The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses

· of special interest:

- Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, Wong JC, Satkunarajah M, Schweneker M, Chapman JM, et al.: **Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection**. *J Exp Med* 2008, **205**:2763-2779.

  In this paper, the authors show that HIV-specific T cells from the peripheral blood of HIV-infected individuals expressed Tim-3 and that blocking Tim-3 signaling enhanced the function of the HIV-specific T cells. Given the recent studies examining PD-1 and CTLA-4 on HIV-specific T cells, this study suggests a third inhibitory molecule that may be important in developing therapies to HIV.

- Cai G, Anumanthan A, Brown JA, Greenfield EA, Zhu B, Freeman GJ: **CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator**. *Nat Immunol* 2008, **9**:176-185.

  This paper demonstrates that CD160 is a receptor for HVEM and that, similar to BTLA, binding of HVEM to CD160 results in negative signaling to the T cell resulting in inhibition of proliferation and cytokine production.

- Rowe JH, Johanns TM, Ertelt JM, Way SS: **PDL-1 blockade impedes T cell expansion and protective immunity primed by attenuated Listeria monocytogenes**. *J Immunol* 2008, **180**:7553-7557.

  Blockade of PD-1 was examined to determine its role in regulating T cell responses to acute infection with Listeria monocytogenes. Surprisingly, blockade of this pathway resulted in reduced primary and secondary T cell responses. This paper is significant important because it not only demonstrated that inhibitory receptors can regulate T cell responses to acute infection but that the role of the PD-1 pathway may not be negative in all cases.

·· of outstanding interest:

- Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, Subramaniam S, Blattman JN, Barber DL, Ahmed R: **Molecular signature of CD8+ T cell exhaustion during chronic viral infection**. *Immunity* 2007, **27**:670-684.

  Gene expression profiles of LCMV-specific T cells from acute and chronic LCMV-infected mice were examined and compared with naïve T cells. A total of 490 genes were upregulated or downregulated by at least 2-fold in the exhausted $CD8^+$ T cells identifying a number of inhibitory receptors, transcription factors and metabolic pathways that may be novel targets for immunotherapy.

- Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, Betts MR, Freeman GJ, Vignali DA, Wherry EJ: **Coregulation of CD8(+) T cell exhaustion by multiple inhibitory receptors during chronic viral infection**. *Nat Immunol* 2008.

  This paper demonstrates that exhausted CD8 T cells from chronically infected LCMV mice upregulate a wide range of inhibitory receptors. Furthermore, *in vivo* blockade of two of the molecules (PD-L1 and LAG3) show that whilst aLAG3 alone had no effect on the frequency of LCMV-specific CD8 T cells, dual blockade had a greater effect than aPD-L1 alone. Moreover, dual blockade also had a greater effect on rescuing of CD8 T cell function than aPD-L1 alone leading to significantly lower viral titers. This study shows inhibitory receptors can work synergistically and suggests that combination therapies have considerable potential.

- Kaufmann DE, Kavanagh DG, Pereyra F, Zaunders JJ, Mackey EW, Miura T, Palmer S, Brockman M, Rathod A, Piechocka-Trocha A, et al.: **Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction**. *Nat Immunol* 2007, **8**:1246-1254.

  Kaufmann et al demonstrated that $CD4^+$ T cells from the blood of HIV-infected individuals co-express PD-1 and CTLA-4. CTLA-4 appears to be upregulated specifically by HIV-specific CD4+ T cells but not CD8+ T cells suggesting that inhibitory receptors can differentially regulate CD4 versus CD8 T cell responses. Both PD-1 and CTLA-4 negatively regulated the function of HIV-specific CD4 T cells.

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Summary

T cell responses are regulated by integrating positive and negative signals from costimulatory and inhibitory receptors. While the function of specific T cell costimulatory molecules during infections has been appreciated for some time, recent observations have now revealed a critical role for inhibitory receptors in regulating T cell responses to pathogens, especially during chronic infections. A key emerging principle is that there is considerable diversity in the number and type of inhibitory receptors that can be expressed by T cells during both acute and chronic infections. These distinct inhibitory pathways appear to cooperate in regulating T cell function, could have distinct mechanisms of action, and are likely to provide novel therapeutic targets during persisting infections and other diseases.

