

Tipping the balance: inhibitory checkpoints in intestinal homeostasis

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Abstract

The small intestinal and colonic lamina propria are populated with forkhead box P3 (FOXP3)⁺CD4⁺ regulatory T cells (Tregs) and interleukin-10-producing T cells that orchestrate intestinal tolerance to harmless microbial and food antigens. Expression of co-inhibitory receptors such as CTLA-4 and PD-1 serve as checkpoints to these cells controlling their T-cell receptor (TCR)- and CD28- mediated activation and modulating the phenotype of neighboring antigen presenting cells. Recent discoveries on the diversity of co-inhibitory receptors and their selective cellular expression has shed new light on their tissue-dependent function. In this review, we provide an overview of the co-inhibitory pathways and checkpoints of Treg and effector T cells and their mechanisms of action in intestinal homeostasis. Better understanding of these inhibitory checkpoints is desired as their blockade harbors clinical potential for the treatment of cancer and their stimulation may offer new opportunities to treat chronic intestinal inflammation such as inflammatory bowel disease.

Introduction

The intestinal mucosa is continuously exposed to exogenous antigens derived from food proteins and microbiota. Upon encounter of foreign antigens, antigen presenting cells (APCs) migrate to draining lymph nodes to present antigens to naive T cells. The interactions between APCs and responding T cells are modulated by additional signals from costimulatory and co-inhibitory receptors. The balance between these signals determines the functional outcome of T-cell receptor (TCR)-mediated activation, including the strength, nature and duration of the T-cell response.¹ In addition to controlling APC-T-cell interactions during the initial activation of naive T cells, costimulatory and co-inhibitory signals also control effector, memory and regulatory T-cell responses.² Under homeostatic circumstances, encounter of innocuous antigens such as food proteins and molecular components of commensal bacteria in mucosa draining lymph nodes result in a preferential tolerogenic T-cell response. Inflammatory T-cell responses to food and microbial antigens can result in allergy and chronic inflammation, such as seen in patients with inflammatory bowel disease (IBD) and celiac disease, and might contribute to autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).³⁻⁷

Over the years, studies in both mice and humans have established a central role for Forkhead box P3 (Foxp3)⁺CD4⁺ regulatory T cells (Tregs) and interleukin 10-producing CD4⁺Foxp3^{neg} T cells in maintaining intestinal tolerance to harmless microbial and food antigens.⁸ Foxp3⁺ Tregs are classified into thymus-derived Treg (tTreg) and peripherally-derived Treg (pTreg). tTreg arise during CD4⁺ T-cell differentiation in the thymus, whereas pTreg differentiate from naive CD4⁺ T cells exposed to antigens under tolerogenic conditions in lymphoid tissue.⁹ Special environmental control in intestinal lymphoid tissues favors *de novo* pTreg development in response to TCR-specific recognition of food- and microbiota-derived antigens.^{8, 10-12} In particular, soluble factors including transforming growth factor- β (TGF- β) and retinoic acid promote intestinal pTreg differentiation.¹³⁻¹⁶ Besides Foxp3-expressing

Tregs, the intestine contains IL-10-secreting CD4⁺Foxp3^{neg} type 1 regulatory T cells (Tr1 cells), differentiation of which is facilitated by the cytokine IL-27.^{17, 18} A common feature of both Foxp3⁺ and Foxp3^{neg} regulatory CD4⁺ T-cell populations is the expression of inhibitory receptors.¹⁹ Recently, immunotherapies directed against co-inhibitory receptors such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1), aiming to enhance anti-tumor T-cell responses in cancer, show that co-inhibitory receptor blockade can lead to the development of immune-mediated intestinal inflammation.²⁰ These findings provide new insight into the critical role of co-inhibitory receptors in the maintenance of intestinal homeostasis, but their involvement in the regulation of mucosal immune responses in the intestine remains poorly understood. In this review, we will discuss the role of co-inhibitory receptors in the maintenance of intestinal homeostasis and how the timing of inhibitory receptor upregulation, ligand distributions and specific effects on different cell-types contribute to this regulation. Better mechanistic understanding of how co-inhibitory receptor pathway modulation leads to intestinal inflammation is desired as stimulating co-inhibitory receptor pathways may offer new opportunities to treat chronic intestinal inflammation as observed in IBD.

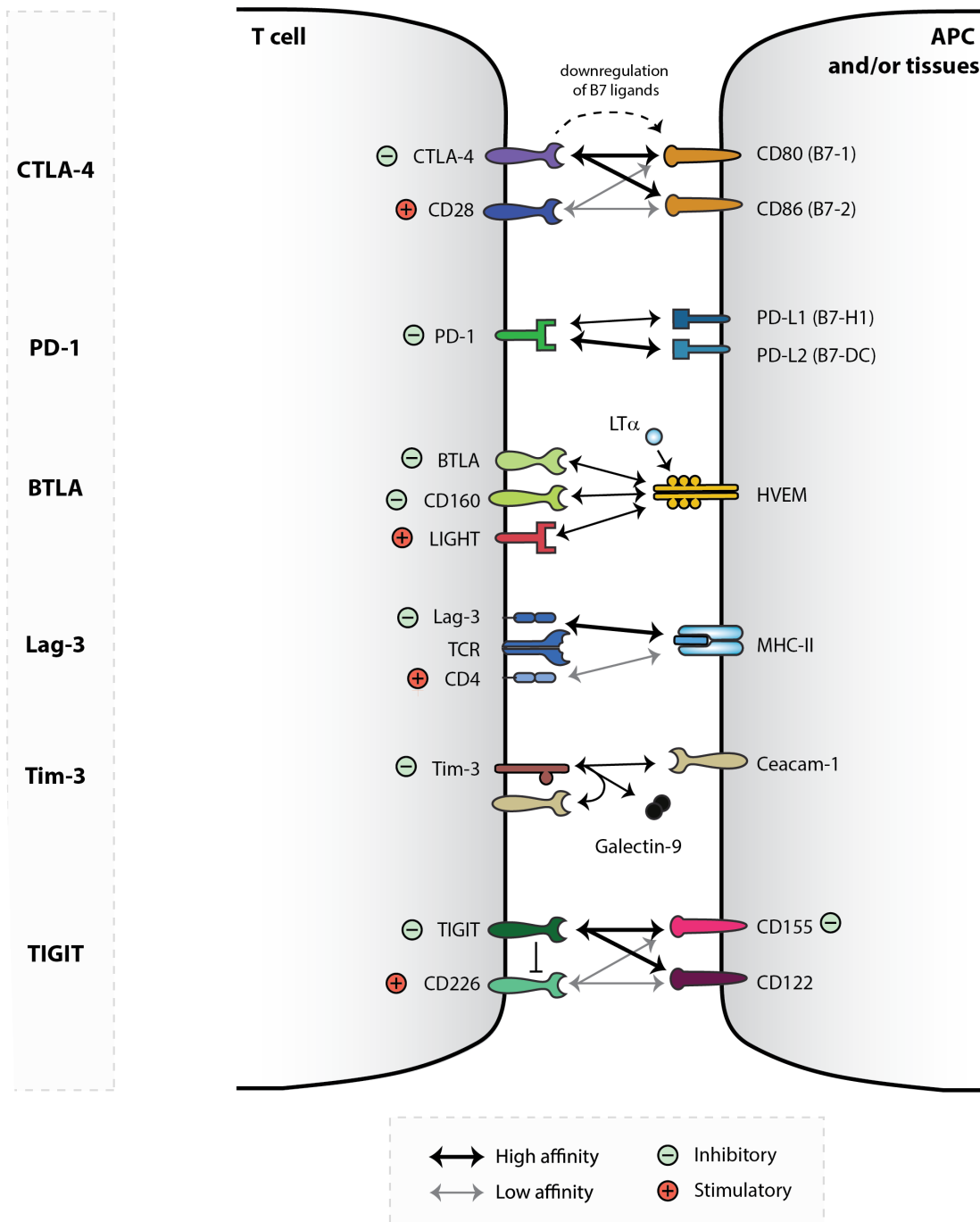


Figure 1. Interactions between co-inhibitory receptors of the immunoglobulin superfamily and their ligands. The majority of T-cell co-inhibitory receptors belong to the immunoglobulin (Ig) superfamily. Many co-inhibitory receptors of the Ig superfamily are expressed on activated T cells, and their specific ligands are expressed in professional antigen presenting cells (APCs), neutrophils, macrophages, or stromal cells. The inhibitory receptor cytotoxic T lymphocyte-associated antigen-4 (**CTLA-4**) is a structural homolog of the costimulatory receptor CD28, but binds B7 ligands CD80 and CD86 with much higher binding affinity. Programmed cell death 1 (**PD-1**) is a member of the B7/CD28 family that has two ligands, PD-L1 and PD-L2. B- and T-lymphocyte attenuator (**BTLA**) is part of a shared receptor-ligand network and binds to the TNF receptor family member herpesvirus entry mediator (HVEM). Lymphocyte activation gene-3 (**Lag-3**) structurally resembles CD4 and binds to MHC-II with higher affinity. T-cell immunoglobulin and mucin domain-containing protein (**Tim-3**) has many ligands, including galectin-9 and Ceacam-1. T-cell immunoreceptor with Ig and ITIM domain (**TIGIT**) and the costimulatory receptor CD226 share the ligands CD112 and CD155. Together they form a pathway reminiscent of the CTLA-4/CD28/B7 pathway, in which TIGIT delivers an inhibitory signal.

