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CD8+ T cell immunity in an encephalitis model of Toxoplasma gondii infection

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Abstract

Toxoplasma gondii infection induces a robust CD8 T cell immunity in the infected host, which is critical for keeping chronic infection under control. IFNγ production and cytolytic activity exhibited by CD8 T cells are critical functions needed to prevent the reactivation of latent infection. Paradoxically, the susceptible mice infected with the parasite develop encephalitis irrespective of the presence of vigorous CD8 T cell immunity. Recent studies from our laboratory have demonstrated that these animals have defect in the memory CD8 T cell population, which become dysfunctional due to exhibition of inhibitory receptors like PD-1. Although the blockade of PD-1-PDL-1 pathway rescues the CD8 response, PD-1^{hi} expressing cells are refractory to the treatment. In this review, we discuss the development of CD8 memory response during chronic infection, mechanism responsible for their dysfunctionality, and possible therapeutic measures that can be taken to reverse the process.

Keywords

Toxoplasma gondii; CD8+ T cells; Cytokines; PD-1; Cytotoxicity

Toxoplasma gondii and protective immunity

Toxoplasma gondii, an obligate intracellular pathogen, is one of the most common parasite infecting humans worldwide [1, 2]. According to CDC estimates, up to one third of the global population is chronically infected with *Toxoplasma gondii*. In the USA, approximately 22 % of the population 12 years and over is afflicted with the parasite, whereas up to 95 % population in the other regions of the world has been shown to be infected (http://www.cdc.gov/parasites/toxoplasmosis/).

Similar to many other intracellular pathogens including viruses, bacteria, and protozoa, the protective immunity against *T. gondii* is highly dependent on the development of robust cell-mediated immunity [3, 4]. In the case of *T. gondii*, although innate immune mechanisms mediated by macrophages and NK cells can restrict acute infection during early stages, the presence of optimal adaptive immunity is essential for keeping the pathogen under control [5, 6]. Among the T cells, CD4 population due to their ability to produce cytokines like

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IFNγ are important for later stages of acute infection [7, 8], while long-term immunity which is essential for the maintenance of chronicity is primarily dependent on CD8 T cells [9-11]. The first report suggesting the importance of CD8 T cells in response to *T. gondii* infection was reported by studies conducted by Khan et.al who demonstrated that mice immunized with the major membrane protein (SAG-1) developed a strong response. Interestingly, immune CD8 T cells from these animals caused lysis of extracellular parasites [12]. Subsequently, it was reported that antigen-specific CD8 cloned T cells raised against the same antigen, upon adoptive transfer protect naive animals against lethal infection [10]. In between this period, there were number of reports by other laboratories which demonstrated the importance of CD8 T cells during Toxoplasma infection [13, 14]. In one of the studies, it was reported that CD8 CTLs (cytotoxic T lymphocytes) generated by the vaccine strain are critical for the protection against a virulent strain of parasite [15]. The importance of CD8 T cells in chronic toxoplasmosis was reported by Brown and McLeod, who demonstrated the role of these cells in determining the cyst burden [16]. Similarly, a number of other studies have further confirmed that CD8 T cells are essential in keeping chronic toxoplasma infection under control, thus establishing them as a dominant component of long-term immunity needed to keep the reactivation process in check [9, 17-19]. In addition to their role in chronic infection, CD8 T cells due to their ability to produce IFNγ may also contribute to protection during acute toxoplasmosis. Nevertheless, in addition to cytokine production, cytotoxic activity of these cells mediated by perforin is critical for preventing encephalitis due to reactivation of latent infection [17, 20]. It is important to state that the importance of CD8 T cells in controlling TE (toxoplasmic encephalitis) can be extended to humans, as the disease in HIV infected population occurred during advanced stages of infection, when CD8 T cell immunity in these patients was weakened [21].

In recent years, multifactorial steps in CD8 T cell activation, effector function acquisition, and memory cell differentiation have unfolded. However, many questions still remain unaddressed and the process may vary with the pathogen involved. Moreover, the process in *T. gondii* may be more complex in mice susceptible to the parasite (which develop TE) where, in spite of a very vigorous CD8 T cell effector immunity, the memory response is severely compromised [22]. In this article, we will discuss available knowledge about the multi-step process involved in CD8 T cell response to *T. gondii* and the factor(s) which inhibit the development of robust long-term immunity in a TE model.

