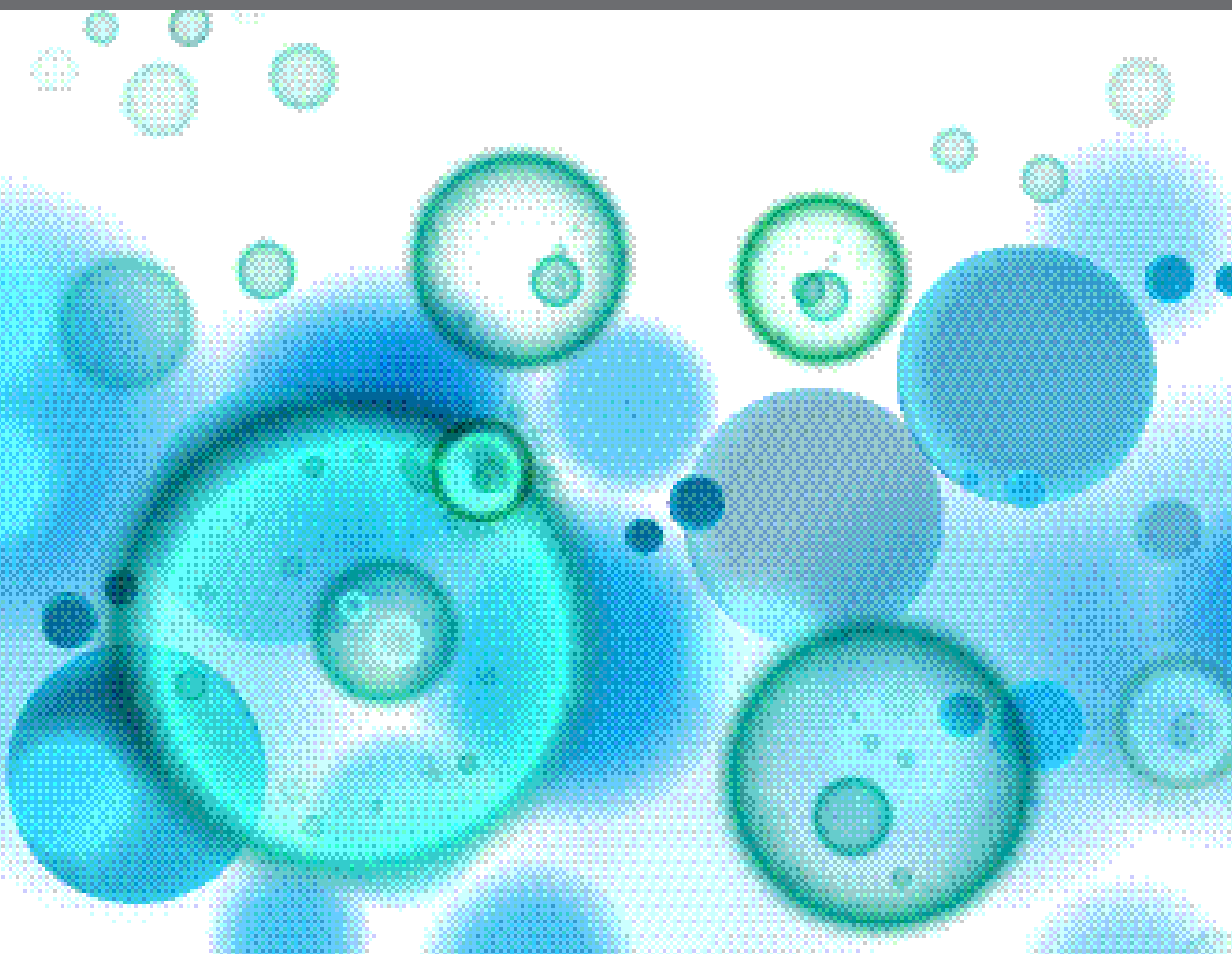
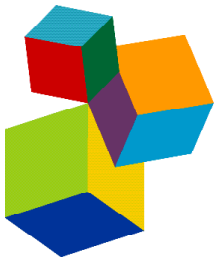
INHIBITORY RECEPTORS AND PATHWAYS OF LYMPHOCYTES

EDITED BY: Alexandre M. Carmo, Paul E. Love and Aaron James Marshall
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics



frontiers

ISSN 1664-8714
ISBN 978-2-88963-993-9
DOI 10.3389/978-2-88963-993-9

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

INHIBITORY RECEPTORS AND PATHWAYS OF LYMPHOCYTES

Topic Editors:
Alexandre M. Carmo, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal
Paul E. Love, National Institutes of Health (NIH), United States
Aaron James Marshall, University of Manitoba, Canada

Citation: Carmo, A. M., Love, P. E., Marshall, A. J., eds. (2020). Inhibitory Receptors and Pathways of Lymphocytes. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-993-9

Table of Contents

frontiers in Immunology

EDITORIAL
published: 22 July 2020
doi: 10.3389/fimmu.2020.01552

Editorial: Inhibitory Receptors and Pathways of Lymphocytes

Alexandre M. Carmo[1,2], *Paul E. Love*[3] *and Aaron J. Marshall*[4*]

[1] IBMC - Instituto de Biologia Molecular e Celular, Porto, Portugal, [2] i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal, [3] Section on Hematopoiesis and Lymphocyte Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, United States, [4] Department of Immunology, Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, MB, Canada

Keywords: lymphocyte, inhibitory receptor, inhibitory signaling, checkpoint blockade, immunotherapy, autoimmunity, cancer

Editorial on the Research Topic

Inhibitory Receptors and Pathways of Lymphocytes

Antigen receptor recognition is a key event that both initiates lymphocyte activation and impacts the fate of immune responses. Co-receptors and co-stimulatory molecules coordinate with antigen receptors to amplify and transduce the initial signals initially triggered at the cell surface, and integrate subsequent signals required to sustain responses. Equally important are the molecular mechanisms that control and terminate activation responses, which act at multiple levels to attenuate activation, modulate signaling thresholds for activation, and shutdown responses in the face of chronic antigen receptor engagement. A very fine line separates activation-mediated clonal expansion from cell death by apoptosis at all stages of lymphocyte development and inhibitory receptors and their associated signaling molecules can determine the difference between life and death. Lymphocyte differentiation and acquisition of effector functions is continuously modulated by a variety of receptors and signaling pathways which balance quiescent, activated and exhausted phenotypes as well as cytolytic activity and cytokine production patterns. Inhibitory receptors and signaling molecules collectively have critical functions to integrate multiple environmental inputs to modulate immune cell activation states in a variety of tissues. As discussed in the reviews in this Topic, inhibitory receptor function can tip the balance between health and disease in a variety of contexts involving chronic lymphocyte activation.

Several reviews in this Topic address the biological functions and clinical applications of Ig superfamily "checkpoint receptors" PD-1, CTLA-4, BTLA, TIGIT, 2B4, Tim3, and LAG-3. De Sousa Linhares et al. provide an excellent overview, summarizing current evidence regarding the unique features and clinical significance of many of these receptors, as well as discussing the remaining open questions. Yasuma-Mitobe and Matsuoka discuss studies implicating these receptors in retroviral infections such as HIV. Morris et al. discuss the function of these inhibitory receptors in the context of CD8+ T cell memory, recall responses and terminal differentiation. Brunner-Weinzierl and Rudd discuss the roles of PD-1 and CTLA-4 in tumor immunotherapy, focussing on their roles in controlling T cell migration via impact on integrin activation and chemokine receptor expression and signaling. Gianchecchi and Fierabracci comprehensively review evidence regarding the roles of PD-1 and its ligand PD-L1 in regulatory T cell (Treg) development and functional activity, and address how this may relate to development of autoimmunity. Paluch et al. address the role of both PD-1 and CTLA-4 in autoimmunity, discuss the rationale for checkpoint receptor agonists as treatment for this class of diseases and outline several different approaches being used in the design of checkpoint agonists.

The article by Georgiev et al. provides a focused review of CD96, another type 1 transmembrane glycoprotein of the Ig superfamily. CD96, together with TIGIT and other receptors, form a distinct sub-group of regulatory Ig superfamily receptors that are relatively less studied, but likely play

OPEN ACCESS

Edited by:
Wanjun Chen,
National Institutes of Health (NIH), United States

Reviewed by:
Wilfried Ellmeier,
Medical University of Vienna, Austria

****Correspondence:***
Aaron J. Marshall
aaron.marshall@umanitoba.ca

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *04 May 2020*
Accepted: *12 June 2020*
Published: *22 July 2020*

Citation:
Carmo AM, Love PE and Marshall AJ (2020) Editorial: Inhibitory Receptors and Pathways of Lymphocytes. Front. Immunol. 11:1552. doi: 10.3389/fimmu.2020.01552

regulatory roles in T cells and NK cells. Kim and Kim also address the role of CD96 in NK cell function and discuss the role of established checkpoint receptors such as PD-1, CTLA-4, TIM-3, and LAG-3 in NK cells. These authors also address the role of killer inhibitory receptors (KIR) and CD94/NKG2A in mediating the unique regulatory mechanisms of NK cells.

Gonçalves et al. and Voisinne et al. address the scavenger receptor cysteine-rich (SRCR) glycoproteins CD5 and CD6 and their differential roles in T cell development and responses. The significance of ligand binding in signal modulation is explored, as well as the inhibitory signaling mechanisms. The therapeutic potential of targeting these receptors for immunotherapy is discussed.

Sialic acid-binding Ig-like lectins (Siglecs) are another important family of inhibitory receptors in lymphocytes. Tsubata has reviewed data regarding the roles of the Siglec receptors CD22, CD72, and SiglecG in development and function of B cells, focusing on how distinct ligand-recognition properties of these receptors determine their functional roles. Clark and Giltiay comprehensively review literature regarding the roles of CD22 in B cell activation, migration, tolerance and autoimmunity, as well as evidence regarding therapeutic targeting of CD22.

Different classes of signaling molecules mediate the inhibitory functions of these receptors. Pike and Tremblay review the roles of protein tyrosine phosphatases such as PTPN22 in controlling CD4+ T cell activation and function, particularly in the context of intestinal inflammation and inflammatory bowel disease. The role of SHP-1 protein tyrosine phosphatase recruitment via receptor ITIM motifs is touched on by several other reviews, while some authors indicate that early implication of this phosphatase turned out to be incorrect (for example, in the case of CD5). The role of cbl family ubiquitin ligases in CD5/6 function is explored by Voisinne et al. and Gonçalves et al.. These authors also touch on the roles of the inhibitory tyrosine kinase Csk which controls the activity of Src kinases.

Rodríguez-Galán et al. uniquely explore the interesting topic of immune regulation mediated by miRNA networks. They discuss evidence that miRNAs can control not only expression of inhibitory receptors such as PD-1 and CTLA-4 in lymphocytes, but also inhibitory protein phosphatases and phosphoinositide phosphatases, critical cell survival and cell cycle regulators as well as co-stimulatory receptors and cytokines.

Together, the excellent reviews collected under this Topic only begin to capture some of the breadth and depth of research in this fascinating and important area. It is clear that "Inhibitory receptors" is somewhat of an over-simplification to describe the nuances of how these receptors function to maintain homeostasis within the immune system. Their roles extend beyond modulation of initial lymphocyte activation signals, with perhaps their greatest impact being in balancing the needs of host defense, avoiding excessive immunopathology, and maintaining normal physiology during infection and chronic inflammation. Future studies will no doubt address how these receptors each function within different tissue contexts and in different contexts of physiological and metabolic stress. This will require better understanding of individual receptor/ligand dynamics and how expression of receptors and ligands are modulated in different locations and contexts.

Therapeutic targeting of inhibitory circuits in lymphocytes will no doubt continue to be a major focus over the coming years. Creative approaches to targeting at the level of receptors, ligands and signaling molecules will be guided by improved understanding of the relevant molecular and cell biology. One issue in therapeutic targeting is the clear functional redundancy among some receptors and ligand systems, which may require targeting multiple molecules to achieve biological impact. Targeting at the level of inhibitory signaling molecules may in some cases offer opportunities to bypass receptors and manipulate lymphocyte functions via small molecule inhibitors or activators of signaling molecules, or perhaps miRNAs. A limitation that has become very apparent with therapeutic targeting of checkpoint receptors is that disturbing the immune ecosystem by blocking inhibitory pathways can have unintended consequences. Freeing the immune system from control networks that have evolved to maintain homeostasis may cure one disease, but create another. This underlines the need for a deeper understanding of how these inhibitory networks function at the molecular, cellular, tissue and whole organism level. Ultimately the goal is to more selectively release or restore these powerful regulatory systems to reset the ecosystem for maximum therapeutic benefit.

AUTHOR CONTRIBUTIONS

AC, PL, and AM served as co-editors of this topic and wrote the editorial. All authors contributed to the article and approved the submitted version.

FUNDING

AC was funded by FEDER through the Operacional Programme for Competitiveness and Internationalization (POCI) and by Portuguese funds through FCT, grant PTDC/MED-IMU/32296/2017. AM was funded by the Canadian Institutes of Health Research (PJT-162268).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a shared affiliation, though no other collaboration, with one of the authors PL at time of review.

frontiers in Immunology

REVIEW
published: 17 May 2018
doi: 10.3389/fimmu.2018.01072

Coming of Age: CD96 Emerges as Modulator of Immune Responses

*Hristo Georgiev, Inga Ravens, Georgia Papadogianni and Günter Bernhardt**

Institute of Immunology, Hannover Medical School, Hannover, Germany

CD96 represents a type I transmembrane glycoprotein belonging to the immunoglobulin superfamily. CD96 is expressed mainly by cells of hematopoietic origin, in particular on T and NK cells. Upon interaction with CD155 present on target cells, CD96 was found to inhibit mouse NK cells, and absence of this interaction either by blocking with antibody or knockout of CD96 showed profound beneficial effects in containment of tumors and metastatic spread in murine model systems. However, our knowledge regarding CD96 functions remains fragmentary. In this review, we will discuss structural features of CD96 and their putative impact on function as well as some unresolved issues such as a potential activation that may be conferred by human but not mouse CD96. This is of importance for translation into human cancer therapy. We will also address CD96 activities in the context of the immune regulatory network that consists of CD155, CD96, CD226, and TIGIT.

Keywords: CD96, immunoglobulin superfamily, CD155, CD226, TIGIT, NK cells, T cells, immune regulation

OPEN ACCESS

Edited by:
Alexandre M. Carmo, i3S, Instituto de Investigação e Inovação em Saúde, Portugal

Reviewed by:
Fabio Malavasi, Università degli Studi di Torino, Italy
Philip Anton Van Der Merwe, University of Oxford, United Kingdom

***Correspondence:**
Günter Bernhardt
bernhardt.guenter@mh-hannover.de

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *28 February 2018*
Accepted: *30 April 2018*
Published: *17 May 2018*

Citation:
Georgiev H, Ravens I, Papadogianni G and Bernhardt G (2018) Coming of Age: CD96 Emerges as Modulator of Immune Responses. Front. Immunol. 9:1072. doi: 10.3389/fimmu.2018.01072

INTRODUCTION

Human CD96 (hCD96) was discovered in 1992 and named originally "T cell activation, increased late expression" (1) (**Figure 1**). Although identified as a marker distinguishing a subset of acute leukemias (2, 3), hCD96 did not receive further attention for more than a decade. This changed when human CD155 (hCD155), formerly addressed as receptor for poliovirus (PVR), was detected as an interaction partner mediating cell adhesion (4). Furthermore, these findings suggested a role of the hCD155/hCD96 axis in target cell elimination by NK cells. Ironically, Wang et al. (1) already mentioned PVR in their publication because it showed up among other polypeptides in a similarity search. Indeed, CD96 (**Figure 1**) and CD155 are membrane bound receptors of the immunoglobulin superfamily (IgSF) and are distantly related to each other (5). However, in contrast to hCD155 that is expressed by a huge variety of cell types, available data indicated that hCD96 expression is largely restricted to cells of hematopoietic origin, in particular to T and NK cells (1, 4). This was confirmed by a study of mouse CD96 (mCD96) (6). Yet attempts to demonstrate a direct role of mCD96 in NK-mediated killing *in vitro* failed (6), a flaw that was resolved later on when it was shown that mCD96 can suppress NK cells *in vivo* (7). Like hCD96, hCD155 initially was an orphan receptor with no known cellular function apart from serving as the cellular receptor for PVR (8). CD155 is related to nectins (nectin 1–4) that mediate homophilic cell adhesion (9). However, in contrast to nectins, CD155 does not interact with itself in *trans*. Instead, it was reported to bind to nectin-3 assisting in the establishment of adherens junctions between tissue cells (10, 11). Moreover, CD155 is engaged in regulation of cell movement and proliferation (12–14) explaining why it was found to be a tumor antigen, first in rodents (15–17), later on also in human (18). Nowadays, hCD155 is firmly established as a marker for various types of cancer, and several reports had shown that the degree of hCD155 overexpression correlates positively with poor prognosis (19). CD96 and especially CD155

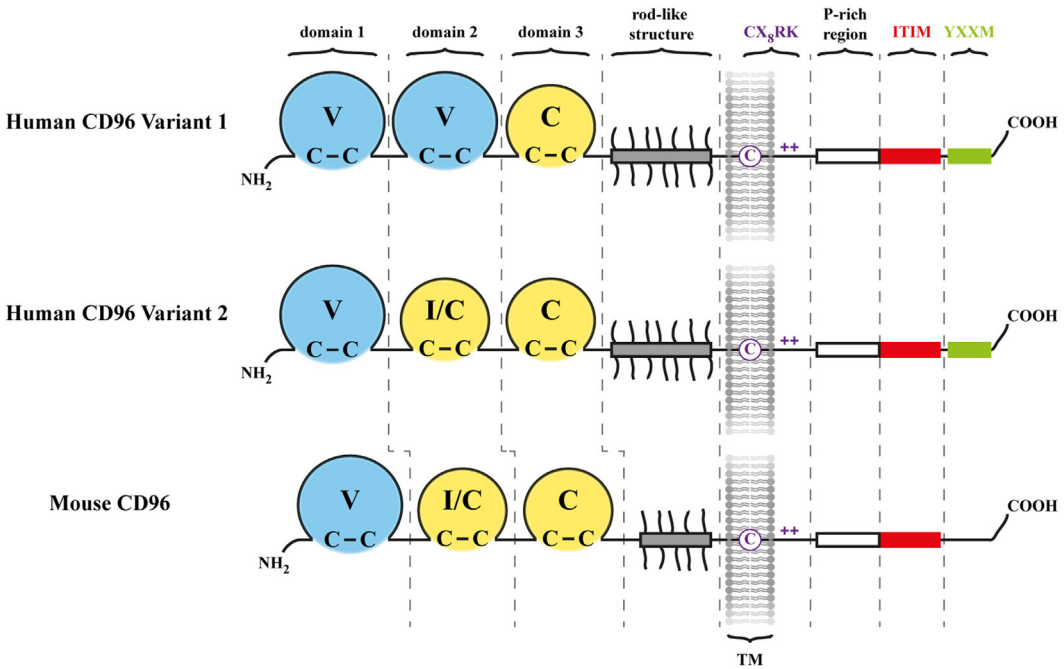


FIGURE 1 | Architecture of CD96. Shown are the two human CD96 (hCD96) isoforms (variant 1 and variant 2) along with mouse CD96 (mCD96). Three Ig-like domains comprise the N-terminal (NH_2) part of CD96 in mouse and hCD96 where V indicates a V-like domain and C indicates a C-like domain. The second domain is predicted to fold as an I-like or C-like domain in hCD96 variant 2 and mCD96. The proline/serine/threonine-rich region (gray bar) contains many potential O-linked sugar modification sites (short protrusions) and may adopt a rod-like shape. The transmembrane (TM) and cytoplasmic domain harbors motifs of potential importance for signaling triggered by CD96 as described in the text and in more detail in **Figure 3**. The C denotes a cysteine residing in the TM region, and the + indicates positively charged amino acid residues.

accumulated considerable sequence diversity at the amino acid level between man and mouse. Nevertheless, the interaction of CD96 with CD155 was preserved and co-evolved with species in that hCD155 only binds hCD96 but not mCD96 and *vice versa* (6, 20, 21). This corroborates the biological significance of this liaison.

In this review, we will focus on common structural and functional aspects of CD96 that are conserved between man and mouse. But we will also highlight species-specific differences as well as gaps in our knowledge illustrating that there is still a way to go to understand comprehensively the role of this receptor in immune regulation and surveillance. By necessity, this will encompass in part a discussion of the functional context into which CD96 is embedded on the molecular level, in particular the receptors that like CD96 interact with CD155 in *trans*: CD226 (DNAM-1) (22) and TIGIT (WUCAM, VSTM3) (23). Like for CD96, binding of TIGIT (23–25) and CD226 (26, 27) to CD155 is well conserved between species. In fact, nectins, CD155, CD96, CD226, and TIGIT represent a subfamily of related IgSF receptors constituting a stimulatory/inhibitory network (**Figure 2**). For convenience, we will address these receptors as CD155 family members here and distinguish between human (h) and mouse (m) receptors whenever appropriate. In addition, a further branch exists consisting of nectin-like molecules (28) that will not be part of the discussion because there is no indication so far that CD96 interacts with them.

STRUCTURE OF CD96

The IgSF-Part of the Ectodomain

CD96 represents a single pass transmembrane receptor that is heavily *N*-glycosylated (1, 6) (**Figure 1**). The crystal structure of the CD96 ectodomain is not resolved wherefore its folding pattern was deduced from comparisons with other IgSF members. According to this, the outermost domain represents a V-like domain in h/mCD96 and mediates binding to h/mCD155 in *trans* (20). A N-terminally located V-like domain is a common feature shared by all CD155 family members and as far as investigated, extracellular binding to themselves or other family members (but also to viruses) is invariantly restricted to this domain (blue in **Figure 2**). Available data from crystal structures of human/mouse nectins, CD155, and TIGIT revealed a consensus binding interface that consists of amino acids residing in the CC′C″FG region of the V-like domain (29–32). The laterally arranged CC′C″FG interfaces contact each other in an almost rectangular orientation forming the binding complex. An alignment of CD96 with its prime binding partner CD155 would suggest that most critical residues of the binding interfaces are conserved predicting that CD96 forms a "standard" dimer in *trans* with CD155 (**Figure 3A**). As a hallmark of these interactions amino acids of the FG loop [TFP in nectins/CD155 and (L/T)YP in CD96/CD226/TIGIT; called the key] of one binding partner come into contact with residues in the C′C″-loop area of the other (AX_6G motif, arrow in

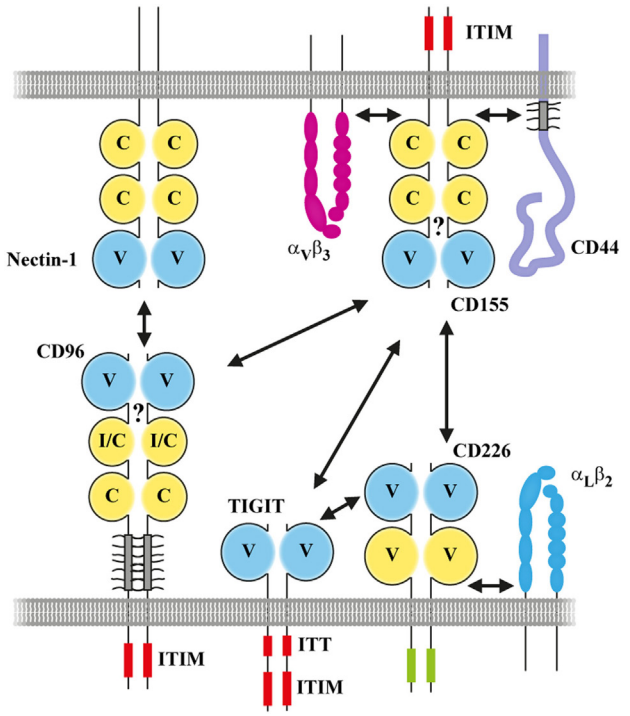


FIGURE 2 | Interactions in *cis* and in *trans* providing the platform for CD96 functions in the context of the CD155 network. Interactions are indicated by two-sided arrows in black. The question mark indicates that it was not shown so far whether CD96 can form a *cis*-homodimer on the cell surface. Red boxes: ITT and ITIM, respectively. Green box: the cytoplasmic domain of CD226 containing a tyrosine and serine residue critically involved in signaling (Y_{322} and S_{329} in human). Not all interactions that can be engaged by CD155 family members are shown. Moreover, the associations shown in *cis* between CD155 family members and partner molecules are cell type specific and/or depend on a cells activation status. Please note that the molecular proportions of the given molecules are not drawn to scale to highlight the interactions between CD155 family members.

Figure 3A, referred to as the lock) that build an acceptor pocket. In addition, residues in the F-strand next to the cysteine (green star in **Figure 3A**) forming the intra-domain disulfide bridge directly face each other and their compatibility impacts on the stability of the respective dimer. Also residues of the C-strand (boxed in **Figure 3A**) locate to the contact area. These residues and those comprising the lock are less well conserved among CD155 family members than those of the key region. The second domain of CD96 adopts an I/C-like folding pattern in mouse and man but in human, a V-like domain can be generated due to alternative splicing of the hCD96 pre-mRNA (20). Thus, in human but not in mouse two variants exist with respect to the ectodomain composition. By contrast, the third domain is a C-like domain in both hCD96 and mCD96.

The Stalk Region

The three Ig-like domains are separated from the transmembrane (TM)-domain by an unusually long region that is rich in proline, serine and threonine (**Figure 1**). This allows for extensive O-linked glyco-modification that would confer to this domain a rod-like structure. As a consequence of this, the Ig-like domains should protrude from the glycocalyx layer markedly exposing them to contacting cells (1). Proline/serine/threonine-rich stalks are also present in other TM receptors like CD44 or CD8α/β. Interestingly, the degree of sialylation of the O-linked oligosaccharides on the CD8β chain impacts on co-receptor function during development of T cells in thymus (35, 36). Therefore, the stalk-like region of CD96 may play a role in orientation/presentation of the Ig-like domains representing a tool how cells could modulate the capacity of CD96 to interact with binding partners.

The TM/Cytoplasmic Domain

The intracellular domain of h/mCD96 is rather short (45 amino acids) but possesses several interesting motifs of potential importance for CD96 function (**Figure 3B**). In accordance with this, there is a high degree of conservation between man and mouse in this domain (80% as compared with 54% for the ectodomain). A split motif consisting of an intra-TM cysteine and charged residues at the TM/cytoplasmic border (CX_8RK) may serve for constitutive association with SRC-like kinases (34). Similarly composed motifs are present in other immune-relevant receptors such as CD28, CD2, CD4, CD8α, FcεRIβ, TIGIT, and CD44 (**Figure 3B** and not depicted). In mCD44, the intra-TM cysteine residue is of critical importance for kinase association (34). Interestingly, the very same residue that is conserved across species was shown to be crucial for homo-dimerization of hCD44 following cell activation (37, 38). Only upon covalent dimerization (not simply clustering), hCD44 can bind efficiently to its ligand hyaluronic acid and initiate signal transduction. Another feature conserved between hCD96 and mCD96 is a proline-rich (P-rich) tandem (RPPPFKPPPPPIK) that is flanked by arginine and lysine residues (**Figure 3B**). A similar but longer P-rich sequence was found in FasL (39). P-rich motifs represent binding sites for SH3 domain containing signaling components (40). In FasL, binding of SRC-like kinases triggers tyrosine phosphorylation and along with mono-ubiquitination of the flanking lysine residues this results of FasL sorting into secretory lysosomes (41). There is a partial overlap of the P-rich stretch with the ITIM-consensus sequence that is also conserved between man and mouse. Remarkably, Wang et al. already stressed the notion that also CD2 harbors P-rich regions in its cytoplasmic tail (1) and one of these (sequence: KGLPPLP) was shown later on to be involved in activation of integrin β1 *via* antibody mediated hCD2 cross-linking (42). This pathway requires recruitment of PI3 kinase. Although the KGLPPLP sequence does not bind to the p85 subunit of PI3 kinase, it is crucial for CD2-triggered PI3-kinase activity. In hCD96 but not mCD96, a binding of the p85 subunit *via* its SH2 domain could be accomplished by the adjacent YXXM motif that is known to bind also other signaling relevant modules in the cytoplasmic domains of CD28, ICOS-1, and CTLA-4 (43). The mutation creating the YXXM motif apparently occurred late during evolution since it is not present in all primate species (**Figure 3B**). Taken together, considering the tight packaging of consensus sequences for cytoplasmic binding partners, surprisingly little is known about their relevance for CD96 function.