Inhibitory receptors in the Ig superfamily that engage B7 ligands: CTLA-4 and PD-1

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4; CD152)

CTLA-4: expression, ligands and function

CTLA-4, a member of the Ig superfamily, is a structural homologue of the costimulatory receptor CD28 and is a ligand for CD80 (B7-1) and CD86 (B7-2) expressed on the surface of APCs.^{21, 22} Unlike CD28, CTLA-4 undergoes constitutive clathrin-mediated endocytosis such that it is rapidly internalized after reaching the cell surface.²³⁻²⁵ CTLA-4 has a higher affinity for CD80 and CD86 than CD28 and can prevent the costimulatory signal normally provided by CD28/B7 interactions (Figure 1).²⁶ As CD28/B7 costimulation is essential to prime naive T cells, the CTLA-4 pathway is thought to inhibit T-cell proliferation early in the immune response, preferentially in the lymph node.^{27, 28} Since CTLA-4 is a target gene of Foxp3²⁹⁻³¹, Tregs have high intracellular stores of CTLA-4 and constitutively traffic high levels of CTLA-4 to their cell surface.^{32, 33} Resting naive T cells can be induced to express CTLA-4 in response to TCR ligation, in particular together with CD28 costimulation, reaching a maximum level after 48 to 72 hours.³⁴⁻³⁶

The critical role of CTLA-4 in controlling T-cell activation and tolerance is well-established. *Ctla4*^{-/-} mice develop a lymphoproliferative disorder within 3 weeks of age that is characterized by the infiltration of CD4⁺ T cells into multiple non-lymphoid tissues, leading to organ destruction and death.^{37, 38} Specific deletion of CTLA-4 in CD4⁺Foxp3⁺ Tregs results in a similar lymphoproliferative disease and multi-organ failure as seen in *Ctla4*^{-/-} mice, but with a delayed onset, demonstrating that expression of CTLA-4 by Foxp3⁺ Tregs is essential to prevent autoimmunity *in vivo*.³⁹ Mice lacking both CTLA-4 and CD28 or their ligands CD80/CD86 show no signs of disease⁴⁰⁻⁴², demonstrating that the main function of CTLA-4 is to control CD28-dependent T-cell activation by competing for CD80 and CD86. This so-called cell extrinsic regulation, in which CTLA-4⁺ cells inhibit the activation of neighboring CTLA-4^{neg} cells, is exerted by conventional T cells as well as by Tregs.⁴³⁻⁴⁶ In addition to competing for CD80/CD86, CTLA-4 can downregulate expression of these ligands on APCs.^{39, 47, 48}

This can occur via transendocytosis, whereby CTLA-4 captures CD80 and CD86 from the surface of APCs and internalizes them for degradation, impairing the capacity of these APCs to provide CD28 costimulation.⁴⁹ The role of transendocytosis in CTLA-4 function offers a plausible biological explanation for the unusual endocytic and recycling behavior of the CTLA-4 molecule. Although it has been suggested that the cytoplasmic domain of CTLA-4 transmits an inhibitory signal leading to cell-intrinsic regulation within CTLA-4 expressing cells, this is not supported by experiments with mixed bone marrow chimeric mice containing a mixture of wildtype and CTLA-4-deficient cells.⁵⁰⁻⁵² Thus while cell intrinsic competition between CTLA-4 and CD28 expressed on the same cell must surely occur, this does not appear to be a major mechanism of CTLA-4 action, at least in conventional T cells [refs 32 and 52]. In sum, CTLA-4 regulates T-cell function primarily by restricting T-cell activation early in the immune response through cell extrinsic ligand competition.

CTLA-4 and intestinal homeostasis

Intestinal CTLA-4 in a nutshell. CTLA-4 uses multiple mechanisms to exert a critical inhibitory effect on intestinal T-cell responses. CTLA-4 favors higher frequencies of pTregs in the intestinal lamina propria.⁵³ Moreover, mouse models of colitis have demonstrated that CTLA-4 expression on Treg is essential to suppress colitogenic CD4⁺ effector T cells.^{32, 45, 54-56} In humans, Tregs of patients with heterozygous mutations in CTLA-4 have impaired suppressive capacity.⁵⁷⁻⁵⁹ Limited data is available on the functional role of CTLA-4 on intestinal regulatory CD4⁺Foxp3^{neg} T-cell populations.

In the intestine, approximately 90% of all Foxp3⁺ Tregs express CTLA-4.⁶⁰ Several murine studies have addressed the role of CTLA-4 in the induction of intestinal Foxp3⁺ cells, its effect on suppressive capacity and its role in maintaining the Foxp3⁺ Tregs pool. CTLA-4 is not required for pTreg induction, as in culture with TGF- β , IL-2 and TCR signals, naive *Ctla4*^{-/-} T cells and *Ctla4*^{+/+} T cells are equally efficient in converting into Foxp3⁺ pTregs.⁵³ However, CTLA-4 does appear to play a role in maintaining the intestinal Treg pool. The intestinal lamina propria of *Rag2*^{-/-} mice reconstituted with a

mixture of *Ctla4*^{-/-} and *Ctla4*-sufficient BALB/c bone marrow cells contains reduced percentages of Foxp3⁺ T cells derived from *Ctla4*^{-/-} bone marrow when compared to Foxp3⁺ T cells derived from BALB/c bone marrow.⁵³ In contrast, in the spleen and mesenteric lymph nodes, CTLA-4-deficient (*Ctla4*^{-/-}) and -sufficient (BALB/c) T cells contributed equally to the Foxp3⁺ and Foxp3^{neg} compartments. This is not because of a general inability of CTLA-4^{-/-} cells to migrate to the gut, as CTLA-4^{-/-} cells in the intestine contribute to the CD4⁺Foxp3^{neg} cell pool to a similar percentage as in other organs.⁵³ These observations thus argue that, *in vivo*, CTLA-4 plays a role in regulating pTreg frequencies in the intestine in particular.⁵³ In sum, CTLA-4 is not essential for intestinal pTreg induction but does regulate the accumulation of intestinal pTreg *in vivo*, hereby regulating the size of the intestinal Foxp3⁺ Treg pool.

CTLA-4 plays a fundamental role in Treg suppressive function during intestinal inflammation.^{33, 40, 55, 56, 61} The majority of data on the role of CTLA-4 in intestinal inflammation has been generated using the T-cell transfer model of colitis. In this model, naive CD4⁺CD45RB^{high} T cells are adoptively transferred into lymphopenic *Scid*^{-/-} or *Rag*^{-/-} mice and cause colitis upon activation in response to intestinal antigens.^{62, 63} Co-transfer of CD4⁺CD25⁺ T cells, enriched in Tregs, with naive CD4⁺CD45RB^{high} T cells prevents development of colitis.⁶⁴ In most studies, CTLA-4 expression on co-transferred CD4⁺CD25⁺ T cells is essential to prevent colitis induced by naive T-cell transfer into *Rag*^{-/-} recipients (Table 1).^{32, 45, 54} Similarly, blocking the interaction of CTLA-4 and its ligands through use of anti-CTLA-4 antibodies or Fab fragments, abrogates CD4⁺CD25⁺ cell mediated suppression of colitis.^{40, 55, 56} This argues that CTLA-4 expression on Tregs is required for colitis suppression (Figure 2.2). It should be noted that genetic deficiency of CTLA-4 in CD4⁺CD25⁺ Tregs has been reported to result in a compensatory IL-10 production in some settings, allowing the prevention of transfer colitis in an IL-10 dependent fashion (Table 1). It is unclear whether CTLA-4 expression by colitogenic naive CD4⁺CD45RB^{high} T cells plays a role in transfer colitis, as anti-CTLA-4 antibody treatment exacerbated colitis in some experimental settings but not others.^{40, 56} An overview of role of CTLA-4 in

colitis suppression in the different transfer colitis studies is provided in Table 1. Altogether, data demonstrate a key role for CTLA-4 in Foxp3⁺ Tregs to prevent T-cell transfer induced colitis.