Factors responsible for elicitation of CD8 T cell response against *T. gondii*

1. Antigen presentation and MHC class I molecules

   The first step for the induction of CD8 T cell response is initiated by interaction of CD8 with antigen presenting cells and subsequent presentation of antigenic peptide [23]. Studies conducted with various mouse strains demonstrated that differences in MHC class I haplotype could influence the outcome of infection [16, 24, 25], thus further emphasizing the importance of CD8 T cells in the immunoprotection against the parasite. Importantly, studies conducted with mice expressing human MHC class I transgenes have demonstrated the allelic

dependence for the control of *T. gondii* infection [26]. Thus, identifying the class I restricted *T. gondii*-specific epitope would be helpful in determining the antigens involved in eliciting CD8 T cell immune response against the parasite. Although several studies identified the antigens and secreted proteins like rhoptry, dense granule and microneme as T cell antigens, whether epitopes contained in these antigens correspond to dominant *T. gondii*-specific CD8 T cell response in the infected mice was not determined till recently [27-31]. While most of the CD8 epitopes are recognized by cells from BALB/C (H-2 L) mice, Wilson et.al identified a H-2 K restricted CD8 T cell epitope derived from Tgd057, a protein of unknown function [32]. However, based on the complex nature of the pathogen studies related to identification of multiple CD8 T cell epitopes responsible for the elicitation of protective immunity against *T. gondii*, needs to be intensified and their specificity in terms of stage-specific recognition determined. This information will be important in designing CD8-based vaccine for acute and chronic stage of infection.

2. Role of co-stimulatory molecules in the development of CD8 T cell immunity

Apart from T cell receptor–peptide interaction, a second cell-cell interplay involving co-stimulatory molecules is important for optimal CD8 T cell activation [33, 34]. Co-stimulation occurs mainly via interaction of two families of proteins, the immunoglobulin (Ig) superfamily members B7/CD28, and the TNF receptor/TNF superfamily [35]. Although the blockade of co-stimulatory molecules like CD80 and CD86 have been shown to dampen T cell activation in human PBMC [36], the mouse model demonstrates that animals lacking CD28 (a receptor for both CD80 and CD86) were not susceptible to *T. gondii* infection, in spite of lower IFNγ production by T cells [37]. However, when infected mice were rechallenged with the virulent (RH) parasite strain, the animals succumbed to infection. Similar observations have been made with CD40-CD40L pathway, and limited data available suggests that although these molecules may play a role in T cell activation, their absence does not profoundly affect the protective immunity against the parasite [38]. Along the same lines studies conducted with CD28 homolog, inducible T cell co-stimulator (ICOS) molecule demonstrated that CD8 T cell immunity in knockout mice was not significantly affected [39]. As the great majority of brain resident CD8 T cells express ICOS during chronic infection, it remains to be seen if the ICOS-ICOSL interaction has differential role in acute versus chronic infection [40]. Overall, a clear picture about the importance of co-stimulatory molecules like CD28-CD80/CD86, CD40-CD40L, ICOS-ICOS-L interaction in the development of CD8 T cell immunity during acute versus chronic infection needs to be investigated. Based on available reports, it may be fair to postulate that during acute infection, the stimulus from the parasite is so strong that requirement for these interactions is bypassed. However, positive signals from these interactions may be needed for the generation/maintenance of adequate CD8 T cell numbers and function. Recent studies performed in our laboratory have demonstrated that reinvigoration of CD8 T cell functionality during chronic toxoplasmosis remains ineffective if

CD40-CD40L interaction is blocked [41]. Thus, in depth studies need to be conducted to determine the role of co-stimulatory molecules in the maintenance of CD8 T cell functionality, which is essential for keeping chronic infection under control.