Introduction

Upon infection, naïve antigen-specific T cells become activated, differentiate and develop into functional effector and memory T cells or, during chronic infections, exhausted T cells. T cells integrate multiple signals during differentiation, but the precise pathways that control optimal T cell memory versus exhaustion remain incompletely understood. This review focuses on our current understanding of inhibitory receptors during T cell responses following acute versus chronic viral infections. In discussing these inhibitory receptors and their roles in regulating functional and dysfunctional T cell responses during infection it is informative to also examine the role of costimulatory pathways.

Initial activation of a naïve T cell occurs upon interaction of the TCR with specific peptide presented by MHC molecules. This is the major signal for T cell activation and differentiation (signal 1), but additional signals from costimulation (signal 2) and inflammation (signal 3) are required for an effective T cell response. In the absence of coordinated stimulation from signals 1, 2, and 3, proper T cell activation and differentiation does not occur [1-3]. For example, TCR signaling in the absence of signal 2 from costimulation induces T cell anergy *in vitro* [2]. *In vivo*, a poor costimulatory environment or a preferential engagement of coinhibitory pathways during T cell priming can lead to tolerance [4],[5]. Several years ago the model for T cell activation versus anergy or tolerization was a simple one. Our developing understanding of signal 3 from inflammation and the increased number and diversity of costimulatory and inhibitory receptors is revealing the decision between full activation and tolerance/dysfunction to be more complex. We are only just beginning to understand the interplay between different costimulatory and inhibitory receptor signals and how they regulate initial T cell activation and division, clonal deletion, the generation and maintenance of memory T cells and T cell exhaustion.

Diversity of costimulatory and inhibitory receptors on T cells

Recent work has highlighted the diversity of costimulatory and inhibitory molecules on T cells (Table 1). Many of these receptors are part of the Ig superfamily and specifically the CD28:B7 subfamily [1]. CD28 family members include the costimulatory receptors CD28 and ICOS and the inhibitory receptors CTLA-4, PD-1 and BTLA. The TNF:TNFR family also contains several costimulatory molecules such as CD27, CD40L, 4-1BB, OX40 and LIGHT that bind the ligands CD70, CD40, 4-1BBL, OX40L and HVEM respectively [6,7]. Interestingly, the CD28 family member BTLA and another Ig superfamily molecule CD160 bind the TNF family member HVEM in unusual examples of cross-family interactions [8,9]. Inhibitory receptors can bind the same ligand as a costimulatory molecule, as is the case for CD28 and CTLA-4

binding B7-1 and B7-2 or LIGHT, BTLA and CD160 binding HVEM or unique ligands not shared with a costimulatory receptor (e.g. PD-1 and PD-L1 and PD-L2) [1]. These interactions can be complex and PD-L1, for example, has recently been demonstrated to bind to B7-1 and deliver a bidirectional inhibitory signal [10].

The receptors and ligands described above are major players in T cell activation and inhibition. However, the number of cell surface molecules that can regulate T cell responses is ever increasing. Recently, molecules including LAG-3 [11], 2B4/CD244 [12,13], CD30-CD30L [6], NK receptors [14], the Tim molecules (Tim1-4) [15,16], CD200R [17], Siglecs [18] and others have been implicated in regulating T cell responses. It is currently unclear to what degree there is functional overlap or specificity in these pathways and whether there are common themes and principles about costimulation and inhibition by these different inhibitory receptors.

Costimulatory and inhibitory receptors during infections

Costimulatory and inhibitory receptors are thought to help the immune system balance productive immune responses to foreign antigens versus tolerance to self-antigens. Indeed, a number of costimulatory pathways can augment T cell responses to acute infections. During some viral infections, the absence of costimulatory pathways can lead to a substantial quantitative reduction in the primary antiviral T cell response [1,19] though the level of costimulation required can depend on the type of infection. Primary T cell responses to influenza virus are substantially diminished in mice lacking various costimulatory molecules while T cell responses to acute LCMV infection are only modestly reduced in the same KO mice [1,6]. Similar examples for other infections also exist [20,21].