In addition to their crucial role in preventing intestinal inflammation, Foxp3⁺ pTregs are required for the induction of oral tolerance.^{10, 65, 66} Feeding of soluble antigen induces systemic immunological hyporesponsiveness that is characterized by a suppressed delayed type hypersensitivity (DTH) reaction after antigen injection in the footpad or the auricle.^{67, 68} At the cellular level, tolerance is a consequence of Foxp3⁺ pTreg mediated suppression of the Th1 and Th2 response to the particular antigen. Several studies have demonstrated a role for CTLA-4 function in the development of oral tolerance, but effects on cytokine secretion varied on the dose of the antigen fed while the DTH response was not monitored.^{69, 70} Administration of anti-CTLA-4 antibodies during 50 mg ovalbumin (OVA) feeding completely abrogated cytokine production by Th1 and Th2 cells.⁷⁰ By contrast, the administration of anti-CTLA-4 antibodies during 1 mg OVA feeding only abrogated suppression of Th2 type immune responses but not Th1.⁶⁹ Co-administration of IL-12 along with anti-CTLA-4 antibodies during 1 mg OVA feed prevented both Th1 and Th2 cytokine secretion. These results indicate that the CTLA-4 pathway is important for establishing oral tolerance. However, whether OVA-specific pTregs require CTLA-4 for full suppression of Th1 and Th2 immune responses in models for low dose oral tolerance remains to be further investigated (Figure 2.2).

In some settings, CTLA-4 deficiency may allow for induction of other subsets of CD4⁺ T cells with the capacity to suppress colitis. Cre-recombinase based deletion of CTLA-4 in adult mice results in significantly increased frequencies of Foxp3^{neg} T cells, that exhibit high levels of IL-10 and increased expression of coinhibitory receptors Lag-3 and PD-1.⁷¹ High IL-10-producing capacity and the expression of co-inhibitory receptors such as Lag-3 and PD-1 are characteristic of IL-10-secreting CD4⁺Foxp3^{neg} Tr1, a cell type that has been shown to inhibit T-cell responses and colitis in an IL-10-dependent manner.⁷²⁻⁷⁴ Along the same lines, it was recently demonstrated that anti-CTLA-4 treatment can induce IL-10-producing ICOS⁺ regulatory CD4⁺ T cells with anti-inflammatory properties that inhibit 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS) induced colitis.⁷⁵ This is in contrast with

the finding that anti-CTLA-4 antibodies abrogated Treg control of colitis in the setting of T-cell transfer experiments (described above), but fits with the observation that IL-10 can compensate for a genetic deficiency of CTLA-4 in some settings (Table 1).⁴⁰ The precise circumstances in which blockade or lack of CTLA-4 allows for the differentiation of immunosuppressive IL-10 secreting CD4⁺Foxp3^{neg} T cells with the ability suppress colitis requires further investigation.

Quantitative deficiencies in CTLA-4 expression in humans

In humans, heterozygous mutations in CTLA-4 resulting in CTLA-4 haploinsufficiency are associated with lymphoproliferation reminiscent of the mouse model, resulting in severe clinical manifestations of autoimmunity and early-onset Crohn's disease.⁵⁷⁻⁵⁹ The organs of affected *CTLA4*^{+/-} individuals, including the intestine and lungs, show extensive CD4⁺ T-cell infiltration.⁵⁹ Frequencies of Tregs within the CD4⁺ T-cell compartment of *CTLA4*^{+/-} individuals are significantly increased compared to healthy *CTLA4*^{+/+} controls and their suppressive function is impaired.⁵⁷⁻⁵⁹ In addition to impaired Treg function, it has been suggested that the *CTLA4*^{+/-} genotype alters naive T-cell responses, as naive T cells obtained from a clinically affected *CTLA4*^{+/-} individual showed hyperproliferation after *in vitro* activation in the presence of autologous T-cell depleted feeder cells.⁵⁷ However, other studies did not observe hyperproliferation in naive T-cell responses isolated from *CTLA4*^{+/-} individuals suggesting that the variations in experimental set-up may be key.^{59, 76} Interestingly, the clinical penetrance of CTLA-4 haploinsufficiency is incomplete, suggesting that additional genetic or environmental factors influence disease susceptibility in individuals with impaired CTLA-4 function. It is currently unknown whether clinically unaffected *CTLA4*^{+/-} individuals are protected from disease by CD4⁺ T cells with suppressive capacity independent of CTLA-4.

Genetic variants in the *CTLA4* gene locus are associated with intestinal inflammation and autoimmune diseases.⁷⁷⁻⁷⁹ The single nucleotide polymorphism (SNP) CT60 (rs3087243; A/G) located in the 3' UTR of the *CTLA4* gene has been associated with IBD in a Slovenian cohort of adult IBD patients.⁸⁰ This variant of the *CTLA4* gene is associated with a functionally altered TCR signaling

in CD4⁺ T cells and decreased production of the soluble CTLA-4 isoform.^{81, 82} However, the association between the CT60 *CTLA4* allele and IBD was not confirmed in a separate cohort of Spanish patients.⁷⁷ Several studies have shown that the CT60 *CTLA4* allele is also weakly associated with celiac disease.^{83, 84} Taken together, these data indicate that quantitative deficiencies in CTLA-4 protein expression can predispose selected subgroups of individuals to autoimmunity, including CD4⁺ T-cell mediated inflammation in the gastrointestinal tract.

Table 1. The role of CTLA-4 in preventing intestinal inflammation in transfer colitis models.

	Colitogenic T-cell population	Recipient	Treg-cell population	Colitis
<i>Ctla4</i>^{-/-} regulatory cells				
Sojka et al. 2009 ⁵⁴	CD4 ⁺ CD25 ^{neg} CD62L ^{high} T cells from WT C57BL/6.CD90.2 mice.	<i>Rag2</i> ^{-/-}	CTLA-4 deficient CD4 ⁺ CD25 ⁺ CD62L ^{high} cells isolated from young <i>Ctla4</i> ^{-/-} mice.	Yes
Jain et al. 2010 ⁴⁵	CD4 ⁺ CD25 ^{neg} cells isolated from WT BALB/c mice.	<i>Rag1</i> ^{-/-}	CTLA-4 deficient CD4 ⁺ CD25 ⁺ cells isolated from transgenic CD57BL/6J mice with CTLA-4 expression restricted to activated T cells (CT4Act mice, <i>Ctla4</i> expression under the control the <i>Il2</i> promoter).	Yes
Tai et al. 2012 ³²	CD4 ⁺ CD45RB ^{high} T cells from WT B6 mice.	<i>Rag2</i> ^{-/-}	CTLA-4 deficient CD4 ⁺ CD25 ⁺ cells isolated from B6+ <i>Ctla4</i> ^{-/-} →B6 mixed BM chimeras.	Yes
Read et al. 2006 ⁴⁰	CD4 ⁺ CD45RB ^{high} T cells from WT BALB/c mice.	<i>Scid</i>	B7/CTLA-4 deficient CD4 ⁺ CD25 ⁺ cells from B7-1/B7-2/CTLA-4 KO mice.	No*
Read et al. 2006 ⁴⁰	CD4 ⁺ CD45RB ^{high} T cells from WT BALB/c mice.	<i>Rag2</i> ^{-/-}	B7-sufficient CTLA-4 deficient CD4 ⁺ CD25 ⁺ cells isolated from BALB/c.CTLA-4 KO mixed BM chimeras.	No*
Anti-CTLA-4 antibodies				
Read et al. 2006 ⁴⁰	<i>CTLA-4</i> deficient CD4 ⁺ CD45RB ^{high} T cells from B7-1/B7-2/CTLA-4 ^{-/-} mice.	<i>Scid</i>	<i>CTLA-4</i> sufficient Transfer of CD4 ⁺ CD25 ⁺ T cells, followed by anti-CTLA-4 mAb i.p. (200 µg), the day after T-cell reconstitution and then on alternate days for 6-8 weeks.	Yes
Read et al. 2006 ⁴⁰	<i>CTLA-4</i> sufficient CD4 ⁺ CD45RB ^{high} T cells from WT mice.	<i>Scid</i>	<i>CTLA-4</i> deficient Transfer of CD4 ⁺ CD25 ⁺ T cells from B7-1/B7-2/CTLA-4 ^{-/-} mice, followed by anti-CTLA-4 mAb i.p. (200 µg), the day after T-cell reconstitution and then on alternate days for 6-8 weeks.	No*
Read et al. 2000 ⁵⁵	Mixture of CD45RB ^{high} and CD45RB ^{low} cells.	<i>Scid</i>	Transfer of CD25 ⁺ CD45RB ^{low} cells, followed by anti-CTLA-4 mAb i.p. (200 µg), the day after T-cell reconstitution and then on alternate days for 6 weeks.	Yes
Liu et al. 2001 ⁵⁶	CD4 ⁺ CD45RB ^{high} T cells from WT mice.	<i>Scid</i>	No Tregs transferred. Anti-CTLA-4 mAb i.p. (250 µg), twice a week starting at the beginning of T-cell transfer up to 8 weeks.	Yes (exacerbated colitis)

An overview of the studies using *Ctla4*^{-/-} CD4⁺ T cells and/or anti-CTLA-4 monoclonal antibodies to investigate the functional effect of CTLA-4 in the T-cell transfer model of colitis. In this model, transfer of naive CD4⁺CD45RB^{high} T cells into lymphopenic recipients causes colitis. Co-transfer of CD4⁺CD25⁺ Tregs prevents development of colitis. In most studies depicted in this table, use of *Ctla4*^{-/-} Tregs or anti-CTLA-4 blocking antibodies abrogates colitis prevention. The * indicates a study using *Ctla4*^{-/-} Tregs in which compensatory IL-10 production by *Ctla4*^{-/-} Tregs was shown to prevent colitis. Additional experiments using anti-CTLA-4 antibodies, indicated in the bottom section of the table, demonstrated that even though *Ctla4*^{-/-} Tregs have compensatory mechanisms, suppression by wildtype Treg is dependent on CTLA-4. Abbreviations: KO = knockout; BM = bone marrow; WT = wildtype; mAb = monoclonal antibody; i.p. = intraperitoneally.