The development of effective CD8 T cell immunity during infection without any immune-pathological consequences relies on a delicate balance on positive signals from co-stimulatory molecules and negative signal from inhibitory receptors like CTLA-4 and PD-1 [42, 43]. Although the role of CTLA-4 on CD8 T cell responses during *T. gondii* infection has not been studied in detail, the importance of PD-1 molecule on these cells has been recently evaluated and will be addressed separately.

3. Cytokines responsible for the development of CD8 T cell immunity against *T. gondii*

Once antigen-specific CD8 T cells are generated, they need to expand and differentiate which is modulated by cytokine milieu [44]. During infection, a number of inflammatory cell types like neutrophils, macrophages, and dendritic cells traffic to the site of infection and release cytokines and chemokines which decides the fate of CD8 T cell response [32, 45-50]. One of the pivotal cytokine which is responsible for the generation of CD8 T cell effectors or short lived effector cells (SLEC) is IL-12 [47, 51, 52]. The biologically active form of IL-12, IL-12p70 (composed of two subunits) [53] produced during *T. gondii* infection can induce CD8 T cells effector response [32]. The production of cytokine is strain dependent which affects CD8 effectors, underlying the importance of the cytokine in their generation [48]. Apart from IL-12, other related cytokines that are known to be important for CD8 T cell immunity are γ chain cytokines like IL-7 and IL-15 [54]. Unlike other pathogens, *T. gondii* does not induce a potent IL-2 response [55-57], which explains the fact that mice lacking CD4 T cells (which is a primary source of IL-2) generate a normal CD8 T cell effector response [58]. However, the studies related to IL-2 need to be investigated further as it has been reported that IL-$2^{-/-}$ mice exhibit poor CD8 T cell immunity [59]. Based on the data obtained from CD4 deficient mice [58], the role of IL-2 during toxoplasma infection may be restricted to maintenance of CD8 T memory and cytokine may be important in programming these cells for long-term survival (Fig. 1).

A possible role of IL-7 in the development of CD8 T cell response was reported several years ago by our laboratory in which it was demonstrated that exogenous treatment of infected animals with cytokine led to augmentation of cytotoxic CD8 T cell responses [55]. In the study conducted few years ago, we observed that in the absence of IL-15, endogenous IL-7 is critical for the development of CD8 T cell memory precursors via expression of anti-apoptotic protein Bcl-2 [54]. In contrast, the role of related γ chain cytokine IL-15, in the maintenance of CD8 T cell memory response has been established by our laboratory [54, 60-63]. Moreover, a dominant role of IL-15 independent of IL-7 in CD8 T cell

recall response during secondary infection has been reported [64]. The importance of IL-15 has also been demonstrated during acute infection, where it regulates CD8 T cell burst size but not per cell function [54]. Interestingly, IL-15$^{-/-}$ mice survive *T. gondii* infection [65], which may be attributed to IL-7, as treatment with anti IL-7 antibody abrogates the protection in knock out animals [54]. These conclusions drawn from these studies is that although the role of both IL-7 and IL-15 is needed for the development of CD8 memory precursors, the later is primarily involved in the maintenance of this response, thus presumably playing an important role during chronic infection (Fig. 1). However, the role of other cytokines involved in the priming and maintenance of CD8 T cells needs to be further studied. In this regard, IL-21, another γc cytokine has been shown to affect the potency of CD8 T cell response in viral infections [66, 67]. Recent unpublished studies from our laboratory have observed the role of this cytokine in the maintenance of CD8 T cell immunity against *T. gondii* and will be discussed later in this review.

