Manipulating the PD-1 pathway to improve immunity

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Abstract

PD-1 is an inhibitory receptor induced in T cells by antigen stimulation and sustained PD-1 expression plays a key role in T cell dysfunction. Blocking PD-1 signaling rescues exhausted T cells and is an effective treatment for chronic infections and cancer. Nonetheless, combining PD-1 pathway blockade to therapeutic vaccination should further improve T cell rescue. PD-1 is induced shortly after T cell priming, but little is known about the role of PD-1 in the initiation of immune responses. In addition, the PD-1 pathway may also modulate humoral responses, since both B cells and Tfh cells express PD-1. Therefore, even though much progress has been achieved by manipulation of the PD-1 pathway to rescue exhausted T cells, this powerful immunotherapy could still be further exploited.

Introduction

Programmed cell death (PD)-1 (CD279) is an inhibitory receptor that belongs to the CD28/CTLA-4 family [1,2]. Activated T cells transiently express PD-1 but sustained PD-1 expression is associated with T cell dysfunction [3]. T cell exhaustion and the role of PD-1 in chronic infection were first described in mice during lymphocytic choriomeningitis virus (LCMV) infection and later shown to occur in several situations of antigen persistence in mice, non-human primates and humans [4]. Importantly, blockade of the PD-1 pathway restores function in exhausted T cells and was recently used to treat patients with advanced cancer with promising results [**5–7].

In this review we will briefly summarize the current understanding on the role of the PD-1 pathway in adaptive immunity. We will then discuss the potential applications for PD-1 pathway blockade to improve immune responses, both in prophylactic and therapeutic settings.

PD-1 and its ligands

PD-1 function has been best characterized in T cells. The cytoplasmic domain of PD-1 contains both an immunoreceptor tyrosine-based inhibitory motif (ITIM) and

immunoreceptor tyrosine-based switch motif (ITSM) [8]. PD-1 conveys negative signals through ITSM recruitment of SH2-domain containing tyrosine phosphatase (SHP2) that dampens TCR signaling [9–11] (Fig. 1). PD-1 expression can also be detected on B cells, NK and NKT cells, as well as on monocytes/macrophages and dendritic cells (DCs) [1,12]. However since no mechanism for PD-1 signaling without association with antigen receptors has been described, the role of PD-1 in non-lymphocytes still requires further study.

PD-1 binds PD-L1 (also know as B7-H1 or CD274) or PD-L2 (also known as B7-DC or CD273). PD-L1 is widely and constitutively expressed on numerous cells such as T and B cells, DCs, macrophages and also in non-hematopoietic cells (e.g. endothelial cells and lymphoid stromal cells). PD-L2 expression is much more restricted: it is expressed on some B cell subsets [13] and can be inducibly expressed on DCs, monocytes and macrophages depending on the cytokine milieu [1,2]. PD-1 ligands have short cytoplasmic tails with no known signaling motifs. However there are some reports showing effects on PD-L1-expressing cells after interaction with PD-1, but how this reverse signaling takes place remains unsolved [2]. Of note, PD-L1 also binds B7-1 (CD80) and PD-L1/B7-1 interactions are reported to be of inhibitory nature [14,15].

PD-1 expression and regulation of T cell responses

PD-1 expression is induced by antigen receptor ligation [9,16]. TCR stimulation leads to nuclear translocation of NFAT, and NFATc1 (NFAT2) binds to the promoter region of the PD-1 gene inducing its transcription [17]. PD-1 is transiently expressed in viral-specific CD8 T cells after infection but once the infection is resolved and there is no more TCR signaling, PD-1 expression decreases [3,18]. Conversely, PD-1 expression is maintained during chronic infections. Sustained PD-1 expression is maintained primarily due to continuous TCR ligation [19,20]. TCR signals promote demethylation of regulatory regions of the PD-l locus. After prolonged antigen stimulation, the PD-1 locus fails to be re-methylated resulting in an open chromatin state poised for expression [18]. Additionally, T-bet binds upstream of the PD-1 gene and represses its transcription. And since persistent TCR stimulation downregulates T-bet, low T-bet expression is another mechanism that helps maintain PD-1 transcription [21]. Cytokines may also further enhance PD-1 expression [22,23].

