P. Monach, et al., 2007; P. A. Monach, Mathis, & Benoist, 2008). K/BxN mice, expressing the KRN T cell receptor transgene and the MHC class II molecule A^{g7}, develop very high titers of arthritogenic autoantibodies against glucose-6-phosphate isomerase (GPI) (Kouskoff, et al., 1996). K/BxN mice develop arthritis spontaneously at 4 weeks of age and arthritis is well established by 5-6 weeks of age (Matsumoto, Staub, Benoist, & Mathis, 1999; P. Monach, et al., 2007). In our study, we treated the K/BxN mice with artesunate either before the disease onset or after the establishment of disease. Artesunate treatment prevented the arthritis development in young K/BxN mice by inhibiting germinal center (GC) formation and the production of autoantibodies. In adult K/BxN mice with established arthritis, artesunate treatment rapidly and dramatically diminished GC B cells in a few days. In contrast to diminishing GC B cells, artesunate treatment did not affect the follicular helper T (T_{fh}) cells. Thus, by using K/BxN mouse model, artesunate was demonstrated to directly act on the germinal center B cell response. A recent study reported that, instead of suppressing GC B cells, SM934 treatment from 9 to 27 weeks of age increased the GC B cell numbers in lupus-prone MRL/lpr mice (Wu, et al., 2015). However, it is difficult to assess direct vs. indirect effects over such a long period of treatment in addition to natural disease progression.

2.3. The efficacy and mechanisms of artemisinin family drugs in treating autoimmune diseases

Due to the prominent suppressive effects on both innate and adaptive immune cells, artemisinin family drugs have been tested for treating various experimental autoimmune models. Dihydroarteannuin was demonstrated to improve lupus syndrome in BXSB mice by

decreasing proinflammatory cytokine TNF- α production from macrophage (W. D. Li, et al., 2006). Subsequently, artesunate was reported to significantly increase the survival rate, reduce the serum levels of anti-dsDNA antibodies, and suppress the proinflammatory cytokine MCP-1 production in female MRL/lpr mice (Jin, et al., 2009). The inhibitory effect of artemisinin family drugs on T cell activation was also investigated in CIA and experimental autoimmune encephalomyelitis (EAE) murine models (X. Li, et al., 2013; J. X. Wang, et al., 2008; Z. Wang, et al., 2007; Zhao, et al., 2012). In CIA model, SM905 was effective in both prevention and therapeutic modes. The beneficial effects of SM905 were accompanied by skewing the T cell subset from pathogenic Th17 to protective Th2 subset. Subsequent *in vitro* T cell differentiation and *in vivo* studies demonstrated that artemisinin family drugs prevent the differentiation of pathogenic Th1 and Th17 cells, and directly induce the expansion of suppressive Treg cells. In EAE models, SM933, SM934, and dihydroartemisinin have been reported to effectively delay the onset of EAE and ameliorated ongoing EAE in mice by independent groups. Among these studies, SM934 and dihydroartemisinin share some common features that they reciprocally regulate Th and Treg cell generation. SM933 was reported to affect NF κ B and the Rig-G/JAB1 signaling pathways (Z. Wang, et al., 2007). SM933 inhibits the activity of NF κ B by up-regulating I κ B. Furthermore, SM933 prevents JAB1, a master cell cycle regulator, from entering the nucleus to promote p27 degradation, resulting in blunted cell cycle progression. This leads to altered cell cycle activity of encephalitogenic T cells as a result of its selective effect on activated, but not resting, T cells.

Given that artemisinin family drugs can affect multiple cell types in these disease models, it has been difficult to assess the relative contribution of each cell type, and direct vs. indirect effects. In this regard, K/BxN mice provide an ideal model with clearly defined initiation phase (autoantibody production by T and B cells) and effector phase (inflammatory response by innate immune cells). Transfer of serum or purified antibodies from arthritic K/BxN mice into healthy animals provokes arthritis quickly and robustly. Taking advantage of the serum transfer model, we were able to test the effect of artesunate on inflammatory phase, which is mediated solely by transferred autoantibodies and host innate immune cells without immunization and boosting. In contrast to the spontaneous K/BxN model, artesunate treatment exerted minor influence on K/BxN serum transfer induced arthritis (L. Hou, et al., 2014), suggesting that *in vivo* artesunate has minor effects on inflammatory responses downstream of immune-complex deposition and complement activation.