Programmed Death 1 (PD-1; CD279)

PD-1: expression, ligands and function

PD-1 is a cell-surface molecule belonging to the B7 receptor family that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic tail.^{85, 86} PD-1 has two ligands, PD-L1 (B7-H1; also termed CD274) and PD-L2 (B7-DC; also termed CD273). Most of the inhibitory roles of PD-1 have been attributed to its interaction with PD-L1 (Figure 1).⁸⁷ Upon PD-L1 or PD-L2 binding, the ITIM or ITSM tyrosine motifs in the cytoplasmic tail of PD-1 are phosphorylated.⁸⁸ This leads to recruitment of Src-homology 2 domain-containing phosphatase 2 (SHP-2) and augments phosphatase and tensin homolog (PTEN) expression, inhibiting phosphatidylinositol 3-kinase (PI3K) and Akt activation.⁸⁹⁻⁹¹ CD28, not the TCR or its associated components, has been reported to be the most sensitive target of PD-1, as shown by the strong degree of CD28-dephosphorylation compared to dephosphorylation of TCR-signaling components after PD-1 activation.^{90, 92} In line with its inhibitory function, PD-1 deficient mice (*Pdcd1*^{-/-}) develop spontaneous autoimmune disease although the incidence of disease depends on the genetic background and symptoms only manifest later in life.⁹³⁻⁹⁵ Moreover, the disease is often tissue-specific which is in contrast to the rapid multi-organ autoimmune disease observed in CTLA-4^{-/-} mice in the first few weeks of life.^{37, 38}

PD-1 is expressed on diverse hematopoietic cells, including T and B cells⁹⁶, natural killer (NK) cells and other innate immune cells.⁹⁷ PD-1 expression is absent on naive T cells but is rapidly upregulated after antigen encounter and can be detected after only 6 hours, with a peak in expression at 48 hours after antigen encounter.^{96, 98-100} Of the two PD-1 ligands, PD-L2 is mainly expressed by DCs and macrophages, whereas PD-L1 is more widely expressed by both hematopoietic and non-hematopoietic cells¹⁰¹⁻¹⁰³ and can be induced by inflammatory cytokines such as interferons.^{104, 105} The broad expression of PD-1 and PD-L1 is in contrast to CTLA-4 and its ligands that are mainly expressed by hematopoietic cells present in the lymph node environment. Therefore, it is often

hypothesized that while CTLA-4 impacts T-cell activation primarily during the priming phase in secondary lymphoid organs, PD-1 plays a dominant role during the maintenance of tolerance in peripheral tissues.^{27, 28} Many murine models for autoimmune disease and cancer have established a role for PD-1/PD-L1 interactions in maintaining tissue tolerance by controlling the effector T-cell responses in non-lymphoid tissues.^{94, 106-110} However, the rapid upregulation of PD-1 after activation suggests that PD-1 can control T-cell activation at the time of initial antigen encounter in the lymph node in addition to modulating effector responses in target tissues.

PD-1 and intestinal homeostasis

Intestinal PD-1 in a nutshell. PD-1/PD-L1 interactions preserve the local homeostasis of the gastrointestinal tract by acting on a wide variety of immune cell types, mostly through interaction with its ligand PD-L1. PD-1 mainly promotes peripheral induction and survival of Foxp3⁺ Tregs¹¹¹⁻¹¹⁴, but does not seem to determine Treg function.¹¹⁴ PD-1 expression on intestinal CD4⁺Foxp3^{neg} Tr1 cells enriches for IL-10-producing capacities.¹¹⁵ Moreover, PD-1 has a nonredundant role in preventing excessive CD8⁺ effector T cell responses to intestinal self-antigens.^{116, 117} Lastly, PD-1 expression on T-follicular helper (Tfh) cells regulates Tfh-cell numbers in Peyer's patches, promoting intestinal tolerance to microbiota through secretory IgA.^{118, 119}

Although Foxp3⁺ Tregs highly express PD-1¹¹¹, the role of PD-1 on Tregs is only beginning to be fully understood. PD-1 regulates the generation of pTreg and PD-L1 synergizes with TGF- β to promote pTreg induction^{111, 112}, suggesting that PD-1/PD-L1-mediated pTreg induction might play a role in TGF- β -rich environments such as the intestinal mucosa-draining lymphoid tissue (Figure 2.1). A role for PD-1 in the maintenance of Tregs has been suggested by several studies. PD-1 maintains the suppressive phenotype of Tregs through inducing PTEN expression.¹²⁰ In line with this finding, PD-1 prevents the conversion of Foxp3⁺ Tregs into pro-inflammatory effector/memory CD4⁺ T cells.¹¹³ Recently, it was shown that low dose IL-2 therapy, used as a therapy to induce expansion of pTregs,

increases PD-1 expression on Tregs of patients treated for graft-versus-host disease.¹¹⁴ Examination of Tregs from IL-2-treated mice demonstrated that PD-1 reduced IL-2-induced Treg proliferation but prevented their terminal differentiation, rendering Tregs less susceptible for apoptosis and promoting their survival and subsequent regulation.¹¹⁴ IL-2-induced Tregs isolated from PD-1^{-/-} mice exhibited normal levels of suppressive activity, indicating that PD-1 does not directly affect Treg function.¹¹⁴ In addition to Foxp3⁺ Tregs, PD-1 expression has recently been described on intestinal Tr1 cells (defined as CD3⁺CD4⁺CD25⁻IL-7R⁺ cells) in both mice and humans.¹¹⁵ Expression of CCR5 and PD-1 allowed enrichment for IL-10⁺ Tr1 cells in the human intestine. In mice, co-transfer of IL-10-producing CCR5⁺PD-1⁺ Tr1 cells strongly inhibited colitis induced by transfer of Th17 cells, whereas IL-10-producing control T cells that lacked CCR5 and PD-1 were less efficient.¹¹⁵ Taken together, PD-1 is expressed on both Foxp3⁺ Treg and CD4⁺Foxp3^{neg} Tr1 cells, and can take part in regulating the induction, survival and IL-10 production of these cells in the intestinal environment.

In addition to PD-1 on regulatory CD4⁺ T-cell populations, PD-1 expression on CD8⁺ effector T cells is involved in preventing responses to intestinal self-antigens (Figure 2.3).^{116, 117} In a transfer model of OVA-specific OT-I CD8⁺ T cells into iFABP-OVA mice that express OVA as a self-antigen on intestinal epithelial cells, blocking PD-1/PD-L1 interaction at the time of OT-I cell transfer prevents tolerance induction and resulted in small intestinal inflammation.^{116, 117} The intestinal pathology was characterized by apoptosis of epithelial cells, villous atrophy and leukocytic infiltration, reminiscent of the pathology in human celiac disease. Crucially, when PD-L1 is blocked 30 days after OT-I cell transfer, mice do not develop intestinal pathology, indicating that PD-1/PD-L1 interaction is crucial for the induction, but not the maintenance of mucosal CD8⁺ T-cell tolerance.¹¹⁷ PD-1 upregulation on CD8⁺ effector T cells upon antigen encounter *in vivo* is highly dependent on the type of antigen recognized. Transfer of CD8⁺ effector T cells specific for influenza hemagglutinin (HA) into mice expressing HA as a self-antigen induces robust PD-1 expression on HA-specific CD8⁺ T cells.⁸⁷ In contrast, when HA-specific CD8⁺ T cells were transferred into mice infected with an HA-expressing *Listeria monocytogenes*, there was virtually no PD-1 expression on HA-specific CD8⁺ T cells,

demonstrating that PD-1 is differentially induced on CD8⁺ effector T cells responding to self-antigen versus microbial antigen.⁸⁷ Of note, the recipient mice in this model expressed HA under the control of the C3 promoter, which directs HA expression on a variety of parenchymal tissues. Whether HA expressed as a self-antigen specifically on intestinal cells induces PD-1 expression on HA-specific T cells remains unknown. Overall, these data demonstrate that the role of PD-1/PD-L1 interactions in CD8⁺ T-cell tolerance to self-antigens and microbial antigens is likely determined by multiple factors, including the timing of the immune response, type of antigen and signals from the tissue microenvironment.