4. Helper role of CD4 T cells in the development of CD8 T cell response

The helper role of CD4 T cells in the induction and maintenance of CD8 T cell response has been debated for quite some time [68]. As regards to *T. gondii*, it was believed that along with CD8, CD4 T cells play a synergistic role in the protection against latent infection [69]. As for the involvement of CD4 T cells specifically in the induction of CD8 T cell response, we reported that robust CD8 T cell immunity in the absence of CD4 T cells can be induced [58]. In subsequent studies, it was demonstrated that in the absence of CD4 T cells, NK cells played an important role in the elicitation of CD8 T cell immunity [70]. Furthermore, depletion of NK cells in the CD4 $^{-/-}$ mice severely compromised the development of CD8 T cell response against the infection. The role of NK cells in helping the generation of CD8 T cell response was demonstrated to be mediated by their interaction with DC via NKG2D molecule [71]. The crosstalk enhanced the IL-12 production by DC leading to generation of strong CD8 T cell response. The role of NK cells in the development of CD8 T cell immunity has potential therapeutic implications in immunocompromised situations like HIV patients who can have severely depleted CD4 T cell numbers. Interestingly, although CD8 T cell response against *T. gondii* in the absence of CD4 T cells could be developed, it was not maintained [58]. These studies imply the importance of CD4 help in the maintenance of CD8 T cell response against the parasite and raise the question about the nature of help provided by these cells for the persistence of protective immunity. The important role of CD4 T cells in the long-term CD8 T cell immunity may be attributed to the programming of these cells at the time of induction. It is possible that in the absence of conventional CD4 T cells, CD8 T cells are not well programmed to maintain their numbers or even functionality, thus resulting in the inability of the susceptible host to prevent encephalitis. However, this cannot be extended to normal wild-type animals that develop encephalitis even when CD4 T cells are present. The loss of functionality, especially in memory CD8 T cells during

chronic toxoplasmosis has been reported and will be discussed in a later part of this review [72]. Although the factors which trigger CD8 T cell dysfunction have not been worked out, loss of CD4 T cells during acute infection, which was reported several years ago, could be an important factor [73] and needs to be studied in detail. The nature of CD4 T cell help needed for development and maintenance of long-term CD8 functionality needs to be characterized and is currently under active investigation in our laboratory. In this regard, as stated above, it is important to note that during primary *T. gondii* infection, the parasite apparently does not induce a potent IL-2 response, which has been recently reported to be important for CD8 T cell functionality during secondary infection [74]. It may be postulated that a lack of IL-2 response results from CD4 T cell loss during acute infection, which ultimately effects the long-term survival of memory CD8 T cell population.

CD8 T cells response during chronic Toxoplasmosis

As stated above, role of CD8 T cells in keeping chronic toxoplasmosis under control is well established. Paradoxically, despite a robust CD8 T cell response during acute phase of infection, long-term immunity against this pathogen is compromised in certain susceptible strains leading to host mortality [75]. Differential susceptibility to *T. gondii* reactivation in AIDS patients was also noted in a study conducted during pre-HAART era, which reported that only 30 % of AIDS patients with low CD4 count and Toxoplasma seropositivity, who were not on effective prophylaxis, developed reactivated toxoplasmosis [76]. Considering that memory CD8 T cells can persist for a lifetime and can mediate a robust recall response, it remains questionable whether the susceptibility to toxoplasmic reactivation is due to potential attrition of memory CD8 T cell response. As mentioned earlier, recent studies from our laboratory have reported that in C57BL/6 (mice susceptible to toxoplasmic encephalitis), CD8 T cells during later phase chronic encephalitis exhibit progressive attrition of functionality, increased apoptosis and poor recall response along with elevated expression of PD-1, an inhibitory receptor, a phenomenon referred to as CD8 exhaustion [22, 75]. Unlike chronic viral models, where this phenomenon has been extensively reported, *T. gondii* model represents an interesting or rather unique situation where despite initial control of parasitemia, the cells eventually get exhausted and lose their functionality.

With regards to *T. gondii* infection, the ability of CD8 T cells to produce IFNγ and exhibit cytotoxicity against the infected targets is critical for maintaining the chronicity of toxoplasma infection [10, 17, 77]. Loss of these functions, presumably even one of them may compromise the host ability to maintain the chronicity of infection. Thus, CD8 T cells possessing polyfunctional properties (like producing IFNγ and exhibiting cytotoxic activity against infected targets) most likely are best equipped to keep infection under control. In recent years, studies with virus models have shown that polyfunctional activity of virus-specific CD8 T cells, rather than the absolute numbers, correlates with the protection against the pathogen [78]. Along the same lines, we observed that concomitant with Toxoplasma reactivation and elevated CD8 mediated PD-1 expression, the frequency of polyfunctional CD8 T cells declined sharply in spleen and brain [22]. Interestingly, inhibitory receptor(s) like PD-1 are preferentially expressed on polyfunctional memory CD8 T cells, which

become apoptotic leading to attrition of this population during chronic toxoplasmosis [22]. Although anti PDL-1 therapy revived CD8 T cell response, some of the functions remained refractory to this treatment [75]. Moreover, continuous anti PDL-1 treatment was needed to prevent the mortality of the chronically infected mice (Khan et.al unpublished observations).