While there is considerable evidence that inhibitory receptors can influence T cell responses to self antigens [5], the role of inhibitory receptors in T cell priming and memory T cell differentiation during acute infection is less clear. CTLA-4 KO CD8 T cells respond normally to acute LCMV infection [22] and CTLA-4 expressing T cells appear to have normal proliferative responses and effector function during acute LCMV or vaccinia virus infection [23,24]. However, inhibitory receptor pathways can negatively regulate immunity to rabies virus, adenovirus and Sendai virus [11,25,26]. During acute infection with rabies virus, the absence of PD-L1 led to better control of virus and a greater accumulation of CD8 T cells in the CNS [25]. When PD-1 KO mice were infected with adenovirus, T cells expanded more in peripheral tissues leading to rapid clearance of the virus [26]. LAG-3 can also regulate acute T cell responses; LAG-3 KO mice infected with Sendai virus had increased numbers of virus-specific CD4 and CD8 T cells 30 days post infection [11]. While much of the data examining the role of inhibitory receptors during acute infection is consistent with a negative role for these pathways, two recent studies with *Listeria monocytogenes* (LM) infection suggest a possible alternative role for the PD-1 pathway in this setting [27,28]. In these studies, PD-L1 blockade delayed the kinetics of CD8$^+$ T cell priming, reduced the magnitude of T cell expansion and diminished secondary T cell responses [27,28]. It is unclear whether this role for the PD-1 pathway during LM infection reflects increased T cell death when PD-1:PD-L1 is blocked or a true positive signal through this pathway under some circumstances. Additional studies should help clarify the precise role of the PD-1 pathway in different infections. However, the majority of studies are consistent with a negative role for most inhibitory pathways during infection.

Costimulatory and inhibitory receptors during chronic infection

Many of the costimulatory receptor KO mice discussed above have profound defects in T cell responses and viral control during chronic infections [29-34] suggesting a critical role for costimulation when pathogens persist. Indeed, enhancing costimulation can in some

circumstances improve T cell response during chronic infections. For example, 4-1BB has a key role during Friend leukemia virus infection [34] and stimulation of the 4-1BB pathway led to expansion of HIV-specific CD8$^+$ T cells *in vitro* most likely by regulating CD8 T cell survival [35]. It will be interesting to define how augmenting other costimulatory signals influences T cell responses during chronic viral infections.

There is now accumulating evidence that inhibitory receptors have a prominent role in regulating T cell responses during chronic viral infections. In contrast to acute infections where T cells expand, contract and form functional, long-lived memory cells, during chronic infection T cells develop functional deficiencies that prevent optimal control of infection [36]. This loss of function or exhaustion is hierarchical, with IL-2, cytotoxicity and robust proliferation lost early, TNFα persisting for longer and IFN-γ lost only at more extreme stages of exhaustion [36]. T cell exhaustion been described during a number of chronic infections in mice as well as human infections with HIV, HBV and HCV [37-39]. These functional defects are likely a key reason for failure of immunological control of these persisting pathogens and recent insights indicate a central role for inhibitory receptors in regulating T cell exhaustion.

A functional role for inhibitory receptors during chronic infection was initially demonstrated during chronic LCMV infection where blockade of the PD-1:PD-L1 pathway rejuvenated CD8 T cell function and enhanced viral control [40]. The PD-1 pathway also appears to regulate T cell responses during other mouse models of chronic infection [41], primate infection with SIV [42,43] and in humans infected with HIV [38,44-48], HBV [37,49,50], HCV [39,51,52]; where disrupting this pathway *in vitro* improves T cell responses. *In vivo* blockade of the PD-1 pathway during SIV infection of macaques not only improves T cell (and B cell) responses, but also lowers viral load and improves survival of the primates receiving treatment [53]. Thus, there is clearly a role for the PD-1:PD-L1 pathway during chronic infections and manipulation of this pathway has therapeutic potential.

There is reason to be cautiously optimistic about the clinical promise of PD-1 pathway blockade. This intervention alone, however, does not completely restore optimal T cell function and the level of functional recovery and even the precise impact of PD-1 blockade on different effector functions and/or apoptosis differs depending on the study [42,46-48]. Some insights into the nature of partial reversal of exhaustion and the range of responses to PD-1 pathway blockade might come from recent studies demonstrating that populations of exhausted CD8 T cells are heterogeneous [54]. For example, exhausted LCMV-specific CD8 T cells with intermediate PD-1 expression were potently re-invigorated by PD-1:PD-L1 blockade, but there was little if any benefit for the PD-1^{Hi} subset of exhausted CD8 T cells [54]. A similar situation might also exist during chronic HCV infection in humans where PD-1^{Lo} HCV-specific CD8 T cells from the blood responded to PD-1 pathway blockade while PD-1^{Hi} HCV-specific CD8 T cells from the livers of the same individuals did not [55].