High PD-1 expression is a hallmark of dysfunctional T cells found in chronic infections and cancer. Importantly, interfering with the PD-1 pathway rescues function in exhausted T cells [2,4]. It was first shown by our group that administration of anti-PD-L1 (or anti-PD-1) blocking antibodies to mice chronically infected with LCMV, increased the number and function of LCMV-specific CD8 T cells and promoted viral control [3]. In the same way, PD-1 blockade improved immunity in macaques chronically infected with simian immunodeficiency virus (SIV) [24]. Likewise, HIV-specific CD8 T cells express PD-1, which correlates with T cell dysfunction and disease progression. And in vitro blockade of the PD-1 pathway improves function of HIV-specific CD8 and CD4 T cells from chronically infected patients [25]. Similar observations were also described in hepatitis C virus infected patients [12,26]. And more recently, in a humanized mouse model of HIV infection, PD-L1 blockade dramatically suppressed HIV viremia and restored CD4 counts, even after

treatment cessation [27]. Besides viral infections, PD-1 signaling plays a major role in other microbial infections [2], such as infection with the pathogenic fungus histoplasma capsulatum [28] and *Plasmodium* parasite during chronic malaria [*29]. Thus PD-1 plays a major role in T cell exhaustion during chronic infections and blockade of the PD-1 pathway can restore T cell function and promote pathogen control.

Dysfunctional CD8 T cells in various cancers also express PD-1 [30–32]. Recently two different antibodies that interfere with the PD-1 pathway have been used to successfully treat patients with advanced cancer. After treatment, durable anti-tumor responses were observed in patients with treatment-refractory melanoma, renal-cell cancer or non-small-cell lung cancer. Remarkably, when stratified, 36% of patients with PD-L1-expressing tumors had an objective response to PD-1 pathway blockade [**5–7]. The results from those clinical trials are extremely promising and subsequent studies involving more patients and the use of predictive biomarkes are highly anticipated.

PD-1 blockade to improve prophylactic vaccination

Interfering with the PD-1/PD-L1 pathway during the early stage of immune responses can result in improved T cell responses. In mice, PD-L1 blockade during acute herpes simplex virus (HSV)-1 infection increases the magnitude and polyfunctionality of effector HSV-specific CD8 responses and improves recall to secondary HSV infection [33]. In rhesus monkeys, blockade of PD-1 during immunization with adenovirus vector type 5 encoding SIV-Gag improves Gag-specific CD8 T cell responses [34].

The hypothesis is that without PD-L1/PD-1 interactions, antigen presenting cells (APCs) can provide stronger stimulation to T cells. This hypothesis is supported by experiments showing a significant enhancement of CD8 and CD4 T cell responses to LCMV infection when hematopoietic cells lack PD-L1 [35]. Costimulatory molecules act like a rheostat to modulate T cell activation: positive costimulatory molecules reduce the TCR signaling threshold necessary for T cell activation, whereas inhibitory molecules restrict T cell activation. So ultimately, T cell activation occurs when there are more positive than negative signals. As a result, blockade of the PD-1 pathway has more significant effects in promoting T cell activation during conditions of sub-optimal antigen presentation such as with low antigen dose or with weak or low numbers of APCs [36,37]. Therefore suboptimal immunization strategies would benefit the most from blockade of the PD-1 pathway.

Even though PD-1 pathway blockade is an attractive strategy to improve prophylactic vaccination, few studies have focused on the PD-1 pathway during early stages of T cell responses. And most importantly, mainly CD8 T cell responses have been assessed. Still, most effective vaccines rely on the development of neutralizing antibodies, and the role of the PD-1 pathway on B cells and CD4 T cell differentiation has been relatively neglected.