Possible mechanism of CD8 T cell dysfunction during chronic infection

Though the mechanism(s) for CD8 T cell dysfunction during viral infections especially LCMV have started to unravel [79], very little information related to this phenomenon during *T. gondii* infection is available. As stated earlier, unlike viral infections where CD8 T cell exhaustion occurs apparently due to persistently high viral load, during toxoplasma infection, the dysfunction is manifested long after the initial control of parasitemia. Recent studies from our laboratory suggest a key role of γ chain cytokine IL-21 in controlling CD8 T cell dysfunction in the mouse model of encephalitis. (Khan et.al, manuscript in preparation). In these animals, unlike those who do not develop encephalitis, a significant dip in cytokine levels was observed. Moreover, significantly enhanced CD8 T cell dysfunction in mice lacking IL-21R was noted. Interestingly, another γ chain cytokine IL-15, which is critical for maintenance of CD8 T cell memory [80], apparently had minimal effect on CD8 T cell exhaustion as treatment with exogenous cytokine did not rescue the cells expressing high levels of PD-1 (Khan et al. manuscript in progress). The question which demands attention is as follows: the kind of effect IL-21 has on memory CD8 T cells that are responsible for the maintenance of their functionality and long-term survival (Fig. 1). The studies conducted in our laboratory have shown that in the absence of functional IL-21, the expression of positive costimulatory molecules on CD8 T cells from infected C57BL/6 is strongly downregulated which may be the cause of their functional impairment during chronic infection (Khan et al. manuscript in progress). The importance of some of the costimulatory molecules in the survival of memory CD8 T cells has been reported [81]. It should be noted that we have demonstrated that reversal of CD8 exhaustion by anti PDL-1 administration is dependent on CD40-CD40L interaction as CD8 T cells lacking CD40 are unresponsive to the treatment [41]. Thus, a balance between positive and negative costimulatory molecules for the development and maintenance of robust CD8 T cell immunity is needed to be studied in further detail.

Defective memory cell development during *T. gondii* infection

Memory CD8 T cells are critical for long-term protection against intracellular pathogens. Although during chronic toxoplasmosis, the majority of *T. gondii*-specific polyfunctional CD8 T cells exhibit cardinal markers of memory phenotype (CD44 and CD127) [22]. They show high PD-1 expression and on subset-specific analysis; they also express high levels of CD43, a marker of effector cell lineage. Memory cells expressing this marker have been reported to exhibit poor recall response [82] which is a hallmark of conventional population. It is important to note that in a recent study using a *Listeria monocytogenes* model, it has been demonstrated that the infection induces a strong central memory ($CD62L^{hi}$) CD8 T cell response and the cells have stem cell-like properties. These central memory cells are highly potent in term of recall response and clearance of infection [83]. Thus, it appears that during chronic infection like *T. gondii*, there is a skewed memory CD8 development which results

in the inefficient control of chronic infection leading to encephalitis in the host. Rather than subscribing to the view that the chronic stage of infection is represented by bradyzoites containing cysts which are quiescent till any severe immune defect occurs in the host [84], we believe that in the presence of inadequate memory response, mild reactivation of infection may be an ongoing process in the hosts suffering from chronic toxoplasmosis. The dissemination of parasites, although controlled by the non-conventional memory CD8 T cells, is not sufficient to clear the infection. Thus, lack of development of conventional central memory population is needed to restrict the chronic infection and has strong therapeutic implications.