Gene expression profiling studies suggested the presence of a number of other known or potential inhibitory receptors on exhausted CD8 T cells [56]. In addition to PD-1, LCMV-specific exhausted CD8 T cells also expressed 2B4, LAG-3, CTLA-4, PirB, GP49 and CD160 [57]. Many of these inhibitory receptors were co-expressed by the same exhausted CD8 T cell and as the severity of the infection increased, both the amount of each receptor and the number of different inhibitory receptors expressed per cell increased [57]. Thus, the PD-1^{Hi} subset of exhausted CD8 T cells also co-expressed multiple other inhibitory receptors. Blockade of the PD-1 pathway together with a second inhibitory receptor LAG-3 demonstrated cooperation between these inhibitory pathways and led to a synergistic restoration of CD8 T cell function and viral control [57]. Several recent studies have demonstrated that HIV-specific T cells can also express more than one inhibitory receptor. Kaufmann et al showed that PD-1 and CTLA-4 are coexpressed by HIV-specific CD4$^+$ T cells [38]. Both PD-1 and CTLA-4 negatively

regulated the function of HIV-specific CD4 T cells and co-expression patterns and combined blockade of these inhibitory receptors *in vitro* suggested cooperative inhibition could occur. One interesting observation was that while CTLA-4 and PD-1 co-regulated HIV-specific CD4 T cells, HIV-specific CD8 T cells expressed substantial amounts of PD-1, but not CTLA-4 suggesting that CD4 and CD8 T cell responses could be regulated by distinct sets of inhibitory receptors. A second study recently demonstrated the expression and inhibitory role of Tim-3 on HIV-specific T cells [15]. Interestingly, Tim-3 and PD-1 were expressed by sometimes distinct and sometimes partially overlapping populations of CD8^{+} T cells depending on the subject [15]. One concept that emerges from these studies is that there is diversity in the cell surface inhibitory pathways available to negatively regulate T cell responses to chronic infections. Specific inhibitory receptors can be expressed by distinct T cell subsets resulting in differential regulation, while other inhibitory receptors are co-expressed and can operate synergistically. Thus, as with costimulatory molecules, the precise inhibitory receptors of therapeutic interest might depend on the type and severity of infection, whether CD4 or CD8 T cells are of interest as well as other factors such as the activation state of the T cell.

Mechanisms of costimulatory and inhibitory receptor pathways

A mechanistic understanding of how inhibitory receptors restrain T cell responses would not only aid in our understanding of memory T cell differentiation following chronic viral infections, but could also provide novel targets for therapeutics. Unfortunately, for many inhibitory receptors there is little information in this area. Again, examining some aspects of costimulation may provide a guide. For the best-studied costimulatory pathways such as CD28, engagement of the costimulatory receptor compliments TCR signaling both quantitatively and qualitatively. For example, CD28 signaling lowers the threshold for activation, enhances IL2 production and CD25 expression, improves survival by upregulating Bcl-$_{XL}$, and promotes progression through the cell cycle [1]. In addition, the coupling of CD28 costimulation to glucose metabolism via AKT [58] is likely a key factor in ensuring robust and sustained T cell responses upon initial activation. While costimulatory molecules of the TNF family can also enhance T cell proliferation and cytokine production and regulate Bcl-2 and Bcl-$_{XL}$ expression, they do so through different signaling pathways. For example, 4-1BB signals via TRAF1, TRAF2 and ERK [6]. Thus, while the end result of costimulation by different pathways can be similar the mechanistic intermediates can be quite distinct.

In contrast to many costimulatory pathways, the coinhibitory molecules CTLA-4 [59], PD-1 [60], BTLA [61] and CD160 [8] all inhibit IL-2 production and these four receptors as well as LAG-3 [11] can negatively regulate cell cycle progression. CTLA-4 inhibits CD28 augmented gene transcription, but does not prevent gene regulation by TCR alone [62]. In contrast, PD-1 appears to block both TCR signals [63,64] and CD28 signals [65]. Interestingly, though PD-1 and CTLA-4 both negatively regulate CD28 signaling and AKT activation, they do so by different mechanisms [65]. While the signaling mechanism for some inhibitory pathways is beginning to be defined, there is far less known about the intracellular mechanisms of action of inhibitory receptors compared to costimulatory receptors. Given the similarities in downstream targets, it is tempting to speculate that molecules such as CTLA-4 and PD-1 have evolved to specifically antagonize CD28 (and perhaps ICOS) mediated positive costimulation. It will be interesting to examine if other costimulatory pathways such as TNF receptor family members are resistant to or can overcome CTLA-4 or PD-1 mediated inhibition.