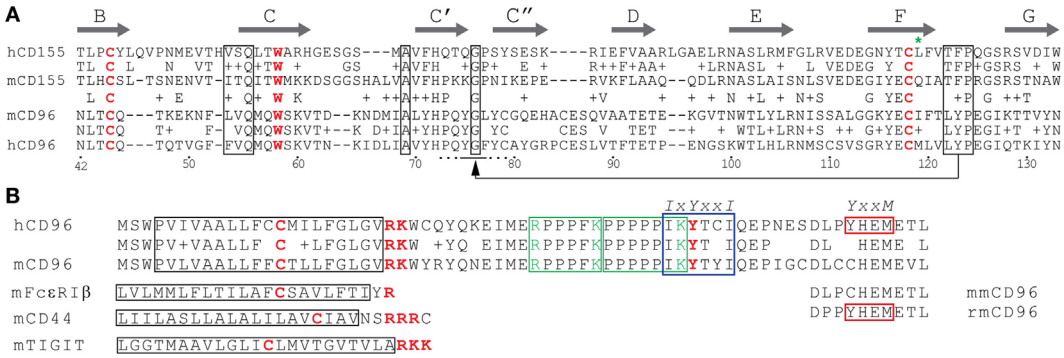


FIGURE 3 | Sequence alignments of CD96 and CD155 domains. **(A)** Alignment of domain 1 of human CD155 (hCD155) and mCD155 as well as human CD96 (hCD96) and mouse CD96 (mCD96). The β-strands (thick arrows) are given according to crystal data for hCD155 (33), but the A strands were not included. A + indicates amino acids with similar chemical properties. Diagnostic residues typical of IgSF members, the cysteines forming the intra-domain disulfide bridge and tryptophan residues, are shown in red. Boxed are conserved sequences among nectins, CD155, and TIGIT that are important for homodimer and heterodimer formation as discussed in the text. The arrow highlights amino acids involved in contact formation where residues of the FG loop of one binding partner contact the C′C″ pocket of the other (located at the AX_6G motif, A and G are boxed). The residues adjacent to the cysteine (green star) in the F strand face each other in the dimers. An additional alignment of mCD155 and mCD96 is superposed to highlight conserved amino acid residues among these distantly related receptors. **(B)** Alignment of the transmembrane (TM) and cytoplasmic domains of hCD96 and mCD96. The TM regions are boxed in black, the tandem proline-rich region in green, the ITIM motif (IXYXXI) in blue, and the YXXM motif in red, respectively. A cysteine residue located in the TM regions is highlighted in red along with the basic residues at the beginning of the cytoplasmic domain. Basic residues flanking the proline-rich motifs are marked in green and the tyrosine of the ITIM motif in red. Shown are also short corresponding amino acid sequences of mFcεRIβ, mCD44, and mTIGIT for comparison on the left side. Peptides representing these regions in mFcεRIβ, mCD44, and mCD28 bound to the SRC-related kinases LCK and LYN (34). To the right, the C-terminal CD96 residues of gray mouse lemur (*Microcebus murinus*, mmCD96) and rhesus monkey (rmCD96) are aligned to demonstrate that the YxxM motif is not conserved among non-human primates. Alignments of the domain 1 and the TM/cytoplasmic domains were done using the NCBI blastp suite applying standard settings, TM regions were predicted by the TMHMM Server v. 2.0.

DISSECTING CD96 FUNCTIONS IN COMPARISON WITH CD226 AND TIGIT: A SNAPSHOT

CD96 belongs to a network of interactions that manipulates in a multifaceted fashion adhesion, activation, and inhibition of participating cells (**Figure 2**). CD226 was reported to activate T and NK cells (22, 44, 45) whereas TIGIT (23, 46, 47) and CD96 (7) act as inhibitors upon interaction with CD155-expressing cells. The described interaction network exists in both mouse and human. Also the functional activities triggered by its engagement appear identical to a large extent despite some black boxes. Most importantly, a direct inhibitory role of CD96 was proven only for murine NK cells and explored *in vivo* mainly in the context of tumor models (next paragraph). Conclusive evidence that this also applies to human NK cells is missing so far (48). In addition, there is currently a wealth of data documenting that CD226 activates T and NK cells but with regard to TIGIT, most publications demonstrate its role in inhibiting T cells, especially CD8 T and regulatory T cells [e.g., Ref. (49–53)]. Less data were presented that documented an inhibition of CD4 T or NK cells by TIGIT (47, 54–56). It remains to be seen whether this illustrates a functional bias of these two inhibitory receptors in that TIGIT predominantly suppresses CD8 T and regulatory T cells whereas CD96 mainly inhibits NK cells. Possibly, this view is misleading and just reflects the current lack of information especially regarding CD96 that was much less thoroughly investigated compared with CD226 or TIGIT.

THE INHIBITORY POTENTIAL OF CD96 PRESENT ON NK CELLS

The first study characterizing hCD96 functionally implied an enhancing effect of the hCD96/hCD155 interaction on NK cell mediated cytotoxicity (4). It was demonstrated that engagement of freshly established polyclonal human NK cell lines *via* an anti-hCD96 monoclonal antibody (mAb) can promote lysis of P815 cells in a redirected killing assay. By contrast, Stanietsky et al. failed to confirm this in a similar setup. Instead, a rather mild boosting effect contributed by hCD96 on 2B4- and NKp30-mediated killing was observed (47). Importantly, attempts to demonstrate a direct role of CD96 as activator for NK cell-mediated cytotoxicity *in vitro* failed because neutralizing anti-CD96 mAb did not reveal any effect of hCD96 in killing of ovarian carcinoma cells (57) or myeloma cell lines (58) and of mCD96 in elimination of RMA, RMA-S, or YAC-1 tumor cells (6). A landmark publication addressing the function of mCD96 was published in 2014 by the group of Smyth (7). In a series of elegant experiments, Chan et al. demonstrated that mCD96 deficient ($CD96^{-/-}$) mice were significantly more sensitive to LPS-induced endotoxicosis than wild-type (WT) mice. This was due to an increased production of IFNγ by NK cells in the $CD96^{-/-}$ animals. Remarkably, this phenotype was not observed in $TIGIT^{-/-}$ mice although the majority of splenic NK cells also express TIGIT (59). This implied a dominant suppressive function of mCD96 on NK cells over mTIGIT under these experimental conditions. The level of IFNγ production by NK cells controlled by mCD96 was also shown to govern the degree

of protection in MCA-induced fibrosarcoma and experimental lung metastases models. In the latter, absence of mTIGIT had no impact on the metastatic burden. The same effects were observed after *in vivo* administration of a blocking anti-mCD96 mAb in WT mice (blocking refers to blocking binding to mCD155). Furthermore, protection was based entirely on an increased IFNγ production in CD96$^{-/-}$ mice and not on enhanced NK cell mediated cytotoxicity. This was demonstrated by *in vivo* administration of a neutralizing anti-IFNγ mAb abolishing the protective effect and by a lack of difference in the killing efficiency of B16F10 cells by CD96$^{-/-}$ or WT NK cells. These findings provided a plausible explanation why earlier attempts to verify a role of h/mCD96 in NK mediated killing *in vitro* had failed. It appears that mCD96 mainly controls the extent of cytokine production by NK cells that critically depends on an interaction with mature dendritic cells (7) while leaving direct killing tested *in vitro* untouched. *Vice versa*, h/mTIGIT may contribute to control the latter (47, 56, 60). Yet, such functional specialization is certainly not absolute and must take into account the specific immunological context as mTIGIT was shown to manipulate IFNγ production by NK cells (54, 60). In continuation of their study, Smyth's group evaluated in more detail *in vivo* the therapeutic potential of anti-mCD96 mAb in murine tumor models (61). Blocking of mCD96 *in vivo* conveyed protective antimetastatic activity against B16F10 melanoma, 3LL lung carcinoma, LWT1 melanoma, and RM-1 prostate carcinoma cells. The antimetastatic activity of mCD96 blocking was largely abolished when mCD226 was neutralized concomitantly corroborating that an imbalance of the CD155/CD226/CD96 axis impacted on metastatic spread. The beneficial effects of mCD96 blockade were independent of antibody-dependent cell-mediated cytotoxicity (ADCC) because they continued to exist in mice lacking Fc receptors. Moreover, the combined administration of anti-CD96 mAb with anti-PD-1 mAb or anti-CTLA-4 mAb, which are therapeutically used as immune checkpoint blockade antibodies, led to significantly reduced numbers of lung metastases and increased survival in comparison with treatment with anti-PD-1 mAb or anti-CTLA-4 mAb alone. Of interest, the antimetastatic treatment was still effective though reduced in power when mAbs were given with delay. Consistent with the previous study by Chan et al. (7), the antitumor effect was mediated by an elevated IFNγ production by NK cells and an increased tissue infiltration rate but was not caused by augmented killing of target cells. This was corroborated by the finding that the antimetastatic effect of CD96 blockage was still present in perforin deficient mice but was completely abolished in the presence of neutralizing anti-IFNγ mAb. Again, TIGIT$^{-/-}$ mice challenged with the same tumor models showed no significant reduction in numbers of tumor metastases in comparison with WT mice. Although there was no evidence proving the direct *in vivo* involvement of mTIGIT alone in controlling tumor metastases in these models, there was a synergistic effect of mCD96 and mTIGIT since blocking of mCD96 with anti-mCD96 mAb in TIGIT$^{-/-}$ animals caused a higher degree of reduction of the numbers of tumor metastases in comparison with anti-mCD96 mAb administration in WT animals (61). The effects of an mCD96 blockade in the context of combined therapeutic approaches were refined further in a recent study utilizing pancreatic ductal adenocarcinoma (PDAC) in mice as a model for highly disseminating cancers which are largely resistant to checkpoint blockage immunotherapies (62). A set of *in vivo* experiments revealed that treatment with an anti-PD-1 mAb as a neoadjuvant in addition to chemotherapy efficiently suppressed local tumor recurrence and improved survival. Still, this approach could not effectively control distant metastases. Remarkably, an additional administration of a blocking anti-mCD96 mAb (clone 6A6) but not of a non-blocking mAb (clone 8B10) as an adjuvant following resection of the primary tumor most significantly improved the long-term survival and reduced the recurrence incidence of PDAC (62). Cytokine production was not evaluated in this study though an abrogation of the protective effect was observed following NK cell depletion. These results demonstrated the importance of a coordinated treatment regimen addressing NK and T cells for a successful therapy. Moreover, disrupting an ongoing functional interaction of mCD96 with mCD155 was crucial for NK-mediated containment of metastatic spread. However, upon transfer of B16F10 cells into mCD155-deficient recipients, the non-blocking mAb 8B10 (but not clone 6A6) retained some antimetastatic activity (63). It should be noted, though, that in this particular setting, the transferred tumor cells express mCD155 and that NK cells in mCD155 knockout hosts possess more mCD226 on their surface than NK cells in WT animals (64). Although these special parameters make an interpretation of the result by Aguilera et al. (63) difficult, it illustrates that the therapeutic effects of individual antibody clones may rely on several mechanisms to a different extent depending on the case under investigation.

NK CELL EXPRESSED hCD96 AS THERAPEUTIC TARGET IN CANCER

Despite the fact that there are increasing numbers of cases documenting mCD96 involvement in controlling tumors and their metastases in mouse models, up to date there is no study translating a concept of an mAb-based neutralization of CD96 into human therapy. However, the design of such treatment strategies is impaired by the lack of conclusive evidence as to whether hCD96 inhibits or activates human NK cells. Since investigations *in vitro* were not helpful in this regard (see above), the *ex vivo* analysis of NK cells obtained from tumor patients could provide at least indirect evidence. This is exemplified by hCD226 that is frequently downregulated as part of an immune evasion mechanism in NK cells controlling tumors overexpressing hCD155 [for example, in ovarian cancer (57), for a review, see Ref. (65)]. Unfortunately, analogous information for hCD96 is very limited yet would suggest that in cases of pancreatic cancer hCD96 rather activates human NK cells (66). However, more studies are required to corroborate this.

hCD96 IN DIAGNOSIS AND POTENTIAL THERAPEUTIC TARGET IN ACUTE MYELOID LEUKEMIA (AML)

In contrast to the role of CD96 participating in immune surveillance of tumors, hCD96 itself was identified as tumor marker.

Indeed, well before first studies deciphered its functions, hCD96 was reported to be upregulated in subpopulations of T-acute lymphoblastic leukemia and AML (2, 3). Increased expression of hCD96 was shown in several subsequent studies to correlate with poor prognosis and enhanced resistance to chemotherapy [see, for example, Ref. (67, 68)] firmly establishing hCD96 as a diagnostic marker. Following the hierarchical theory of cancer development (69), it is assumed that in leukemia the disease-causing incident(s) occur among stem cells generating a leukemic stem cell (LSC) that shares self-renewal potency with the stem cells (70, 71). In line with this, Hosen et al. identified hCD96 as a potential target in an LSC-specific therapy to treat AML (72). In approximately two-thirds of the AML cases analyzed, the majority of AML-LSC was found to be $hCD96^+$ whereas only a small fraction of approximately 5% was $hCD96^+$ among hematopoietic stem cells from healthy donors. A promising treatment strategy would therefore be to sort out hCD96-expressing stem cells before autologous transplantation of AML patients. A classical approach of an hCD96-based therapy would engage mechanism such as ADCC and complement dependent-cytotoxicity to eliminate AML cells but must take into account that this might affect other hCD96-expressing cells as well (72–74). The functional role hCD96 plays in AML-LSC biology remains elusive, and its expression may turn out irrelevant or of inferior importance for the neoplastic properties of these cells but raises the question whether hCD96 would exert inhibition as observed for mCD96 in NK cells.

FUNCTION OF CD96 IN T CELLS

Although identified originally as a human T cell antigen (1), not much is known about CD96 function in CD4 and CD8 T cells. Recently, the level of hCD96 expression on CD8 T cells from HIV-1-infected patients with high and low viral loads was analyzed (75). Interestingly, a dowregulation of hCD96 on a fraction of CD8 T cells present in the patients with high viral loads was found. Functional characterization of the $hCD96^+$ and $hCD96^-$ CD8 T cells showed that both are potent producers of IFNγ but that the $hCD96^-$ cells also produced perforin. This raises the possibility that in chronic infection hCD96 negatively regulates perforin production in human CD8 T cells. Dissimilar effector functions were also observed among $mCD96^{hi}$ and $mCD96^{lo}$ TH9 cells generated *in vitro* (76). The $mCD96^{hi}$ subpopulation was found to be less pathogenic, produced less cytokines, and propagated less efficiently when compared with $mCD96^{lo}$ TH9 cells. These observations would be in line with the assumption that CD96 inhibits selective T cell effector functions. But again, more information is required to draw more general conclusions.

UNRESOLVED ISSUES, FUTURE CHALLENGES

Interaction Partners of CD96 in *Cis*

Despite the existence of various consensus binding sites, the nature of the *cytoplasmic* interaction partners binding to CD96 remains a subject of speculation. The elucidation of the signaling pathways triggered upon CD96 engagement will be crucial for a better understanding of the CD96 biology. But functions of CD96 may also be regulated by *extracellular* proteins complexing *in cis* thereby creating more or less heterogeneous membrane complexes. The most simple higher order structure would be a homo-dimeric CD96 receptor. To manipulate the monomer/dimer balance represents a well-known tool how cells can control the functional status of receptors that depend on *cis*-dimerization (e.g., CD44 as discussed earlier). Experimental evidence would suggest that dimerization of CD155 in *cis* is required for functionality (11, 28) and *cis*-dimerization appears to be a common theme for CD155 family members. Interestingly, a high molecular weight complex (~240 kDa) in addition to the presumptive monomeric hCD96 (~160 kDa) was described by Wang et al. (1) investigating hCD96 by SDS-PAGE analysis under non-reducing conditions following immunoprecipitation. However, the precipitated material obtained from the human T cell lines migrated too fast for a hypothetical homo-dimer (~320 kDa) raising doubts regarding its composition. Thus, it remains unclear whether membrane-bound CD96 forms dimers in *cis* and whether this is required for functionality. As described, CD155 family members possess a binding interface in domain one that is used for complex formation with other members in *trans*. The very same CC′C″FG interface can be utilized by nectins and most likely also by CD226 to form homo-dimers in *cis*. However, in contrast to nectins, available data suggest that the CC′C″FG interface of CD155 is ineligible to perform homo-dimerization (11, 30). This fits the observation that, unlike nectins, CD155 does not mediate homophilic cell adhesion. Although not proven, it is plausible to assume that this characteristic is also shared by CD96 that like CD155 lacks self-adhesive capacity (6). Therefore, any potential *cis*-dimerization must utilize alternative mechanisms to accomplish this such as the TM cysteine (**Figure 3B**) that may serve to form stable CD96 dimers. Its genetically engineered replacement by another residue might inform whether the high molecular weight component observed by Wang et al. represented indeed a dimer (1) or whether another component stably associated with hCD96. The integration of CD96 into a hetero-dimeric/-oligomeric structure on a cell surface is quite likely considering other CD155 family members. CD155 was found to be associated with the integrin $\alpha_v\beta_3$ in fibroblasts (11, 77) or hCD44 on monocytes (78) (**Figure 2**) and CD226 complexes to LFA-1 in NK and activated T cells (79, 80). Integrin association in *cis* with CD155 (11, 81) or CD226 (80, 82, 83) is of functional relevance. Fuchs et al. (4) reported that in their redirected killing assays using an anti-hCD96 mAb activated polyclonal NK cells but not the cell line NK92 was stimulated to kill target cells. This illustrates that hCD96 expressed by NK92 cell differs functionally from that of the freshly isolated NK cells (4). Bearing in mind that hCD226 requires co-activity of β_2-integrin for NK cell function (79), the authors speculated that a similar mode of regulation might also apply for hCD96. Integrins of the β1 family might represent candidates taking this role. The incorporation of CD96 into complex membrane-bound structures could be specific for the type of cell or its activation status (like in case of CD226). This would enable a context-dependent tuning of CD96 functions. In addition, this might also force the receptors to preferentially engage in

interactions in *trans* and help avoid that, for example, CD155 and CD96 neutralize each other in *cis* since both are usually present simultaneously on the surface of T and NK cells.

Functional Differences Between hCD96 and mCD96

An important issue that directly would affect the translation of results obtained in mouse into therapeutic approaches for treatment of diseases in human relates to the structural differences between mCD96 and hCD96 and the resulting potential functional divergences. Of note, mCD96 but not hCD96 binds to nectin-1. Overexpression of nectin-1 in tumor cells is not described, but nectin-1 serves as an entry receptor for herpesviruses in human and mouse (84, 85) and therefore control of infection *via* CD96 expressed by NK cells may differ between species. It is surprising that human nectin-1 (hnectin-1) does not bind to hCD96 because mnectin-1 and hnectin-1 are highly conserved possessing an identical CC′C″FG interface in their domain one. mnectin-1 also binds to the first domain of mCD96 wherefore it is likely that subtle differences in the CC′C″FG interface of mCD96 compared with hCD96 (**Figure 3A**) account for the divergent binding specificity. Also effects from outside the binding interface can contribute substantially to modulate or alter binding of CD96 to ligands and thus illustrate the complexity of the CC′C″FG interface in mediating binding. The second domain of hCD96 (but not mCD96) can adopt a V-like folding pattern due to alternative splicing, and the presence of this domain instead of the I/C-like second domain modulates binding strength to hCD155 (20). The functional significance of the two existing variants in human compared with mouse remains elusive. But quantitative PCR data would indicate that the I/C-like domain variant that binds stronger to hCD155 and that corresponds to the domain one present in mCD96 is predominantly expressed in all normal cells and tissues tested (20). Also a described point mutation in the most distant third domain of hCD96 that was linked to a rare form of trigonocephaly weakens the binding to hCD155 (20). Along with other results, this suggested that the first domain of hCD96 but not of mCD96 is quite susceptible in its binding characteristics to even remotely located anomalies. This also increases the likelihood that a modified rigidity of the stalk region due to altered glyco-modification as mentioned earlier modulates ligand binding. Last not least, reminiscent of the scenario for h/mCD96 itself, the hCD96 interaction partner hCD155 can be expressed in four different isoforms due to alternative splicing (86) whereas alternative mRNA splice variants for mCD155 were not observed (21). Two hCD155 isoforms represent secreted receptors lacking the TM domain and of the two membrane-bound versions only the α-isoform (that corresponds to mCD155) harbors an ITIM motif (**Figure 2**) (87). Thus, human but not murine cells expressing CD155 could create a balance between an hCD155 isoform serving as an adhesion and signaling receptor and another one that only mediates adhesion.

A critical point that awaits elucidation relates to the issue whether hCD96 possesses an inhibitory potential as revealed for mCD96 (7). The key to this is buried in the short cytoplasmic domains. Despite a high degree of conservation they differ in the absence/presence of the YXXM motif. The importance of this binding site for actual performance of hCD96 cannot be predicted due to its low degree of specificity. Thus, although both can recruit p85 of PI3 kinase, the YXXM in CD28 triggers IL-2 production upon tyrosine phosphorylation but YXXM in ICOS-1 fails to do so because GRB2 cannot be bound (88). Taken together, there might be a "worst case" scenario, and hCD96 exerts inhibition or activation depending on the cell type.

CONCLUDING REMARKS

The regulatory network built by the CD155-family members attracted increasing attention during the past decade. However, despite its early identification, CD96 represents the least well-investigated building block of this system. Considering the importance of the CD155-driven regulatory circuits in immune surveillance in general and in particular in tumor biology, it is of upmost interest to learn more about the pathways governing the functions of hCD96. Current evidence brings to mind that the CD155 network is rather complex, and many factors contribute to the net inhibitory/activating outcome of its engagement (7, 19, 59, 89–91): participating cell types, divergent affinities of the receptors among each other, splice variants, the variegated expression dynamics that change with cell status, the accessory molecules that may associate with family members in a cell type- and status-specific pattern. This listing is certainly incomplete. This illustrates that the biological significance of CD96 can only be apprehended adequately when studied as part of this network.

AUTHOR CONTRIBUTIONS

HG and GB designed the concept and wrote the manuscript. IR and GP contributed to the overall concept of the manuscript, helped designing figures, and assisted in editing the manuscript.

FUNDING

This work was supported by DFG grants BE1886/2-1, BE1886/2-2, BE1886/2-3, and BE1886/5-1 to GB.

REFERENCES

1. Wang PL, O'Farrell S, Clayberger C, Krensky AM. Identification and molecular cloning of tactile. A novel human T cell activation antigen that is a member of the Ig gene superfamily. *J Immunol* (1992) 148:2600–8.
2. Burger R, Hansen-Hagge TE, Drexler HG, Gramatzki M. Heterogeneity of T-acute lymphoblastic leukemia (T-ALL) cell lines: suggestion for classification by immunophenotype and T-cell receptor studies. *Leuk Res* (1999) 23:19–27. doi:10.1016/S0145-2126(98)00133-7
3. Gramatzki M, Ludwig WD, Burger R, Moos P, Rohwer P, Grunert C, et al. Antibodies TC-12 ("unique") and TH-111 (CD96) characterize T-cell acute lymphoblastic leukemia and a subgroup of acute myeloid leukemia. *Exp Hematol* (1998) 26:1209–14.
4. Fuchs A, Cella M, Giurisato E, Shaw AS, Colonna M. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). *J Immunol* (2004) 172:3994–8. doi:10.4049/jimmunol.172.7.3994
5. Du Pasquier L, Zucchetti I, De Santis R. Immunoglobulin superfamily receptors in protochordates: before RAG time. *Immunol Rev* (2004) 198:233–48. doi:10.1111/j.0105-2896.2004.00122.x

6. Seth S, Maier MK, Qiu Q, Ravens I, Kremmer E, Forster R, et al. The murine pan T cell marker CD96 is an adhesion receptor for CD155 and nectin-1. *Biochem Biophys Res Commun* (2007) 364:959–65. doi:10.1016/j.bbrc.2007.10.102
7. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, et al. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. *Nat Immunol* (2014) 15:431–8. doi:10.1038/ni.2850
8. Mendelsohn CL, Wimmer E, Racaniello VR. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell* (1989) 56:855–65. doi:10.1016/0092-8674(89)90690-9
9. Takai Y, Irie K, Shimizu K, Sakisaka T, Ikeda W. Nectins and nectin-like molecules: roles in cell adhesion, migration, and polarization. *Cancer Sci* (2003) 94:655–67. doi:10.1111/j.1349-7006.2003.tb01499.x
10. Ikeda W, Kakunaga S, Itoh S, Shingai T, Takekuni K, Satoh K, et al. Tage4/Nectin-like molecule-5 heterophilically trans-interacts with cell adhesion molecule Nectin-3 and enhances cell migration. *J Biol Chem* (2003) 278:28167–72. doi:10.1074/jbc.M303586200
11. Mueller S, Wimmer E. Recruitment of nectin-3 to cell-cell junctions through trans-heterophilic interaction with CD155, a vitronectin and poliovirus receptor that localizes to alpha(v)beta3 integrin-containing membrane microdomains. *J Biol Chem* (2003) 278:31251–60. doi:10.1074/jbc.M304166200
12. Erickson BM, Thompson NL, Hixson DC. Tightly regulated induction of the adhesion molecule necl-5/CD155 during rat liver regeneration and acute liver injury. *Hepatology* (2006) 43:325–34. doi:10.1002/hep.21021
13. Minami Y, Ikeda W, Kajita M, Fujito T, Monden M, Takai Y. Involvement of up-regulated Necl-5/Tage4/PVR/CD155 in the loss of contact inhibition in transformed NIH3T3 cells. *Biochem Biophys Res Commun* (2007) 352:856–60. doi:10.1016/j.bbrc.2006.11.089
14. Ogita H, Ikeda W, Takai Y. Roles of cell adhesion molecules nectin and nectin-like molecule-5 in the regulation of cell movement and proliferation. *J Microsc* (2008) 231:455–65. doi:10.1111/j.1365-2818.2008.02058.x
15. Chadeneau C, Le Moullac B, Cornu G, Meflah K, Denis MG. Glycosylation of a novel member of the immunoglobulin gene superfamily expressed in rat carcinoma cell lines. *Int J Cancer* (1995) 61:87–91. doi:10.1002/ijc.2910610115
16. Chadeneau C, LeCabellec M, LeMoullac B, Meflah K, Denis MG. Overexpression of a novel member of the immunoglobulin superfamily in Min mouse intestinal adenomas. *Int J Cancer* (1996) 68:817–21. doi:10.1002/(SICI)1097-0215(19961211)68:6<817::AID-IJC21>3.0.CO;2-W
17. Chadeneau C, LeMoullac B, Denis MG. A novel member of the immunoglobulin gene superfamily expressed in rat carcinoma cell lines [published erratum appears in J. Biol. Chem. 1995 270(35):20870]. *J Biol Chem* (1994) 269:15601–5.
18. Masson D, Jarry A, Baury B, Blanchardie P, Laboisse C, Lustenberger P, et al. Overexpression of the CD155 gene in human colorectal carcinoma. *Gut* (2001) 49:236–40. doi:10.1136/gut.49.2.236
19. Martinet L, Smyth MJ. Balancing natural killer cell activation through paired receptors. *Nat Rev Immunol* (2015) 15:243–54. doi:10.1038/nri3799
20. Meyer D, Seth S, Albrecht J, Maier MK, Pasquier L, Ravens I, et al. CD96 interaction with CD155 via its first Ig-like domain is modulated by alternative splicing or mutations in distal Ig-like domains. *J Biol Chem* (2009) 284:2235–44. doi:10.1074/jbc.M807698200
21. Ravens I, Seth S, Forster R, Bernhardt G. Characterization and identification of Tage4 as the murine orthologue of human poliovirus receptor/CD155. *Biochem Biophys Res Commun* (2003) 312:1364–71. doi:10.1016/j.bbrc.2003.11.067
22. Shibuya A, Campbell D, Hannum C, Yssel H, Franz-Bacon K, McClanahan T, et al. DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity* (1996) 4:573–81. doi:10.1016/S1074-7613(00)70060-4
23. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* (2009) 10:48–57. doi:10.1038/ni.1674
24. Boles KS, Vermi W, Facchetti F, Fuchs A, Wilson TJ, Diacovo TG, et al. A novel molecular interaction for the adhesion of follicular CD4 T cells to follicular DC. *Eur J Immunol* (2009) 39:695–703. doi:10.1002/eji.200839116
25. Levin SD, Taft DW, Brandt CS, Bucher C, Howard ED, Chadwick EM, et al. Vstm3 is a member of the CD28 family and an important modulator of T-cell function. *Eur J Immunol* (2011) 41:902–15. doi:10.1002/eji.201041136
26. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med* (2003) 198:557–67. doi:10.1084/jem.20030788
27. Tahara-Hanaoka S, Miyamoto A, Hara A, Honda S, Shibuya K, Shibuya A. Identification and characterization of murine DNAM-1 (CD226) and its poliovirus receptor family ligands. *Biochem Biophys Res Commun* (2005) 329:996–1000. doi:10.1016/j.bbrc.2005.02.067
28. Takai Y, Miyoshi J, Ikeda W, Ogita H. Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. *Nat Rev Mol Cell Biol* (2008) 9:603–15. doi:10.1038/nrm2457
29. Deuss FA, Gully BS, Rossjohn J, Berry R. Recognition of nectin-2 by the natural killer cell receptor T cell immunoglobulin and ITIM domain (TIGIT). *J Biol Chem* (2017) 292:11413–22. doi:10.1074/jbc.M117.786483
30. Harrison OJ, Vendome J, Brasch J, Jin X, Hong S, Katsamba PS, et al. Nectin ectodomain structures reveal a canonical adhesive interface. *Nat Struct Mol Biol* (2012) 19:906–15. doi:10.1038/nsmb.2366
31. Samanta D, Ramagopal UA, Rubinstein R, Vigdorovich V, Nathenson SG, Almo SC. Structure of Nectin-2 reveals determinants of homophilic and heterophilic interactions that control cell-cell adhesion. *Proc Natl Acad Sci U S A* (2012) 109:14836–40. doi:10.1073/pnas.1212912109
32. Stengel KF, Harden-Bowles K, Yu X, Rouge L, Yin J, Comps-Agrar L, et al. Structure of TIGIT immunoreceptor bound to poliovirus receptor reveals a cell-cell adhesion and signaling mechanism that requires cis-trans receptor clustering. *Proc Natl Acad Sci U S A* (2012) 109:5399–404. doi:10.1073/pnas.1120606109
33. Zhang P, Mueller S, Morais MC, Bator CM, Bowman VD, Hafenstein S, et al. Crystal structure of CD155 and electron microscopic studies of its complexes with polioviruses. *Proc Natl Acad Sci U S A* (2008) 105:18284–9. doi:10.1073/pnas.0807848105
34. Rozsnyay Z. Signaling complex formation of CD44 with src-related kinases. *Immunol Lett* (1999) 68:101–8. doi:10.1016/S0165-2478(99)00037-1
35. Casabo LG, Mamalaki C, Kioussis D, Zamoyska R. T cell activation results in physical modification of the mouse CD8 beta chain. *J Immunol* (1994) 152:397–404.
36. Moody AM, Chui D, Reche PA, Priatel JJ, Marth JD, Reinherz EL. Developmentally regulated glycosylation of the CD8alphabeta coreceptor stalk modulates ligand binding. *Cell* (2001) 107:501–12. doi:10.1016/S0092-8674(01)00577-3
37. Liu D, Sy MS. A cysteine residue located in the transmembrane domain of CD44 is important in binding of CD44 to hyaluronic acid. *J Exp Med* (1996) 183:1987–94. doi:10.1084/jem.183.5.1987
38. Liu D, Sy MS. Phorbol myristate acetate stimulates the dimerization of CD44 involving a cysteine in the transmembrane domain. *J Immunol* (1997) 159:2702–11.
39. Blott EJ, Bossi G, Clark R, Zvelebil M, Griffiths GM. Fas ligand is targeted to secretory lysosomes via a proline-rich domain in its cytoplasmic tail. *J Cell Sci* (2001) 114:2405–16.
40. Kay BK, Williamson MP, Sudol M. The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *FASEB J* (2000) 14:231–41. doi:10.1096/fasebj.14.2.231
41. Zuccato E, Blott EJ, Holt O, Sigismund S, Shaw M, Bossi G, et al. Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation. *J Cell Sci* (2007) 120:191–9. doi:10.1242/jcs.03315
42. Kivens WJ, Hunt SW III, Mobley JL, Zell T, Dell CL, Bierer BE, et al. Identification of a proline-rich sequence in the CD2 cytoplasmic domain critical for regulation of integrin-mediated adhesion and activation of phosphoinositide 3-kinase. *Mol Cell Biol* (1998) 18:5291–307. doi:10.1128/MCB.18.9.5291
43. Chambers CA. The expanding world of co-stimulation: the two-signal model revisited. *Trends Immunol* (2001) 22:217–23. doi:10.1016/S1471-4906(01)01868-3
44. Gilfillan S, Chan CJ, Cella M, Haynes NM, Rapaport AS, Boles KS, et al. DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *J Exp Med* (2008) 205:2965–73. doi:10.1084/jem.20081752

45. Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S, et al. Accelerated tumor growth in mice deficient in DNAM-1 receptor. *J Exp Med* (2008) 205:2959–64. doi:10.1084/jem.20081611
46. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol* (2011) 186:1338–42. doi:10.4049/jimmunol.1003081
47. Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A, et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc Natl Acad Sci U S A* (2009) 106:17858–63. doi:10.1073/pnas.0903474106
48. Bernhardt G. TACTILE becomes tangible: CD96 discloses its inhibitory peculiarities. *Nat Immunol* (2014) 15:406–8. doi:10.1038/ni.2855
49. Chauvin JM, Pagliano O, Fourcade J, Sun Z, Wang H, Sander C, et al. TIGIT and PD-1 impair tumor antigen-specific CD8(+) T cells in melanoma patients. *J Clin Invest* (2015) 125:2046–58. doi:10.1172/JCI80445
50. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell* (2014) 26:923–37. doi:10.1016/j.ccell.2014.10.018
51. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* (2014) 40:569–81. doi:10.1016/j.immuni.2014.02.012
52. Kong Y, Zhu L, Schell TD, Zhang J, Claxton DF, Ehmann WC, et al. T-cell immunoglobulin and ITIM domain (TIGIT) associates with CD8+ T-cell exhaustion and poor clinical outcome in AML patients. *Clin Cancer Res* (2016) 22:3057–66. doi:10.1158/1078-0432.CCR-15-2626
53. Kurtulus S, Sakuishi K, Ngiow SF, Joller N, Tan DJ, Teng MW, et al. TIGIT predominantly regulates the immune response via regulatory T cells. *J Clin Invest* (2015) 125:4053–62. doi:10.1172/JCI81187
54. Bi J, Zheng X, Chen Y, Wei H, Sun R, Tian Z. TIGIT safeguards liver regeneration through regulating natural killer cell-hepatocyte crosstalk. *Hepatology* (2014) 60:1389–98. doi:10.1002/hep.27245
55. Lozano E, Dominguez-Villar M, Kuchroo V, Hafler DA. The TIGIT/CD226 axis regulates human T cell function. *J Immunol* (2012) 188:3869–75. doi:10.4049/jimmunol.1103627
56. Liu S, Zhang H, Li M, Hu D, Li C, Ge B, et al. Recruitment of Grb2 and SHIP1 by the ITT-like motif of TIGIT suppresses granule polarization and cytotoxicity of NK cells. *Cell Death Differ* (2013) 20:456–64. doi:10.1038/cdd.2012.141
57. Carlsten M, Bjorkstrom NK, Norell H, Bryceson Y, van Hall T, Baumann BC, et al. DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells. *Cancer Res* (2007) 67:1317–25. doi:10.1158/0008-5472.CAN-06-2264
58. El-Sherbiny YM, Meade JL, Holmes TD, McGonagle D, Mackie SL, Morgan AW, et al. The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. *Cancer Res* (2007) 67:8444–9. doi:10.1158/0008-5472.CAN-06-4230
59. Georgiev H, Danisch S, Chambers BJ, Shibuya A, Forster R, Bernhardt G. To the editor: TIGIT versus CD226: hegemony or coexistence? *Eur J Immunol* (2014) 44:307–8. doi:10.1002/eji.201343925
60. Stanietsky N, Rovis TL, Glasner A, Seidel E, Tsukerman P, Yamin R, et al. Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *Eur J Immunol* (2013) 43:2138–50. doi:10.1002/eji.201243072
61. Blake SJ, Stannard K, Liu J, Allen S, Yong MC, Mittal D, et al. Suppression of metastases using a new lymphocyte checkpoint target for cancer immunotherapy. *Cancer Discov* (2016) 6:446–59. doi:10.1158/2159-8290.CD-15-0944
62. Brooks J, Fleischmann-Mundt B, Woller N, Niemann J, Ribback S, Peters K, et al. Perioperative, spatiotemporally coordinated activation of T and NK cells prevents recurrence of pancreatic cancer. *Cancer Res* (2017) 78(2):475–88. doi:10.1158/0008-5472.CAN-17-2415
63. Aguilera AR, Lutzky VP, Mittal D, Li X-Y, Stannard K, Takeda K, et al. CD96 targeted antibodies need not block CD96-CD155 interactions to promote NK cell anti-metastatic activity. *Oncoimmunology* (2018) 7:e1424677. doi:10.1080/2162402X.2018.1424677
64. Seth S, Qiu Q, Danisch S, Maier MK, Braun A, Ravens I, et al. Intranodal interaction with dendritic cells dynamically regulates surface expression of the co-stimulatory receptor CD226 protein on murine T cells. *J Biol Chem* (2011) 286:39153–63. doi:10.1074/jbc.M111.264697
65. Gao J, Zheng Q, Xin N, Wang W, Zhao C. CD155, an onco-immunologic molecule in human tumors. *Cancer Sci* (2017) 108:1934–8. doi:10.1111/cas.13324
66. Peng YP, Xi CH, Zhu Y, Yin LD, Wei JS, Zhang JJ, et al. Altered expression of CD226 and CD96 on natural killer cells in patients with pancreatic cancer. *Oncotarget* (2016) 7:66586–94. doi:10.18632/oncotarget.11953
67. Du W, Hu Y, Lu C, Li J, Liu W, He Y, et al. Cluster of differentiation 96 as a leukemia stem cell-specific marker and a factor for prognosis evaluation in leukemia. *Mol Clin Oncol* (2015) 3:833–8. doi:10.3892/mco.2015.552
68. Jiang Y, Xu P, Yao D, Chen X, Dai H. CD33, CD96 and death associated protein kinase (DAPK) expression are associated with the survival rate and/or response to the chemotherapy in the patients with acute myeloid leukemia (AML). *Med Sci Monit* (2017) 23:1725–32. doi:10.12659/MSM.900305
69. Dick JE. Stem cell concepts renew cancer research. *Blood* (2008) 112:4793–807. doi:10.1182/blood-2008-08-077941
70. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* (1997) 3:730–7. doi:10.1038/nm0797-730
71. Vadillo E, Dorantes-Acosta E, Pelayo R, Schnoor M. T cell acute lymphoblastic leukemia (T-ALL): new insights into the cellular origins and infiltration mechanisms common and unique among hematologic malignancies. *Blood Rev* (2018) 32:36–51. doi:10.1016/j.blre.2017.08.006
72. Hosen N, Park CY, Tatsumi N, Oji Y, Sugiyama H, Gramatzki M, et al. CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proc Natl Acad Sci U S A* (2007) 104:11008–13. doi:10.1073/pnas.0704271104
73. Majeti R. Monoclonal antibody therapy directed against human acute myeloid leukemia stem cells. *Oncogene* (2011) 30:1009–19. doi:10.1038/onc.2010.511
74. Mohseni Nodehi S, Repp R, Kellner C, Brautigam J, Staudinger M, Schub N, et al. Enhanced ADCC activity of affinity maturated and Fc-engineered mini-antibodies directed against the AML stem cell antigen CD96. *PLoS One* (2012) 7:e42426. doi:10.1371/journal.pone.0042426
75. Eriksson EM, Keh CE, Deeks SG, Martin JN, Hecht FM, Nixon DF. Differential expression of CD96 surface molecule represents CD8(+) T cells with dissimilar effector function during HIV-1 infection. *PLoS One* (2012) 7:e51696. doi:10.1371/journal.pone.0051696
76. Stanko K, Iwert C, Appelt C, Vogt K, Schumann J, Strunk FJ, et al. CD96 expression determines the inflammatory potential of IL-9-producing Th9 cells. *Proc Natl Acad Sci U S A* (2018) 115:E2940–9. doi:10.1073/pnas.1708329115
77. Minami Y, Ikeda W, Kajita M, Fujito T, Amano H, Tamaru Y, et al. Necl-5/poliovirus receptor interacts in cis with integrin alphaVbeta3 and regulates its clustering and focal complex formation. *J Biol Chem* (2007) 282:18481–96. doi:10.1074/jbc.M611330200
78. Freistadt MS, Eberle KE. Physical association between CD155 and CD44 in human monocytes. *Mol Immunol* (1997) 34:1247–57. doi:10.1016/S0161-5890(98)00003-0
79. Shibuya K, Lanier LL, Phillips JH, Ochs HD, Shimizu K, Nakayama E, et al. Physical and functional association of LFA-1 with DNAM-1 adhesion molecule. *Immunity* (1999) 11:615–23. doi:10.1016/S1074-7613(00)80136-3
80. Shirakawa J, Wang Y, Tahara-Hanaoka S, Honda S, Shibuya K, Shibuya A. LFA-1-dependent lipid raft recruitment of DNAM-1 (CD226) in CD4+ T cell. *Int Immunol* (2006) 18:951–7. doi:10.1093/intimm/dxl031
81. Ikeda W, Kakunaga S, Takekuni K, Shingai T, Satoh K, Morimoto K, et al. Nectin-like molecule-5/Tage4 enhances cell migration in an integrin-dependent, Nectin-3-independent manner. *J Biol Chem* (2004) 279:18015–25. doi:10.1074/jbc.M312969200
82. Shibuya K, Shirakawa J, Kameyama T, Honda S, Tahara-Hanaoka S, Miyamoto A, et al. CD226 (DNAM-1) is involved in lymphocyte function-associated antigen 1 costimulatory signal for naive T cell differentiation and proliferation. *J Exp Med* (2003) 198:1829–39. doi:10.1084/jem.20030958
83. Shirakawa J, Shibuya K, Shibuya A. Requirement of the serine at residue 329 for lipid raft recruitment of DNAM-1 (CD226). *Int Immunol* (2005) 17:217–23. doi:10.1093/intimm/dxh199

84. Geraghty RJ, Krummenacher C, Cohen GH, Eisenberg RJ, Spear PG. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* (1998) 280:1618–20. doi:10.1126/science.280.5369.1618
85. Menotti L, Lopez M, Avitabile E, Stefan A, Cocchi F, Adelaide J, et al. The murine homolog of human Nectin1delta serves as a species nonspecific mediator for entry of human and animal alpha herpesviruses in a pathway independent of a detectable binding to gD. *Proc Natl Acad Sci U S A* (2000) 97:4867–72. doi:10.1073/pnas.97.9.4867
86. Koike S, Horie H, Ise I, Okitsu A, Yoshida M, Iizuka N, et al. The poliovirus receptor protein is produced both as membrane-bound and secreted forms. *EMBO J* (1990) 9:3217–24.
87. Oda T, Ohka S, Nomoto A. Ligand stimulation of CD155alpha inhibits cell adhesion and enhances cell migration in fibroblasts. *Biochem Biophys Res Commun* (2004) 319:1253–64. doi:10.1016/j.bbrc.2004.05.111
88. Harada Y, Ohgai D, Watanabe R, Okano K, Koiwai O, Tanabe K, et al. A single amino acid alteration in cytoplasmic domain determines IL-2 promoter activation by ligation of CD28 but not inducible costimulator (ICOS). *J Exp Med* (2003) 197:257–62. doi:10.1084/jem.20021305
89. Chan CJ, Andrews DM, Smyth MJ. Receptors that interact with nectin and nectin-like proteins in the immunosurveillance and immunotherapy of cancer. *Curr Opin Immunol* (2012) 24:246–51. doi:10.1016/j.coi.2012.01.009
90. Dougall WC, Kurtulus S, Smyth MJ, Anderson AC. TIGIT and CD96: new checkpoint receptor targets for cancer immunotherapy. *Immunol Rev* (2017) 276:112–20. doi:10.1111/imr.12518
91. Stanietsky N, Mandelboim O. Paired NK cell receptors controlling NK cytotoxicity. *FEBS Lett* (2010) 584:4895–900. doi:10.1016/j.febslet.2010.08.047

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 08 August 2018
doi: 10.3389/fimmu.2018.01810

Influence of T Cell Coinhibitory Molecules on CD8+ Recall Responses

*Anna B. Morris, Layne E. Adams and Mandy L. Ford**

Department of Surgery, Emory University, Atlanta, GA, United States

T cell co-signaling molecules play an important role in fine-tuning the strength of T cell activation during many types of immune responses, including infection, cancer, transplant rejection, and autoimmunity. Over the last few decades, intense research into these cosignaling molecules has provided rich evidence to suggest that cosignaling molecules may be harnessed for the treatment of immune-related diseases. In particular, coinhibitory molecules such as programmed-death 1, 2B4, BTLA, TIGIT, LAG-3, TIM-3, and CTLA-4 inhibit T cell responses by counteracting TCR and costimulatory signals, leading to the inhibition of proliferation and effector function and the downregulation of activation and adhesion molecules at the cell surface. While many reviews have focused on the role of coinhibitory molecules in modifying primary CD8+ T cell responses, in this review, we will consider the complex role of coinhibitory molecules in altering CD8+ T cell recall potential. As memory CD8+ T cell responses are critical for protective memory responses in infection and cancer and contribute to potentially pathogenic memory responses in transplant rejection and autoimmunity, understanding the role of coinhibitory receptor control of memory T cells may illuminate important aspects of therapeutically targeting these pathways.

Keywords: recall, CD8, coinhibitory, memory, cancer, transplant, vaccine, autoimmunity

OPEN ACCESS

Edited by:
Paul E. Love, National Institutes of Health (NIH), United States

Reviewed by:
Lawrence Kane, University of Pittsburgh, United States
Koji Yasutomo, Tokushima University, Japan

***Correspondence:**
Mandy L. Ford
mandy.ford@emory.edu

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *31 May 2018*
Accepted: *23 July 2018*
Published: *08 August 2018*

Citation:
Morris AB, Adams LE and Ford ML (2018) Influence of T Cell Coinhibitory Molecules on CD8+ Recall Responses. Front. Immunol. 9:1810. doi: 10.3389/fimmu.2018.01810

INTRODUCTION

Recently, there has been an explosion of research on the function of coinhibitory receptors on CD8+ T cells, mostly focusing on their role during primary responses [reviewed in Ref. (1–6)]. Here, we will discuss the roles of individual coinhibitory molecules specifically on the recall response of CD8+ T cells. Memory cells typically express one or more coinhibitory receptors (7), and as memory cells are important protective regulators against infections and cancer and can be pathogenic in autoimmunity and transplantation, understanding the role of coinhibitory molecules on their recall potential has numerous implications in vaccine design and therapeutics. Two perspectives will be reviewed here: first, that coinhibitory molecules limit recall potential by inhibiting proliferation and activation of secondary effectors, and second, that coinhibitory molecules limit terminal differentiation to preserve recall potential, a process that leads to a stable population of memory T cells that are able to provide a sustained, protective memory response.

COINHIBITORY MOLECULES LIMIT RECALL POTENTIAL

Infection-Elicited T Cells

Programmed-death 1 (PD-1) is a hallmark coinhibitory receptor that has been implicated in limiting recall potential in models of viral infection. PD-1 belongs to the Ig superfamily, is expressed by activated T and B cells and constitutively expressed by natural killer (NK) cells and macrophages (8–10). PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor

tyrosine-based switch motif (ITSM) that both contribute to its inhibitory signaling mechanism.

Programmed-death 1 was implicated in limiting $CD8^+$ recall responses in studies aiming to understand the high incidence of reinfection of lower respiratory infections in children, which typically indicates poorly generated immunity (11, 12). Interestingly, it was found that dysfunction of pulmonary antigen-specific $CD8^+$ T cells generated from influenza and human metapneumonovirus (HMPV) infection in both the primary and secondary effector phase express high levels of PD-1 (13). Upon blockade of PD-1, lytic granule release and antiviral cytokine production were restored, indicating the functional impairment conferred by PD-1 expression. Further, they found that in a model of HMPV reinfection in which B-cell deficient hosts are used to enable reinfection, antigen-specific $CD8^+$ T cells further upregulated PD-1, LAG-3, Tim-3, and 2B4 over that of primary effectors (14). Additionally, PD-1 upregulation following primary infection limited recall potential (degranulation and cytokine production) that could be restored with *in vivo* PD-1 blockade at the time of reinfection.

The cosignaling molecule CD244, or 2B4, was also found to play a role in memory $CD8^+$ T cell functionality. 2B4, a CD2 family member expressed by NK cells and $CD8^+$ T cells, has the unique ability to be costimulatory or coinhibitory due to its ITSM in the cytoplasmic domain (15, 16). Interestingly, microarray data following LCMV Clone 13 infection showed that while some "exhaustive" coinhibitory molecules are similarly expressed in primary and secondary effectors, 2B4 was more highly expressed in the latter (17). Further, studies using antigen-specific $CD8^+$ T cells that were genetically deficient in 2B4 revealed that 2B4 expression was associated with lack of survival of secondary effectors in chronic LCMV infection. These data imply that 2B4 limits the recall response of $CD8^+$ secondary effector T cells in chronic infection by inhibiting their proliferation and functionality.

Similarly, CTLA-4 blockade during a memory response to *Listeria monocytogenes* enhances $CD8^+$ memory T cell recall with greater production of IFNγ and TNF (18). The coinhibitory molecule CTLA-4 outcompetes the costimulatory molecule CD28 for the shared ligands CD80 and CD86 due to its higher affinity (19–21). Importantly, CTLA-4 inhibits T cell activation by numerous mechanisms, including intrinsically *via* interaction with the signaling modalities SHP-2 and PP2A and extrinsically *via* competition for the ligands of CD28 (22–25). Pedicord et al. found that not only does blockade of CTLA-4 during a memory response lead to a better $CD8^+$ recall response against bacterial infection but also that anti-CTLA-4 given during the primary response results in an enhanced $CD8^+$ memory recall response, suggesting that CTLA-4 upregulation during priming imprints a differentiation program that impedes memory function. These data suggest that PD-1, 2B4, and CTLA-4 all have the ability to inhibit protective memory responses and implicate these inhibitory molecules as potential targets in vaccination strategies to enhance $CD8^+$ memory T cell formation and recall potential.

Vaccination-Elicited T Cells

In the setting of vaccination, inhibitory receptor expression, specifically Tim-3 and PD-1, has been associated with poor protective potential and unsuccessful vaccination strategies. Tim-3, or T cell immunoglobulin mucin-3, is expressed by a myriad of immune cells, including $CD8^+$ T cells (26, 27), and its inhibitory function has been identified in models of autoimmunity (26, 28, 29). Vaccination with the Adenovirus5 vector (Ad5), although highly immunogenic, induced higher expression of PD-1 and Tim-3 on memory $CD8^+$ T cells and inhibited recall upon boosting compared with alternative Ad vectors (30). Interestingly, when lower doses of Ad5 were administered, the expression of PD-1 and Tim-3 was lowered and overall expansion of $CD8^+$ T cells was higher, demonstrating that a more robust $CD8^+$ recall potential correlated with lower coinhibitory expression. This also suggests that antigen dose could play a role in the differentiation of $CD8^+$ memory T cells that results in upregulation of coinhibitory molecules and inhibition of recall.

Another study corroborated these results using LPG (*Leishmania* lipophosphaglycan) as a vaccine candidate against *Leishmania* infections (31). Vaccination with LPG did not protect mice from *Leishmania mexicana* infection, and LPG vaccination resulted in upregulation of PD-1 on $CD8^+$ T cells. They also found, like the study discussed above, that PD-1 upregulation was dose dependent based on the amount of LPG given. They hypothesized that PD-1 could lead to repressed IFNγ production and cytotoxicity, which are important protective modulators in *Leishmania* infections. These studies highlight the role of PD-1 in inhibiting $CD8^+$ recall potential, and a possible strategy to avert PD-1 expression with lower antigen doses.

T Cells in Transplantation and Autoimmunity

Studies in transplantation and autoimmunity have likewise revealed similar associations between coinhibitory molecule expression and $CD8^+$ memory recall potential. In both transplantation and autoimmunity, it is beneficial to inhibit allo- or autoreactive $CD8^+$ memory T cells to prevent rejection or pathogenic T cell responses, but also to maintain memory $CD8^+$ T cell populations to respond to subsequent infections. In work assessing liver transplant patients, it was found that there was an association in the pre-transplant frequency of PD-1 and Tim-3 double-positive $CD8^+$ effector memory T cells in patients who would go on to develop liver infections (32), suggesting that these coinhibitory molecules could inhibit the recall potential of these $CD8^+$ memory T cells. Furthermore, the frequency of $PD\text{-}1^+$ and $Tim\text{-}3^+$ cells also negatively correlated with IFNγ production, indicative of lack of function. Although coinhibitory molecules are typically beneficial in graft survival, this study provided evidence that coinhibitory molecules, especially memory cells expressing both PD-1 and Tim-3, could inhibit the recall potential and memory function of protective antigen-specific cells, leading to increased infections posttransplant.

Additionally, the coinhibitory molecule CTLA-4 has been associated with diminished recall potential of $CD8^+$ memory T cells in transplantation and autoimmunity. Studies have used therapeutics to target costimulatory molecules; for instance, CTLA-4Ig, which binds CD80 and CD86 and prevents their binding to CD28 and CTLA-4, and anti-CD28 domain

antibodies (anti-CD28 dAb) that specifically block CD28 but preserve CTLA-4-mediated coinhibition (33–35). A recent study by Liu et al. revealed differential outcomes of graft-specific CD8+ memory T cells upon treatment with CTLA-4 Ig and anti-CD28 dAb in a murine model of skin transplantation (36). Interestingly, the selective CD28 domain antibodies more potently attenuated graft rejection mediated by memory T cells over CTLA-4 Ig, indicating that CTLA-4 and CD28 are important modulators of memory CD8+ T cell recall responses. Although the number of CD8+ secondary effectors was similar with either treatment, the cytokine production of the effectors generated with anti-CD28dAb treatment was much lower than that in the CTLA-4 Ig group, signifying a necessary inhibitory role of CTLA-4 in controlling the cytokine production of CD8+ secondary effectors. A corroborative study analyzing pathogenic memory CD8+ T cells in autoimmunity recapitulated these results (37). Using both human memory CD8+ T cells and non-human primate recall studies, they showed that use of a selective CD28 antagonist prevented reactivation and controlled both cellular and humoral memory recall. The results of these studies provide evidence that CTLA-4 has a unique functional role in modulating memory CD8+ T cell recall responses.

Moreover, additional studies in a murine transplant model found that 2B4 is also associated with a diminished recall response (Laurie et al., in press). In this model, *L. monocytogenes*-infected animals were rechallenged with a skin graft. Interestingly, the 2B4-deficient CD8+ secondary effectors had a significantly higher frequency of IFN-γ and IL-2-secreting cells, as compared to their wild-type counterparts, indicating that 2B4 expressed on CD8+ secondary effectors inhibits their ability to secrete cytokines under these conditions. Altogether, these data have provided evidence that coinhibitory molecules, including 2B4, CTLA-4, Tim-3, and PD-1, can selectively limit recall responses *via* inhibition of proliferation, cytokine production, and cytotoxic granule release (**Figure 1A**).

COINHIBITORY MOLECULES MAY LIMIT TERMINAL DIFFERENTIATION TO PRESERVE RECALL POTENTIAL

Contrary to the concept that coinhibitory receptors have a negative impact on recall potential, recent studies also suggest that coinhibitory receptors may not necessarily negatively impact CD8+ memory T cell responses. Although PD-1 is the most well-known exhaustion marker in chronic infection and cancer, and above, we have provided evidence of its ability to inhibit memory CD8+ T cell responses, studies revealed that healthy human adults harbor populations of CD8+ effector memory T cells that have high levels of PD-1 on their surface, and that these cells were less terminally differentiated (38). Further studies of healthy human CD8+ T cells that analyzed multiple inhibitory receptors, including PD-1, CTLA-4, Tim-3, LAG3, 2B4, BTLA, and CD160 found that the expression of inhibitory receptors is not as tightly linked to exhaustion as it is to T cell differentiation or activation status (39), reviewed in Ref. (40). Unlinking coinhibitory molecules and exhaustion status may be an important aspect of understanding the function of these inhibitory molecules on CD8+ T cells. Instead of dictating exhaustion status, terminal differentiation, or lack of function, coinhibitory molecules under some conditions limit

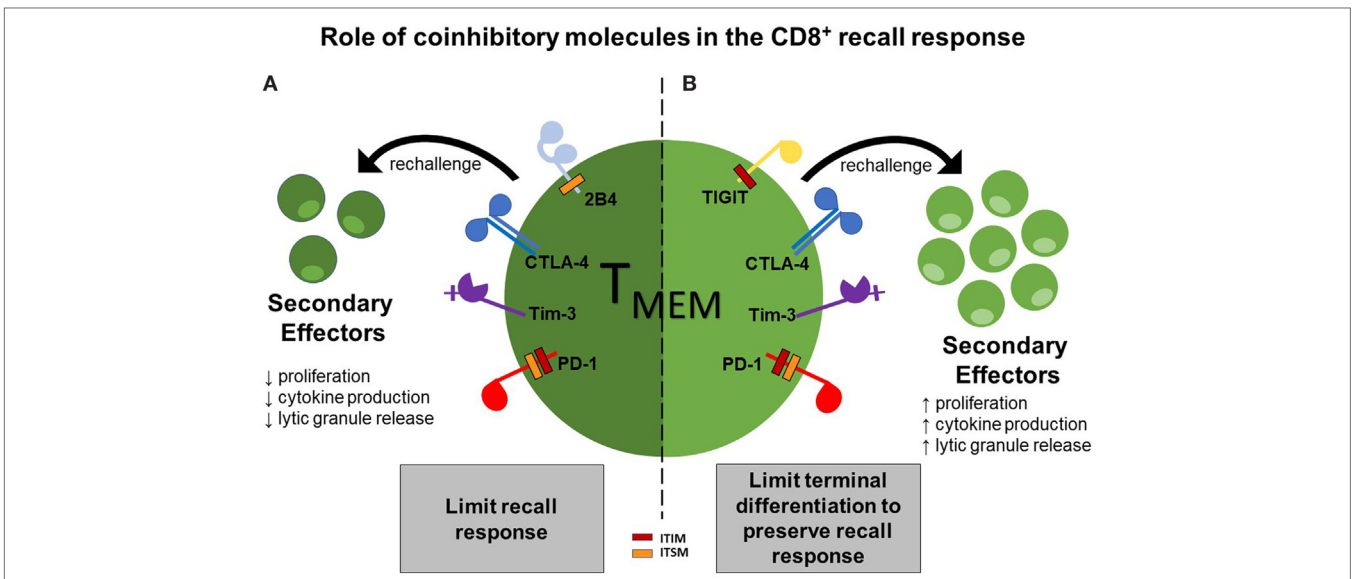


FIGURE 1 | Two functions of coinhibitory molecules in modulating the CD8+ recall response. Functionality of CD8+ T cell secondary effectors can be limited by ligation of the coinhibitory molecules 2B4, CTLA-4, Tim-3, and programmed-death 1 (PD-1), thus dampening the recall response **(A)**; however, ligation of the coinhibitory molecules TIGIT, Tim-3, CTLA-4, and PD-1 can function to preserve secondary recall responses by inhibiting terminal differentiation, thus leading to a more stable memory population **(B)**.

terminal differentiation and facilitate the CD8+ T cell population to be stably maintained.

Resident Memory T Cells

Interestingly, recent findings suggest that resident memory CD8+ T cells (Trm) express coinhibitory molecules in their core gene signature (41, 42). For instance, brain CD8+ Trm have not only been shown to express PD-1, but the promotor of *Pdcd1* is epigenetically fixed in a demethylated state, indicating its significance as part of the core gene signature (42). CD8+ Trm cells are sentinels for immune surveillance and protection, yet, their secondary effector function has been debated due to their slow turnover and expression of inhibitory molecules, typically indicative of terminal differentiation. In a recent study, it was found that although CD8+ Trm maintain high amounts of coinhibitory receptors (i.e., 2B4, CTLA-4, LAG3, PD-1, and Tim-3) relative to circulating memory cells in the spleen, they were still able to undergo local proliferation after secondary rechallenge (43). These findings indicate that the cells expressing these coinhibitory molecules were not terminally differentiated and instead maintained recall potential. Additionally, the findings that coinhibitory molecules are in the gene signature and that the PD-1 promotor is in an epigenetically fixed state indicate a potential function of coinhibitory molecules to modulate CD8+ Trm cells in a manner that allows them to be maintained as a stable population capable of recall.