Among conventional CD4 T cells, follicular helper cells (Tfh) express the highest levels of PD-1. Tfh are key cells to provide B cell help and promote the germinal center (GC) reaction. Tfh cells are required for differentiation of long-lived plasma cells and production of high affinity antibodies [38,39] (Figure 2A). Although Tfh cells have been extensively

studied in recent years, the role of PD-1 in CD4 T cell differentiation remains largely unexplored. The current thought is that Tfh cells express high levels of PD-1 due to continuous TCR triggering by interactions with cognate B cells. In Tfh cells, PD-1 most likely also reduces TCR signaling and duration of cognate interactions, however this has not been formally demonstrated. Interestingly, PD-1 may aid Tfh cells by limiting IL-2 production. IL-2 is deleterious to Tfh because it induces Blimp-1, which in turn antagonizes Bcl6, the Tfh master regulator [40,41]. It has been shown that PD-1 deficient Tfh cells have altered cytokine secretion, with decreased IL-21 production [13,42]. And mice deficient in PD-1 have dysfunctional Tfh cells that cannot appropriately select IgA B cells in germinal center of Peyer's patches [42]. Thus PD-1 signaling contributes to CD4 T cell differentiation and function of Tfh cells.

The in vivo role of PD-1 signaling specifically in B cells also deserves further investigation. PD-1 is upregulated in activated B cells and germinal center B cells, and GC B cells also express PD-L2 and PD-L1 [13,16,43] (Figure 2A). Similar to T cells, PD-1 ligation inhibits B cell receptor (BCR) signaling by recruitment of the phosphatase SHP2 [8] and PD-1 deficient B cells have increased proliferation upon BCR ligation [44].

It was originally reported that PD-1 KO mice produce higher levels of antibodies after immunization with a T-cell independent antigen, but no differences are observed between wild type and PD-1 deficient mice after immunization with a T-cell dependent antigen [44]. On the contrary, it was more recently shown that mice deficient in components of the PD-1 pathway have diminished B cell responses to alum/protein T-cell dependent immunization. In this study, long-lived plasma cells were reduced when B cells lacked PD-L1 and/or PD-L2, or when T cells lacked PD-1 [13]. However, another study found increased germinal center responses (and Tfh cells) in PD-L1 deficient mice after infection with the parasite *Schistosoma mansoni* or immunization with protein in complete Freund's adjuvant [45]. Different requirements for Tfh differentiation depending on the immunization protocol might explain these conflicting data. Alternatively, differences in gut flora on PD-1 deficient mice may also distinctively affect general immunoreactivity of different mouse colonies [42].

Importantly, experiments with mice deficient in components of the PD-1 pathway or with antibody blockade cannot clearly address the mechanisms whereby PD-1 signaling modulates humoral responses. Thus more complicated experiments, such as those performed by Good-Jacobson et al. [13], are necessary to address the intrinsic role of PD-1 in specific cell types. Additionally, it is important to consider that PD-1 pathway blockade may affect B cell responses in a different way whether the blockade happens at early (during Tfh differentiation and GC reaction) or late stage (memory) of immune responses.

Moreover, regulatory CD4 T cells (Tregs) express both PD-1 and PD-L1 and those molecules may play a role in Treg suppressive activity [46]. For example it has been shown that Tregs suppress autoreactive PD-1+ B cells through interactions with PD-1 ligands [47]. Importantly, some Tregs can express CXCR5 and suppress germinal center reactions [48] (Figure 2B). And it was recently reported that PD-1 inhibits CXCR5+ Tregs, suggesting that

PD-1 pathway blockade may decrease humoral responses through enhancement of CXCR5+ Treg function [43].

In conclusion, the role of PD-1 pathway on humoral responses is complex and may depend on the model or disease as well as the stage of the response, and further studies need to be performed to clarify the mechanisms involved.