It is currently unclear whether inhibitory receptors merely block the induction of gene expression by TCR/costimulation or whether inhibitory receptor signaling could actually induce alternative gene transcription. Several models for how inhibitory receptor signals might alter gene expression in T cells are possible and are described in Figure 1. First, inhibitory receptor signaling could simply block all signals from the TCR and costimulation leading to

an attenuation or elimination of TCR induced gene expression. The effect of this inhibition would be a broad quantitative reduction in gene expression. A temporal delay in expression of inhibitory versus costimulatory receptors would likely be more consistent with an abbreviated activation signal rather than a complete block if this model were intact. Second, inhibitory receptors might selectively block some, but not all signals from TCR and costimulation. For example, while CTLA-4 and/or PD-1 could block CD28-mediated AKT activation and could recruit Shp-1, Shp-2 (and/or SHIP) to dephosphorylate LCK and other substrates, some partial TCR signaling could still occur. The effect might be the induction of only a subset of genes normally associated with T cell activation and perhaps a qualitatively altered response. A third possibility is that ligation of one inhibitory receptor (e.g. PD-1) might lead to inhibition of a specific costimulatory pathway (e.g. CD28), but leave other costimulatory pathways intact. In this situation, qualitative differences in costimulation could alter the course of T cell activation and differentiation. A fourth possibility is that inhibitory receptors actively induce or regulate gene expression independent of their inhibition of TCR or costimulatory signaling. For example, inhibitory molecules might recruit and/or activate selective signaling pathways that lead to new gene transcription (or active repression). This latter model would likely occur with some partial TCR signaling since there is little evidence that inhibitory receptor signaling has in impact without TCR engagement. These models are not mutually exclusive and these and other potential mechanisms could occur for different inhibitory receptor pathways.

Conclusion and future directions

T cells can express a diverse array of both costimulatory and inhibitory receptors and these molecules can positively or negatively regulate T cell function. In some cases pathogens have exploited negative regulatory pathways to facilitate persistence and inhibitory receptors appear to play a prominent role in T cell dysfunction during persisting infections. *In vitro* and *in vivo* blockade studies have revealed the therapeutic potential of modulating costimulatory and inhibitory receptors on T cells. A better understanding of the complexity of co-expression of inhibitory and costimulatory receptors on T cells in different settings is likely to reveal T cell functions and pathways regulated by specific receptors. It is likely there will be opportunities to therapeutically "tune" T cell responses by modulating specific receptor:ligand pathways. Since these same costimulatory and inhibitory pathways are used not only during T cell responses to pathogens, but also in anti-tumor immunity and also autoimmunity, a better mechanistic understanding of the regulation of T cell responses by diverse and co-expressed positive and negative regulatory molecules could have broad therapeutic implications.