Conclusions and perspectives

There is little doubt that CD8 T cells are critical for long-term protection against *T. gondii* infection, especially during the chronic phase of the disease. Although the infection generates a robust CD8 T cell effector response during acute infection, long-term immunity is compromised due to exhaustion of memory cells. For vaccine-based studies, CD8 T cell population needs to be targeted and the measures that will allow to maintain the functionality of memory cells need to be investigated. It is also very important to identify dominant class I restricted epitopes for *T. gondii* recognized during acute and chronic toxoplasmosis. Some of the work in this direction is being conducted in McLeod laboratory using human transgenic mice approach [85, 86]. However, the biggest challenge in the encephalitis model remains the dysfunctionality of memory CD8 T cell population. Without reversing the phenomenon of exhaustion, robust long-term CD8 T cell immunity against chronic infection will not be achievable and host will remain susceptible to encephalitis. Although blockade PD-1 receptor (a major inhibitor molecule) ameliorates the dysfunctionality of CD8 T cells and ensures the survival of infected animals, long-term treatment is needed, which can have severe consequences like autoimmune reactions in the host. However, recent studies in our laboratory have shown that combinatorial treatment with anti PDL-1 and antibody to other inhibitory receptor LAG-3 is more effective (Hwang et al. manuscript in progress). Thus, it is very essential to evaluate the pattern of inhibitory receptors expressed by highly exhausted CD8 (PD-1^{hi}) expressing memory T cell population during chronic toxoplasmosis. Most likely, cocktail of antibodies against multiple inhibitory receptors like PD-1, LAG-3, and Tim3 may be needed to rescue this population (Fig. 2). Thus, short and effect treatment with antibody cocktail against these multiple inhibitory receptors, which restore memory CD8 T cell population in chronically, infected hosts' needs to be worked out.

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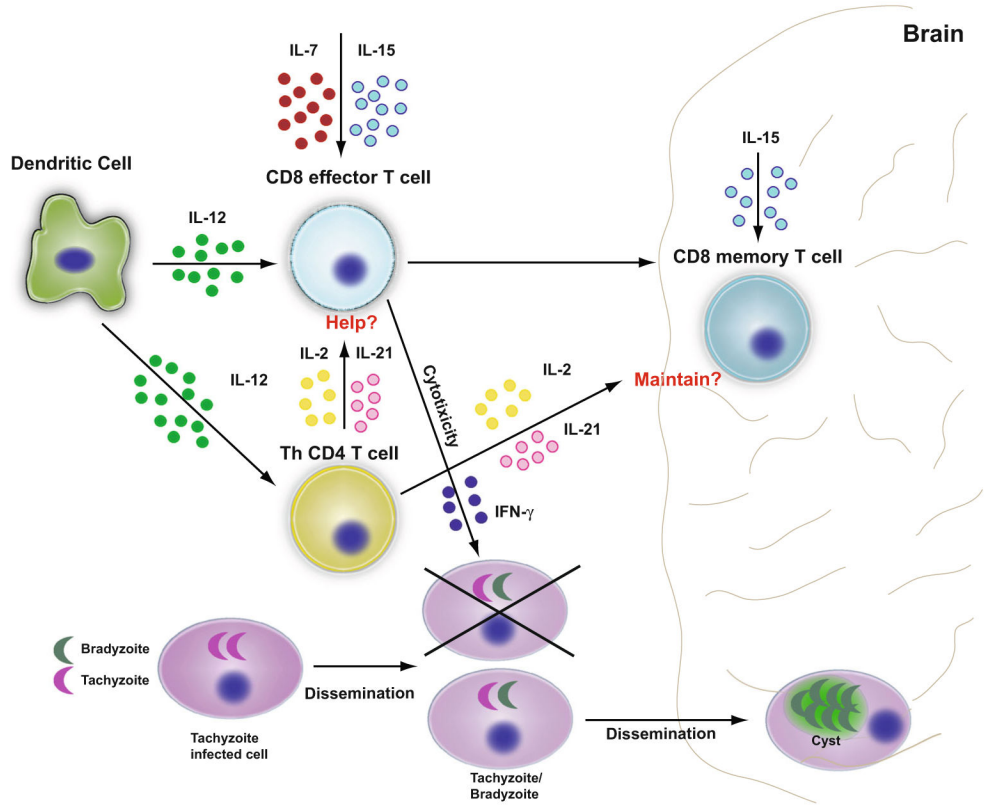

Fig. 1.