Additionally, the benefits of PD-1 pathway blockade during prophylactic vaccination would have to outweigh the therapy costs and also risks of potential autoimmune side effects. Hence, more studies have focused on blockade of the PD-1 pathway in a therapeutic setting, in situations of cancer or chronic infections.

PD-1 blockade to improve therapeutic vaccination

During chronic infections and cancer several immunosuppressive mechanisms are in place. Increased expression of immunosuppressive molecules (such as IL-10 and TGF-β), as well as increase recruitment and differentiation of regulatory T cells and myeloid-derived suppressor cells have all been shown to contribute to inhibition of T cell responses [49*]. In addition, besides PD-1, antigen-specific T cells co-express other inhibitory receptors such as Tim-3, LAG-3, 2B4 and CTLA-4, which maintain T cell exhaustion [50].

To overcome immunosuppression and elicit an effective immune response capable of controlling pathogens and tumors is not an easy task and will probably require combination therapies. For example, blockade of PD-1 signaling in combination with blockade of other inhibitory receptors has shown additive effects in different models of chronic infection and cancer [*29,51–54]. Likewise, in chronic LCMV infection, blockade of PD-L1 and IL-10 was more effective than either therapy alone at rescuing function of exhausted CD8 T cell responses and promoting viral control [55].

Another approach to reinvigorate the immune system consists of combining blockade of suppressive pathways to strategies that boost immune responses, such as therapeutic vaccination. Therapeutic vaccination is based on the assumption that introducing antigens in an immunogenic form can stimulate immune responses to clear infected or tumor cells. Strategies used in therapeutic vaccination to introduce antigen include: DCs, DNA, modified recombinant viruses, and peptides or proteins with adjuvants [49*].

However, therapeutic vaccination in cancer or chronic infections has so far only shown very modest results. Perhaps not surprisingly, given the number of immunosuppressive mechanisms that preserve immune escape of tumors and infected cells. Furthermore, even if therapeutic vaccination strategies would achieve effective activation of antigen-specific T cells, target cells would still need to be eliminated by PD-1 expressing T cells. Since PD-L1 is overexpressed during inflammation associated with chronic infections and tumors [56–59], only improving T cell responses, without manipulation of the PD-1 pathway may not be enough to successfully control infections or tumors.

Distinctively, blockade of the PD-1 pathway improves effector responses, not only by increasing T cell activation by APCs, but also by unleashing their ability to act upon target

cells (Figure 3). To investigate the differential role of PD-L1 on hematopoietic cells and nonhematopoietic cells, Mueller et al. generated bone marrow chimera mice where either hematopoietic cells or nonhematopoietic cells were genetically deficient in PD-L1 expression. In this system, after chronic LCMV infection, CD8 T cells had a greater ability to control viral replication in mice where nonhematopoietic cells lacked PD-L1 [35]. Furthermore, PD-L1 expression on target cells has been shown to directly inhibit the lytic activity of cytotoxic CD8 T cells [57,60].

Thus combining PD-1 blockade to therapeutic vaccination would improve T cell priming and control of tumors or infected cells. Confirming this conceptual idea, therapeutic vaccination, of LCMV chronically infected mice, with recombinant vaccinia virus expressing LCMV-GP33 peptide was highly effective to stimulate CD8 T cell responses and reduce viral load, when combined with anti-PD-L1 antibodies [61]. Likewise, PD-1 pathway blockade improved immunotherapy consisting of irradiated tumor cells secreting granulocyte macrophage colony stimulating factor (GM-CSF) in mouse models of melanoma and colon carcinoma [62]. And complete melanoma regression in mice was only achieved when therapeutic vaccination with adenovirus encoding tumor antigen and 4-1BB co-stimulation were combined with blockade of the PD-1 pathway [63].