Decidual T Cells

Coinhibitory molecules have also been associated with a special type of CD8+ T cell at the maternal–fetal interface. These decidual CD8+ T cells are effector-memory T cells critical to maintain immunity to infection and provide tolerance against the foreign fetus. Recently, it has been shown that these decidual CD8+ T cells express little perforin or granzyme B but can respond to viral and bacterial antigens (44–46). Further analysis revealed that although these CD8+ effector memory T cells express high levels of PD-1, CTLA-4, TIGIT, and LAG3, they were still able to produce TNF and IFNγ and upregulate perforin and granzyme upon *ex vivo* stimulation. Moreover, although they were slower to begin proliferating, they reached a similar proliferation index as the peripheral CD8+ T cells (47). These data indicate that although these memory CD8+ T cells express coinhibitory molecules, these receptors do not render them nonfunctional and exhausted. Rather, these CD8+ T cells are adequately able to respond to antigen. In the setting of maternal–fetal interface in which tolerance must be maintained to the fetus but protection against infections must also be maintained, the expression of these coinhibitory molecules does not fully render these CD8+ T cells exhausted, indicating that expression of coinhibitory molecules could raise the threshold of activation while preventing terminally differentiation or exhaustion.

Tumor-Infiltrating and Infection-Elicited T Cells

The same phenomenon in which CD8+ T cells express coinhibitory molecules that do not necessarily result in exhaustion has been observed in the field of tumor immunology as well. In a study analyzing the molecular signature of tumor-infiltrating lymphocytes (TILs) of non-small cell lung carcinoma, it was found that the CD8+ TILs that infiltrated tumors at a high density had high levels of PD-1 and the costimulatory molecule 4-1BB (48), molecules that are upregulated upon TCR engagement and have been associated with both exhaustion and activation (4, 49, 50). These antigen-specific CD8+ TILs also exhibited high expression of Tim-3, LAG3, and TIGIT. For instance, it is known that patients with tumors containing a high density of TILs have better survival (48), even if those cells express high levels of coinhibitory molecules. This observation suggests that, in some instances, TILs expressing coinhibitory molecules are still able to elicit antitumor effector function. These data could be reflective of the fact that many exhaustion markers are upregulated as a result of antigen recognition, thus serving as a marker of T cell activation. Alternatively, they could indicate that Tim-3, LAG3, and TIGIT might have additional positive roles on T cell effector function. Support for this hypothesis comes from recent research on Tim-3 in the setting of infection, where a positive impact of Tim-3 on T cell effector function was identified [reviewed in Ref. (51)]. Likewise, in this infection model, expression of Tim-3 on T cells increased signaling downstream of the TCR (52), and Tim-3 deficiency led to impaired CD8+ recall responses (53).

Further, in studies assessing the function of CD8+ T cells in patients with stage IV advanced metastatic melanoma, TIGIT was found to be co-expressed with PD-1 on tumor-specific effector memory T cells, and TIGIT-expressing cells represented an activated T cell phenotype with high expression of HLA-DR and CD38 (54). The T cell immunoglobulin and ITIM domain (TIGIT) is a member of the Ig superfamily and functions as a coinhibitory molecule on activated T cells, memory T cells, some Tregs, Tfh, and NK cell (55–57). Signaling of TIGIT in T cells leads to the downregulation of the TCR and CD3 molecules and other internal signaling molecules necessary for T cell activation (57). When assessing cytokine production and the ability of these memory T cells to respond to antigen, $TIGIT^+PD\text{-}1^+$, $TIGIT^-PD\text{-}1^+$, and $TIGIT^+PD\text{-}1^-$ all had similar cytokine-producing abilities, whereas Tim3 expression was associated with lower IL-2 and TNF production. These results indicate that TIGIT itself or with PD-1 was not a marker of dysfunction in melanoma, unlike Tim-3. Interestingly, dual blockade of TIGIT and PD-1 led to increased proliferation and cytokine production, indicating both the inhibitory role of TIGIT on CD8+ T cells and the elasticity of TIGIT-expressing cells to produce cytokine, indicating a non-terminally differentiated state. Upregulation of TIGIT itself does not lead to decreased cytokine production and recall potential, but could be acting to inhibit T cell activation in a manner that prevents activation-induced cell death while maintaining basal levels of cytokine production.

Consequently, these data have provided evidence that coinhibitory molecules, including TIGIT, Tim-3, CTLA-4, and PD-1, can function to preserve recall response by potentially limiting terminal differentiation, allowing for sufficient cytokine production and cytotoxic granule release (**Figure 1B**).

CONCLUSION

Here, we have discussed the evidence that coinhibitory molecules limit recall potential of $CD8^+$ memory T cells by inhibiting expansion and function, but also that coinhibitory molecules can allow for the maintenance of stable memory populations that can respond to rechallenge. Interestingly, certain coinhibitory molecules have been reported to do both, including PD-1 and CTLA-4. The difference in function of the coinhibitory molecule—whether it limits recall potential or maintains stable recall potential—could depend on many factors including the environment in which these cells develop and differentiate, genetic programming imprinted upon priming, duration of antigen exposure, number of coinhibitory molecules, and epigenetic modulations. Understanding the context in which these coinhibitory molecules function is important to instruct better vaccine strategies and immunotherapies for cancer, transplant, and autoimmune diseases.

AUTHOR CONTRIBUTIONS

AM and MF conceived of the manuscript focus, researched papers, and wrote the manuscript. LA created the figure. AM, LA, and MF edited the manuscript.

FUNDING

The authors are supported by National Institutes of Health NIAID Grants AI073707 and AI104699 (to MF).

REFERENCES

1. Zhang Q, Vignali DA. Co-stimulatory and co-inhibitory pathways in autoimmunity. *Immunity* (2016) 44:1034–51. doi:10.1016/j.immuni.2016.04.017
2. Attanasio J, Wherry EJ. Costimulatory and coinhibitory receptor pathways in infectious disease. *Immunity* (2016) 44:1052–68. doi:10.1016/j.immuni.2016.04.022
3. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. *Cell* (2009) 138:30–50. doi:10.1016/j.cell.2009.06.036
4. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, et al. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* (2007) 27:670–84. doi:10.1016/j.immuni.2007.09.006
5. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity* (2016) 44:989–1004. doi:10.1016/j.immuni.2016.05.001
6. Zhu C, Sakuishi K, Xiao S, Sun Z, Zaghouani S, Gu G, et al. An IL-27/NFIL3 signalling axis drives Tim-3 and IL-10 expression and T-cell dysfunction. *Nat Commun* (2015) 6:6072. doi:10.1038/ncomms7072
7. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* (2009) 10:29–37. doi:10.1038/ni.1679
8. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol* (2005) 23:515–48. doi:10.1146/annurev.immunol.23.021704.115611
9. Liang SC, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, et al. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol* (2003) 33:2706–16. doi:10.1002/eji.200324228
10. Pilat N, Sayegh MH, Wekerle T. Costimulatory pathways in transplantation. *Semin Immunol* (2011) 23:293–303. doi:10.1016/j.smim.2011.04.002
11. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. *J Infect Dis* (1991) 163:693–8. doi:10.1093/infdis/163.4.693
12. Pavlin JA, Hickey AC, Ulbrandt N, Chan YP, Endy TP, Boukhvalova MS, et al. Human metapneumovirus reinfection among children in Thailand determined by ELISA using purified soluble fusion protein. *J Infect Dis* (2008) 198:836–42. doi:10.1086/591186
13. Erickson JJ, Gilchuk P, Hastings AK, Tollefson SJ, Johnson M, Downing MB, et al. Viral acute lower respiratory infections impair CD8+ T cells through PD-1. *J Clin Invest* (2012) 122:2967–82. doi:10.1172/JCI62860
14. Erickson JJ, Rogers MC, Hastings AK, Tollefson SJ, Williams JV. Programmed death-1 impairs secondary effector lung CD8(+) T cells during respiratory virus reinfection. *J Immunol* (2014) 193:5108–17. doi:10.4049/jimmunol.1302208
15. McNerney ME, Lee KM, Kumar V. 2B4 (CD244) is a non-MHC binding receptor with multiple functions on natural killer cells and CD8+ T cells. *Mol Immunol* (2005) 42:489–94. doi:10.1016/j.molimm.2004.07.032
16. Mooney JM, Klem J, Wulfing C, Mijares LA, Schwartzberg PL, Bennett M, et al. The murine NK receptor 2B4 (CD244) exhibits inhibitory function independent of signaling lymphocytic activation molecule-associated protein expression. *J Immunol* (2004) 173:3953–61. doi:10.4049/jimmunol.173.6.3953
17. West EE, Youngblood B, Tan WG, Jin HT, Araki K, Alexe G, et al. Tight regulation of memory CD8(+) T cells limits their effectiveness during sustained high viral load. *Immunity* (2011) 35:285–98. doi:10.1016/j.immuni.2011.05.017
18. Pedicord VA, Montalvo W I, Leiner M, Allison JP. Single dose of anti-CTLA-4 enhances CD8+ T-cell memory formation, function, and maintenance. *Proc Natl Acad Sci U S A* (2011) 108:266–71. doi:10.1073/pnas.1016791108
19. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* (2001) 1:220–8. doi:10.1038/35105024
20. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol* (2002) 2:116–26. doi:10.1038/nri727
21. Rothstein DM, Sayegh MH. T-cell costimulatory pathways in allograft rejection and tolerance. *Immunol Rev* (2003) 196:85–108. doi:10.1046/j.1600-065X.2003.00088.x
22. Martin M, Schneider H, Azouz A, Rudd CE. Cytotoxic T lymphocyte antigen 4 and CD28 modulate cell surface raft expression in their regulation of T cell function. *J Exp Med* (2001) 194:1675–81. doi:10.1084/jem.194.11.1675
23. Chikuma S, Imboden JB, Bluestone JA. Negative regulation of T cell receptor-lipid raft interaction by cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* (2003) 197:129–35. doi:10.1084/jem.20021646
24. Lee KM, Chuang E, Griffin M, Khattri R, Hong DK, Zhang W, et al. Molecular basis of T cell inactivation by CTLA-4. *Science* (1998) 282:2263–6. doi:10.1126/science.282.5397.2263
25. Chuang E, Fisher TS, Morgan RW, Robbins MD, Duerr JM, Vander Heiden MG, et al. The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity* (2000) 13:313–22. doi:10.1016/S1074-7613(00)00031-5
26. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* (2002) 415:536–41. doi:10.1038/415536a
27. Freeman GJ, Casasnovas JM, Umetsu DT, DeKruyff RH. TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. *Immunol Rev* (2010) 235:172–89. doi:10.1111/j.0105-2896.2010.00903.x
28. Sabatos CA, Chakravarti S, Cha E, Schubart A, Sanchez-Fueyo A, Zheng XX, et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat Immunol* (2003) 4:1102–10. doi:10.1038/ni988
29. Sanchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, et al. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. *Nat Immunol* (2003) 4:1093–101. doi:10.1038/ni987
30. Penaloza-MacMaster P, Alayo QA, Ra J, Provine NM, Larocca R, Lee B, et al. Inhibitory receptor expression on memory CD8 T cells following Ad vector immunization. *Vaccine* (2016) 34:4955–63. doi:10.1016/j.vaccine.2016.08.048

31. Martinez Salazar MB, Delgado Dominguez J, Silva Estrada J, Gonzalez Bonilla C, Becker I. Vaccination with *Leishmania mexicana* LPG induces PD-1 in CD8(+) and PD-L2 in macrophages thereby suppressing the immune response: a model to assess vaccine efficacy. *Vaccine* (2014) 32:1259–65. doi:10.1016/j.vaccine.2014.01.016
32. Mysore KR, Ghobrial RM, Kannanganat S, Minze LJ, Graviss EA, Nguyen DT, et al. Longitudinal assessment of T cell inhibitory receptors in liver transplant recipients and their association with posttransplant infections. *Am J Transplant* (2018) 18:351–63. doi:10.1111/ajt.14546
33. Judge TA, Tang A, Spain LM, Deans-Gratiot J, Sayegh MH, Turka LA. The in vivo mechanism of action of CTLA4Ig. *J Immunol* (1996) 156:2294–9.
34. Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Sci Transl Med* (2010) 2:17ra10. doi:10.1126/scitranslmed.3000116
35. Liu D, Krummey SM, Badell IR, Wagener M, Schneeweis LA, Stetsko DK, et al. 2B4 (CD244) induced by selective CD28 blockade functionally regulates allograft-specific CD8+ T cell responses. *J Exp Med* (2014) 211:297–311. doi:10.1084/jem.20130902
36. Liu D, Badell IR, Ford ML. Selective CD28 blockade attenuates CTLA-4-dependent CD8+ memory T cell effector function and prolongs graft survival. *JCI Insight* (2018) 3:e96378. doi:10.1172/jci.insight.96378
37. Poirier N, Chevalier M, Mary C, Hervouet J, Minault D, Baker P, et al. Selective CD28 antagonist blunts memory immune responses and promotes long-term control of skin inflammation in nonhuman primates. *J Immunol* (2016) 196:274–83. doi:10.4049/jimmunol.1501810
38. Duraiswamy J, Ibegbu CC, Masopust D, Miller JD, Araki K, Doho GH, et al. Phenotype, function, and gene expression profiles of programmed death-1(hi) CD8 T cells in healthy human adults. *J Immunol* (2011) 186:4200–12. doi:10.4049/jimmunol.1001783
39. Legat A, Speiser DE, Pircher H, Zehn D, Fuertes Marraco SA. Inhibitory receptor expression depends more dominantly on differentiation and activation than "exhaustion" of human CD8 T cells. *Front Immunol* (2013) 4:455. doi:10.3389/fimmu.2013.00455
40. Fuertes Marraco SA, Neubert NJ, Verdeil G, Speiser DE. Inhibitory receptors beyond T cell exhaustion. *Front Immunol* (2015) 6:310. doi:10.3389/fimmu.2015.00310
41. Kumar BV, Ma W, Miron M, Granot T, Guyer RS, Carpenter DJ, et al. Human tissue-resident memory T cells are defined by core transcriptional and functional signatures in lymphoid and mucosal sites. *Cell Rep* (2017) 20:2921–34. doi:10.1016/j.celrep.2017.08.078
42. Shwetank, Abdelsamed HA, Frost EL, Schmitz HM, Mockus TE, Youngblood BA, et al. Maintenance of PD-1 on brain-resident memory CD8 T cells is antigen independent. *Immunol Cell Biol* (2017) 95:953–9. doi:10.1038/icb.2017.62
43. Park SL, Zaid A, Hor JL, Christo SN, Prier JE, Davies B, et al. Local proliferation maintains a stable pool of tissue-resident memory T cells after antiviral recall responses. *Nat Immunol* (2018) 19:183–91. doi:10.1038/s41590-017-0027-5
44. Tilburgs T, Schonkeren D, Eikmans M, Nagtzaam NM, Datema G, Swings GM, et al. Human decidual tissue contains differentiated CD8+ effector-memory T cells with unique properties. *J Immunol* (2010) 185:4470–7. doi:10.4049/jimmunol.0903597
45. Clark DR, Chaturvedi V, Kinder JM, Jiang TT, Xin L, Ertelt JM, et al. Perinatal *Listeria monocytogenes* susceptibility despite preconceptual priming and maintenance of pathogen-specific CD8(+) T cells during pregnancy. *Cell Mol Immunol* (2014) 11:595–605. doi:10.1038/cmi.2014.84
46. Constantin CM, Masopust D, Gourley T, Grayson J, Strickland OL, Ahmed R, et al. Normal establishment of virus-specific memory CD8 T cell pool following primary infection during pregnancy. *J Immunol* (2007) 179:4383–9. doi:10.4049/jimmunol.179.7.4383
47. van der Zwan A, Bi K, Norwitz ER, Crespo AC, Claas FHJ, Strominger JL, et al. Mixed signature of activation and dysfunction allows human decidual CD8(+) T cells to provide both tolerance and immunity. *Proc Natl Acad Sci U S A* (2018) 115:385–90. doi:10.1073/pnas.1713957115
48. Ganesan AP, Clarke J, Wood O, Garrido-Martin EM, Chee SJ, Mellows T, et al. Tissue-resident memory features are linked to the magnitude of cytotoxic T cell responses in human lung cancer. *Nat Immunol* (2017) 18:940–50. doi:10.1038/ni.3775
49. Gros A, Robbins PF, Yao X, Li YF, Turcotte S, Tran E, et al. PD-1 identifies the patient-specific CD8(+) tumor-reactive repertoire infiltrating human tumors. *J Clin Invest* (2014) 124:2246–59. doi:10.1172/JCI73639
50. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med* (2010) 207:2175–86. doi:10.1084/jem.20100637
51. Ferris RL, Lu B, Kane LP. Too much of a good thing? Tim-3 and TCR signaling in T cell exhaustion. *J Immunol* (2014) 193:1525–30. doi:10.4049/jimmunol.1400557
52. Lee J, Su EW, Zhu C, Hainline S, Phuah J, Moroco JA, et al. Phosphotyrosine-dependent coupling of Tim-3 to T-cell receptor signaling pathways. *Mol Cell Biol* (2011) 31:3963–74. doi:10.1128/MCB.05297-11
53. Avery L, Filderman J, Szymczak-Workman AL, Kane LP. Tim-3 co-stimulation promotes short-lived effector T cells, restricts memory precursors, and is dispensable for T cell exhaustion. *Proc Natl Acad Sci U S A* (2018) 115:2455–60. doi:10.1073/pnas.1712107115
54. Chauvin JM, Pagliano O, Fourcade J, Sun Z, Wang H, Sander C, et al. TIGIT and PD-1 impair tumor antigen-specific CD8(+) T cells in melanoma patients. *J Clin Invest* (2015) 125:2046–58. doi:10.1172/JCI80445
55. Boles KS, Vermi W, Facchetti F, Fuchs A, Wilson TJ, Diacovo TG, et al. A novel molecular interaction for the adhesion of follicular CD4 T cells to follicular DC. *Eur J Immunol* (2009) 39:695–703. doi:10.1002/eji.200839116
56. Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A, et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc Natl Acad Sci U S A* (2009) 106:17858–63. doi:10.1073/pnas.0903474106
57. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol* (2011) 186:1338–42. doi:10.4049/jimmunol.1003081

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 31 August 2018
doi: 10.3389/fimmu.2018.01909

Not All Immune Checkpoints Are Created Equal

*Annika De Sousa Linhares[1], Judith Leitner[1], Katharina Grabmeier-Pfistershammer[2] and Peter Steinberger[1]**

[1] Division of Immune Receptors and T Cell Activation, Medical University of Vienna, Vienna, Austria, [2] Division of Clinical and Experimental Immunology, Center for Pathophysiology, Infectiology, and Immunology, Institute of Immunology, Medical University of Vienna, Vienna, Austria

Antibodies that block T cell inhibition via the immune checkpoints CTLA-4 and PD-1 have revolutionized cancer therapy during the last 15 years. T cells express additional inhibitory surface receptors that are considered to have potential as targets in cancer immunotherapy. Antibodies against LAG-3 and TIM-3 are currently clinically tested to evaluate their effectiveness in patients suffering from advanced solid tumors or hematologic malignancies. In addition, blockade of the inhibitory BTLA receptors on human T cells may have potential to unleash T cells to effectively combat cancer cells. Much research on these immune checkpoints has focused on mouse models. The analysis of animals that lack individual inhibitory receptors has shed some light on the role of these molecules in regulating T cells, but also immune responses in general. There are current intensive efforts to gauge the efficacy of antibodies targeting these molecules called immune checkpoint inhibitors alone or in different combinations in preclinical models of cancer. Differences between mouse and human immunology warrant studies on human immune cells to appreciate the potential of individual pathways in enhancing T cell responses. Results from clinical studies are not only highlighting the great benefit of immune checkpoint inhibitors for treating cancer but also yield precious information on their role in regulating T cells and other cells of the immune system. However, despite the clinical relevance of CTLA-4 and PD-1 and the high potential of the emerging immune checkpoints, there are still substantial gaps in our understanding of the biology of these molecules, which might prevent the full realization of their therapeutic potential. This review addresses PD-1, CTLA-4, BTLA, LAG-3, and TIM-3, which are considered major inhibitory immune checkpoints expressed on T cells. It provides summaries of our current conception of the role of these molecules in regulating T cell responses, and discussions about major ambiguities and gaps in our knowledge. We emphasize that each of these molecules harbors unique properties that set it apart from the others. Their distinct functional profiles should be taken into account in therapeutic strategies that aim to exploit these pathways to enhance immune responses to combat cancer.

Keywords: cancer immunotherapy, inhibitory receptors, immune checkpoints, PD-1, BTLA, LAG-3, CTLA-4

OPEN ACCESS

Edited by:
Alexandre M. Carmo, i3S, Instituto de Investigação e Inovação em Saúde, Portugal

Reviewed by:
Christopher E. Rudd, Université de Montréal, Canada
Lewis Zhichang Shi, Case Western Reserve University, United States

***Correspondence:**
Peter Steinberger
peter.steinberger@meduniwien.ac.at

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *30 May 2018*
Accepted: *02 August 2018*
Published: *31 August 2018*

Citation:
De Sousa Linhares A, Leitner J, Grabmeier-Pfistershammer K and Steinberger P (2018) Not All Immune Checkpoints Are Created Equal. Front. Immunol. 9:1909. doi: 10.3389/fimmu.2018.01909

INTRODUCTION

Although T cells can recognize tumor antigens, they depend on therapeutic intervention to effectively combat malignant cells in cancer patients. While many attempts with antigen-based therapies failed, antigen-independent strategies that enhance T cell responses by blocking inhibitory pathways have been shown to be effective in a significant proportion of treated patients. This therapeutic success is achieved by antibodies often referred to as immune checkpoint inhibitors (ICIs) (1). CTLA-4 was the first immune checkpoint that was targeted to enhance T cell responses in patients suffering from melanoma (2). Antibodies interfering with PD-1 mediated inhibition of T cells and potentially other immune cells were introduced a few years later and have had the greatest success so far (3–6). Inhibitory immune checkpoints help maintaining tolerance and consequently a broad spectrum of side effects–immune-related adverse events (IRAEs)–are observed in treated patients (7). Moreover, monotherapy with ICIs that are currently in use is only beneficial in a subset of cancer patients and frequently leads to acquired resistance (8–10). Consequently, there have been many attempts to evaluate the efficacy of combining PD-1 blockers with conventional cancer treatments (chemotherapy, radiation, surgery) or targeted therapies. Co-administration of PD-1 and CTLA-4 antibodies to patients with melanoma was shown to increase therapeutic efficacy, whereas adverse events were only moderately increased as compared to CTLA-4 blockade alone (11). It is possible that blocking other inhibitory receptors might also augment the therapeutic benefit of PD-1 blockade. Antibodies targeting BTLA, TIM-3, and LAG-3 are promising candidates to boost T cell responses alone or in combination with ICIs disrupting PD-1 mediated inhibition.

Consequently, there is great interest to understand the biology of these inhibitory receptors, which, like CTLA-4, are clearly distinct from PD-1. The original concept of inhibitory receptors was shaped by a group of receptors described on NK cells over 20 years ago (12, 13). These molecules were shown to contain inhibitory motifs (ITIMs), which upon engagement by their ligands, counteract activating signaling processes mediated by ITAM-containing receptors (14). These classical inhibitory receptors exert their function by recruiting SH2-containing phosphatases SHP-1 and SHP-2, which dephosphorylate signaling molecules, thereby directly interfering with activating signaling processes. It was later established that receptors can exert inhibitory functions independent of ITIM motifs. Therefore, inhibitory receptors are now defined by their function rather than by the presence of an ITIM motif in their cytoplasmic domain (13, 15). It is quite clear that BTLA, LAG-3, TIM-3, and CTLA-4 deviate from the classical inhibitory receptors described above. They also differ considerably from PD-1, which can be regarded as the prototypic T cell-expressed immune checkpoint that induces inhibitory intracellular signaling upon engagement with its non-signaling ligands that are preferentially expressed on antigen-presenting cells (APC) and tumor cells.

Here, we want to illustrate that each of these inhibitory receptors has unique properties and we want to draw attention to important open questions regarding BTLA, LAG-3, TIM-3 and CTLA-4 (**Figure 1**). Distinct features of each immune checkpoint should be accounted for when developing strategies to exploit these pathways therapeutically and unresolved issues and controversies need to be addressed to better validate their potential as targets in cancer immunotherapy.

PROGRAMMED CELL DEATH PROTEIN-1 (PD-1)

Programmed cell death-1 (PD-1) is a type 1 transmembrane receptor that belongs to the immunoglobulin superfamily (Ig-SF). Its cytoplasmic domain contains two inhibitory motifs: an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (**Figure 2**). Following TCR-ligation, the phosphatase SHP-2 associates with the intracellular domain of PD-1 via these motifs (16). However, PD-1 ligation is required for inhibition, suggesting that PD-1 must co-localize with the TCR-CD3 complex or CD28 to exert its function (17). Whereas earlier work has suggested that strong CD28 costimulation can override PD-1 costimulation (18), two recent studies reported that CD28 is a major target of PD-1 signaling (19, 20). The B7-family members programmed cell death ligand-1 (PD-L1) and−2 (PD-L2) are ligands for PD-1. PD-L2 expression is mainly restricted to professional APCs such as dendritic cells (DCs) and macrophages, whereas PD-L1 is broadly expressed on cells of the hematopoietic lineage including activated T cells (21). Inflammatory stimuli induce PD-L1 expression and this ligand is also expressed in a wide variety of non-hematopoietic tissues and importantly in many different types of tumor cells (22, 23). PD-1 is a potent negative regulator of T cell activation and studies on PD-$1^{-/-}$ mice highlighted an essential role of PD-1 in maintaining tolerance and preventing autoimmunity. Mice deficient in PD-1 develop features of a lupus-like disease and autoimmunity is promoted in NOD and MLR mice (24, 25). The interaction of PD-1 with its ligands promotes tolerance and dampens T cell immunity at several levels. PD-1 helps to maintain central tolerance by regulating positive and negative selection (26). It critically contributes to peripheral tolerance, e.g., by promoting Treg induction, expression of PD-ligands on resting DCs and upregulation of PD-L1 on host tissues and endothelial cells during inflammation (27–29). However, PD-1 also limits productive T cell immunity against pathogens and tumor cells (30). PD-1 is induced upon T cell activation, and PD-ligands are constitutively expressed on APCs such as DCs. Consequently, PD-1 is broadly engaged on T cells responding to their cognate antigens. Importantly, PD-1 gains importance on T cells that are exposed to persistent antigenic challenge through antigens derived from chronic viruses or tumor cells. Such T cells enter a state of functional impairment that is often described as exhaustion (31). It was shown that PD-1 is constitutively expressed on mouse, macaque and human CD8 T cells specific for LCMV and HIV antigens, respectively (32–34). Importantly, blockade of PD-1 signaling reverts the functional impairment of exhausted T cells in both models. Signs of exhaustion are frequently observed in tumor resident T cells (35) and their capability to combat tumor cells

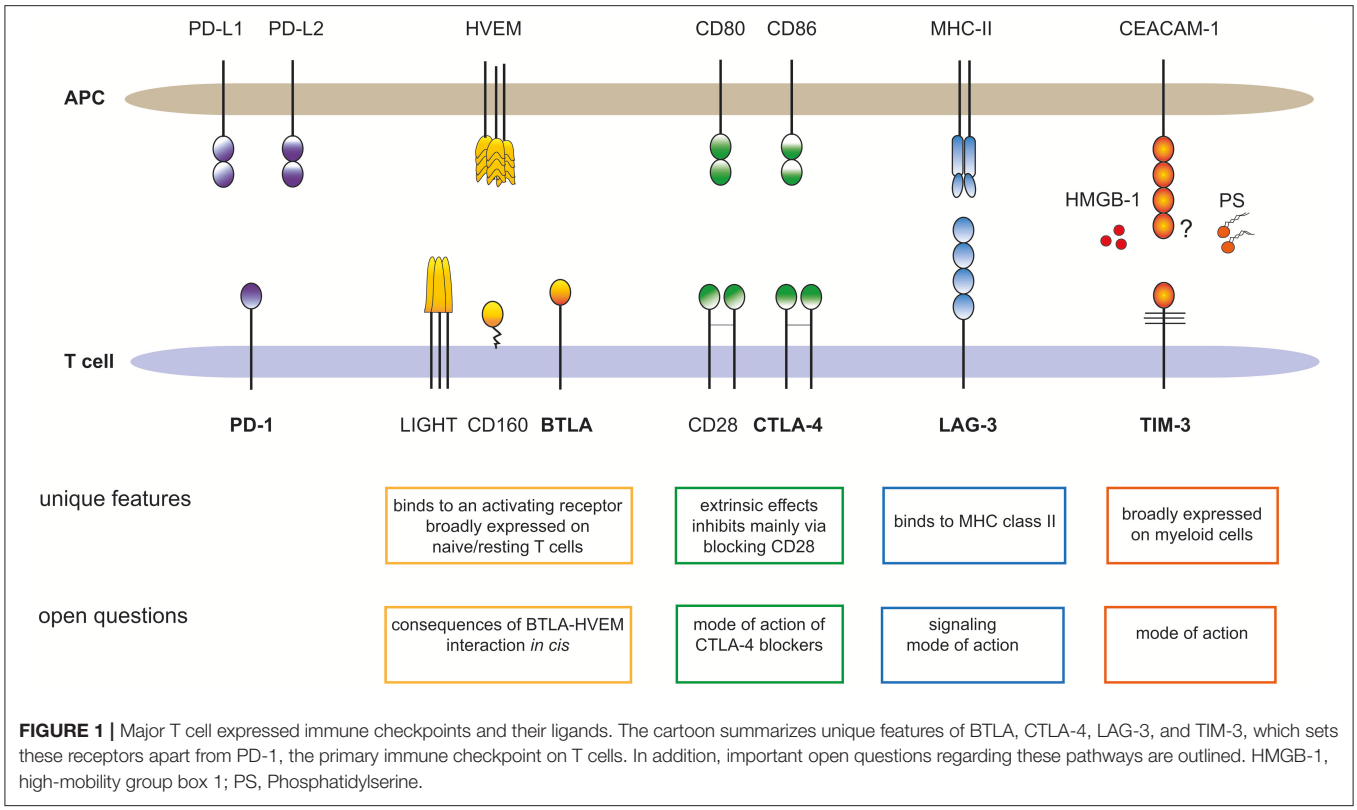


FIGURE 1 | Major T cell expressed immune checkpoints and their ligands. The cartoon summarizes unique features of BTLA, CTLA-4, LAG-3, and TIM-3, which sets these receptors apart from PD-1, the primary immune checkpoint on T cells. In addition, important open questions regarding these pathways are outlined. HMGB-1, high-mobility group box 1; PS, Phosphatidylserine.

is frequently impaired by the presence of PD-L1 on their targets. Moreover, blocking PD-1 or PD-L1 was demonstrated to enhance anti-tumor responses in murine models of cancer (36–38). Taken together, these findings provided a rationale for targeting PD-1 to enhance anti-tumor responses in humans. Several antibodies blocking PD-1 signaling by either binding to PD-1 or to PD-L1 have shown clinical efficacy in solid tumors and hematological malignancies such as melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), head, and neck squamous cell carcinoma, cervical cancer, uterine cancer, breast cancer, Merkel cell carcinoma, Hodgkin's lymphoma, diffuse large B cell lymphoma, and follicular lymphoma (39). Although these antibodies represent a great advance in cancer treatment, there is a great variation in patient response to PD-1 blockade with a significant proportion not responding. Consequently, there are intense efforts underway to combine PD-1 blockers with conventional therapies or targeting of other inhibitory receptors to further increase the response rate in cancer patients.