Acknowledgments

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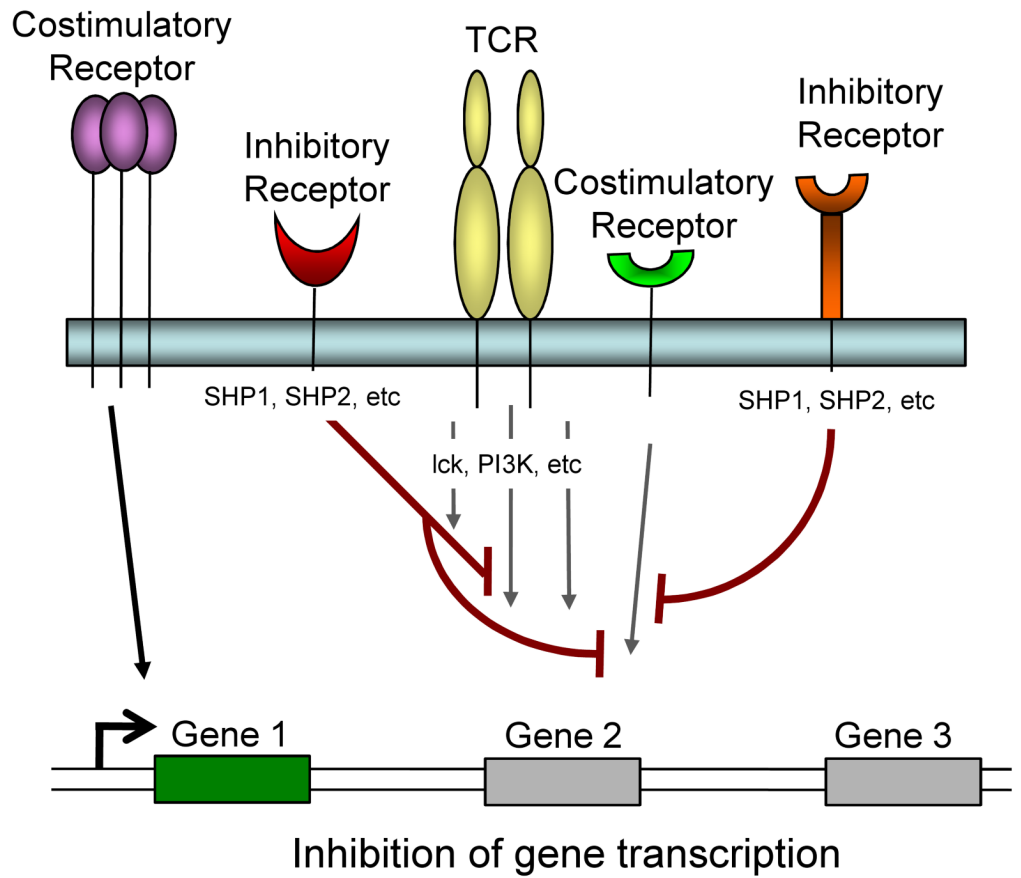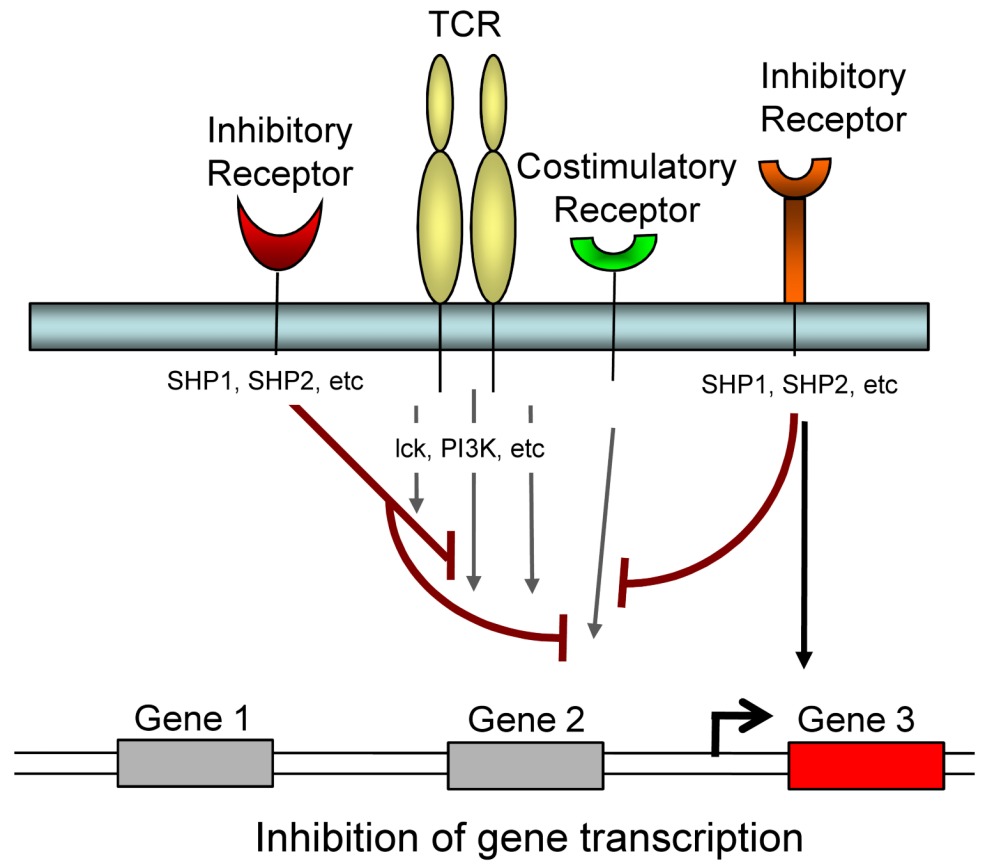
Model 1