Following this same direction, several clinical trials have been planned to test the safety and effects on tumors of PD-1 pathway blockade combined to therapeutic vaccination. For example, PD-1 pathway blockade will be given in conjunction to dendritic cell/tumor fusion vaccines in the treatment of renal cell carcinoma and multiple myeloma (NCT: NCT01441765, NCT01067287). Also, a combination of PD-1 pathway blockade with tumor peptides in oil-based adjuvants will be tested in advanced melanoma patients (NCT: NCT01176474, NCT01176461). The results from those clinical trials should further advance the use of combinatorial immunotherapy for the treatment of cancer patients.

Conclusions

Blockade of the PD-1 pathway has proved to be one of the most effective strategies to rescue function of exhausted T cells. The recent cancer clinical trials with PD-1 pathway blockade are very promising and should drive the use of this therapy into other clinical applications. The PD-1 pathway suppresses T cells responses in lymphoid organs but also in the periphery, since non-hematopoietic cells also express PD-L1. This important and unique feature of PD-1/PD-L1 interactions probably underlines the positive results observed in advanced cancer patients subjected to PD-1 blockade therapy. Although there is an understanding on the role of the PD-1 pathway in CD8 T cell exhaustion during chronic stimulation, much less is known about the early phase of immune responses or in other cell types. Thus, manipulation of the PD-1 pathway to improve prophylactic vaccination remains to be fully explored. Likewise a better understanding of the role of the PD-1 pathway on CD4 T cells and B cells is imperative to fully exploit this powerful therapy.

Acknowledgments

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Highlights

- Blocking PD-1 signaling rescues exhausted T cells in chronic infections and cancer

- PD-1 pathway blockade unleashes effector T cell functions on target cells

- Combining PD-1 pathway blockade to therapeutic vaccination improves T cell rescue

- PD-1 pathway may regulate humoral immunity, but mechanisms are not well established

- Further studies on how PD-1 modulates initiation of immune responses are needed
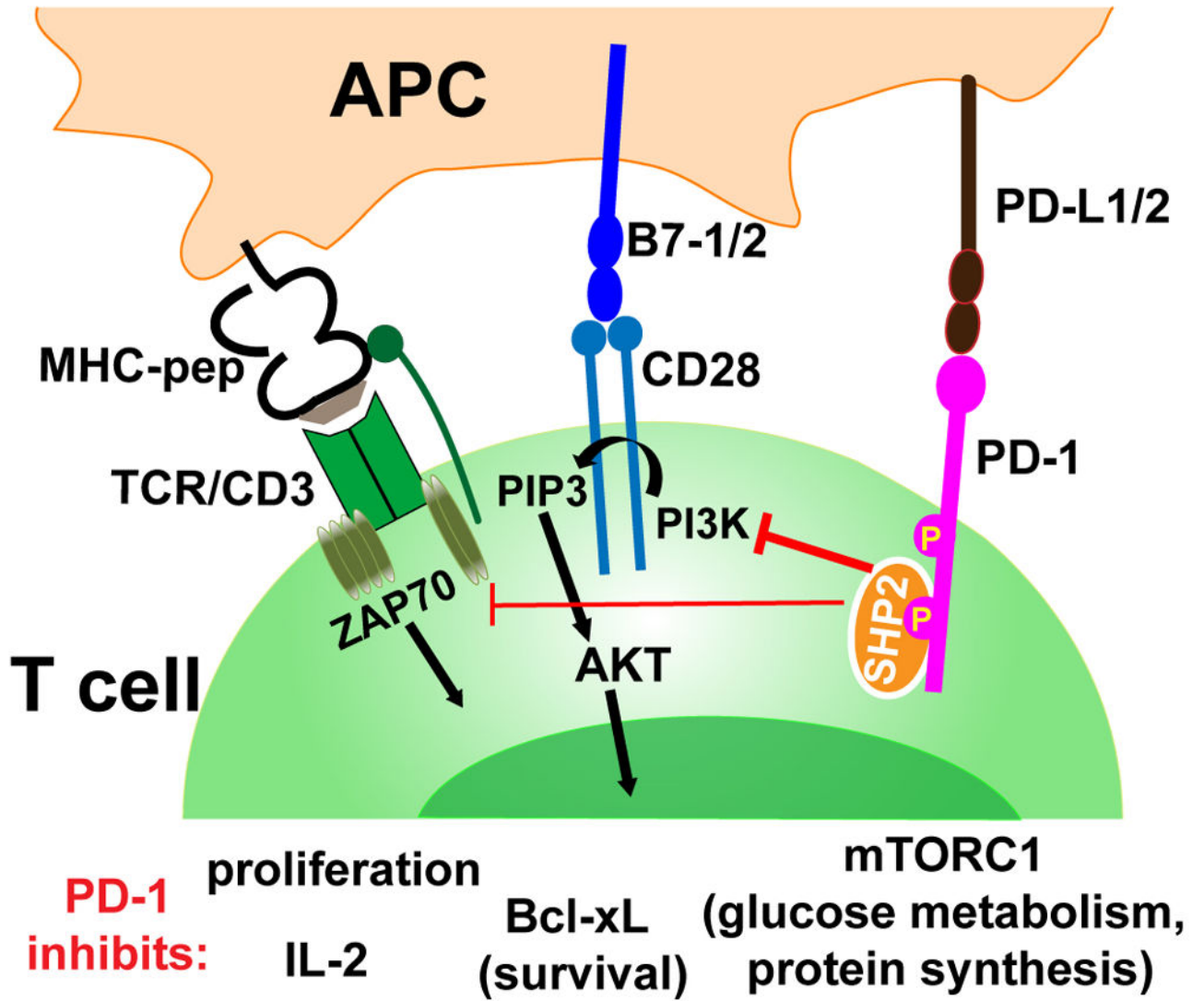
Figure 1. PD-1 inhibits TCR/CD28 signaling