B AND T LYMPHOCYTE ATTENUATOR (BTLA)

B and T lymphocyte attenuator (BTLA) is a type I transmembrane receptor belonging to the Ig-superfamily. It bears similarities to PD-1; its extracellular domain has an IgV-like fold and its cytoplasmic domain also harbors an ITIM and an ITSM motif, two classical inhibitory motifs (**Figure 2**). Engagement of BTLA was reported to lead to the recruitment of the SH2-domain containing phosphatases SHP-1 and SHP-2, which subsequently mediate the inhibitory effects of this receptor (40, 41). However, BTLA, which has a long cytoplasmic tail of 111 amino acids can also engage activating signaling pathways via a putative Grb-2 binding motif located upstream of ITIM/ITSM sequences (42) (**Figure 2**). As implied by its name, BTLA is preferentially expressed on B and T cells, but it is also present on innate immune cells such as monocytes and DCs (43). The only known ligand expressed in human cells is the Herpes virus entry mediator (HVEM), a member of the tumor necrosis factor receptor superfamily (TNFR-SF) (44). HVEM is an activating receptor that also interacts with members of the TNF-SF, LIGHT, and LT-α (43). In addition, HVEM is a binding partner of CD160, which is also a member of the Ig-SF (45). The role of CD160 in T cell activation processes is controversial because both activating and inhibitory effects have been reported (45–47). CD160 is mainly expressed as a GPI-linked molecule and it is currently unclear how this receptor engages the intracellular signaling machinery of T cells.

In mice, BTLA deficiency is associated with hyper-reactive B and T cells and enhanced susceptibility to autoimmunity (43). Interestingly, HVEM-deficiency results in a similar phenotype, indicating that inhibitory BTLA signaling might play a dominant role in the HVEM network (48). Several reports have found that BTLA blockers can enhance human T cell responses when used alone or in combination with antibodies against PD-1 (49–52). Work by Derré and colleagues demonstrated that although BTLA is down-regulated during activation and differentiation, this receptor is prominently expressed on human T cells in the

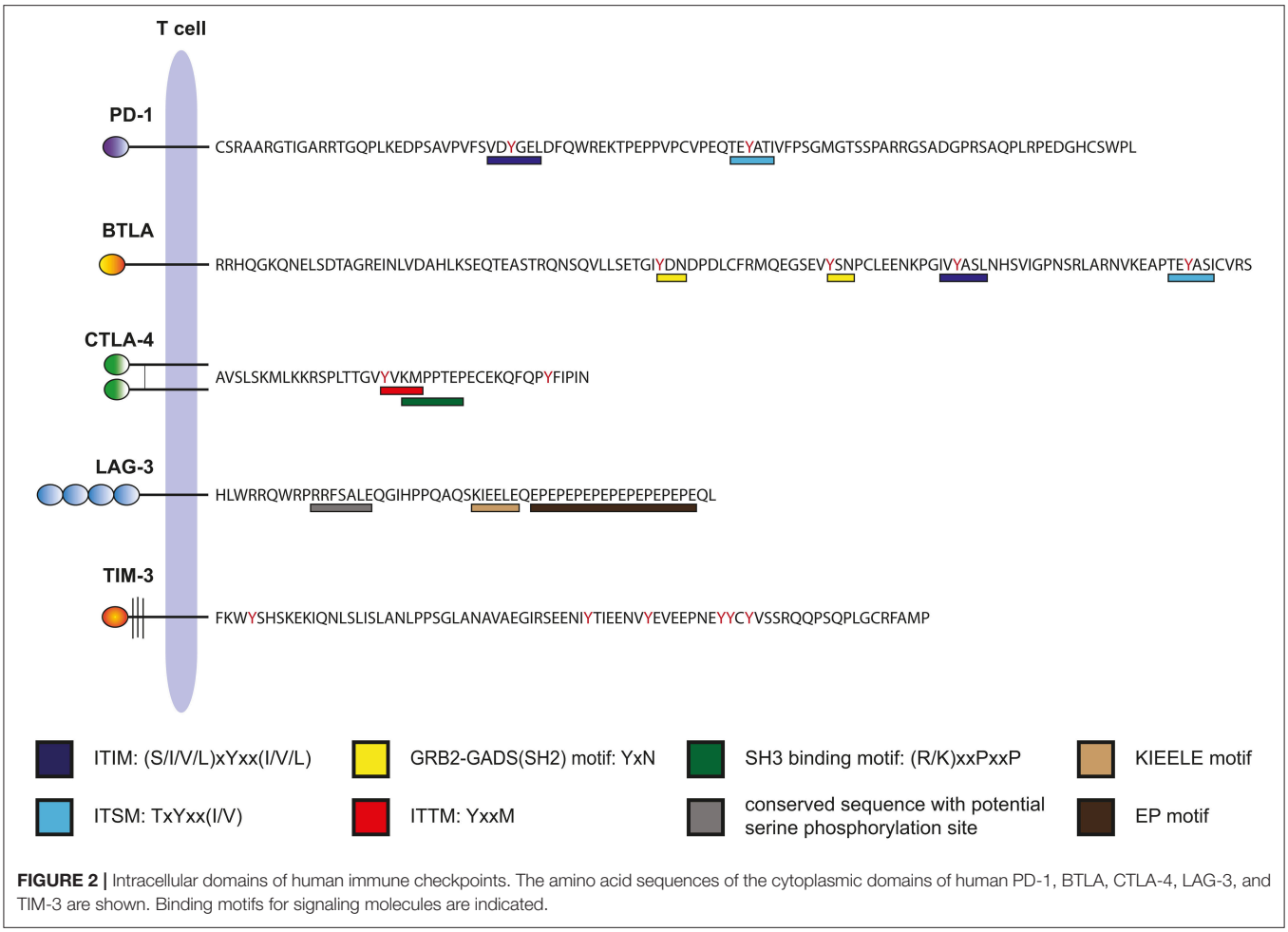


FIGURE 2 | Intracellular domains of human immune checkpoints. The amino acid sequences of the cytoplasmic domains of human PD-1, BTLA, CTLA-4, LAG-3, and TIM-3 are shown. Binding motifs for signaling molecules are indicated.

tumor microenvironment and can function to inhibit tumor-specific T cells (53). However, recent studies indicate that the role of BTLA in tumor-resident T cells is complex, as engagement by its ligand HVEM inhibits proliferation and cytokine production but promotes survival of tumor-infiltrating lymphocytes (TILs) (54). Signaling mediated via PI3K recruitment to the Grb-2 binding motif of BTLA has been implicated in these activating effects (54, 55).

Unique Features of BTLA

The intracellular domain of BTLA bears classical inhibitory motifs and it is well established that BTLA mainly functions as a negative regulator of lymphocytes. However, two striking features set BTLA apart from other inhibitory immune checkpoints expressed on T cells. One peculiarity is that it has a ligand that functions as activating receptor. Therefore, the interaction of BTLA with the TNFR-family member HVEM not only generates inhibitory signals in BTLA expressing cells but also stimulatory signals in the cells that express HVEM. Therefore, both BTLA and HVEM have a dual role as a ligand and receptor when they interact with each other (43, 56, 57). It seems counter-intuitive that upon engagement of an inhibitory receptor also an activating signal is generated, and currently the significance of this phenomenon is not understood.

Another unique feature of BTLA is that it is prominently expressed on naïve T cells and tentatively down-regulated upon activation and differentiation (58). This is in stark contrast to other inhibitory immune checkpoints, which are largely absent on naïve cells. PD-1, CTLA-4, or LAG-3 expression is associated with activation and persistent stimulation, which is consistent with a role of these molecules in limiting and terminating immune reactions. Currently the significance of the unusual expression pattern of BTLA expression is not clear.

Important Open Questions About BTLA

Elegant work by Cheung and colleagues has shown that BTLA and HVEM interact with each other on cells co-expressing these molecules (59). The majority of human T cells harbor HVEM, and thus BTLA and its ligand HVEM are extensively co-expressed in these cells. It has not yet been addressed whether such *in cis* engagement of BTLA and HVEM during the activation of T cells results in signaling by either of these molecules. However, there is evidence that *in cis* engagement of HVEM prevents the interaction of this receptor with ligands *in trans*, thereby precluding HVEM signaling (59). To date, no

studies have addressed whether engagement of BTLA by co-expressed HVEM also attenuates BTLA signaling by interfering with interaction with HVEM *in trans*. The majority of co-inhibitory receptors expressed on T cells is tightly regulated and can only be detected on the surface of activated or "exhausted" T cells. In contrast, BTLA is broadly expressed on T cells and it is tempting to speculate that its function is controlled by co-expression of HVEM. Future studies should test this hypothesis and aim to reveal the interrelationship of BTLA and HVEM on T cells. Eventually, these studies might help to gauge the potential of BTLA as a target of tumor immunotherapy and to devise immune checkpoint inhibitors that optimally target this pathway.

CYTOTOXIC T LYMPHOCYTE ANTIGEN-4 (CTLA-4)

Cytotoxic T cell lymphocyte antigen-4 (CTLA-4) is a type-1 transmembrane protein harboring a IgV-like Ig-domain. Conventional T cells express CTLA-4 upon activation, whereas Tregs express it constitutively (60). CTLA-4 resides in intracellular vesicles and is quickly exported to the surface upon activation. Importantly, CD28, the primary costimulatory receptor, and CTLA-4 share their extracellular ligands CD80 and CD86, but CTLA-4 binds both molecules with higher affinity. Two papers on CTLA-4-deficient mice published in 1995 clearly established that CTLA-4 functions as a negative regulator of T cell responses. These studies demonstrated that mice lacking CTLA-4 suffer from autoimmune phenomena and immune dysregulation which results in early death (61, 62).

The 36 amino acid cytoplasmic tail of CTLA-4 is highly conserved and interaction with several intracellular signaling molecules has been reported (63). Interestingly, CTLA-4 and its activating counterpart CD28 have common intracellular binding partners including the p85 subunit of PI3K and the phosphatase PP2A (63). In Tregs, the protein kinase C-η (PKC-η) associates with CTLA-4 and signaling via the CTLA-4–PKC-η axis was found to be required for contact-dependent suppression (64). Arguably, the best-established relationship of a binding motif within the cytoplasmic tail of CTLA-4 with a function is the YVKM motif that interacts with the clathrin adaptor complex AP-2, thereby promoting internalization and localization of CTLA-4 in intracellular vesicles (**Figure 2**) (65, 66). For surface expression of CTLA-4 a molecular complex comprised of TRIM, LAX and Rab8 is formed, which shuttle CTLA-4 from the trans-Golgi networt to the surface (67). It has been suggested that the function of the cytoplasmic domain of CTLA-4 is to control the turnover and cellular location of this molecule rather than transmitting inhibitory signals (68).

Unique Features of CTLA-4

Initial research focused on the contribution of the cytoplasmic tail of CTLA-4 to T cell inhibition. These studies revealed that several intracellular signaling molecules can interact with motifs contained in the intracellular domain of CTLA-4. In addition to "classical" effects like recruitment of enzymes that counteract TCR mediated downstream signaling processes (69, 70), it was found that CTLA-4 mediates a reversal of the "stop-signal" initiated upon cognate T cell-APC interaction and thereby prevents efficient cytokine production and proliferation (71). Although these mechanisms contribute to T cell inhibition, there is increasing evidence that CTLA-4 exhibits inhibitory functions that are independent of its intracellular moiety (72–74). Therefore, one unique property of CTLA-4 is that "extrinsic effects," specifically its capacity to interfere with CD28 costimulation, critically contribute to its function as an attenuator of T cell immunity. Two major mechanisms have been demonstrated in this context. First, CTLA-4, which has a higher affinity for CD80 and CD86 than CD28, binds these ligands and thereby prevents CD28 costimulation (73, 75, 76). More recently, it was shown that CTLA-4 depletes B7 molecules on APC by literally ripping out these costimulatory ligands, a process termed trans-endocytosis (77). However, it is currently not clear to which extend this process contributes to the extrinsic function of CTLA-4. Results of a study were a transgene encoding tail-less CTLA-4 and full length CTLA-4 was introduced into CTLA-4 deficient mice only mice expressing the full length molecule were completely healthy, whereas expression of a tail-less molecule only partly restored immune function. This indicated that both intrinsic and extrinsic effects contribute to maintenance of immune homeostastis by CTLA-4 (78). An important function of CTLA-4 on conventional T cells and Tregs may be the regulation of activating signals via the primary costimulatory receptor CD28. Indeed, induction of autoimmune disease in CTLA-$4^{-/-}$ mice is only observed when *in vivo* CD28 costimulation is in place (79).

Important Open Questions About CTLA-4

As outlined above CTLA-4 has been implicated to mediate T cell inhibition by numerous quite distinct mechanisms. Although there is mounting evidence that signaling-independent processes have a major role, the contribution of individual mechanisms is a matter of ongoing debate. Tregs, which have a variety of mechanisms to inhibit immune responses, are characterized by constitutive and high CTLA-4 expression. Studies in mouse tumor models showing that CTLA-4 antibodies can function by depleting intratumoral Tregs via Fc-receptor dependent mechanisms have received much attention (80–82). Recent work by Romano and colleagues demonstrated that patients responding to ipilimumab have higher frequencies of non-classical monocytes and that ipilimumab can mediate killing of CTLA-4^{high} cells by these cells (83). In addition, there is evidence that in melanoma patients response to ipilimumab was associated with the CD16a-V158F high affinity polymorphism (84). Taken together, these results suggest that ipilimumab, which is an IgG_1 antibody that is fully capable of interacting with Fc-receptors, may mediate killing of Tregs *in vivo*. However, more investigations are required to substantiate that Treg depletion is a major mechanism of ipilimumab action in cancer patients. Such studies might also help determine potential of strategies aiming at Treg depletion in cancer therapy.

LYMPHOCYTE ACTIVATION GENE-3 (LAG-3)

Lymphocyte activation gene-3 is a type 1 transmembrane protein that has significant homology to CD4 and was first described by Triebel and colleagues in 1990 (85). Expression of LAG-3 has been described on activated T cells, B cells, and NK cells but also on plasmacytoid DCs (85–87). Its extracellular part contains four Ig-like domains and shares high structural homology to CD4. Like CD4, LAG-3 binds to MHC class II molecules, albeit with much higher affinity (88). LAG-3 is heavily glycosylated and interacts with the lectins galectin 3 and the cell surface resident liver sinusoidal endothelial lectin (LSECtin), which is a member of the DC-SIGN family (89, 90).

The 54 amino acid cytoplasmic tail of LAG-3 is devoid of classical motifs involved in the recruitment of inhibitory phosphatases. Instead, it contains a potential serine phosphorylation motif (S454), an unusual sequence consisting of glutamic acid and proline dipeptide motifs (EP motif) and a highly conserved KIEELE motif (**Figure 2**). A protein termed LAG-3-associated protein (LAP) was shown to bind to the repeated EP motif of LAG-3 but functional effects of this interaction were not studied (91). Follow-up studies on this finding are lacking. The role of the cytoplasmic tail in the function of LAG-3 has to date only been addressed in a singular study by Workman and colleagues published more than 15 years ago (92). The authors expressed wild type and mutated variants of LAG-3 in a murine hen egg lysozyme-specific LAG-3-negative $CD4^+$ T cell hybridoma line. They found that wildtype, but not tailless, LAG-3 inhibited IL-2 production in response to antigen. Moreover, the authors reported that LAG-3 inhibition depended on its ligation to MHC class II as well as on the presence of CD4, since a CD4-deficient subline was not inhibited (92). Different LAG-3 mutants were tested and it was found that the KIEELE motif was required, whereas S454 and the EP motif were dispensable for LAG-3 function (92). Collectively, these data suggest that although the presence of CD4 is required for LAG-3 inhibition, this receptor does not simply function by interfering with MHC class II–CD4 interaction, since the intracellular motifs of LAG-3 are required for inhibition. Several studies have shown that LAG-3 functions as an intrinsic negative regulator of $CD4^+$ but also $CD8^+$ T cells (92–95). In addition, LAG-3 is constitutively expressed on Tregs and can contribute to Treg mediated inhibition (96, 97). Interaction of Treg expressed LAG-3 with MHC class II molecules was shown to induce inhibitory signaling pathways in DCs (98). A number of studies in murine tumor models have provided a rationale for LAG-3 blockade to limit tumor growth. It was shown that LAG-3 antibodies alone or in combination with PD-1 blockers curtailed growth of malignant cells and promoted tumor clearance (99–102). Several antibodies targeting LAG-3, including the bispecific agent MGD013 that simultaneously binds LAG-3 and PD-1, are in clinical development. Most of these aim at enhancing T cell responses, but a depleting antibody that should function by killing activated effector memory T cells, thus reducing unwanted T cell responses, is also being developed (103, 104). In addition, IMP321, a LAG-3 immunoglobulin fusion protein that exerts immune potentiating functions by activating APCs via MHC class II molecules, is being tested in several clinical trials (103, 105).

Unique Features of LAG-3

A striking feature of LAG-3 is that it ligates to MHC class II molecules rather than to a generic co-inhibitory ligand. Related to this, LAG-3 has a large extracellular domain compared to other T cell expressed co-inhibitory molecules like PD-1, BTLA, and CTLA-4. In addition, LAG-3 has an unusual cytoplasmic tail containing motifs that are not found in other co-inhibitory receptors. Therefore, inhibitory mechanisms of LAG-3 are likely to be unique and clearly distinct from those exerted by other immune checkpoints; potential extrinsic effects will affect the antigen-specific signals rather than costimulatory signals (signal 1 rather than signal 2) and intrinsic effects will engage unique pathways that are not used by other inhibitory receptors.

Important Open Questions About LAG-3

The mode of action of LAG-3 mediated inhibition is currently incompletely understood. LAP binds to the EP motifs of LAG-3 but the consequences of this interaction are not known (91). The KIEELE domain is highly conserved and was described to be required for the inhibitory function of LAG-3. However, there is a complete lack of data showing how the intracellular signaling machinery of T cells connects with this motif to counteract activating T cell signaling processes. Workman and colleagues have described that LAG-3 inhibits CD4-dependent, but not CD4-independent T cell function (92). Thus, it is possible that the role of the intracellular domain of LAG-3 is to promote the extrinsic effects of LAG-3 for instance by ensuring optimal spatial orientation of LAG-3 in the immunological synapse. However, direct inhibition of $CD8^+$ T cells by LAG-3 has also been described and distinct mechanisms have to be involved for such a function of LAG-3 (93–95). We have found that blocking LAG-3 alone or in combination with PD-1 on T cells stimulated with allogeneic DC or virus antigens had limited efficacy (49, 50). In general, there are scarce data describing a robust effect of LAG-3 on human T cell responses *in vitro*. Establishing stimulation conditions for primary human T cells where LAG-3 blockade exerts a strong and reproducible effect would be valuable to further our understanding of LAG-3 function and aid the development of improved therapeutic strategies targeting this immune checkpoint in T cells.

Another important issue is the consequence of MHC class II engagement by LAG-3. The LAG-3 fusion protein IMP321 shows adjuvant properties and enhances immunogenicity of tumor vaccines (105). Induction of DC maturation via engagement of MHC class II has been proposed as a mechanism underlying this effect (106, 107). Interestingly, binding of MHC class II molecules on a CD4 T cell clone by a LAG-3 fusion protein inhibited proliferation and cytokine production upon stimulation with antigen (108). Moreover, Tregs were shown to inhibit DC maturation via LAG-3 (98). Thus it appears that engagement of MHC class II molecules by membrane-bound or soluble LAG-3 can transduce either activating or inhibitory signals and

dissecting the mechanisms behind this functional dichotomy will certainly help to understand the complex pathways used by LAG-3 to regulate immune responses. LAG-3 is also released from CD4 T cells after activation but it is not known whether this has a role in immune regulation (109).

T CELL IMMUNOGLOBULIN AND MUCIN-DOMAIN CONTAINING PROTEIN-3 (TIM-3)

T cell immunoglobulin and mucin-domain containing protein-3 (TIM-3) is a member of the TIM family, which has two additional members in humans: TIM-1 and TIM-4. TIM-molecules are type I transmembrane proteins that contain an N-terminal IgV-like domain and a mucin domain (110). TIM-3 is constitutively expressed on innate immune cells such as monocytes/macrophages, DCs, mast cells, and mature NK cells, whereas on T cells its expression is associated with activated and terminally differentiated states (110–113). Several ligands have been proposed for TIM-3. Like all TIMs, it binds phosphatidylserine (PtdSer), yet compared to TIM-1 and TIM-4, its capacity to interact with these molecules appears to be considerably lower (110, 114). TIM-3 also binds to high-mobility group box 1 (HMGB-1), a damage-associated molecular pattern protein that is released from stressed innate immune cells and can interact with different molecules including nucleic acids and lipopolysaccharide (LPS) (115). Based on intracellular binding experiments Galectin-9 (Gal-9) was reported to serve as a binding partner for TIM-3 (116). The galectins are a family of beta-galactoside-binding proteins and Gal-9 was also implicated in binding 4-1BB, CD40, CD44, and Dectin-1 (117–120). We performed a series of experiments that produced no evidence for a specific interaction of human or mouse TIM-3 with Gal-9 (121). CEACAM-1, a co-inhibitory molecule expressed on T cells that functions as a self-ligand, was reported as another ligand for TIM-3 (122). An interaction between TIM-3 and CEACAM-1 on cell surfaces was not shown in this study. Instead, co-precipitation experiments were performed and the crystal structure of a heterodimer of the V-domains of human CEACAM-1 and human TIM-3 was published (122). The heterodimer models have since been withdrawn and further work is required to establish an interaction between CEACAM-1 and TIM-3 (123).

Human TIM-3 has a cytoplasmic tail of 71 amino acids that lacks classical activating or inhibitory signaling motifs like ITAMs or ITIMs (**Figure 2**). However, several studies report that the cytoplasmic domain of TIM-3 can mediate intracellular signaling in T cells and myeloid cells. Two tyrosines (Y256 and Y263 in human TIM-3) whose phosphorylation enables interaction with SH2 domain containing molecules appear to have a significant role in this process. Intracellular signaling proteins that have been reported to interact with TIM-3 include p85 of PI3K, PLC-γ, ZAP-70, Lck, and SLP-76 (124). Rangachari et al. found that HLA-B-associated transcript 3 (Bat3) associates with the cytoplasmic domain of TIM-3, thereby preventing T cell dysfunction and exhaustion (125). Recent work by Avery and colleagues showed that TIM-3 promotes Akt/mTOR signaling and is essential for optimal effector T cell responses (126).

An autoimmune phenotype of mice lacking TIM-3 was not described but consistent for an inhibitory role of TIM-3 in immunity these animals were found to be refractory to tolerance induction (127). Gorman et al. however found that TIM-3 knockout mice had reduced magnitudes of both primary and secondary CD8 T cell responses. They showed that this effect was cell intrinsic, suggesting that TIM-3 can mediate a stimulatory effect on CD8 T cell responses (128).

Unique Features of TIM-3

Unlike the other immune checkpoints described in this review, TIM-3 is constitutively expressed on several cell types of the myeloid lineage. TIM-3 acts as a receptor for ligands like HMGB-1 and phosphatidylserine, which is consistent with a molecule that is primarily expressed on innate immune cells. CTLA-4, PD-1, LAG-3, and BTLA interact with cell surface molecules preferentially expressed on professional APCs, whereas APC-expressed membrane-bound ligands for TIM-3 have not been reported. Although TIM-3 is present on activated and exhausted T cells, a recent study reported that TIM-3-positive cells in breast cancer cell samples were of myeloid rather than T cell origin (129). Thus, therapeutic approaches targeting TIM-3 are likely to have a strong impact on APCs such as macrophages and DCs.

Important Open Questions About TIM-3

TIM-3 is expressed in many immune cells and activating as well as inhibitory functions have been ascribed to this receptor. Phosphatidylserine, HMGB-1, Galectin-9, and CEACAM-1 were proposed as binding partners for this molecule, but it is currently not clear whether all of these molecules act as *bona fide* TIM-3 ligands. In many studies, TIM-3 function was not linked to a specific TIM-3 ligand, and Galectin-9 and CEACAM-1 can regulate T cells independent of TIM-3 (120, 130–133).

Several reports found that antibodies against human TIM-3 enhance T cells responses alone or in combination with PD-1 blockers and thus provide a rationale to explore strategies to enhance anti-cancer immunity by targeting TIM-3 (49, 50, 113, 134, 135). TIM-3 antibodies could directly act on T cells or indirectly by potentiating APC functions, which in turn could enhance T cell responses. In this context, it should be noted that TIM-3 antibodies were shown to induce activating signals in human DCs (5, 111). Gain of function studies on TIM-3 in human T cell lines have yielded conflicting results; while one group obtained results that point to an activating role of TIM-3 (124), others have observed effects that are consistent with an inhibitory role of TIM-3 (136). T cell reporter systems based on the human T cell line Jurkat are powerful tools to assess mechanisms of co-inhibition and to test immune checkpoint inhibitors. Although such reductionist assay systems for evaluating antibodies against PD-1, CTLA-4, BTLA, and LAG-3 are commercially available and have been described in the literature (72, 137–140), a validated test system for antibodies targeting TIM-3 has not yet been described

to our knowledge. A recent report by Sabins and colleagues demonstrated that a TIM-3 antibody that was used in several studies to target human TIM-3 could function as an agonist and promoted CD8 T cell differentiation through activation of mTORC1 (141). Thus, it will be necessary to address whether functionally active antibodies to human TIM-3 act as agonists or antagonists to understand the role of TIM-3 in human T cell responses.

GENERAL OPEN QUESTIONS AND OUTLOOK

Exhaustion and Immune Checkpoints

It is generally accepted that persistent stimulation with an antigen can result in a state of functional impairment referred to as exhaustion in T cells specific for virus and tumor antigens. A landmark paper by Blackburn and colleagues showed that exhausted T cells can upregulate several co-inhibitory receptors (142). Subsequently, it was shown that Melan-A-specific T cells in patients with melanoma resemble exhausted T cells in chronic infections (143). Importantly, several studies have demonstrated that PD-1 antagonists can revert dysfunction in exhausted T cells (32, 33, 144, 145). Based on these findings, immune checkpoint receptors have been phenotypically and functionally linked to T cell exhaustion (146, 147). Consequently, it is often inferred that immune checkpoint inhibitors mainly function to reinvigorate exhausted T cells. Although inhibitory receptors are involved in T cell exhaustion, it is important to emphasize that the expression of immune checkpoint inhibitors on T cells is by no means limited to exhausted populations (147, 148). In addition, the presence of a particular inhibitory receptor on exhausted T cells neither proves that the receptor is the cause of their state nor that it critically contributes to their functional impairment (13). A better understanding on the relationship of inhibitory immune checkpoints and their role in exhaustion is highly desired and will help to understand the potential but also the limitations of ICIs in targeting exhausted tumor specific T cells.

Tregs and Inhibitory Immune Checkpoints

Tregs and T cell-expressed inhibitory immune checkpoints play important roles in maintaining peripheral tolerance. However, they can both limit protective immunity against pathogens and tumor cells. As summarized in a recent review addressing the immune checkpoint inhibitors in Tregs, these cells constitutively express immune checkpoints like CTLA-4 but also PD-1, BTLA, LAG-3, and TIM-3, and upregulate inhibitory receptors during activation and at tumor sites (1). Although there is ample evidence for a role of co-inhibitory receptors in Treg function (96, 149, 150), many aspects of the interrelation between these two pillars of tolerance are incompletely understood. Specifically, it is not clear how immune checkpoints that inhibit T cells by downregulating intracellular signaling pathways function in Tregs: is engagement of such receptors on Tregs mainly attenuating or enhancing their regulatory function? If the former would be true, ICI-therapy would potentiate Treg function, which could result in reduced efficacy of such regimens. Whereas in the latter case, immune checkpoint inhibitors might exert their beneficial function at least in part by targeting Tregs.