Inhibition of gene transcription

Model 2

Inhibition of gene transcription
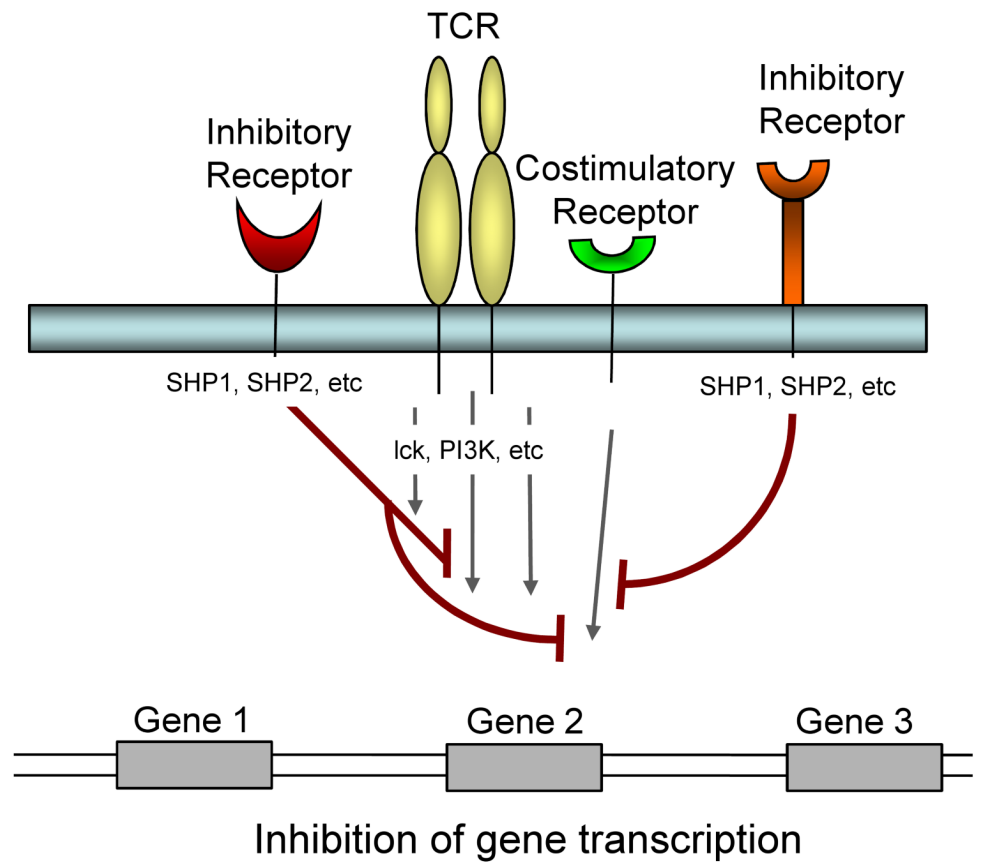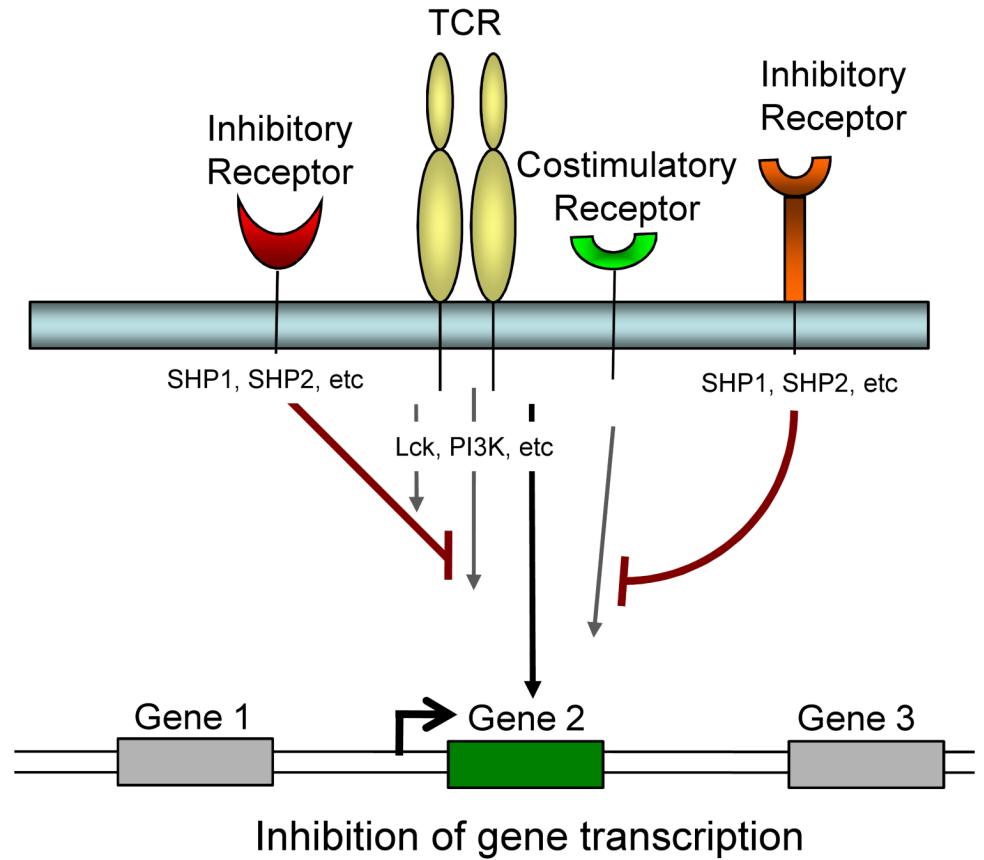
Model 3