Upon engagement with PD-L1 or PD-L2, PD-1 is phosphorylated at both thyrosine containing motifs ITIM and ITSM. The phosphatase SHP2 (and possible SHP1 as well) is recruited to phosphorylated ITSM. SHP2 dephosphorylates phosphatidylinositol-3-kinase (PI3K) and ZAP70/CD3ζ, ultimately attenuating TCR/CD28 signaling and T cell activation [9–11].
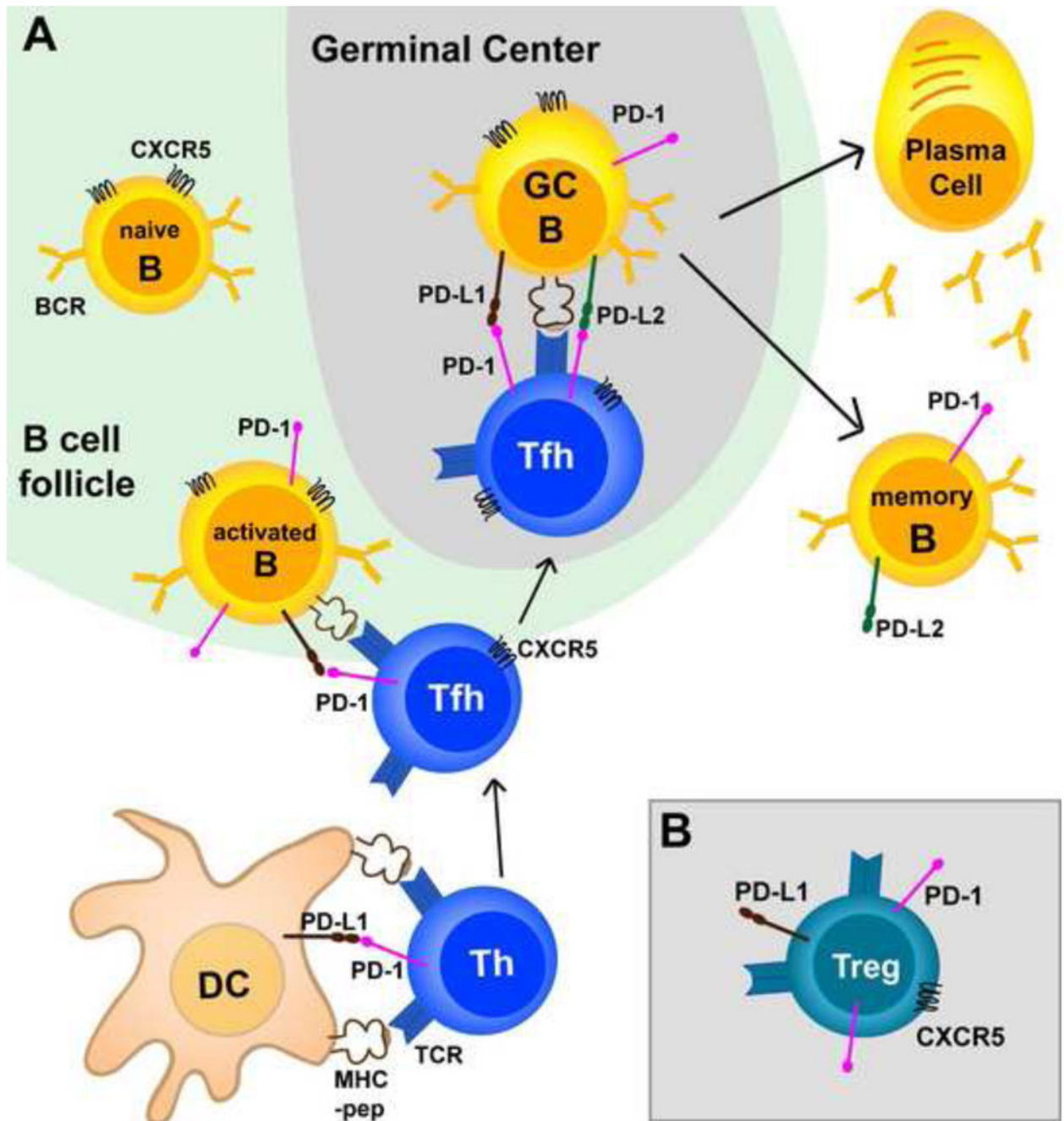
Figure 2. Potential role of the PD-1 pathway in B cell responses
A. Naïve T cells interact with dendritic cells (DCs) presenting cognate antigen and initiate commitment to different CD4 T helper (Th) lineages. Upon activation all CD4 cells express PD-1, but PD-1 expression is maintained and further increase in CD4 T cells that interact with cognate B cells and fully commit to Tfh lineage. Tfh cells express CXCR5 and migrate to the germinal center (GC) where they select high affinity B cells by providing IL-21 and CD40L. GC B cells that receive T cell help survive and differentiate into memory and plasma cells. Reports have shown that B cells express PD-1 upon activation, and PD-1