3. Cellular and molecular mechanisms of artemisinin family drugs

Artemisinin is a sesquiterpene lactone containing peroxide bridge that plays an essential role in anti-malarial effects by acting as an alkylating agent to attack macromolecular bearing electrophilic groups or centers, eventually leading to parasitic death (Golenser, et al., 2006; Olliaro, et al., 2001; Robert, et al., 2005; Vennerstrom, et al., 2004). However, the precise molecular targets of artemisinin family drugs are not well understood. One previous elegant study showed that artemisinin inhibits endoplasmic reticulum Ca²⁺-ATPase (SERCA) of *Plasmodium falciparum* (Eckstein-Ludwig, et al., 2003; Jung, Kim, Nam, & No, 2005). Interestingly, two most recent studies demonstrated that artemisinin is able to bind to a broad spectrum of targets simultaneously, and fatally disrupting the biochemistry of the parasite

(Ismail, et al., 2016; J. Wang, et al., 2015). In one of these studies, Wang et al developed an unbiased chemical proteomics approach, and systematically identified 124 artemisinin covalent binding proteins from *Plasmodium falciparum* (J. Wang, et al., 2015). Many of the newly-identified protein targets are involved in essential biological processes in the parasite. By using an artemisinin activity-based probe, the other independent study by Ismail et al also revealed that artemisinin targets a broad pathways involved in the glycolytic, hemoglobin degradation, antioxidant defense, and protein synthesis, processes essential for parasite survival (Ismail, et al., 2016).

Due to the need for heme, artemisinin is extremely effective in killing *Plasmodium falciparum*-infected red blood cells, but only shows marginal effects on resting red blood cells (Golenser, et al., 2006; J. Wang, et al., 2015). Similarly, artemisinin family drugs selectively inhibit the proliferation and survival of activated T cells, but not the resting ones. In 2009, Hou et al reported that artemisinin analogues can induce apoptosis of activated T cells (L. F. Hou, et al., 2009). In this study, T cells were *in vivo* activated by TCR engagement and then co-stained by CD69 and apoptotic marker annexin V. Results showed that only activated T cells (CD69-positive), but not the resting T cells (CD69-negative), are annexin V positive when treated with artemisinin analogue SM934. In the same study, authors also reported that SM934 can directly inhibit the proliferation of activated T cells using CFSE labeling. The same mechanism might underlie the effect on germinal center B cells that are highly proliferative and metabolically active.

4. Future directions

Artemisinin family drugs are potent anti-malarial agents with high efficacy and low toxicity. Besides the outstanding antimalarial activity, artemisinin and its derivatives are also able to regulate various aspects of immune responses, such as macrophage activation, T cell activation and proliferation, T cell subsets differentiation (Th1, Th17, Treg). Furthermore, the recent finding that artesunate can regulate GC B cells highlights artesunate's role in humoral immune response (L. Hou, et al., 2014). Artemisinin family drugs were also reported to be effective in murine allergic asthma models, and inhibit proliferation of primary human cultured airway smooth muscle cells (Cheng, et al., 2011; Cheng, et al., 2013; Ho, et al., 2012; Tan, et al., 2014). Collectively, artemisinin family drugs act on multiple components of immune system creating a synergistic immune suppressive effect in inflammation and autoimmune diseases (Summarized in Table 1). Artemisinin, a compound with sesquiterpene lactone bearing a peroxy group, is a promising leading compound for further development of novel immunoregulatory therapeutics to treat various autoimmune diseases and immune disorders. In parallel, advanced chemical biology strategy combined with proteomics could provide better understanding of the cellular and molecular mechanisms of artemisinin family drugs, thus to facilitate the discovery of novel drug targets to treat autoimmune diseases.

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Abbreviations

CD	cluster of differentiation
CIA	collagen-induced arthritis
EAE	Experimental allergic encephalomyelitis
IL	Interleukin
TCR	T cell receptor
Th	T helper
Treg	regulatory T cell

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Table 1

The effects of artemisinin derivatives in various autoimmune disease models

Disease	Animal model	Compound tested	Effects
RA	CIA	SM905	↓Th17, ↑Th2
	K/BxN	artesunate	↓GC B cell
SLE	MRL/lpr	SM934, artesunate	↓Th17 and Th1, ↑ Treg ↓NFkB,
	NZBW/F1	SM934	↓Th17 and Th1, ↑ Treg
	BXSB	dihydroarteannuin	↓NFkB
MS	EAE	SM933	↓NFkB and the Rig-G/JAB1 signaling pathways
		SM934	↓Th17 and Th1, ↑ Treg
		DHA	↓Th17, ↑Treg
Asthma	OVA immunization	Artesunate	↓PI3K/Akt, ↓ oxidative damage ↓Smooth muscle proliferation