Emerging Immune Checkpoints

There are several additional co-inhibitory pathways that are implicated in limiting T cell responses and thus might have potential in cancer immunotherapy. One such protein is TIGIT (T cell immunoreceptor with Ig and ITIM domains), which bears similarities to CTLA-4 as it shares binding partners with an activating receptor (CD226), which binds these ligands with lower affinity (151–154).

V-domain Ig suppressor of T cell activation (VISTA), also known as B7-H5, PD-1H, and Gi24, is expressed on T cells, myeloid cells, and NK-cells (155). VISTA, an orphan receptor on T cells, can also function as a ligand for an unknown receptor on T cells (156–158). Studies in mice and murine cells indicate that VISTA has an inhibitory role in immunity. VISTA was knocked out in mice by two independent approaches and both showed signs of enhanced immune activity and autoimmunity albeit to different degrees (159, 160). In addition, there are several studies in mice that suggest that blocking VISTA might enhance tumor immunity (155, 156, 158, 161, 162). In addition several studies show VISTA expression in tumors and treatment with ipilimumab was found to upregulate VISTA in patients with prostate cancer (162–165). Therefore, antibodies blocking VISTA on T cells as well as the interaction of APC-expressed VISTA with its unknown receptor expressed on T cells may have potential in cancer immunotherapy since they could enhance T cell responses by disrupting two inhibitory signaling pathways in T cells.

Surprisingly few studies have addressed the role of VISTA in human T cells and myeloid cells. Lines et al. reported that a VISTA immunoglobulin fusion protein blocks T cell activation and promotes the generation of human Tregs (166). By contrast Baraj and colleagues found that overexpression of VISTA on human monocytes promoted their activation and subsequently enhanced T cell responses (167). To date there is a lack of information not only regarding receptors and ligands on T cells and APC, respectively, that mediate the proposed effects of VISTA but also on downstream signaling events induced upon VISTA engagement. Despite, this and based on promising result in mice, a clinical trial with a monoclonal antibody to VISTA was initiated (NCT02671955).

B7-H7, also known as HERV-H LTR associating 2 (HHLA2), is a member of the extended B7 family and inhibits proliferation and cytokine production of human CD4 and CD8 T cells (168). B7-H7 is expressed on human APCs such as monocytes or B cells, but it is also widely expressed in non-hematopoietic tissues and cancers (168, 169). CD28H, also known as TMIGD2, was shown to function as a binding partner for HHLA2 (170). HHLA2 was designated as B7-H5 in this publication and it thus should be stressed that HHLA2 is distinct from VISTA, which has also been referred to as B7-H5. The interaction of TMIGD2 with HHLA2 has since been confirmed by an independent study (169). Interestingly, engagement of TMIGD2 was found to costimulate cytokine production and proliferation in human T cells (170).

Thus, it is possible that HHLA2 interacts with another yet-unidentified inhibitory receptor on T cells. Interestingly, neither HHLA2 nor TMIGD2 are expressed in mice and rats (168, 170).

In addition, there are orphan ligand molecules including B7-H3 (CD276), B7-H4 (also known as B7S1, B7x or VTCN1), and ILDR2 that have been reported to inhibit T cell responses (171–177). The identification of receptors for orphan ligands like B7-H3 will be mandatory to target pathways involving these molecules to enhance T cell responses (178). B7-H3 is broadly expressed in cancer cells and B7-H3 antibodies targeting B7-H3$^+$ tumors are currently being tested in several clinical trials (179). It is currently not known whether these B7-H3 antibodies interfere with T cell inhibitory effects of this molecule.

The Future: Novel Immune Checkpoint Inhibitors and Beyond

The arrival of ICIs has dramatically changed the therapeutic landscape of cancer. Despite the enormous success of these immunotherapies, it is becoming increasingly clear that combining ICIs with a second drug may have superior potential to combat cancer. Currently, numerous clinical trials testing combinations of established immune checkpoint inhibitors (PD-1 and PD-L1 antibodies) with conventional treatments (chemotherapy, radiation, or targeted therapy) are carried out and are likely to result in improved treatment modalities for many different types of cancer (180). It is noteworthy to mentioned that ICIs are not the only mean to target inhibitory receptors. Taylor et al. have recently shown that glycogen synthase kinase 3 (GSK-3) has a key role in the regulation of PD-1 expression in CD8$^+$T cells (181). Follow up work by the same group has demonstrated that GSK-inhibitors are as effective in enhancing anti-tumor responses in preclinical models as PD-1 and PD-L1 antibodies (182).

Promising clinical data were obtained upon co-administration of antibodies targeting PD-1 and CTLA-4 (11, 183). These two immune checkpoints might mediate anti-tumor effects through distinct non-redundant mechanisms (184, 185). The CTLA-4 antibody ipilimumab acts early during T cell activation and mainly exerts extrinsic effects by outcompeting the primary costimulatory receptor CD28. It promotes the expansion of Th1-like CD4 T cells and potentially the deletion of tumor-resident Tregs. In contrast, PD-1 blockade mainly acts intrinsically on tumor-infiltrating exhausted-like CD8 T cells (184). These cells expand but maintain PD-1 expression indicating that PD-1 blockade does not reprogram them into a non-exhausted state, which is consistent with a epigenetic regulation of exhaustion (184). In addition, these results suggests that despite their exhausted like phenotype these cells are capable to expert potent anti-tumor activity following PD-1 blockade. Since PD-L1 is frequently expressed on tumor cells, PD-1 blockade can have a dual role in the tumor microenvironment–expansion of effector cells and also promoting anti-tumor effector functions.

The successful co-targeting of PD-1 and CTLA-4 and encouraging results obtained in preclinical models that combined PD-1 antibodies with other immune checkpoint inhibitors has fostered strategies to combine immune checkpoint inhibitors to enhance anti-tumor responses in patients with cancer. Several clinical trials where PD-1 antibodies are tested in combination with antibodies targeting TIM-3 and LAG-3 are ongoing (https://clinicaltrials.gov). Distinct properties of PD-1 versus TIM-3 and LAG-3 might result in synergistic effects of such combinations.

Adoptive therapy with T cells genetically engineered to express chimeric antigen receptors (CARs) or TCRs specific for tumor antigens is able to induce impressive anti-tumor responses. However, the upregulation of inhibitory receptors in genetically engineered T cells following transfer reduces their efficacy (186, 187). Multiple clinical trials investigating combinations of CAR T cells with antibodies to PD-1 or PD-L1 are ongoing (188). Engineering CAR T cells or TCR-transgenic T cells that are refractory to inhibition by immune checkpoints represents a promising future avenue to specifically protect engineered tumor-specific T cells against functional impairment through inhibitory pathways such as PD-1/PD-L1. This could be achieved by silencing or knocking out inhibitory receptors but also by co-introducing genes encoding PD-1/PD-L1 antibodies or so-called chimeric switch receptors (188–190).

The triumph of immune checkpoint inhibitors has underpinned that T cells have the potential to efficiently fight tumor cells, but in the majority of cases can only do so upon therapeutic intervention. Immune checkpoint blockade is effective but associated with severe side effects since it interferes with vital mechanisms of peripheral tolerance. Recent work by the Schreiber group has identified T cells that are reactivated upon immune checkpoint inhibitor treatment and mediate tumor rejection in a mouse model. The authors went on to show that tumor rejection can also be achieved by specifically boosting these T cells by peptide vaccination (191). The identification of antigens that are recognized by T cells in patients responding to immune checkpoint therapy might thus offer possibilities to target these antigens by vaccination or introduction of TCR-transgenic autologous T cells. Such approaches may increase the specificity of tumor targeting, thereby potentially enhancing therapy effects while reducing autoimmune toxicity.

Specificity is a hallmark of adaptive immunity and it seems paradoxical that immune checkpoint inhibition, which is an antigen-independent approach, has had the most spectacular success in cancer immunotherapy to date. Recent technological progress has facilitated the identification of mutations, which give rise to neoantigens in the tumors of individual cancer patients (192, 193). Studies in mouse models have demonstrated that vaccination with neoantigens can result in tumor control (191, 194). Strategies that combine patient-tailored approaches aimed at enhancing immune responses to individual neoantigens (e.g., by synthetic vaccines, oncolytic viruses or tumor radiation therapy but also adoptive therapy with *in vitro* expanded neoantigen-specific T cells) and interference with inhibitory pathways might represent particular promising avenues to improve anti-cancer immunotherapy (195, 196).

AUTHOR CONTRIBUTIONS

AD, JL, KG-P, and PS wrote the manuscript. AD and JL prepared figures.

FUNDING

This work was supported by the Austrian Science Fund FWF (DK W 1248-B30). The funder had no role in study design, deceision to publish or preparation of the manuscript.

ACKNOWLEDGMENTS

Daniel Speiser critically read the manuscript. We wish to thank him for his invaluable comments and suggestions.

REFERENCES

1. Sasidharan Nair V, Elkord E. Immune checkpoint inhibitors in cancer therapy: a focus on T-regulatory cells. *Immunol Cell Biol.* (2018) 96:21–33. doi: 10.1111/imcb.1003
2. Korman AJ, Peggs KS, Allison JP. Checkpoint blockade in cancer immunotherapy. *Adv Immunol.* (2006) 90:297–339. doi: 10.1016/S0065-2776(06)90008-X
3. Hamanishi J, Mandai M, Ikeda T, Minami M, Kawaguchi A, Murayama T, et al. Safety and antitumor activity of anti-PD-1 antibody, nivolumab, in patients with platinum-resistant ovarian cancer. *J Clin Oncol.* (2015) 33:4015–22. doi: 10.1200/JCO.2015.62.3397
4. Iwai Y, Hamanishi J, Chamoto K, Honjo T. Cancer immunotherapies targeting the PD-1 signaling pathway. *J Biomed Sci.* (2017) 24:26. doi: 10.1186/s12929-017-0329-9
5. Nghiem PT, Bhatia S, Lipson EJ, Kudchadkar RR, Miller NJ, Annamalai L, et al. PD-1 blockade with pembrolizumab in advanced merkel-cell carcinoma. *N Engl J Med.* (2016) 374:2542–52. doi: 10.1056/NEJMoa1603702
6. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med.* (2012) 366:2443–54. doi: 10.1056/NEJMoa1200690
7. Marrone KA, Ying W, Naidoo J. Immune-related adverse events from immune checkpoint inhibitors. *Clin Pharmacol Ther.* (2016) 100:242–51. doi: 10.1002/cpt.394
8. Pitt JM, Vetizou M, Daillere R, Roberti MP, Yamazaki T, Routy B, et al. Resistance mechanisms to immune-checkpoint blockade in cancer: tumor-intrinsic and -extrinsic factors. *Immunity* (2016) 44:1255–69. doi: 10.1016/j.immuni.2016.06.001
9. Jenkins RW, Barbie DA, Flaherty KT. Mechanisms of resistance to immune checkpoint inhibitors. *Br J Cancer* (2018) 118:9–16. doi: 10.1038/bjc.2017.434
10. Gide TN, Wilmott JS, Scolyer RA, Long GV. Primary and acquired resistance to immune checkpoint inhibitors in metastatic melanoma. *Clin Cancer Res.* (2018) 24:1260–70. doi: 10.1158/1078-0432.CCR-17-2267
11. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med.* (2013) 369:122–33. doi: 10.1056/NEJMoa1302369
12. Lanier LL. NK cell receptors. *Annu Rev Immunol.* (1998) 16:359–93. doi: 10.1146/annurev.immunol.16.1.359
13. Fuertes Marraco SA, Neubert NJ, Verdeil G, Speiser DE. Inhibitory receptors beyond T cell exhaustion. *Front. Immunol.* (2015) 6:310. doi: 10.3389/fimmu.2015.00310
14. Long EO. Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol.* (1999) 17:875–904. doi: 10.1146/annurev.immunol.17.1.875
15. Odorizzi PM, Wherry EJ. Inhibitory receptors on lymphocytes: insights from infections. *J Immunol.* (2012) 188:2957–65. doi: 10.4049/jimmunol.1100038
16. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med.* (2012) 209:1201–17. doi: 10.1084/jem.20112741
17. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol.* (2004) 173:945–54.
18. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med.* (2000) 192:1027–34.
19. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* (2017) 355:1428–33. doi: 10.1126/science.aaf1292
20. Kamphorst AO, Wieland A, Nasti T, Yang S, Zhang R, Barber DL, et al. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. *Science* (2017) 355:1423–27. doi: 10.1126/science.aaf0683
21. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev.* (2010) 236:219–42. doi: 10.1111/j.1600-065X.2010.00923.x
22. Selenko-Gebauer N, Majdic O, Szekeres A, Hofler G, Guthann E, Korthauer U, et al. B7-H1 (programmed death-1 ligand) on dendritic cells is involved in the induction and maintenance of T cell anergy. *J Immunol.* (2003) 170:3637–44.
23. Wang X, Teng F, Kong L, Yu J. PD-L1 expression in human cancers and its association with clinical outcomes. *Onco Targets Ther.* (2016) 9:5023–39. doi: 10.2147/OTT.S105862
24. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* (1999) 11:141–51.
25. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* (2001) 291:319–22. doi: 10.1126/science.291.5502.319
26. Nishimura H, Honjo T, Minato N. Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice. *J Exp Med.* (2000) 191:891–8.
27. Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol.* (2005) 6:280–6. doi: 10.1038/ni1165
28. Rodig N, Ryan T, Allen JA, Pang H, Grabie N, Chernova T, et al. Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. *Eur J Immunol.* (2003) 33:3117–26. doi: 10.1002/eji.200324270
29. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med.* (2006) 203:883–95. doi: 10.1084/jem.20051776
30. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol.* (2007) 8:239–45. doi: 10.1038/ni1443
31. Wherry EJ. T cell exhaustion. *Nat Immunol.* (2011) 12:492–9. doi: 10.1038/ni.2035
32. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* (2006) 439:682–7. doi: 10.1038/nature04444
33. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* (2006) 443:350–4. doi: 10.1038/nature05115
34. Petrovas C, Price DA, Mattapallil J, Ambrozak DR, Geldmacher C, Cecchinato V, et al. SIV-specific CD8+ T cells express high levels of PD1 and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection. *Blood* (2007) 110:928–36. doi: 10.1182/blood-2007-01-069112
35. Baitsch L, Legat A, Barba L, Fuertes Marraco SA, Rivals JP, Baumgaertner P, et al. Extended co-expression of inhibitory receptors by human CD8 T-cells depending on differentiation, antigen-specificity and anatomical localization. *PLoS ONE* (2012) 7:e30852. doi: 10.1371/journal.pone.0030852
36. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and

tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci USA*. (2002) 99:12293–7. doi: 10.1073/pnas.192461099
37. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med.* (2002) 8:793–800. doi: 10.1038/nm730
38. Blank C, Brown I, Peterson AC, Spiotto M, Iwai Y, Honjo T, et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. *Cancer Res.* (2004) 64:1140–5.
39. Bardhan K, Anagnostou T, Boussiotis VA. The PD1:PD-L1/2 pathway from discovery to clinical implementation. *Front Immunol.* (2016) 7:550. doi: 10.3389/fimmu.2016.00550
40. Gavrieli M, Watanabe N, Loftin SK, Murphy TL, Murphy KM. Characterization of phosphotyrosine binding motifs in the cytoplasmic domain of B and T lymphocyte attenuator required for association with protein tyrosine phosphatases SHP-1 and SHP-2. *Biochem Biophys Res Commun.* (2003) 312:1236–43.
41. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol.* (2003) 4:670–9. doi: 10.1038/ni944
42. Gavrieli M, Murphy KM. Association of Grb-2 and PI3K p85 with phosphotyrosile peptides derived from BTLA. *Biochem Biophys Res Commun.* (2006) 345:1440–5. doi: 10.1016/j.bbrc.2006.05.036
43. Murphy TL, Murphy KM. Slow down and survive: enigmatic immunoregulation by BTLA and HVEM. *Annu Rev Immunol.* (2010) 28:389–411. doi: 10.1146/annurev-immunol-030409-101202
44. Sedy JR, Gavrieli M, Potter KG, Hurchla MA, Lindsley RC, Hildner K, et al. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat Immunol.* (2005) 6:90–8. doi: 10.1038/ni1144
45. 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) 9:176–85. doi: 10.1038/ni1554
46. Peretz Y, He Z, Shi Y, Yassine-Diab B, Goulet JP, Bordi R, et al. CD160 and PD-1 Co-Expression on HIV-Specific CD8 T Cells Defines a Subset with Advanced Dysfunction. *PLoS Pathog.* (2012) 8:e1002840. doi: 10.1371/journal.ppat.1002840
47. El-Far M, Pellerin C, Pilote L, Fortin JF, Lessard IA, Peretz Y, et al. CD160 isoforms and regulation of CD4 and CD8 T-cell responses. *J Trans Med.* (2014) 12:217. doi: 10.1186/s12967-014-0217-y
48. Wang Y, Subudhi SK, Anders RA, Lo J, Sun Y, Blink S, et al. The role of herpesvirus entry mediator as a negative regulator of T cell-mediated responses. *J Clin Invest.* (2005) 115:711–7. doi: 10.1172/JCI22982
49. Stecher C, Battin C, Leitner J, Zettl M, Grabmeier-Pfistershammer K, Holler C, et al. PD-1 blockade promotes emerging checkpoint inhibitors in enhancing T cell responses to allogeneic dendritic cells. *Front Immunol.* (2017) 8:572. doi: 10.3389/fimmu.2017.00572
50. Grabmeier-Pfistershammer K, Stecher C, Zettl M, Rosskopf S, Rieger A, Zlabinger GJ, et al. Antibodies targeting BTLA or TIM-3 enhance HIV-1 specific T cell responses in combination with PD-1 blockade. *Clin Immunol.* (2017) 183:167–73. doi: 10.1016/j.clim.2017.09.002
51. Fourcade J, Sun Z, Pagliano O, Guillaume P, Luescher IF, Sander C, et al. CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer Res.* (2012) 72:887–96. doi: 10.1158/0008-5472.CAN-11-2637
52. Hobo W, Norde WJ, Schaap N, Fredrix H, Maas F, Schellens K, et al. B and T lymphocyte attenuator mediates inhibition of tumor-reactive CD8+ T cells in patients after allogeneic stem cell transplantation. *J Immunol.* (2012) 189:39–49. doi: 10.4049/jimmunol.1102807
53. Derre L, Rivals JP, Jandus C, Pastor S, Rimoldi D, Romero P, et al. BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination. *J Clin Invest.* (2010) 120:157–67. doi: 10.1172/JCI40070
54. Haymaker CL, Wu RC, Ritthipichai K, Bernatchez C, Forget MA, Chen JQ, et al. BTLA marks a less-differentiated tumor-infiltrating lymphocyte subset in melanoma with enhanced survival properties. *Oncoimmunology* (2015) 4:e1014246. doi: 10.1080/2162402X.2015.1014246
55. Ritthipichai K, Haymaker CL, Martinez M, Aschenbrenner A, Yi X, Zhang M, et al. Multifaceted role of BTLA in the control of CD8(+) T-cell fate after antigen encounter. *Clin Cancer Res.* (2017) 23:6151–64. doi: 10.1158/1078-0432.CCR-16-1217
56. Cheung TC, Steinberg MW, Oborne LM, Macauley MG, Fukuyama S, Sanjo H, et al. Unconventional ligand activation of herpesvirus entry mediator signals cell survival. *Proc Natl Acad Sci USA.* (2009) 106:6244–9. doi: 10.1073/pnas.0902115106
57. Cai G, Freeman GJ. The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation. *Immunol Rev.* (2009) 229:244–58. doi: 10.1111/j.1600-065X.2009.00783.x
58. Serriari NE, Gondois-Rey F, Guillaume Y, Remmerswaal EB, Pastor S, Messal N, et al. B and T lymphocyte attenuator is highly expressed on CMV-specific T cells during infection and regulates their function. *J Immunol.* (2010) 185:3140–8. doi: 10.4049/jimmunol.0902487
59. Cheung TC, Oborne LM, Steinberg MW, Macauley MG, Fukuyama S, Sanjo H, et al. T cell intrinsic heterodimeric complexes between HVEM and BTLA determine receptivity to the surrounding microenvironment. *J Immunol.* (2009) 183:7286–96. doi: 10.4049/jimmunol.0902490
60. Rowshanravan B, Halliday N, Sansom DM. CTLA-4: a moving target in immunotherapy. *Blood* (2018) 131:58–67. doi: 10.1182/blood-2017-06-741033
61. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* (1995) 270:985–8.
62. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* (1995) 3:541–7.
63. Rudd CE, Schneider H. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat Rev Immunol.* (2003) 3:544–56. doi: 10.1038/nri1131
64. Kong KF, Fu G, Zhang Y, Yokosuka T, Casas J, Canonigo-Balancio AJ, et al. Protein kinase C-eta controls CTLA-4-mediated regulatory T cell function. *Nat Immunol.* (2014) 15:465–72. doi: 10.1038/ni.2866
65. Schneider H, Martin M, Agarraberes FA, Yin L, Rapoport I, Kirchhausen T, et al. Cytolytic T lymphocyte-associated antigen-4 and the TCR zeta/CD3 complex, but not CD28, interact with clathrin adaptor complexes AP-1 and AP-2. *J Immunol.* (1999) 163:1868–79.
66. Shiratori T, Miyatake S, Ohno H, Nakaseko C, Isono K, Bonifacino JS, et al. Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2. *Immunity* (1997) 6:583–9.
67. Banton MC, Inder KL, Valk E, Rudd CE, Schneider H. Rab8 binding to immune cell-specific adaptor LAX facilitates formation of trans-Golgi network-proximal CTLA-4 vesicles for surface expression. *Mol Cell Biol.* (2014) 34:1486–99. doi: 10.1128/MCB.01331-13
68. Walker LS. Treg and CTLA-4: two intertwining pathways to immune tolerance. *J Autoimmun.* (2013) 45:49–57. doi: 10.1016/j.jaut.2013.06.006
69. Lee KM, Chuang E, Griffin M, Khattri R, Hong DK, Zhang W, et al. Molecular basis of T cell inactivation by CTLA-4. *Science* (1998) 282:2263–6.
70. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol.* (2001) 1:220–8. doi: 10.1038/35105024
71. Schneider H, Downey J, Smith A, Zinselmeyer BH, Rush C, Brewer JM, et al. Reversal of the TCR stop signal by CTLA-4. *Science* (2006) 313:1972–5. doi: 10.1126/science.1131078
72. Jutz S, Hennig A, Paster W, Asrak O, Dijanovic D, Kellner F, et al. A cellular platform for the evaluation of immune checkpoint molecules. *Oncotarget* (2017) 8:64892–906. doi: 10.18632/oncotarget.17615
73. Corse E, Allison JP. Cutting edge: CTLA-4 on effector T cells inhibits in trans. *J Immunol.* (2012) 189:1123–7. doi: 10.4049/jimmunol.1200695
74. Walker LS, Sansom DM. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nat Rev Immunol.* (2011) 11:852–63. doi: 10.1038/nri3108
75. Read S, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L, et al. Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function *in vivo*. *J Immunol.* (2006) 177:4376–83.

76. Sojka DK, Hughson A, Fowell DJ. CTLA-4 is required by CD4+CD25+ Treg to control CD4+ T-cell lymphopenia-induced proliferation. *Eur J Immunol.* (2009) 39:1544–51. doi: 10.1002/eji.200838603
77. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* (2011) 332:600–3. doi: 10.1126/science.1202947
78. Masteller EL, Chuang E, Mullen AC, Reiner SL, Thompson CB. Structural analysis of CTLA-4 function *in vivo*. *J Immunol.* (2000) 164:5319–27.
79. Tai X, Van Laethem F, Sharpe AH, Singer A. Induction of autoimmune disease in CTLA-4-/- mice depends on a specific CD28 motif that is required for *in vivo* costimulation. *Proc Natl Acad Sci USA*. (2007) 104:13756–61. doi: 10.1073/pnas.0706509104
80. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med.* (2013) 210:1695–710. doi: 10.1084/jem.20130579
81. Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, et al. Activating Fc gamma receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. *J Exp Med.* (2013) 210:1685–93. doi: 10.1084/jem.20130573
82. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, et al. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol Res.* (2013) 1:32–42. doi: 10.1158/2326-6066.CIR-13-0013
83. Romano E, Kusio-Kobialka M, Foukas PG, Baumgaertner P, Meyer C, Ballabeni P, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells *ex vivo* by nonclassical monocytes in melanoma patients. *Proc Natl Acad Sci USA*. (2015) 112:6140–5. doi: 10.1073/pnas.1417320112
84. Arce Vargas F, Furness AJS, Litchfield K, Joshi K, Rosenthal R, Ghorani E, et al. Fc Effector function contributes to the activity of human anti-CTLA-4 antibodies. *Cancer Cell* (2018) 33:649-63 e4. doi: 10.1016/j.ccell.2018.02.010
85. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med.* (1990) 171:1393–405.
86. Kisielow M, Kisielow J, Capoferri-Sollami G, Karjalainen K. Expression of lymphocyte activation gene 3 (LAG-3) on B cells is induced by T cells. *Eur J Immunol.* (2005) 35:2081–8. doi: 10.1002/eji.200526090
87. Workman CJ, Wang Y, El Kasmi KC, Pardoll DM, Murray PJ, Drake CG, et al. LAG-3 regulates plasmacytoid dendritic cell homeostasis. *J Immunol.* (2009) 182:1885–91. doi: 10.4049/jimmunol.0800185
88. Baixeras E, Huard B, Miossec C, Jitsukawa S, Martin M, Hercend T, et al. Characterization of the lymphocyte activation gene 3-encoded protein. a new ligand for human leukocyte antigen class II antigens. *J Exp Med.* (1992) 176:327–37.
89. Kouo T, Huang L, Pucsek AB, Cao M, Solt S, Armstrong T, et al. Galectin-3 shapes antitumor immune responses by suppressing CD8+ T cells via LAG-3 and inhibiting expansion of plasmacytoid dendritic cells. *Cancer Immunol Res.* (2015) 3:412–23. doi: 10.1158/2326-6066.CIR-14-0150
90. Xu F, Liu J, Liu D, Liu B, Wang M, Hu Z, et al. LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. *Cancer Res.* (2014) 74:3418–28. doi: 10.1158/0008-5472.CAN-13-2690
91. Iouzalen N, Andreae S, Hannier S, Triebel F. LAP, a lymphocyte activation gene-3 (LAG-3)-associated protein that binds to a repeated EP motif in the intracellular region of LAG-3, may participate in the down-regulation of the CD3/TCR activation pathway. *Eur J Immunol.* (2001) 31:2885–91. doi: 10.1002/1521-4141(2001010)31:10<2885::AID-IMMU2885>3.0.CO;2-2
92. Workman CJ, Dugger KJ, Vignali DA. Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. *J Immunol.* (2002) 169:5392–5.
93. Macon-Lemaitre L, Triebel F. The negative regulatory function of the lymphocyte-activation gene-3 co-receptor (CD223) on human T cells. *Immunology* (2005) 115:170–8. doi: 10.1111/j.1365-2567.2005.02145.x
94. Cook KD, Whitmire JK. LAG-3 confers a competitive disadvantage upon antiviral CD8+ T cell responses. *J Immunol.* (2016) 197:119–27. doi: 10.4049/jimmunol.1401594
95. Grosso JF, Kelleher CC, Harris TJ, Maris CH, Hipkiss EL, De Marzo A, et al. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J Clin Invest.* (2007) 117:3383–92. doi: 10.1172/JCI31184
96. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. *Immunity* (2004) 21:503–13. doi: 10.1016/j.immuni.2004.08.010
97. Durham NM, Nirschl CJ, Jackson CM, Elias J, Kochel CM, Anders RA, et al. Lymphocyte Activation Gene 3 (LAG-3) modulates the ability of CD4 T-cells to be suppressed *in vivo*. *PLoS ONE* (2014) 9:e109080. doi: 10.1371/journal.pone.0109080
98. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol.* (2008) 180:5916–26.
99. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res.* (2012) 72:917–27. doi: 10.1158/0008-5472.CAN-11-1620
100. Goding SR, Wilson KA, Xie Y, Harris KM, Baxi A, Akpinarli A, et al. Restoring immune function of tumor-specific CD4+ T cells during recurrence of melanoma. *J Immunol.* (2013) 190:4899–909. doi: 10.4049/jimmunol.1300271
101. Huang RY, Eppolito C, Lele S, Shrikant P, Matsuzaki J, Odunsi K. LAG3 and PD1 co-inhibitory molecules collaborate to limit CD8+ T cell signaling and dampen antitumor immunity in a murine ovarian cancer model. *Oncotarget* (2015) 6:27359–77. doi: 10.18632/oncotarget.4751
102. Wierz M, Pierson S, Guyonnet L, Viry E, Lequeux A, Oudin A, et al. Dual PD1/LAG3 immune checkpoint blockade limits tumor development in a murine model of chronic lymphocytic leukemia. *Blood* (2018) 131:1617–21. doi: 10.1182/blood-2017-06-792267
103. Andrews LP, Marciscano AE, Drake CG, Vignali DA. LAG3 (CD223) as a cancer immunotherapy target. *Immunol Rev.* (2017) 276:80–96. doi: 10.1111/imr.12519
104. He Y, Rivard CJ, Rozeboom L, Yu H, Ellison K, Kowalewski A, et al. Lymphocyte-activation gene-3, an important immune checkpoint in cancer. *Cancer Sci.* (2016) 107:1193–7. doi: 10.1111/cas.12986
105. Sierro S, Romero P, Speiser DE. The CD4-like molecule LAG-3, biology and therapeutic applications. *Expert Opin Ther Targets* (2011) 15:91–101. doi: 10.1517/14712598.2011.540563
106. Andreae S, Buisson S, Triebel F. MHC class II signal transduction in human dendritic cells induced by a natural ligand, the LAG-3 protein (CD223). *Blood* (2003) 102:2130–7. doi: 10.1182/blood-2003-01-0273
107. Nguyen LT, Ohashi PS. Clinical blockade of PD1 and LAG3–potential mechanisms of action. *Nat Rev Immunol.* (2015) 15:45–56. doi: 10.1038/nri3790
108. Huard B, Prigent P, Pages F, Bruniquel D, Triebel F. T cell major histocompatibility complex class II molecules down-regulate CD4+ T cell clone responses following LAG-3 binding. *Eur J Immunol.* (1996) 26:1180–6. doi: 10.1002/eji.1830260533
109. Annunziato F, Manetti R, Tomasevic I, Guidizi MG, Biagiotti R, Gianno V, et al. Expression and release of LAG-3-encoded protein by human CD4+ T cells are associated with IFN-gamma production. *FASEB J.* (1996) 10:769–76.
110. Freeman GJ, Casasnovas JM, Umetsu DT, DeKruyff RH. TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. *Immunol Rev.* (2010) 235:172–89. doi: 10.1111/j.0105-2896.2010.00903.x
111. Anderson AC, Anderson DE, Bregoli L, Hastings WD, Kassam N, Lei C, et al. Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. *Science* (2007) 318:1141–3. doi: 10.1126/science.1148536
112. Ocana-Guzman R, Torre-Bouscoulet L, Sada-Ovalle I. TIM-3 regulates distinct functions in macrophages. *Front Immunol.* (2016) 7:229. doi: 10.3389/fimmu.2016.00229
113. Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, 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) 205:2763–79. doi: 10.1084/jem.20081398