expression is maintained in GC and memory B cells. In addition, it has been reported that GC and memory B cells express PD-L2. PD-L1 expression is ubiquitous and was shown in the figure only in situations where engagement with PD-1 has been demonstrated to occur [13,38–43] **B.**PD -1 is expressed by CXCR5+ Tregs that limit GC reactions, and PD-1 engagement suppresses Tregs [43,48].
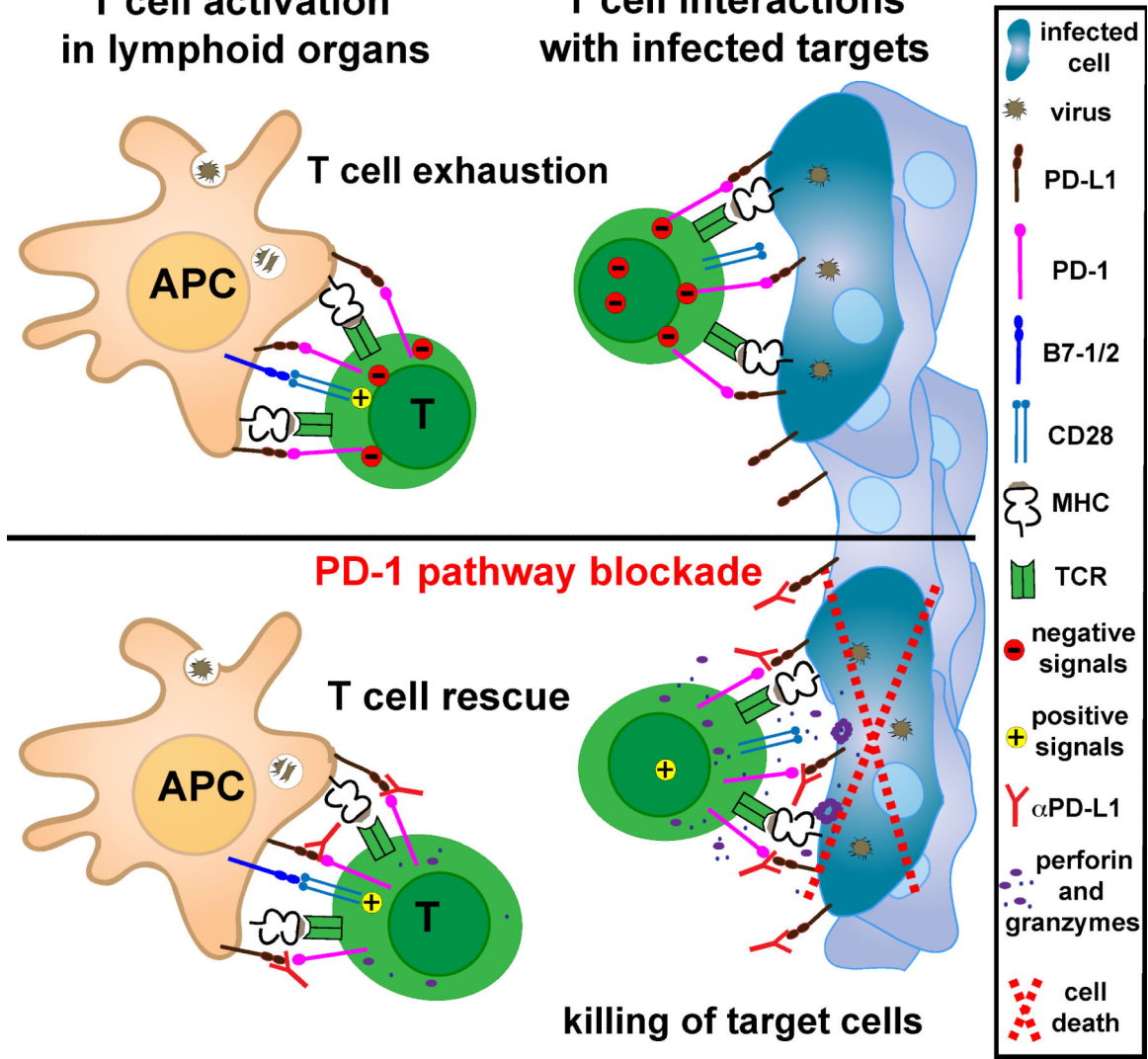
Figure 3. PD-1 pathway blockade promotes T cell activation and elimination of infected cells
During chronic infections, antigen specific T cells are exhausted due to PD-1 inhibitory signals and lack of positive co-stimulation. PD-1 pathway blockade promotes T cell activation by shifting the balance of signals delivered by cognate APCs from suppressive to activating. When rescued T cells recognize antigen in the periphery, in the absence of PD-1 engagement, T cells can assume full effector function and eliminate target cells [35]. Similar mechanisms also occur in cancer patients.