Besides CD8⁺ effector T cells, PD-1-expressing Tfh cells are involved in promoting intestinal tolerance to microbiota through secretory IgA (Figure 2.4). PD-1 is highly expressed on cells in the germinal centers of Peyer's patches¹²¹, the major sites for induction of mucosal secretory IgA antibody responses.¹²² Secretory IgA plays a key role in regulating host-microbe interactions at the mucosal surface by maintaining a balanced and highly diverse microbial communities in the gut.¹²³ In humans, the protective role of IgA is illustrated by the fact that IgA-deficiency is associated with increased susceptibility to autoimmunity and gastrointestinal infections.¹²⁴⁻¹²⁶ It has been demonstrated that PD-1 deficient mice (*Pdcd1*^{-/-}) have significantly more Tfh cells in Peyer's patches compared to wildtype mice.¹¹⁸ The production of IL-21, the cytokine that promotes proliferation and differentiation of IgA⁺ B cells into plasma cells^{127, 128}, is reduced in Tfh cells from *Pdcd1*^{-/-} mice causing an impaired ability to support the generation of IgA plasma cells in gut.^{118, 119} As a result, *Pdcd1*^{-/-} mice have lower proportions of bacteria coated with IgA and altered microbial communities in the intestine¹¹⁸, which resemble alterations of the microbiome observed in several pathological conditions including human IBD.¹²⁹ Serum from *Pdcd1*^{-/-} mice contains increased titers of commensal-specific IgG¹¹⁸, indicating a breach of normal host-microbe interaction in the absence of PD-L1/PD-1 interactions.

Besides a role for the PD-1/PD-L1 pathway in adaptive immune cells, PD-L1 serves as an essential ligand for innate immune cells in the intestine to prevent intestinal inflammation.¹³⁰

Experiments with bone marrow chimeras have demonstrated that PD-L1 expression on intestinal epithelial cells reduces dextran sulfate sodium (DSS)-induced intestinal inflammation.¹³⁰ This protective effect was mediated through inhibition of TNF α secretion of CD11c⁺CD11b⁺ lamina propria cells and was independent of adaptive immunity, as PD-L1-deficient *Rag1*^{-/-} mice exhibited a significantly higher morbidity and mortality than *Rag1*^{-/-} mice after DSS administration.¹³⁰ In humans, PD-L1 is expressed by intestinal epithelial cells of IBD patients but not of healthy controls¹³¹, and PD-1 expression is increased on lamina propria mononuclear cells¹³², which might reflect an effort to promote protective intestinal immune responses through PD-1.

Lessons learned from CTLA-4 and PD-1 blockade in cancer

Over the past decade, a role for co-inhibitory receptors in the maintenance of intestinal homeostasis in humans has been widely appreciated following the implementation of anti-CTLA-4 and anti-PD-1/PD-L1 immunotherapies to promote anti-tumor T-cell responses and tumor regression in cancer patients. Blockade of CTLA-4 and PD-1/PD-L1 is thought to activate a wide repertoire of T cells, not only tumor-specific T cells. In consequence, these therapies have a broad range of adverse effects, of which diarrhea and colitis are very frequently observed.^{133, 134} The incidence of diarrhea and colitis is 35.4% and 8.8%, respectively with CTLA-4 inhibitors and 13.7% and 1.6%, respectively for PD-1 inhibitors.^{135, 136} Combining CTLA-4 and PD-1 inhibitors may increase the risk of diarrhea but not colitis.^{135, 136} Colonic bowel perforation is the most common cause of fatal immune-related adverse events in patients who develop immunotherapy-induced colitis, but the incidence of life-threatening colon perforation is low (<1% of patients).²⁰ Thus, although blockade of co-inhibitory receptors is a promising new approach to improve tumor control, it can cause severe life-threatening immune-related adverse events, most often through a dysregulation of intestinal homeostasis.

In contrast to the chronic colitis-associated histological alterations observed in IBD, anti-CTLA-4 and anti-PD-1 colitis are characterized by neutrophilic inflammation or a mononuclear expansion in the lamina propria and increased intraepithelial lymphocytes.¹³⁷⁻¹³⁹ In most patients with

immunotherapy-induced colitis, disease remission is achieved by discontinuing the drug¹³⁴, but patients are likely to relapse when restarting the same drug.^{137, 140} Development of recurrent colitis has been observed in patients without a past medical history of intestinal inflammation who had stopped anti-CTLA-4 or anti-PD-1 therapy for multiple months.^{137, 140} The incidence of recurrent colitis is unknown. Colonic biopsies in these patients often demonstrated features of chronicity such as crypt architectural irregularity, suggesting that immunotherapy-induced colitis may progress to chronic intestinal inflammation as seen in IBD. Further studies are needed to understand how short-term co-inhibitory receptor blockade can result in long-term effects.

The exact mechanisms of immunotherapy-induced colitis are still elusive. There is evidence that Treg-cell depletion contributes to the pathogenesis of CTLA-4 induced colitis in some studies^{141, 142}, but not in others.^{143, 144} More investigation is required to establish a role for Tregs in immunotherapy-induced colitis. Other possible mechanisms of immunotherapy-induced colitis include the priming of naïve lymphocytes with reactivity to intestinal bacteria, self-antigens or cross-reactivity with tumor antigens, and the worsening of a pre-existing colitogenic immune response upon co-inhibitory receptor blockade. Interestingly, the risk of immunotherapy-related colitis is increased in patients with lower abundance of intestinal bacteria belonging to the Bacteroidetes phylum before start of anti-CTLA-4 therapy, which is reminiscent of the dysbiosis observed in IBD patients.¹⁴⁵⁻¹⁴⁷ It has been suggested that the risk of immunotherapy-related colitis may be depend on a patient's co-inhibitory receptor allele polymorphisms,¹⁴⁸ although in general SNPs associated with autoimmune disease do not appear useful in predicting the side-effects of immunotherapy.¹⁴⁹ Moreover, the majority of individuals experience no immunotherapy-related adverse events on anti-CTLA-4 or anti-PD-(L)1 therapy. Therefore, it is likely that multiple factors contribute to intestinal disease development during co-inhibitory receptor blockade.

Other members of the Ig superfamily: BTLA, Lag-3, Tim-3 and TIGIT

The success of immunotherapies directed against CTLA-4 and PD-1 in enhancing anti-tumor activity has prompted intense investigation into the targeting of other co-inhibitory receptors in order to broaden the therapeutic repertoire. A next generation immune checkpoint inhibitors directed against co-inhibitory receptors BTLA, Lag-3, Tim-3 and TIGIT, are currently being explored in clinical trials and may emerge soon.^{150, 151} It is expected that many of these new checkpoint inhibitors will be used in combination with the already approved checkpoint inhibitors against CTLA-4 and PD-1.¹⁵² In order to maximize success of novel combination therapies while minimizing gastro-intestinal adverse effects, it is crucial to increase our current knowledge on the basic molecular mechanisms of these co-inhibitory molecules and their tissue-specific functions. In the paragraphs below, we highlight the unique tissue-specific functions in the intestine of four newly emerging immune checkpoints.

HVEM ligands: B- and T-lymphocyte attenuator (BTLA; also known as CD272) and CD160

BTLA is a co-inhibitory receptor that is expressed on a wide range of hematopoietic cells, including CD4⁺ and CD8⁺ T cells, $\gamma\delta$ T cells, B cells, innate lymphoid cells (ILCs), NK cells and DCs.^{153, 154} In contrast to other co-inhibitory receptors that are induced upon TCR ligation, BTLA is constitutively expressed on naive CD4⁺ T cells and its expression increases upon T-cell activation, with the highest level of BTLA observed 3 days after TCR stimulation.¹⁵⁵ BTLA is part of a shared receptor-ligand network and binds to the TNF receptor family member Herpesvirus entry mediator (HVEM), a receptor that is constitutively expressed on the surface of various cell types, including hematopoietic cells and non-hematopoietic cells.¹⁵⁴ HVEM also binds to CD160, an Ig superfamily member that can engage HVEM on the same cell surface as BTLA and also functions as a co-inhibitory receptor (Figure 1).¹⁵⁶ However, HVEM also triggers costimulatory signals by acting as a receptor for tumor necrosis factor (TNF) superfamily members, including the TNF superfamily members LIGHT and LT α .^{157, 158} As a result, the functional outcome of BTLA and CD160 engagement is determined by a complex balance of costimulatory and co-inhibitory signals delivered to T cells through HVEM.^{1, 154}