CD8 T cells immunity during *T. gondii* infection. During acute toxoplasma infection, strong IL-12 release by dendritic cells generates robust CD8 T cell effector response. The cytokine is also responsible for polarized Th1 response, which most likely helps in the expansion of effector CD8+ T cell immunity. While IL-7 and IL-15 (both γ chain cytokines) are required for the CD8 T cell effector development, maintenance of long-term memory response is dependent on IL-15. CD8 T cells secret IFNγ, which can inhibit the parasites replication and also eliminate the infected cells by their cytolytic ability. Under immunological pressure the disseminated tachyzoites convert to bradyzoites and remain apparently in a quiescent state in the tissues especially brain. Possible role of cytokines like IL-2 and IL-21 produced primarily by CD4 in this scenario needs further elucidation

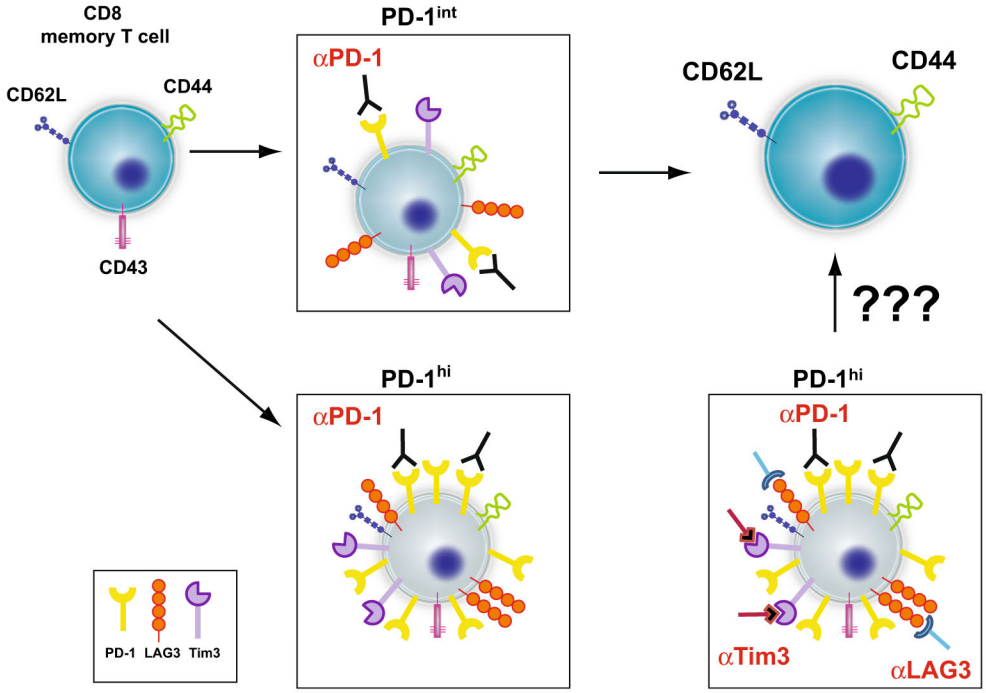

Fig. 2.

Restoration of CD8 T cell memory against chronic toxoplasmosis by inhibitory receptor blockade. CD8 memory Tcells ($CD62L^{hi}$ $CD44^{hi}$ $CD43^{hi}$) generated during chronic toxoplasma infection exhibit increased expression of inhibitory molecule, especially PD-1. The blockade of PD-1-PDL-1 interaction by antibody treatment is able to restore the functionality of $PD\text{-}1^{inter}$ memory cells but fail to reverse the exhaustion in $PD\text{-}1^{hi}$ expressing population. Treatment with cocktail, comprising of antibodies against multiple receptors TIM-3 and LAG-3 in addition to anti PDL-1 may be needed for rescue of highly exhausted CD8 T cells ($PD\text{-}1^{hi}$) population