114. DeKruyff RH, Bu X, Ballesteros A, Santiago C, Chim YL, Lee HH, et al. T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells. *J Immunol.* (2010) 184:1918–30. doi: 10.4049/jimmunol.0903059
115. Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, et al. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. *Nat Immunol.* (2012) 13:832–42. doi: 10.1038/ni.2376
116. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol.* (2005) 6:1245–52. doi: 10.1038/ni1271
117. Madireddi S, Eun SY, Lee SW, Nemcovicova I, Mehta AK, Zajonc DM, et al. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J Exp Med.* (2014) 211:1433–48. doi: 10.1084/jem.20132687
118. Vaitaitis GM, Wagner DH Jr. Galectin-9 controls CD40 signaling through a Tim-3 independent mechanism and redirects the cytokine profile of pathogenic T cells in autoimmunity. *PLoS ONE* (2012) 7:e38708. doi: 10.1371/journal.pone.0038708
119. Katoh S, Ishii N, Nobumoto A, Takeshita K, Dai SY, Shinonaga R, et al. Galectin-9 inhibits CD44-hyaluronan interaction and suppresses a murine model of allergic asthma. *Am J Respir Crit Care Med.* (2007) 176:27–35. doi: 10.1164/rccm.200608-1243OC
120. Daley D, Mani VR, Mohan N, Akkad N, Ochi A, Heindel DW, et al. Dectin 1 activation on macrophages by galectin 9 promotes pancreatic carcinoma and peritumoral immune tolerance. *Nat Med.* (2017) 23:556–67. doi: 10.1038/nm.4314
121. Leitner J, Rieger A, Pickl WF, Zlabinger G, Grabmeier-Pfistershammer K, Steinberger P. TIM-3 does not act as a receptor for galectin-9. *PLoS Pathog.* (2013) 9:e1003253. doi: 10.1371/journal.ppat.1003253
122. Huang YH, Zhu C, Kondo Y, Anderson AC, Gandhi A, Russell A, et al. CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. *Nature* (2015) 517:386-90. doi: 10.1038/nature13848
123. Huang YH, Zhu C, Kondo Y, Anderson AC, Gandhi A, Russell A, et al. Corrigendum: CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. *Nature* (2016) 536:359. doi: 10.1038/nature17421
124. Lee J, Su EW, Zhu C, Hainline S, Phuah J, Moroco JA, et al. Phosphotyrosine-dependent coupling of Tim-3 to T-cell receptor signaling pathways. *Mol Cell Biol.* (2011) 31:3963–74. doi: 10.1128/MCB.05297-11
125. Rangachari M, Zhu C, Sakuishi K, Xiao S, Karman J, Chen A, et al. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3-mediated cell death and exhaustion. *Nat Med.* (2012) 18:1394–400. doi: 10.1038/nm.2871
126. Avery L, Filderman J, Szymczak-Workman AL, Kane LP. Tim-3 co-stimulation promotes short-lived effector T cells, restricts memory precursors, and is dispensable for T cell exhaustion. *Proc Natl Acad Sci USA.* (2018) 115:2455–60. doi: 10.1073/pnas.1712107115
127. Sabatos CA, Chakravarti S, Cha E, Schubart A, Sanchez-Fueyo A, Zheng XX, et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat Immunol.* (2003) 4:1102–10. doi: 10.1038/ni988
128. Gorman JV, Starbeck-Miller G, Pham NL, Traver GL, Rothman PB, Harty JT, et al. Tim-3 directly enhances CD8 T cell responses to acute Listeria monocytogenes infection. *J Immunol.* (2014) 192:3133–42. doi: 10.4049/jimmunol.1302290
129. de Mingo Pulido A, Gardner A, Hiebler S, Soliman H, Rugo HS, Krummel MF, et al. TIM-3 Regulates CD103(+) Dendritic cell function and response to chemotherapy in breast cancer. *Cancer Cell* (2018) 33:60–74 e6. doi: 10.1016/j.ccell.2017.11.019
130. Su EW, Bi S, Kane LP. Galectin-9 regulates T helper cell function independently of Tim-3. *Glycobiology* (2011) 21:1258–65. doi: 10.1093/glycob/cwq214
131. Oomizu S, Arikawa T, Niki T, Kadowaki T, Ueno M, Nishi N, et al. Galectin-9 suppresses Th17 cell development in an IL-2-dependent but Tim-3-independent manner. *Clin Immunol.* (2012) 143:51–8. doi: 10.1016/j.clim.2012.01.004
132. Chen CJ, Shively JE. The cell-cell adhesion molecule carcinoembryonic antigen-related cellular adhesion molecule 1 inhibits IL-2 production and proliferation in human T cells by association with Src homology protein-1 and down-regulates IL-2 receptor. *J Immunol.* (2004) 172:3544–52.
133. Li Y, Shively JE. CEACAM1 regulates Fas-mediated apoptosis in Jurkat T-cells via its interaction with beta-catenin. *Exp Cell Res.* (2013) 319:1061–72. doi: 10.1016/j.yexcr.2013.02.020
134. McMahan RH, Golden-Mason L, Nishimura MI, McMahon BJ, Kemper M, Allen TM, et al. Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed *in vitro* cytotoxicity. *J Clin Invest.* (2010) 120:4546–57. doi: 10.1172/JCI43127
135. Golden-Mason L, Palmer BE, Kassam N, Townshend-Bulson L, Livingston S, McMahon BJ, et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol.* (2009) 83:9122–30. doi: 10.1128/JVI.00639-09
136. Tomkowicz B, Walsh E, Cotty A, Verona R, Sabins N, Kaplan F, et al. TIM-3 Suppresses Anti-CD3/CD28-induced TCR activation and IL-2 expression through the NFAT signaling pathway. *PLoS ONE* (2015) 10:e0140694. doi: 10.1371/journal.pone.0140694
137. Bhagwat B, Cherwinski H, Sathe M, Seghezzi W, McClanahan TK, de Waal Malefyt R, et al. Establishment of engineered cell-based assays mediating LAG3 and PD1 immune suppression enables potency measurement of blocking antibodies and assessment of signal transduction. *J Immunol Methods* (2018) 456:7–14. doi: 10.1016/j.jim.2018.02.003
138. Wang L, Yu C, Yang Y, Gao K, Wang J. Development of a robust reporter gene assay to measure the bioactivity of anti-PD-1/anti-PD-L1 therapeutic antibodies. *J Pharm Biomed Anal.* (2017) 145:447–53. doi: 10.1016/j.jpba.2017.05.011
139. Rosskopf S, Jutz S, Neunkirchner A, Candia MR, Jahn-Schmid B, Bohle B, et al. Creation of an engineered APC system to explore and optimize the presentation of immunodominant peptides of major allergens. *Sci Rep.* (2016) 6:31580. doi: 10.1038/srep31580
140. Jutz S, Leitner J, Schmetterer K, Doel-Perez I, Majdic O, Grabmeier-Pfistershammer K, et al. Assessment of costimulation and coinhibition in a triple parameter T cell reporter line: simultaneous measurement of NF-kappaB, NFAT and AP-1. *J Immunol Methods* (2016) 430:10–20. doi: 10.1016/j.jim.2016.01.007
141. Sabins NC, Chornoguz O, Leander K, Kaplan F, Carter R, Kinder M, et al. TIM-3 engagement promotes effector memory T cell differentiation of human antigen-specific CD8 T cells by activating mTORC1. *J Immunol.* (2017) 199:4091–102. doi: 10.4049/jimmunol.1701030
142. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol.* (2009) 10:29–37. doi: 10.1038/ni.1679
143. Baitsch L, Baumgaertner P, Devevre E, Raghav SK, Legat A, Barba L, et al. Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients. *J Clin Invest.* (2011) 121:2350–60. doi: 10.1172/JCI46102
144. Lee J, Ahn E, Kissick HT, Ahmed R. Reinvigorating exhausted T cells by blockade of the PD-1 pathway. *For Immunopathol Dis Therap.* (2015) 6:7–17. doi: 10.1615/ForumImmunDisTher.2015014188
145. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, et al. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med.* (2006) 12:1198–202. doi: 10.1038/nm1482
146. Bengsch B, Seigel B, Ruhl M, Timm J, Kuntz M, Blum HE, et al. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog.* (2010) 6:e1000947. doi: 10.1371/journal.ppat.1000947
147. Haymaker C, Wu R, Bernatchez C, Radvanyi L. PD-1 and BTLA and CD8(+) T-cell "exhaustion" in cancer: "Exercising" an alternative viewpoint. *Oncoimmunology* (2012) 1:735–38. doi: 10.4161/onci.20823
148. Legat A, Speiser DE, Pircher H, Zehn D, Fuertes Marraco SA. Inhibitory receptor expression depends more dominantly on differentiation and activation than "Exhaustion" of human CD8 T cells. *Front Immunol.* (2013) 4:455. doi: 10.3389/fimmu.2013.00455
149. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T

cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med.* (2000) 192:303–10.
150. Zhang B, Chikuma S, Hori S, Fagarasan S, Honjo T. Nonoverlapping roles of PD-1 and FoxP3 in maintaining immune tolerance in a novel autoimmune pancreatitis mouse model. *Proc Natl Acad Sci USA.* (2016) 113:8490–5. doi: 10.1073/pnas.1608873113
151. Dougall WC, Kurtulus S, Smyth MJ, Anderson AC. TIGIT and CD96: new checkpoint receptor targets for cancer immunotherapy. *Immunol Rev.* (2017) 276:112–20. doi: 10.1111/imr.12518
152. Lozano E, Dominguez-Villar M, Kuchroo V, Hafler DA. The TIGIT/CD226 axis regulates human T cell function. *J Immunol.* (2012) 188:3869–75. doi: 10.4049/jimmunol.1103627
153. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol.* (2011) 186:1338–42. doi: 10.4049/jimmunol.1003081
154. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory receptors with specialized functions in immune regulation. *Immunity* (2016) 44:989–1004. doi: 10.1016/j.immuni.2016.05.001
155. Nowak EC, Lines JL, Varn FS, Deng J, Sarde A, Mabaera R, et al. Immunoregulatory functions of VISTA. *Immunol Rev.* (2017) 276:66–79. doi: 10.1111/imr.12525
156. Flies DB, Han X, Higuchi T, Zheng L, Sun J, Ye JJ, et al. Coinhibitory receptor PD-1H preferentially suppresses CD4(+) T cell-mediated immunity. *J Clin Invest.* (2014) 124:1966–75. doi: 10.1172/JCI74589
157. Flies DB, Wang S, Xu H, Chen L. Cutting edge: a monoclonal antibody specific for the programmed death-1 homolog prevents graft-versus-host disease in mouse models. *J Immunol.* (2011) 187:1537–41. doi: 10.4049/jimmunol.1100660
158. Wang L, Rubinstein R, Lines JL, Wasiuk A, Ahonen C, Guo Y, et al. VISTA, a novel mouse Ig superfamily ligand that negatively regulates T cell responses. *J Exp Med.* (2011) 208:577–92. doi: 10.1084/jem.20100619
159. Wang L, Le Mercier I, Putra J, Chen W, Liu J, Schenk AD, et al. Disruption of the immune-checkpoint VISTA gene imparts a proinflammatory phenotype with predisposition to the development of autoimmunity. *Proc Natl Acad Sci USA.* (2014) 111:14846–51. doi: 10.1073/pnas.1407447111
160. Yoon KW, Byun S, Kwon E, Hwang SY, Chu K, Hiraki M, et al. Control of signaling-mediated clearance of apoptotic cells by the tumor suppressor p53. *Science* (2015) 349:1261669. doi: 10.1126/science.1261669
161. Le Mercier I, Chen W, Lines JL, Day M, Li J, Sergent P, et al. VISTA Regulates the Development of Protective Antitumor Immunity. *Cancer Res.* (2014) 74:1933–44. doi: 10.1158/0008-5472.CAN-13-1506
162. Kondo Y, Ohno T, Nishii N, Harada K, Yagita H, Azuma M. Differential contribution of three immune checkpoint (VISTA, CTLA-4, PD-1) pathways to antitumor responses against squamous cell carcinoma. *Oral Oncol.* (2016) 57:54–60. doi: 10.1016/j.oraloncology.2016.04.005
163. Kuklinski LF, Yan S, Li Z, Fisher JL, Cheng C, Noelle RJ, et al. VISTA expression on tumor-infiltrating inflammatory cells in primary cutaneous melanoma correlates with poor disease-specific survival. *Cancer Immunol Immunother.* (2018) 67:1113–21. doi: 10.1007/s00262-018-2169-1
164. Gao J, Ward JF, Pettaway CA, Shi LZ, Subudhi SK, Vence LM, et al. VISTA is an inhibitory immune checkpoint that is increased after ipilimumab therapy in patients with prostate cancer. *Nat Med.* (2017) 23:551–55. doi: 10.1038/nm.4308
165. Villarroel-Espindola F, Yu X, Datar I, Mani N, Sanmamed M, Velcheti V, et al. Spatially resolved and quantitative analysis of VISTA/PD-1H as a novel immunotherapy target in human non-small cell lung cancer. *Clin Cancer Res.* (2018) 24:1562–73. doi: 10.1158/1078-0432.CCR-17-2542
166. Lines JL, Sempere LF, Broughton T, Wang L, Noelle R. VISTA is a novel broad-spectrum negative checkpoint regulator for cancer immunotherapy. *Cancer Immunol Res.* (2014) 2:510–7. doi: 10.1158/2326-6066.CIR-14-0072
167. Bharaj P, Chahar HS, Alozie OK, Rodarte L, Bansal A, Goepfert PA, et al. Characterization of programmed death-1 homologue-1 (PD-1H) expression and function in normal and HIV infected individuals. *PLoS ONE* (2014) 9:e109103. doi: 10.1371/journal.pone.0109103
168. Zhao R, Chinai JM, Buhl S, Scandiuzzi L, Ray A, Jeon H, et al. HHLA2 is a member of the B7 family and inhibits human CD4 and CD8 T-cell function. *Proc Natl Acad Sci USA.* (2013) 110:9879–84. doi: 10.1073/pnas.1303524110
169. Janakiram M, Chinai JM, Fineberg S, Fiser A, Montagna C, Medavarapu R, et al. Expression, clinical significance, and receptor identification of the newest B7 family member HHLA2 protein. *Clin Cancer Res.* (2015) 21:2359–66. doi: 10.1158/1078-0432.CCR-14-1495
170. Zhu Y, Yao S, Iliopoulou BP, Han X, Augustine MM, Xu H, et al. B7-H5 costimulates human T cells via CD28H. *Nat Commun.* (2013) 4:2043. doi: 10.1038/ncomms3043
171. Leitner J, Klauser C, Pickl WF, Stockl J, Majdic O, Bardet AF, et al. B7-H3 is a potent inhibitor of human T-cell activation: no evidence for B7-H3 and TREML2 interaction. *Eur J Immunol.* (2009) 39:1754–64. doi: 10.1002/eji.200839028
172. Li J, Lee Y, Li Y, Jiang Y, Lu H, Zang W, et al. Co-inhibitory molecule B7 superfamily member 1 expressed by tumor-infiltrating myeloid cells induces dysfunction of anti-tumor CD8(+) T cells. *Immunity* (2018) 48:773–86.e5. doi: 10.1016/j.immuni.2018.03.018
173. Steinberger P, Majdic O, Derdak SV, Pfistershammer K, Kirchberger S, Klauser C, et al. Molecular characterization of human 4Ig-B7-H3, a member of the B7 family with four Ig-like domains. *J Immunol.* (2004) 172:2352–9.
174. Hecht I, Toporik A, Podojil JR, Vaknin I, Cojocaru G, Oren A, et al. ILDR2 is a novel B7-like protein that negatively regulates T cell responses. *J Immunol.* (2018) 200:2025–37. doi: 10.4049/jimmunol.1700325
175. Prasad DV, Nguyen T, Li Z, Yang Y, Duong J, Wang Y, et al. Murine B7-H3 is a negative regulator of T cells. *J Immunol.* (2004) 173:2500–6.
176. Sica GL, Choi IH, Zhu G, Tamada K, Wang SD, Tamura H, et al. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* (2003) 18:849–61.
177. Zang X, Loke P, Kim J, Murphy K, Waitz R, Allison JP. B7x: a widely expressed B7 family member that inhibits T cell activation. *Proc Natl Acad Sci USA.* (2003) 100:10388–92. doi: 10.1073/pnas.1434299100
178. Steinberger P. B7-H3 ameliorates GVHD. *Blood* (2015) 125:3219–21. doi: 10.1182/blood-2015-04-638304
179. Janakiram M, Shah UA, Liu W, Zhao A, Schoenberg MP, Zang X. The third group of the B7-CD28 immune checkpoint family: HHLA2, TMIGD2, B7x, and B7-H3. *Immunol Rev.* (2017) 276:26–39. doi: 10.1111/imr.12521
180. Hughes PE, Caenepeel S, Wu LC. Targeted therapy and checkpoint immunotherapy combinations for the treatment of cancer. *Trends Immunol.* (2016) 37:462–76. doi: 10.1016/j.it.2016.04.010
181. Taylor A, Harker JA, Chanthong K, Stevenson PG, Zuniga EI, Rudd CE. Glycogen synthase kinase 3 inactivation drives T-bet-mediated downregulation of Co-receptor PD-1 to enhance CD8(+) cytolytic T cell responses. *Immunity* (2016) 44:274–86. doi: 10.1016/j.immuni.2016.01.018
182. Taylor A, Rothstein D, Rudd CE. Small-molecule inhibition of PD-1 transcription is an effective alternative to antibody blockade in cancer therapy. *Cancer Res.* (2018) 78:706–17. doi: 10.1158/0008-5472.CAN-17-0491
183. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. *J Clin Oncol.* (2015) 33:1974–82. doi: 10.1200/JCO.2014.59.4358
184. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAS, Andrews MC, et al. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. *Cell* (2017) 170:1120-33 e17. doi: 10.1016/j.cell.2017.07.024
185. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science* (2018) 359:1350–55. doi: 10.1126/science.aar4060
186. Cherkassky L, Morello A, Villena-Vargas J, Feng Y, Dimitrov DS, Jones DR, et al. Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. *J Clin Invest.* (2016) 126:3130–44. doi: 10.1172/JCI83092
187. Moon EK, Ranganathan R, Eruslanov E, Kim S, Newick K, O'Brien S, et al. Blockade of programmed death 1 augments the ability of human T cells engineered to target NY-ESO-1 to control tumor growth after adoptive transfer. *Clin Cancer Res.* (2016) 22:436–47. doi: 10.1158/1078-0432.CCR-15-1070
188. Yoon DH, Osborn MJ, Tolar J, Kim CJ. Incorporation of immune checkpoint blockade into chimeric antigen receptor T cells (CAR-Ts): combination or built-in CAR-T. *Int J Mol Sci.* (2018) 19: E340. doi: 10.3390/ijms19020340
189. Ankri C, Shamalov K, Horovitz-Fried M, Mauer S, Cohen CJ. Human T cells engineered to express a programmed death 1/28 costimulatory retargeting

molecule display enhanced antitumor activity. *J Immunol.* (2013) 191:4121–9. doi: 10.4049/jimmunol.1203085
190. Rosskopf S, Leitner J, Paster W, Morton LT, Hagedoorn RS, Steinberger P, et al. A Jurkat 76 based triple parameter reporter system to evaluate TCR functions and adoptive T cell strategies. *Oncotarget* (2018) 9:17608–19. doi: 10.18632/oncotarget.24807
191. Gubin MM, Zhang X, Schuster H, Caron E, Ward JP, Noguchi T, et al. Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. *Nature* (2014) 515:577–81. doi: 10.1038/nature 13988
192. Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, et al. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med.* (2013) 19:747–52. doi: 10.1038/nm.3161
193. Duan F, Duitama J, Al Seesi S, Ayres CM, Corcelli SA, Pawashe AP, et al. Genomic and bioinformatic profiling of mutational neoepitopes reveals new rules to predict anticancer immunogenicity. *J Exp Med.* (2014) 211:2231–48. doi: 10.1084/jem.20141308
194. Castle JC, Kreiter S, Diekmann J, Lower M, van de Roemer N, de Graaf J, et al. Exploiting the mutanome for tumor vaccination. *Cancer Res.* (2012) 72:1081–91. doi: 10.1158/0008-5472.CAN-11-3722
195. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science* (2015) 348:69–74. doi: 10.1126/science.aaa4971
196. Cohen CJ, Gartner JJ, Horovitz-Fried M, Shamalov K, Trebska-McGowan K, Bliskovsky VV, et al. Isolation of neoantigen-specific T cells from tumor and peripheral lymphocytes. *J Clin Invest.* (2015) 125:3981–91. doi: 10.1172/JCI82416

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 10 September 2018
doi: 10.3389/fimmu.2018.02041

Targeting Checkpoint Receptors and Molecules for Therapeutic Modulation of Natural Killer Cells

Nayoung Kim[1,2]* *and Hun Sik Kim*[3,4,5]*

[1] Department of Convergence Medicine, University of Ulsan College of Medicine, Seoul, South Korea, [2] Asan Institute for Life Sciences, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea, [3] Department of Biomedical Sciences, University of Ulsan College of Medicine, Seoul, South Korea, [4] Department of Microbiology, University of Ulsan College of Medicine, Seoul, South Korea, [5] Stem Cell Immunomodulation Research Center (SCIRC), Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

Among the most promising therapeutic modalities for cancer treatment is the blockade of immune checkpoint pathways, which are frequently co-opted by tumors as a major mechanism of immune escape. CTLA-4 and PD-1 are the representative examples, and their blockade by therapeutic antibodies leads to enhanced anti-tumor immunity with durable clinical responses, but only in a minority of patients. This has highlighted the need to identify and target additional immune checkpoints that can be exploited to further enhance immune responses to refractory cancers. These emerging targets include natural killer (NK) cell-directed checkpoint receptors (KIR and CD94/NKG2A) as well as the NK- and T cell-expressed checkpoints TIM-3, TIGIT, CD96, and LAG-3. Interestingly, the potentiation of anti-tumor immunity by checkpoint blockade relies not only on T cells but also on other components of the innate immune system, including NK cells. NK cells are innate lymphoid cells that efficiently kill tumor cells without MHC specificity, which is complementary to the MHC-restricted tumor lysis mediated by cytotoxic T cells. However, the role of these immune checkpoints in modulating the function of NK cells remains unclear and somewhat controversial. Unraveling the mechanisms by which these immune checkpoints function in NK cells and other immune cells will pave the way to developing new therapeutic strategies to optimize anti-tumor immunity while limiting cancer immune escape. Here, we focus on recent findings regarding the roles of immune checkpoints in regulating NK cell function and their potential application in cancer immunotherapy.

Keywords: NK cells, immune checkpoints, checkpoint blockade, combined targeting, cancer immunotherapy

OPEN ACCESS

Edited by:
Alexandre M. Carmo, i3S, Instituto de Investigação e Inovação em Saúde, Portugal

Reviewed by:
Michael G. Brown, University of Virginia, United States
Ennio Carbone, Università degli Studi Magna Græcia di Catanzaro, Italy

***Correspondence:**
Nayoung Kim
naykim@amc.seoul.kr
Hun Sik Kim
hunkim@amc.seoul.kr

Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *08 June 2018*
Accepted: *20 August 2018*
Published: *10 September 2018*

Citation:
Kim N and Kim HS (2018) Targeting Checkpoint Receptors and Molecules for Therapeutic Modulation of Natural Killer Cells. Front. Immunol. 9:2041. doi: 10.3389/fimmu.2018.02041

INTRODUCTION

Natural killer (NK) cells express an array of inhibitory receptors, such as killer immunoglobulin (Ig)-like receptors (KIRs), CD94/NKG2A, programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3), T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif (ITIM) domains (TIGIT), CD96, and lymphocyte activation gene 3 (LAG-3) (1–5). The primary mechanism of NK cell activation is governed by the "missing-self hypothesis." NK cells do not attack healthy cells when their inhibitory receptors

(KIRs and CD94/NKG2A on human NK cells and Ly49 family members on mouse NK cells) are engaged by MHC class I molecules on target cells, but downregulation of MHC class I, as frequently occurs in virally transformed or neoplastic cells, results in NK cell activation (6). In addition, activation of resting NK cells is rarely triggered by the ligation of a single activating receptor (2). Instead, effective cytotoxicity against tumor cells requires co-engagement of specific activating receptors or pre-activation by cytokines (e.g., IL-2 or IL-15) (7). This additional checkpoint is mediated by common signaling molecules [e.g., c-Cbl, glycogen synthase kinase (GSK)-3β, diacylglycerol kinase (DGK)ζ, or cytokine-inducible Src homology-2 (SH2)-containing protein (CIS)] downstream of diverse activating receptors (1), which provides an additional strategy to enhance NK cell reactivity against tumor cells. Since landmark publications have shown the significant clinical efficacy of PD-1 and/or CTLA-4 blockade in patients with melanoma and other non-treatable cancers (8, 9), much attention has been drawn to immune checkpoint receptors and their cognate ligands. Here, we focus on inhibitory receptors that serve as checkpoints in human NK cell activation, focusing on the key signaling pathways mediated by these receptors and their clinical relevance.

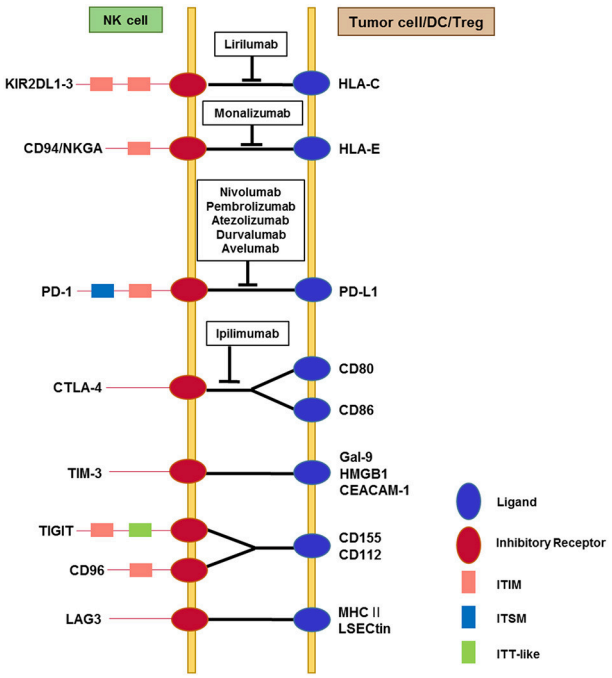


FIGURE 1 | Interactions among immune checkpoint receptors and ligands affecting NK cell function. NK cells express multiple immune checkpoint receptors, which can interact with their cognate ligands on tumor cells or on other immune cells, particularly dendritic cells and Tregs. The red circles represent immune checkpoint receptors and the blue circles represent ligands. The pink squares represent classical ITIM motifs and the light blue squares represent ITSM motifs, both of which mediate inhibitory signals. TIGIT contains an ITT-like motif in addition to the ITIM motif in its cytoplasmic tail. Phosphorylation of the ITT-like motif upon ligand binding plays a critical role in inhibitory signaling via the recruitment of SHIP-1. Cytoplasmic domains of other immune checkpoint receptors contain less well-known motifs (not shown). TIM-3 contains five conserved tyrosine residues in the cytoplasmic tail, among which Y256 and Y263 in mouse (Y265 and Y272 in human) are phosphorylated upon ligand binding. This triggers the dissociation of Bat3 from the cytoplasmic tail of TIM-3, thereby promoting TIM-3-mediated T cell inhibition via the recruitment of Fyn to the same region in place of Bat3. LAG-3 contains a unique KIEELE motif in its cytoplasmic tail that is indispensable for the inhibitory function of LAG-3 in effector $CD4^+$ T cells. Blocking antibodies that target immune checkpoints and are being developed for clinical use are displayed in the boxes.