Antagonistic anti-human BTLA antibodies are currently in clinical development for cancer treatment. To understand the effect of BTLA blockade on maintenance of intestinal homeostasis, it is pivotal to understand which receptor-ligand pairs are involved in engaging HVEM in the intestine. Due to the opposing effects of the BTLA-HVEM and LIGHT-HVEM interactions, HVEM can mediate both pro- and anti-inflammatory signals during intestinal inflammation. In line with a pro-inflammatory role, *Hvem*^{-/-} mice are resistant to DSS colitis^{159, 160} and HVEM expression on transferred naive CD4⁺CD45RB^{high} T cells is required for the induction of T-cell mediated colitis in recipient *Rag*^{-/-} mice.^{155, 159} In contrast, absence of HVEM expression in *Rag*^{-/-} recipients (*Hvem*^{-/-}*Rag*^{-/-} mice) resulted in a dramatic acceleration of colitis development compared to *Rag*^{-/-} recipients, suggesting that recipient HVEM exerts an anti-inflammatory role during T-cell transfer colitis.¹⁵⁵ In this model, HVEM-BTLA interactions were required to prevent colitis acceleration, as treatment of *Hvem*^{-/-}*Rag*^{-/-} recipients with an agonistic anti-BTLA antibody prevented disease acceleration. This identified BTLA as the main ligand engaging HVEM in the mucosa during T-cell transfer colitis, but a possible role for HVEM-CD160 interactions could not be excluded.¹⁵⁵ Using the *Citrobacter rodentium*-induced colitis model, the Kronenberg group demonstrated that CD160 is the non-redundant ligand engaging HVEM in the mucosa during intestinal anti-bacterial responses.¹⁶¹ HVEM-CD160 interactions at the mucosal surface enhanced IL-22R mediated STAT-3 activation in epithelial cells and promoted their innate response to acute bacterial infection (Figure 2.5).¹⁶¹ In both uninfected and infected mice, CD160 was expressed by several intraepithelial lymphocyte (IEL) subpopulations, particularly CD8 $\alpha\alpha$ -expressing IEL. These CD160⁺CD8 $\alpha\alpha$ ⁺ IEL rapidly increased during the early stages of *C. Rodentium* infection.¹⁶¹ Given their close contact with intestinal epithelial cells, CD160⁺CD8 $\alpha\alpha$ ⁺ IEL seem likely candidates to interact with HVEM, which is known to be highly expressed on intestinal epithelial cells.^{155, 161} Human CD160 expression on intestinal IEL has been described¹⁶², but further studies are needed to decipher whether it functions to prevent intestinal inflammation as shown in mice. Taken together, BTLA-HVEM and CD160-HVEM interactions help maintain mucosal immune homeostasis

and protect against mucosal infections. Caution is warranted when blocking BTLA-HVEM interactions due to the potential interaction of HVEM with costimulatory ligands present in the intestine.

Lymphocyte activation gene-3 (Lag-3; also known as CD223)

Lag-3 is a co-inhibitory receptor that is expressed on activated CD4⁺ and CD8⁺ T cells and subsets of NK cells.^{163, 164} Lag-3 structurally resembles CD4 and binds to MHC-II with higher affinity than CD4 (Figure 1)¹⁶⁵, resulting in downregulation of antigen-specific CD4⁺ T-cell responses.¹⁶⁶⁻¹⁶⁸ Lag-3 also interferes with CD8⁺ T-cell function¹⁶⁹, suggesting that Lag-3 has other unidentified ligands in addition to MHC-II.¹⁷⁰ In addition to activated T cells¹⁷¹, Lag-3 is expressed on Tregs and is often used as a marker for IL-10-secreting CD4⁺Foxp3^{neg} Tr1 cells.¹⁷²

Multiple companies have developed Lag-3 specific antagonist antibodies that are currently being tested in phase I clinical trials, but it remains to be seen what the effects on gastro-intestinal homeostasis will be. Lag-3 expression in Tregs is critical for Treg-mediated suppression of colitogenic T-cell responses.¹⁷³ Lag-3 expression on Tregs is induced by IL-27, a cytokine that is mainly produced by APCs upon activation by microbial products.¹⁷⁴ IL-27 stimulation has been shown to enhance Treg-suppressive function through a Lag-3-dependent mechanism (Figure 2.6).¹⁷³ In contrast to wildtype Tregs, *Lag3*^{-/-} Tregs failed to suppress effector T-cell expansion and cytokine expression in mesenteric lymph nodes, even after IL-27 stimulation.¹⁷³ In addition to inhibiting effector T-cell responses through enhancing Treg-suppressive function, Lag-3 can prevent T-cell activation through inhibition of DC maturation (Figure 2.6).^{173, 175}

Lag-3 on CD4⁺Foxp3^{neg} T cells also has a role in regulating or suppressing other cells in the intestine. CD4⁺CD25^{neg}Lag-3⁺ T cells prevent colitis induced in *Rag1*^{-/-} recipients by the transfer of naive CD4⁺CD25^{neg}CD45RB^{high} T cells in a Foxp3-independent, IL-10-dependent manner.¹⁷⁶ In a murine model for SLE, CD4⁺CD25^{neg}Lag-3⁺ T cells suppressed B-cell activation and antibody production through TGF-β3.¹⁷⁷ As frequencies of CD4⁺CD25^{neg}Lag-3⁺ T cells are high in Peyer's patches¹⁷⁶, it can be expected that intestinal Lag-3⁺ T-cells regulate intestinal humoral immune

responses, but further studies are needed to prove this hypothesis (Figure 2.7). In sum, Lag-3-dependent mechanisms contribute to intestinal homeostasis influencing the function of both intestinal Tregs and suppressive CD4⁺Foxp3^{neg} T-cell subsets. However, whether Lag-3-driven regulation also modulates effector T cells or B-cell responses in the intestine has not yet been determined.

T-cell immunoglobulin and mucin domain-containing protein-3 (Tim-3)

Tim-3 is a co-inhibitory receptor initially identified on interferon-gamma (IFN γ)-producing CD4⁺ T cells and CD8⁺ T cells¹⁷⁸, but is also expressed on innate immune cells including DCs, NK cells and monocytes.¹⁷⁹⁻¹⁸¹ TCR ligation induces Tim-3 expression on conventional CD4⁺ T cells and Tregs, although with different kinetics.¹⁸² Activated human CD4⁺ T cells that express Tim-3 secrete reduced levels of IFN γ and IL-17A¹⁸³, and Tim-3⁺ Tregs have been shown to specifically suppress Th17 cells.¹⁸² Over the past decade, Tim-3 expression on CD4⁺ and CD8⁺ T cells has especially been associated with exhausted and dysfunctional T-cell phenotypes¹⁸⁴⁻¹⁸⁶, suggesting that Tim-3 negatively regulates T cells that have previously undergone activation. Tim-3 has many ligands, including galectin-9¹⁸⁷ and Ceacam-1¹⁸⁸, and can bind to its ligands both *in cis* and *in trans* (Figure 1).^{183, 188} Therefore, Tim-3 has the capacity to function via both autocrine and paracrine mechanisms to inhibit T-cell responses.

Several Tim-3 antagonists are currently being tested in phase I clinical trials. Data available from animal models support a role for Tim-3 in inhibiting intestinal inflammation. Tim-3 inhibits polarization of pathogenic pro-inflammatory M1 macrophages by preventing phosphorylation of IRF3, a transcriptional factor downstream of TLR-4 that regulates macrophage polarization.¹⁸⁰ In consequence, blockade of Tim-3 exacerbates DSS-induced colitis in wildtype mice, but not in *Tlr4*^{-/-} mice (Figure 2.8).¹⁸⁹ In addition to a role in macrophages, IL-27 greatly enhances TCR-mediated induction of Tim-3 on naïve CD4⁺ T cells¹⁸⁷ but not on Tregs.¹⁷³ IL-27-conditioned Th1 cells induce

less severe colitis in *Rag1^{-/-}* recipients compared to unconditioned Th1 cells, suggesting that IL-27 can directly suppress Th1 cell-mediated colitis via the induction of Tim-3 (Figure 2.9).^{180, 187}

In humans, Tim-3 expression is strongly enriched on CD4⁺ T cells isolated from the intestinal mucosa compared to CD4⁺ T cells in peripheral blood.¹⁹⁰ In contrast, IBD patients had significantly lower frequencies of Tim-3⁺ cells in CD4⁺ T-cell populations isolated from peripheral blood and intestinal biopsies when compared to healthy individuals, possibly suggesting that decreased Tim-3 expression or blockade of Tim-3 may contribute to intestinal inflammation.^{190, 191} Whether restoration of Tim-3 expression in patients with IBD harbors potential clinical benefit needs to be further investigated.

T-cell immunoreceptor with Ig and ITIM domain (TIGIT)

TIGIT is a co-inhibitory receptor specifically expressed by immune cells, including NK cells, memory CD4⁺ T cells and subsets of Tregs.^{192, 193} TIGIT binds to CD155 and CD112, which are expressed on the surface of APCs, T cells and non-hematopoietic cells.^{192, 194} The costimulatory receptor CD226 also binds to the same ligands¹⁹⁵ and together with TIGIT forms a pathway in which CD226 enhances the activation of T cells while TIGIT inhibits their activation¹⁹⁶, which is reminiscent of the CTLA-4/CD28/B7 pathway (Figure 1). On effector T cells, TIGIT directly targets molecules in the TCR signaling pathway, dampening effector T-cell activation, proliferation and inflammatory cytokine secretion.¹⁹⁷ Moreover, TIGIT modifies DC function via bi-directional co-signaling through CD155, promoting the generation of immunoregulatory DCs with decreased IL-12 and increased IL-10 production.¹⁹²

In addition to effector cells, TIGIT promotes the suppressive function of both Foxp3⁺ Treg and regulatory CD4⁺Foxp3^{neg} T cells. TIGIT expression on CD4⁺Foxp3^{neg} T cells discriminates IL-10-secreting CD4⁺ T cells induced by immunotherapy.¹⁹⁸ On Tregs, TIGIT ligation induces the expression of IL-10 and other effector molecules, such as fibrinogen-like protein 2 (Fgl2).¹⁹⁹ TIGIT identifies a Treg subset that specifically suppresses pro-inflammatory Th1 and Th17 but not Th2

responses (Figure 2.10).¹⁹⁹ The *TIGIT* gene is a direct target of Foxp3 and TIGIT expression on Foxp3⁺ Tregs results in higher levels of Treg signature genes.^{199, 200} Moreover, TIGIT⁺ Tregs show enhanced demethylation of Treg-specific demethylated regions (TSDR) ensuring stable Foxp3 expression.¹⁹⁹ Combined, these data show that TIGIT can promote the stability and function of various subsets of regulatory T cells.