Figure 1.
Model showing possible mechanisms of T cell regulation by inhibitory receptors. **Model 1**: Both TCR-mediated signals and costimulation are inhibited by inhibitory receptors resulting in a termination or quantitative reduction in gene transcription. **Model 2**: Only some of the TCR and costimulatory-induced signals are inhibited resulting in qualitatively altered gene transcription. **Model 3**: Inhibitory receptors may regulate some costimulatory pathways while allowing others to signal normally resulting in qualitatively different costimulatory signals. **Model 4**: Inhibitory receptor signaling could block costimulation-induced signaling and partially block TCR signaling but also induce the transcription of novel genes.

Table 1

Costimulatory and inhibitory receptors

Receptor or Ligand on T cell	Superfamily	Cellular Expression pattern	T cell expression	Binds	Costimulatory or Inhibitory	References
CD28	Ig (CD28 family)	T cells	Constitutive on Naïve	B7.1, B7.2	Costimulatory	[1]
ICOS	Ig (CD28 family)	T cells, NKT cells, NK cells	Induced upon activation	ICOSL (B7RP1)	Costimulatory	[1]
CD40L	TNF/TNFR	T cells	Induced upon activation	CD40	Costimulatory	[6]
OX40	TNF/TNFR	T cells	Induced upon activation	OX40L	Costimulatory	[6]
4-1BB	TNF/TNFR	T cells, DC subset, FDCs, eosinophils, NK cells	Induced upon activation	4ABBL	Costimulatory	[6]
CD27	TNF/TNFR	T cells, NK cells, B cells	Constitutive on Naïve	CD70	Costimulatory	[6]
LIGHT	TNF/TNFR	T cells, monocytes, NK cells, immature DC	Induced upon activation	HVEM	Costimulatory	[6]
CTLA4	Ig (CD28 family)	T cells	Induced upon activation	B7.1/B7.2	Inhibitory	[1]
PD-1	Ig (CD28 family)	T cells, NKT cells, B cells monocytes	Induced upon activation	PD-L1/PD-L2	Inhibitory	[1]
PD-L1	Ig (CD28 family)	T cells, NKT cells, B cells monocytes, DC, endothelial cells, hepatocytes, etc.	Constitutive and upregulated upon activation	B7-1	Inhibitory	[1,10]
BTLA	Ig (CD28 family)	T cells, B cells, DC (macs + NK in B6 mice only)	Constitutive on Naïve	HVEM	Inhibitory	[61]
CD160	Ig	HVEM and MHCI (low affinity)	Induced upon activation	HVEM	Inhibitory	[8]
TIM3	Ig	T cells, macs, DCs	Induced upon activation	GALECTIN 9, phosphatidyl serine	Inhibitory	[16]
2B4	Ig (CD2 family)	T cells, NK cells, dendritic epidermal T cells, monocytes	Induced upon activation	CD48	Costimulatoryan d Inhibitory	[12]
LAG3	Ig	T cell, NK cells, B cells	Induced upon activation	MHC-II	Inhibitory	[11,58]
CD200R	Ig	T cells, macrophages, DCs, neutrophils, basophils, mast cells	Constitutive on Naïve	CD200	Inhibitory?	[17]
PIR-B	?	T cell, B cells, DCs	?	MHC-I	Inhibitory	[57]
NKG2A	C-lectin	T cells, NK cells	Induced upon activation	Qa-1B, non-classical MHC-I, stress induced molecules (e.g. Rae-1, MICA, MICB)	Inhibitory	[57]
Ly49 family (mice)	C-lectin	T cells, NK cells, NK T cells monocytes/ macrophages	?	MHC-I	Costimulatory and Inhibitory	[14]
KIRs (human)	Ig	T cells	Induced upon activation	HLA-1	Inhibitory	[14]
GP49B	Ig	T cells, macrophages, neutrophils, and NK cells, mast cells	?	$\alpha_v\beta_3$	Inhibitory	[57]