In line with its inhibitory functions, TIGIT deficiency or blockade exacerbates disease in models for autoimmune disease.^{197, 201} However, little is known regarding the role of TIGIT in intestinal homeostasis. One study has investigated TIGIT expression in human colonic tissue, and demonstrated that TIGIT is expressed by approximately 30% of intestinal CD4⁺Foxp3^{neg} T cells and virtually all Tregs.⁶⁰ Recently, our group has identified TIGIT as a key regulatory molecule in circulating CD38⁺ effector T cells, a population enriched for T-cells with specificity for mucosal antigens.²⁰² Frequencies of TIGIT⁺CD38⁺ effector T cells were decreased in a subgroup of pediatric IBD patients before start of treatment and TIGIT percentages below 25% identified patients with a shorter duration of clinical remission.²⁰² Mechanistic studies are needed to establish whether TIGIT contributes to regulatory T-cell function in the intestine and whether reduced functioning of the TIGIT pathway accelerates or exacerbates intestinal inflammation.

Future perspectives: targeting co-inhibitory receptors in intestinal inflammation

Over the past decades, the concepts of T-cell costimulation and co-inhibition have substantially increased our understanding of the mechanisms controlling the development and maintenance of intestinal homeostasis (Table 2, Figure 2). In autoimmune diseases such as RA and SLE, mechanistic analysis of co-inhibitory pathways has already led to the identification of potential therapeutic targets and initiation of clinical trials.³ As such, co-inhibitory receptors may represent potential novel therapeutic targets to treat chronic intestinal inflammation as seen in patients with IBD or intestinal graft-versus-host disease. Moreover, studies have only now begun to identify altered expression of co-inhibitory receptors in peripheral blood and intestinal tissue of IBD patients, raising

the possibility that their expression could serve as predictive biomarkers to identify patients that are most likely benefit from targeted therapies.

Increasing the expression levels of co-inhibitory receptors is one possible mechanism to reduce intestinal inflammation. In peripheral blood of IBD patients, expression of Tim-3 is significantly decreased compared to healthy controls and preliminary data indicate that Tim-3 expression increases after anti-TNF α therapy.²⁰³ Similar results have been obtained in patients with multiple sclerosis (MS).^{204, 205} Tim-3 expression is decreased on T cells in peripheral blood of MS patients during active disease and is induced specifically in responders to IFN- β therapy^{206, 207}, a potent inducer of IL-27.²⁰⁸ This suggests that IL-27 administration might be a promising therapy to treat chronic inflammatory disease, possibly by overcoming intrinsic defects in the upregulation co-inhibitory receptor expression during inflammation.^{204, 209} In humans, IL-27 polymorphisms are associated with susceptibility to IBD.²¹⁰ Individuals homozygous for the IBD risk allele near the *IL27* gene express less colonic IL-27 than individuals homozygous for the protective allele.²¹¹ Although multiple studies in mice have identified IL-27 as a suppressor of intestinal inflammation^{173, 212-215}, investigations are ongoing to establish whether IL-27 administration is effective in IBD. In this context, it will be interesting to see whether decreased co-inhibitory receptor expression in intestinal tissue or peripheral blood are predictive biomarkers to identify patients that are most likely benefit from IL-27-directed therapy, or from other cytokines involved in inducing co-inhibitory receptor expression.

In addition to restoration of co-inhibitory receptor expression, co-inhibitory receptor stimulation or mimicking co-inhibitory receptor function could be beneficial to treat intestinal inflammation. An example of such an approach is abatacept, a chimeric CTLA-4 and IgG-Fc fusion protein that mimics the function of CTLA-4 by blocking CD28 costimulation.²¹⁶ CTLA-4-Fc results in downregulation of T-cell activation and proliferation and has demonstrated efficacy in the treatment of autoimmune diseases such as RA and psoriatic arthritis.²¹⁷ However, abatacept was not effective treating intestinal inflammation in IBD patients²¹⁸, which could reflect the relative lack of dependence of CD28

costimulation in memory T cells in the intestine. Moreover, as costimulation through CD28 is also required for the maintenance of Tregs in the periphery²¹⁹, abatacept may impede intestinal Treg survival by inhibiting essential costimulation mediated by CD28.²²⁰ Co-inhibitory receptor fusion proteins other than CTLA-4-Fc might be more successful to treat intestinal inflammation. As an example, the therapeutic potential of targeting the TIGIT/CD226/CD155 pathway by using a TIGIT-Fc fusion protein has been demonstrated *in vivo* in collagen-induced arthritis²⁰¹, but its efficacy in models of IBD remains to be tested.

Co-inhibitory molecules often have synergistic effects to dampen effector T-cell responses and enhance regulatory T-cell function. This is illustrated by models for T-cell exhaustion, where co-inhibitory receptors, such as TIGIT and PD-1, are often co-expressed on exhausted virus-specific CD8⁺ T cells.²²¹ Expression of PD-1, Lag-3, Tim-3 and TIGIT all correlate with IL-10 production by human CD4⁺ T cells, although none of these inhibitory receptors are exclusively expressed on IL-10-producing T cells.¹⁹⁸ This suggests that cooperative function of co-inhibitory receptor molecules is needed to achieve optimal T-cell regulation.¹⁹⁸ In consequence, targeting multiple co-inhibitory receptors simultaneously might be needed for effective treatment of intestinal inflammation. Bi-specific antibodies can be used to target more than one co-inhibitory receptor and could co-ligate co-inhibitory receptors and stimulatory receptors simultaneously. As an example of the latter, crosslinking of Lag-3 and CD3 inhibits T-cell proliferation¹⁶⁹, suggesting that Lag-3-CD3 bispecific antibodies could harbor potential clinical efficacy in T-cell mediated inflammatory diseases, including inflammatory disease of the intestine.

Taken together, accumulating evidence suggests that targeting co-inhibitory receptors is a promising approach to treat intestinal inflammation that warrants further investigation. To define co-inhibitory targets for successful treatment of intestinal inflammation, it is essential to acknowledge their tissue-dependent functions and distinct responses based on cell type and associated kinetics.

Conclusion

This review highlights the emerging role of co-inhibitory receptors in intestinal homeostasis and elucidates many potential prospects for translation to human disease, such as IBD. Despite the substantial insights into the cell-specific expression and function of co-inhibitory receptors in the intestine over the past decades, there is still much to be learned. Key issues remaining to be resolved include the mechanisms of co-inhibitory molecule induction in the intestinal environment, how co-inhibitory signaling pathways integrate to achieve intestinal homeostasis, and whether memory T cells that reside in the intestinal mucosa require specific co-inhibitory signals. In addition, there is a need for a better mechanistic understanding of why inhibitory receptor pathway blockade leads to intestinal inflammation in some individuals but not in others. This should help us to develop more effective therapies while guaranteeing their safety profiles and obtain a better understanding of chronic intestinal inflammation.

Table 2. Role of coinhibitory receptors on intestinal homeostasis.

	Treg differentiation	Treg suppressive capacity	Lineage stability	Regulatory CD4 ⁺ Foxp3 ^{neg} T cells	Inflammatory effector T cells	Other effects	Human intestinal disease
CTLA-4	CTLA-4 is not required for intestinal pTreg induction but does regulate the accumulation of intestinal pTreg <i>in vivo</i> . ⁵³	CTLA-4 is essential for Treg function; <i>Ctla4</i> ^{-/-} CD4 ⁺ CD25 ⁺ T cells fail to prevent colitis induced by naive T-cell transfer into <i>Rag</i> ^{-/-} recipients in most studies. ^{32, 40, 45, 54-56}		Limited data available on the functional role of CTLA-4 on intestinal CD4 ⁺ Foxp3 ^{neg} T cells. Blockade or lack of CTLA-4 in adulthood might allow for the differentiation of immunosuppressive CD4 ⁺ Foxp3 ^{neg} T cells. ^{40, 71, 75}	CTLA-4 prevents hyperactivation of colitogenic naive CD4 ⁺ CD45RB ^{high} T cells ⁴⁰ ; CTLA-4 is essential to suppress systemic Th1 and Th2 responses in high-dose oral tolerance. ⁷⁰ In low dose oral tolerance, other factors than CTLA-4 are required for the suppression of Th1 cell responses. ⁶⁹	CTLA-4 limits Treg expansion ^{47, 71, 72} ; CTLA-4 regulates pTreg frequencies in the intestinal lamina propria. ⁵³	Patients with CTLA-4 haploinsufficiency have impaired Treg function ⁵⁷⁻⁵⁹ ; The CTLA4/CT60 polymorphism that increases production of a soluble CTLA-4 isoform, has been associated with IBD.
PD-1	Synergizes with TGF-β to promote pTreg differentiation. ^{111, 112}	PD-1 does not directly affect pTreg function, as Tregs from PD-1 ^{-/-} mice exhibited normal levels of suppressive activity. ¹¹⁴	PD-1 prevents terminal differentiation of Tregs, rendering Tregs less susceptible for apoptosis and promoting their survival. ^{113, 114, 120}	PD-1 on intestinal CD4 ⁺ Foxp3 ^{neg} T cells enriches for IL-10-producing cells. ¹¹⁵	PD-1 expression on CD8 ⁺ effector T cells is involved in preventing responses to intestinal self-antigens. ^{87, 116, 117}	PD-1 ⁺ Tfh cells in Peyer's patches promote secretory IgA production ^{118, 119} ; PD-1 inhibits TFNα secretion by CD11c ⁺ CD11b ⁺ lamina propria cells in the setting of DSS colitis. ¹³⁰	Increased PD-L1 expression on intestinal epithelial cells of IBD patients. ¹³¹
BTLA					HVEM/BTLA interactions are required to prevent colitis acceleration in T-cell transfer colitis. ¹⁵⁵	HVEM/CD160 interactions promote innate responses to bacterial infection. ¹⁶¹	
Lag-3		Lag-3 expression in Tregs is critical to mediate Treg suppression of colitogenic responses. ¹⁷³		CD4 ⁺ CD25 ⁺ Lag-3 ⁺ T cells prevent colitis induced in <i>Rag1</i> ^{-/-} recipients by the transfer of naive CD4 ⁺ CD25 ⁺ CD45RB ^{high} T cells in a Foxp3-independent, IL-10-dependent manner. ¹⁷⁶	Prevents effector T-cell activation through inhibition of DC maturation. ^{173, 175}		
Tim-3				Tim-3 expression correlates with IL-10 production by Tr1-like human T cells generated <i>in vitro</i> from CD4 ⁺ memory T cells. ²²²	Induction of Tim-3 on effector T cells via IL-27 directly suppress Th1 cell-mediated colitis. ^{180, 187}	Inhibits DC maturation and polarization of pathogenic pro-inflammatory M1 macrophages. ^{180, 189}	IBD patients have lower frequencies of intestinal Tim-3 ⁺ CD4 ⁺ T cells when compared to healthy individuals. ^{190, 191}
TIGIT		TIGIT identifies a Treg subset that specifically suppresses pro-inflammatory Th1 and Th17 but not Th2 responses through effector molecule Fgl-2. ¹⁹⁹	TIGIT ⁺ Tregs show enhanced demethylation of Treg-specific demethylated regions (TSDR) ensuring stable Foxp3 expression. ^{199, 200}	TIGIT is expressed by approximately 30% of intestinal CD4 ⁺ Foxp3 ^{neg} T cells. ⁶⁰	TIGIT inhibits IFNγ production by CD4 ⁺ and CD8 ⁺ effector T cells. ¹⁹⁶		

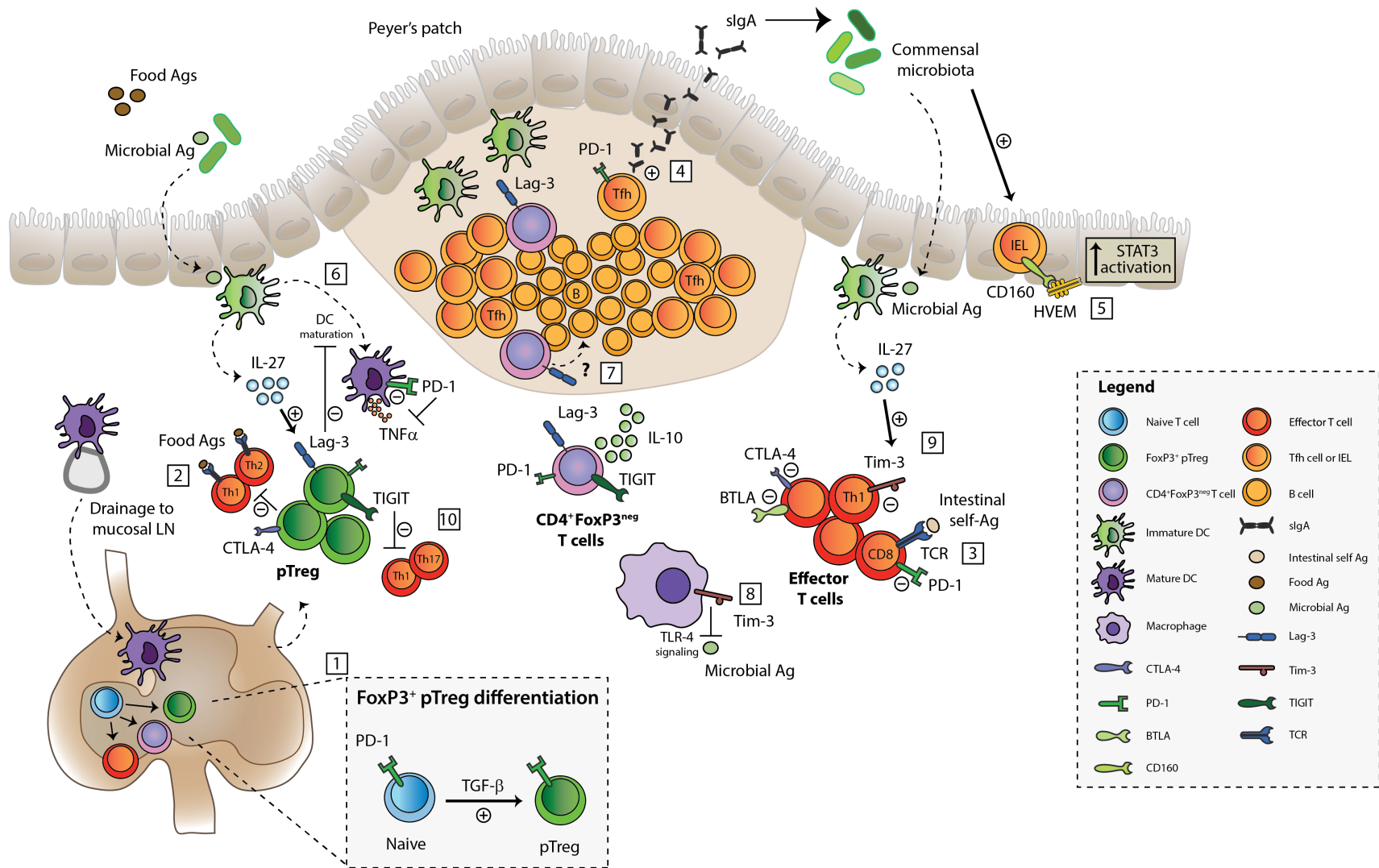


Figure 2. Functional effects of co-inhibitory receptors in the intestine. (1) PD-1 promotes peripheral Foxp3 induction in naive CD4⁺ T cells, especially in TGF-β rich environments. (2) CTLA-4 enhances the suppressive function of Tregs to suppress colitogenic CD4⁺ effector T cells. In addition, the CTLA-4 pathway is important in establishing oral tolerance through suppression of Th1 and/or Th2 responses. (3) PD-1 expression on CD8⁺ effector T cells is involved in preventing responses to intestinal self-antigens. (4) PD-1 expression on Tfh cells regulates Tfh-cell numbers in Peyer's patches, promoting intestinal tolerance to microbiota through secretory IgA. (5) HVEM-CD160 interactions at the mucosal surface enhance IL-22R mediated STAT-3 activation in epithelial cells. (6) IL-27 is

produced by APCs upon activation by microbial products. IL-27 induces Lag-3 expression on Tregs and inhibiting effector T-cell responses through enhancing Treg-suppressive function. Additionally, Lag-3 can prevent T-cell activation through inhibition of DC maturation. **(7)** Frequencies of CD4⁺CD25^{neg}Lag-3⁺ T cells are high in Peyer's patches, but their role in regulating intestinal humoral immune responses remains unknown. **(8)** Tim-3 inhibits polarization of pathogenic pro-inflammatory M1 macrophages. **(9)** IL-27 enhances TCR-mediated induction of Tim-3 on naïve CD4⁺ T cells and can directly suppress Th1 cell-mediated colitis via the induction of Tim-3. **(10)** TIGIT identifies a Treg subset that specifically suppresses pro-inflammatory Th1 and Th17 but not Th2 responses.

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