IMMUNE CHECKPOINT RECEPTORS

KIR and CD94/NKG2A

The KIR family molecules include inhibitory KIRs, which have long cytoplasmic tails harboring two ITIMs (**Figure 1**), as well as activating KIRs that interact with DAP12 or FcRγ (10). The inhibitory KIRs are KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1, KIR3DL2, and KIR3DL3, and these receptors recognize HLA-A, -B, or -C. They have highly polymorphic Ig domains that confer specificity for HLA molecules (11). CD94/NKG2A, a heterodimeric inhibitory receptor related to C-type lectins, recognizes HLA-E, while CD94/NKG2C is an activating receptor. NKG2A, but not CD94, has two ITIMs in its cytoplasmic tail (**Figure 1**). The ITIMs are phosphorylated upon receptor ligation and recruit the tyrosine phosphatases SH2 domain-containing phosphatase (SHP)-1 and SHP-2 (12, 13). SHP-1 dephosphorylates Vav1, a critical mediator downstream of various activating receptors on NK cells (14, 15). Crk phosphorylation also contributes to the inhibition of NK cells following ligation of NKG2A by HLA-E (16). ITIM-based inhibition is dominant over activation in NK cells. Recruitment of SHP-1 by ITIM-bearing receptors appears to inhibit signaling at a proximal step, such that most downstream signals are blocked (2). Interaction of NK cell inhibitory receptors with MHC I ligands on target cells results in complete inhibition of polarization and release of cytotoxic granules (17). Besides their inhibitory function, the interaction of KIRs with MHC I ligands during NK cell development is crucial for their education against self-recognition (2, 18, 19). Accordingly, NK cells can maintain their intrinsic responsiveness against MHC I-deficient target cells, a process referred to as licensing.

As tumor cells exhibit variable expression of MHC I ligands, adoptive transfer of alloreactive NK cells has emerged as a promising strategy that overcomes this checkpoint and creates a condition of "missing-self" recognition. Some solid tumors and leukemias/lymphomas also use the upregulation of HLA-E to evade killing by NK and T cells (20–22). In this respect, another approach that mimics missing-self recognition is treatment with blocking antibodies against KIRs and/or NKG2A on autologous NK cells. Lirilumab (IPH2102) and monalizumab (IPH2201) are IgG4 monoclonal antibodies (mAbs) currently in clinical development that target KIR2DL1-3 and NKG2A, and antagonize the inhibition of NK cells mediated by HLA-C and HLA-E on tumor cells, respectively (3, 23). The anti-KIR antibody (IPH2101) had acceptable safety without significant toxicity or autoimmunity in multiple myeloma (MM) and acute myeloid leukemia (AML) patients (24, 25). IPH2101 treatment

enhanced *ex vivo* NK cell cytotoxicity in MM patients, but did not increase NK cell numbers or cytotoxicity in AML patients. A phase II clinical trial of lirilumab in MM was stopped due to a lack of efficacy, presumably because of decreased responsiveness of KIR2D+ NK cells, accompanied by a loss of KIR2D expression (26). As pan-KIR2D blockade with IPH2101 as a monotherapy was not effective (26, 27), it is currently being widely tested in combination with other therapeutics, including lenalidomide, tumor-targeting monoclonal antibodies (mAbs) such as elotuzumab (an anti-SLAMF7 antibody) or rituximab (an anti-CD20 antibody), and other forms of immune checkpoint blockade (3, 28–31). MM cells upregulate MHC class I; thus blocking inhibitory KIRs could enhance the anti-tumor effect of NK cells in combination with lenalidomide, which is currently used with steroids (28). In combination with anti-CD20 mAbs, anti-KIR treatment (IPH2101) enhances NK cell-mediated, rituximab-dependent cytotoxicity against lymphoma *in vitro* and *in vivo* in KIR transgenic and syngeneic murine lymphoma models (29). Elotuzumab has also been developed to target MM in combination with other therapies, although it has no single-agent activity in advanced MM (32). Monalizumab improves NK cell dysfunction in chronic lymphocytic leukemia (CLL) (33). Moreover, multiple studies demonstrated the expression of NKG2A on tumor-infiltrating NK and T cells in various cancers, including breast cancer (34), cervical cancer (35), lung cancer (36), and hepatocellular carcinoma (37). Given the association between HLA-E overexpression and a poor prognosis in solid tumors (37–39), these studies support NKG2A blockade as a promising strategy to enhance anti-tumor immune responses. Monalizumab is currently under clinical investigation as a single agent in ovarian cancer or in combination with cetuximab (anti-EGFR) and durvalumab (anti-PD-L1) for advanced-stage solid cancers (3, 31). Taken together, combining anti-KIR or anti-NKG2A mAbs with chemotherapy or other mAbs targeting tumor antigens or immune checkpoint molecules may be a promising strategy to achieve clinical efficacy.

CTLA-4 and PD-1

Co-inhibitory signaling molecules are well-described for T cells, particularly in the context of cancer immunology. The most notable examples are CTLA-4 and PD-1. CTLA-4 is a key regulator of T cell expansion, while PD-1 plays an important role in regulating T cell effector function. As of March 2018, six antibodies targeting these immune checkpoint pathways have been approved for clinical use: ipilimumab (anti-CTLA-4), nivolumab (anti-PD-1), pembrolizumab (anti-PD-1), atezolizumab (anti-PD-L1), durvalumab (anti-PD-L1), and avelumab (anti-PD-L1) (40). Therapeutic strategies targeting the CTLA-4 or PD-1 pathway restore T cell function in the cancer microenvironment and lead to durable clinical responses in various cancer types (8, 41–43). Further, combined blockade of both pathways has an additive therapeutic benefit but could come at the cost of a higher rate of adverse effects (44, 45). Various combination strategies employing PD-1 and CTLA-4 blockade are currently under investigation. The therapeutic efficacy of PD-1 and/or CTLA-4 blockade is thought to rely largely on the rescue of tumor-specific T cells from exhaustion and restoration of their effector functions.

The co-stimulatory receptor CD28 and the co-inhibitory receptor CTLA-4 compete for the same ligands, CD80 (B7-1) and CD86 (B7-2; **Figure 1**). CTLA-4 is a structural homolog of CD28, but binds CD80/CD86 with greater avidity and affinity. Unlike many other inhibitory receptors, CTLA-4 lacks a classical signaling motif such as an ITIM in its cytoplasmic tail. CTLA-4 activates the serine/threonine phosphatase PP2A, which inhibits Akt activation without affecting PI3K activity (46). CTLA-4 is found on activated mouse NK cells, and its engagement with B7-1 inhibits IFN-γ production in response to mature dendritic cells (47). CTLA-4$^+$ regulatory T cells (Tregs) suppress NK cell cytotoxicity in cetuximab-treated head and neck cancer patients (48). Of interest, in melanoma, the activity of anti-CTLA-4 antibodies is also attributed to the selective depletion of Tregs mediated by Fc receptors (49, 50). Clinical outcome of anti-CTLA-4 treatment in melanoma patients correlates with low expression of TIM-3 on circulating T and NK cells prior to and during therapy, and correlates with an increased frequency of mature circulating CD3$^-$CD56^{dim}CD16$^+$ NK cells during treatment (51). Survival also correlates with low serum IL-15 levels, which raises a concern regarding treating cancer patients with IL-15, which may lead to the upregulation of PD-1 and TIM-3 on T and NK cells (51). However, B7.1-CD28/CTLA-4 was not required to trigger human NK cell activation in a previous study (52). Furthermore, CD28/B7 co-stimulation was not required for peripheral NK cells to control murine cytomegalovirus infection (53). Thus, it is possible to speculate that anti-CTLA-4 therapy improves NK cell function indirectly via blockade of suppressive CTLA-4$^+$ Tregs (50) and/or restoration of CTLA-4$^+$ T cell function (54, 55).

The ligands of PD-1 are PD-L1 (B7-H1) and PD-L2 (B7-DC), which are upregulated in diverse tumor cells (56, 57). Their engagement of PD-1 on T cells mediates potent inhibition of T cell receptor (TCR) signaling and effector functions, thus allowing tumor cells to escape immunosurveillance (58, 59). Accordingly, blockade of PD-1/PD-L1 interactions rescues PD-1$^+$ T cells from exhaustion and restores their anti-tumor function (60, 61). The cytoplasmic domain of PD-1 contains one ITIM and one immunoreceptor tyrosine-based switch motif (ITSM); the latter interacts with the phosphatases SHP-1/2. Specifically, Y248 of the PD-1 ITSM associates with SHP-2 and is required for the inhibition of PI3K/Akt activation (62). In healthy humans, PD-1 is expressed on approximately one-fourth of peripheral blood NK cells (**Figure 1**). Its expression is confined to CD56^{dim}NKG2A$^-$KIR$^+$CD57$^+$ mature NK cells, and is not expressed on CD56^{bright} NK cells (63). PD-1$^+$ NK cells are thought to be memory-like NK cells (64) or functionally exhausted, given their impaired cytotoxicity and cytokine production (65, 66). PD-1 is upregulated on NK cells from ascites of ovarian cancer patients and on peripheral blood NK cells from Kaposi sarcoma patients, which suggests impaired NK cell function (66, 67). Treatment with an anti-PD-1 antibody increases NK cell cytotoxicity against autologous MM cells *in vitro* (68). Activated primary human NK cells efficiently kill colorectal cancer cells in organoid cultures

independently of PD-L1 expression (69). Tumor-associated macrophage-like monocytes suppress activation of PD-1^+ NK cells from patients with Hodgkin's lymphoma and diffuse large B cell lymphoma, and this suppression is reversed by PD-1 blockade *in vitro* (70). Moreover, PD-1 blockade can induce the expression of genes typically involved in cytolysis and cytokine production, including IFN-γ, in T lymphocytes (71). Because these factors can also boost NK cell function, this might be an additional mechanism underlying the clinical efficacy of PD-1 antibodies, in addition to their direct effects on NK cells. In summary, PD-1 and CTLA-4 blockade may enhance the anti-tumor activity of NK cells both directly and indirectly, via other immune cells such as tumor-specific T cells.

TIM-3

TIM-3 is an activation-induced checkpoint receptor that was originally identified on activated $CD4^+$ T helper 1 (Th1) and $CD8^+$ T cytotoxic 1 (Tc1) T cells (72, 73). TIM-3 is also expressed in Th17 cells and Tregs, and on diverse innate immune cells including NK cells, NKT cells, and myeloid cells (31). The expression of TIM-3 is low on resting T cells, but strongly upregulated on activated and exhausted T cells. TIM-3 is often co-expressed with PD-1 and has been implicated in T cell exhaustion during chronic viral infection and cancer (74, 75). Blockade of TIM-3 alone or in combination with PD-1 reversed T cell exhaustion and reduced tumor growth by restoring T cell effector function in several preclinical mouse models (74, 76, 77). In contrast to T cells, NK cells express TIM-3 basally, and their expression of TIM-3 is the highest among human PBMCs (**Figure 1**) (78). TIM-3 is expressed on all mature $CD56^{dim}CD16^+$ NK cells and is further upregulated upon stimulation with the cytokines IL-12, IL-15, and/or IL-18 (79, 80). In addition, cytokine activation induces TIM-3 expression on immature $CD56^{bright}CD16^-$ NK cells (79), suggesting TIM-3 as a marker for mature and/or activated NK cells. The cognate ligands for TIM-3 include galectin-9 (Gal-9) (81), phosphatidylserine (PtdSer) on apoptotic cells (82), high mobility group box (HMGB)1 (83), and carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1 (5, 84). TIM-3 does not have a classical signaling motif in its cytoplasmic tail such as an ITIM or ITSM. Instead, TIM-3 has five conserved tyrosine residues in its cytoplasmic tail, among which Y256 and Y263 (in mouse) are important for TIM-3 signaling through regulated interaction with HLA-B-associated transcript 3 (Bat3) (5, 85). Bat3 is bound to TIM-3 at the steady state and recruits catalytically active Lck, which can promote T cell signaling. Upon binding of TIM-3 to its cognate ligands (e.g., Gal-9 and CEACAM-1), Y256 and Y263 are phosphorylated, leading to the dissociation of Bat3, thereby promoting T cell inhibition (84, 85). Bat3 and Fyn, a Src kinase that mediates T cell anergy (86), compete for the same binding domain in TIM-3. Thus, Bat3 might be a key determinant of TIM-3 function via regulation of the recruitment of certain signaling components.

Compared to the conserved role of TIM-3 in the suppression of activated T cells, the functional role of TIM-3 on NK cells is controversial. TIM-3 engagement has been shown to have opposing effects on NK cell activation depending on the experimental design. Cross-linking of TIM-3 with an agonistic antibody significantly decreased the cytotoxicity of primary NK cells and the NK cell line NKL (79), whereas stimulation of TIM-3 via Gal-9 selectively enhanced the production of IFN-γ by NK cells (80). Nonetheless, Gal-9 can inhibit the function of human and murine NK cells independently of TIM-3 (87). TIM-3 is upregulated in peripheral blood NK cells from patients with advanced gastric cancer (88), lung adenocarcinoma (89), and advanced melanoma (90), and this sustained increase in TIM-3 expression is associated with NK cell exhaustion and dysfunction. It remains unclear whether this dysfunction of TIM-3^+ NK cells is related to specific or multiple ligands on these cancers, and this merits further investigation. TIM-3 is also found on tumor-infiltrating NK cells in approximately 75% of patients with gastrointestinal stromal tumors (GIST) (91). Of interest, TIM-3^+ tumor-infiltrating NK cells in GIST do not co-express PD-1 (91). However, in a mouse model using lung tumor cells (TC-1) that express human papillomavirus oncoproteins and are MHC class I-deficient, TIM-3^+PD-1^+ NK cells could be detected and were functionally exhausted (65). Blockade of TIM-3 on NK cells from patients with advanced melanoma and lung adenocarcinoma rescues exhausted NK cells and results in increased NK cell cytotoxicity and IFN-γ production (89, 90, 92). TIM-3 expression has also been found to correlate with advanced disease and poor prognosis. These studies suggest that TIM-3 serves as a prognostic biomarker for cancer and is a potential therapeutic target to restore NK cell reactivity against cancer. However, TIM-3 blockade reduces NK cell-mediated killing of pancreatic cancer cell lines (93), and blocking Gal-9 reduces IFN-γ production by NK cells from healthy donors upon incubation with primary AML blasts (94). The promiscuous binding of TIM-3 to multiple ligands may account for its controversial effects on NK cell function. In summary, given the conflicting effects of TIM-3 modulation on NK cell function, further studies will be necessary to determine the precise role of TIM-3 in cancer surveillance by NK cells and to better harness the therapeutic potential of TIM-3 blockade in NK cell-mediated cancer therapy.

TIGIT and CD96

TIGIT and CD96 are inhibitory receptors that compete with an activating receptor DNAM-1 (CD226) for binding to nectin and nectin-like ligands (e.g., CD155 and CD112; **Figure 1**) (4). CD155 is the main ligand for TIGIT and CD96, and is highly expressed on many types of tumor cells (95–97). TIGIT contains ITIM and immunoreceptor tyrosine tail (ITT)-like motifs in its cytoplasmic tail. ITT-like motifs play an important role in mediating inhibitory signaling (98, 99). Engagement of TIGIT by CD155 induces its phosphorylation through Fyn and Lck, resulting in recruitment of SHIP-1, which downregulates the PI3K, MAPK, and NF-κB signaling pathways (5). The cytoplasmic tail of CD96 has an ITIM-like motif for inhibitory signaling, but human CD96 differs from mouse CD96 by the presence of a YXXM motif, similar to that found in activating receptors (e.g., NKG2D and CD28).

TIGIT is readily detectable on resting human NK cells but not on mouse NK cells, and is upregulated upon NK cell activation (4, 100). By contrast, CD96 is constitutively expressed on both resting human and mouse NK cells (4, 101). Engagement of TIGIT by CD155 inhibits human NK cell cytotoxicity and cytokine production by counterbalancing DNAM-1-mediated activation, and this can be reversed by antibody-mediated TIGIT blockade (100, 102). TIGIT blockade also renders NK cells resistant to inhibition by myeloid-derived suppressor cells (103). CD96 binding to CD155 inhibits IFN-γ production by NK cells in mice (104). Accordingly, antibody blockade of CD96 promotes NK cell production of IFN-γ and leads to improved tumor control of lung metastases in three different mouse models, both alone or, more effectively, in combination with anti-CTLA-4, anti-PD-1, or doxorubicin (105). Combined blockade of TIGIT and PD-1 also resulted in significant tumor clearance via enhanced $CD8^+$ T cell effector function (106). It remains unclear why both TIGIT and CD96 are required to counteract DNAM-1-mediated NK cell activation. One possibility is that they play a complementary role in the control of NK cell effector function; TIGIT mainly regulates cytotoxicity, whereas CD96 controls IFN-γ production, as described above. In support of this hypothesis, CD96 blockade in $Tigit^{-/-}$ mice results in better control of B16F10 lung metastasis compared with wild-type mice (107), although lung metastasis is unaffected in $Tigit^{-/-}$ mice (104). Differences in ligand specificity and affinity may also contribute to the net signaling outcome of these paired receptors in a context-dependent manner (4, 105). Despite efficacy in certain preclinical tumor models, whether blockade of TIGIT and/or CD96 modulates NK cell effector function and results in clinical responses in human cancer patients remains to be seen.

LAG-3

LAG-3 is structurally similar to CD4 but binds to MHC class II molecules with a higher affinity than CD4 (108, 109). It is expressed on activated T and NK cells (**Figure 1**) (109). Another potential ligand for LAG-3 is LSECtin, a member of the DC-SIGN family that is expressed on many tumors and is involved in the inhibition of anti-tumor T cell responses (110). The cytoplasmic tail of LAG-3 has three unique regions that are conserved in humans and mice: the serine phosphorylation site, a KIEELE motif, and glutamic acid-proline (EP) repeats (111). Of these, the KIEELE motif is required for the inhibitory function of LAG-3 in $CD4^+$ T cells. T cell effector function is inhibited by engagement of LAG-3 and is improved by LAG-3 blockade (111–113). Of interest, LAG-3 is involved in T cell exhaustion, and therefore combined blockade of LAG-3 and PD-1 synergize to restore T cell function (114, 115). However, the role of LAG-3 in the regulation of NK cell function remains unclear and requires further investigation. NK cells from LAG-3-deficient mice show defects in killing of certain tumor targets, whereas lysis of MHC class I-mismatched cells was not affected by LAG-3 deletion (116). In addition, blocking the LAG-3 pathway with an anti-LAG-3 antibody or soluble LAG-3 has no effect on human NK cell cytotoxicity (117). In conclusion, LAG-3 could be a good candidate for immunotherapy because of its potential to activate both T and NK cells, but further studies on its specific role in NK cells are necessary.

CHECKPOINT MOLECULES IN NK CELL ACTIVATION

In contrast to the MHC-restricted activation of T cells, NK cell activation does not require the recognition of specific antigen presented on MHC molecules. Rather, NK cells have an array of activating receptors with unique ligand specificity and signaling properties: receptors containing immunoreceptor tyrosine-based activation motifs (ITAMs; e.g., CD16, NKp30, and NKp46), the DAP10-associated receptor NKG2D, receptors of the signaling lymphocytic activation molecule (SLAM) family (e.g., 2B4), and other receptors (e.g., DNAM-1) (2, 118). Given the expression of multiple and heterogeneous ligands on tumor cells, it would be desirable to target common signaling molecules that restrain NK cell activation via multiple activating receptors. Modulation of these molecules, which serve as checkpoints, may provide an additional strategy to improve NK cell function. We and others recently described this class of signaling molecules, which includes c-Cbl and GSK-3β (1).

Cbl family members, including c-Cbl and Cbl-b, primarily serve as negative regulators of signaling associated with activating receptors on various lymphocytes (119). In human NK cells, knockdown of c-Cbl rather than Cbl-b augments cytotoxicity and cytokine production by NK cells via multiple activating receptors in a Vav1-dependent manner (15). In addition, c-Cbl serves as a checkpoint for NK cell activation through different activating receptors by imposing a requirement for receptor co-engagement in resting NK cells. In mouse models, Cbl-b-deficiency enhances NK cell function and results in better control of lung metastases (120). Although the therapeutic potential of Cbl-b in human NK cells requires further investigation, modulation of Cbl proteins may provide a promising therapeutic strategy to increase NK cell reactivity against tumor cells.

Using a model of NK cell activation via different activating receptors, GSK-3β was identified as a common downstream signaling molecule in NK cell activation (121). GSK-3β inhibits NK cell function, including cytotoxicity and cytokine production, because its kinase activity is critically involved in these pathways. Accordingly, knockdown or pharmacologic inhibition of GSK-3β increases NK cell function via different activating receptors, suggesting GSK-3β as a checkpoint molecule involved in diverse NK cell activation pathways. Likewise, the NK cell dysfunction observed in AML patients could be reversed by genetic or pharmacologic GSK-3β inactivation (122). Furthermore, NK cells expanded *ex vivo* in the presence of a GSK-3β inhibitor exhibit a more mature phenotype and significantly higher anti-tumor activity (123), suggesting GSK-3β as a promising therapeutic target for NK cell-based therapy.

DGKζ is a negative regulator of diacylglycerol-mediated signaling, which is triggered by diverse activating receptors. DGKζ deficiency in mice increases NK cell function in an extracellular-related kinase (ERK)-dependent manner (124). DGKζ-deficient mice reject tumors more efficiently *in vivo*,

although the deficiency does not affect the expression or function of NK cell inhibitory receptors. DGKζ is expressed in macrophages and dendritic cells (DCs), where it regulates microbial recognition (125). DGKζ also limits the generation of natural Tregs by inhibiting their development (126, 127).

Two members of the suppressor of cytokine signaling (SOCS) family, CIS and SOCS2, are reported to control NK cell differentiation and activity (128). Importantly, CIS serves as a novel checkpoint in NK cell-mediated anti-tumor responses by targeting IL-15 signaling. The gene encoding CIS, *cish*, is highly induced by IL-15, and the deletion of *cish* rendered NK cells hypersensitive to IL-15 (129). $Cish^{-/-}$ mice show reduced metastasis in various tumor models, likely due to upregulation of Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling in activated NK cells.

CONCLUSIONS AND PERSPECTIVES

NK cells are innate lymphoid cells with an intrinsic ability to kill diverse tumor cells without MHC restriction. Thus, NK cells are now considered promising therapeutic targets for cancer immunotherapy, particularly for the control of metastases and leukemia/lymphoma. Recent studies have demonstrated the clinical efficacy of NK cell-based therapies in the treatment of various cancer types. However, success is still limited, and there is substantial interest in identifying therapeutic targets to improve NK cell reactivity against tumor cells. Initially discovered as a safeguard mechanism to ensure self-tolerance and prevent autoimmunity, immune checkpoint receptors have been explored as attractive therapeutic targets to enhance anti-tumor immunity, including that mediated by NK cells. As many of these immune checkpoint receptors are not specific to NK cells, it will be important to determine the contribution of NK cells to the clinical benefit of blockade of these molecules. For example, the therapeutic benefit of blockade of PD-1 and CTLA-4 is largely thought to be due to actions on T cells rather than NK cells. A notable feature of targeting NK cell-specific checkpoints (e.g., inhibitory KIRs and NKG2A), alone or in combination with others (e.g., PD-1 blockade by nivolumab), is the lack of severe toxicity (3, 25), which could provide strategic flexibility for NK cell-based therapy. Checkpoint receptors often cooperate to impair T cell responses, which can be overcome by combined targeting (e.g., blockade of PD-1 and CTLA-4), resulting in improved clinical outcomes. Given the disappointing clinical efficacy of molecules targeting NK cell-specific checkpoints as a monotherapies, therapies that target both NK cells and other effector cells, such as T cells, can be pursued. Recent studies also suggest that NK cell effector function relies on the modulation of various molecular checkpoints (e.g., Cbl, GSK-3β, DGKζ, or CIS) in diverse activation pathways, which may provide an additional strategy to enhance NK cell function. Blockade of these molecular checkpoints could facilitate the activation of NK cells by lowering the activation threshold in response to activating receptors and/or cytokines. Although the therapeutic benefit of targeting these checkpoints needs to be assessed, this information will provide new therapeutic options to improve NK cell activation, possibly in combination with other therapies, for better outcomes in the clinic.

AUTHOR CONTRIBUTIONS

Both NK and HSK conceived, wrote the manuscript, and approved it for publication.

FUNDING

This work was supported by the Intelligent Synthetic Biology Center of the Global Frontier Project, funded by the Ministry of Education, Science, and Technology of the Republic of Korea (2013-0073185); by grants from the National Research Foundation of Korea (2016R1A2B4010300); and by an MRC grant (2018R1A5A2020732) funded by the Ministry of Science and Information Technology (MSIT) of the Korean government.

REFERENCES

1. Kwon HJ, Kim N, Kim HS. Molecular checkpoints controlling natural killer cell activation and their modulation for cancer immunotherapy. *Exp Mol Med.* (2017) 49:e311. doi: 10.1038/emm.2017.42
2. Long EO, Kim HS, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses, integration of signals for activation and inhibition. *Ann Rev Immunol.* (2013) 31:227–58. doi: 10.1146/annurev-immunol-020711-075005
3. Chiossone L, Vienne M, Kerdiles YM, Vivier E. Natural killer cell immunotherapies against cancer: checkpoint inhibitors and more. *Semin Immunol.* (2017) 31:55–63. doi: 10.1016/j.smim.2017.08.003
4. Martinet L, Smyth MJ. Balancing natural killer cell activation through paired receptors. *Nat Rev Immunol.* (2015) 15:243–54. doi: 10.1038/nri3799
5. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity* (2016) 44:989–1004. doi: 10.1016/j.immuni.2016.05.001
6. Long EO. Negative signaling by inhibitory receptors: the NK cell paradigm. *Immunol Rev.* (2008) 224:70–84. doi: 10.1111/j.1600-065X.2008.00660.x
7. Kwon HJ, Choi G, E, Ryu S, Kwon SJ, Kim SC, Booth C, et al. Stepwise phosphorylation of p65 promotes NF-kappaB activation and NK cell responses during target cell recognition. *Nat Commun.* (2016) 7:11686. doi: 10.1038/ncomms11686
8. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med.* (2012) 366:2455–65. doi: 10.1056/NEJMoa1200694
9. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med.* (2015) 373:23–34. doi: 10.1056/NEJMoa1504030
10. Thielens A, Vivier E, Romagne F. NK cell MHC class I specific receptors (KIR): from biology to clinical intervention. *Curr Opin Immunol.* (2012) 24:239–45. doi: 10.1016/j.coi.2012.01.001
11. Saunders PM, Vivian JP, O'Connor GM, Sullivan LC, Pymm P, Rossjohn J, et al. A bird's eye view of NK cell receptor interactions with their MHC class I ligands. *Immunol Rev.* (2015) 267:148–66. doi: 10.1111/imr.12319
12. Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, Yi T, et al. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity* (1996) 4:77–85. doi: 10.1016/S1074-7613(00)80300-3

13. Olcese L, Lang P, Vely F, Cambiaggi A, Marguet D, Blery M, et al. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. *J Immunol.* (1996) 156:4531–4.
14. Stebbins CC, Watzl C, Billadeau DD, Leibson PJ, Burshtyn DN, Long EO. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Mol Cell Biol.* (2003) 23:6291–9. doi: 10.1128/MCB.23.17.6291-6299.2003
15. Kim HS, Das A, Gross CC, Bryceson YT, Long EO. Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase. *Immunity* (2010) 32:175–86. doi: 10.1016/j.immuni.2010.02.004
16. Liu D, Peterson ME, Long EO. The adaptor protein Crk controls activation and inhibition of natural killer cells. *Immunity* (2012) 36:600–11. doi: 10.1016/j.immuni.2012.03.007
17. Das A, Long EO. Lytic granule polarization, rather than degranulation, is the preferred target of inhibitory receptors in NK cells. *J Immunol.* (2010) 185:4698–704. doi: 10.4049/jimmunol.1001220
18. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart CA, et al. Human NK cell education by inhibitory receptors for MHC class I. *Immunity* (2006) 25:331–42. doi: 10.1016/j.immuni.2006.06.013
19. Elliott JM, Yokoyama W M. Unifying concepts of MHC-dependent natural killer cell education. *Trends Immunol.* (2011) 32:364–72. doi: 10.1016/j.it.2011.06.001
20. Huang R, Zhang D, Li F, Xiao Z, Wu M, Shi D, et al. Loss of Fas expression and high expression of HLA-E promoting the immune escape of early colorectal cancer cells. *Oncol Lett.* (2017) 13:3379–86. doi: 10.3892/ol.2017.5891
21. Lo Monaco E, Tremante E, Cerboni C, Melucci E, Sibilio L, Zingoni A, et al. Human leukocyte antigen E contributes to protect tumor cells from lysis by natural killer cells. *Neoplasia* (2011) 13:822–30. doi: 10.1593/neo.101684
22. Ruggeri L, Urbani E, Andre P, Mancusi A, Tosti A, Topini F, et al. Effects of anti-NKG2A antibody administration on leukemia and normal hematopoietic cells. *Haematologica* (2016) 101:626–33. doi: 10.3324/haematol.2015.135301
23. Muntasell A, Ochoa MC, Cordeiro L, Berraondo PA. Lopez-Diaz de Cerio, Cabo M, et al. Targeting NK-cell checkpoints for cancer immunotherapy. *Curr Opin Immunol.* (2017) 45:73–81. doi: 10.1016/j.coi.2017.01.003
24. Benson DM Jr, Hofmeister CC, Padmanabhan S, Suvannasankha A, Jagannath S, Abonour R, et al. A phase 1 trial of the anti-KIR antibody IPH2101 in patients with relapsed/refractory multiple myeloma. *Blood* (2012) 120:4324–33. doi: 10.1182/blood-2012-06-438028
25. Vey N, Bourhis JH, Boissel N, Bordessoule D, Prebet T, Charbonnier A, et al. A phase 1 trial of the anti-inhibitory KIR mAb IPH2101 for AML in complete remission. *Blood* (2012) 120:4317–23. doi: 10.1182/blood-2012-06-437558
26. Carlsten M, Korde N, Kotecha R, Reger R, Bor S, Kazandjian D, et al. Checkpoint Inhibition of KIR2D with the Monoclonal Antibody IPH2101 Induces Contraction and Hyporesponsiveness of NK Cells in Patients with Myeloma. *Clin Cancer Res.* (2016) 22:5211–22. doi: 10.1158/1078-0432.CCR-16-1108
27. Korde N, Carlsten M, Lee MJ, Minter A, Tan E, Kwok M, Manasanch E, et al. A phase II trial of pan-KIR2D blockade with IPH2101 in smoldering multiple myeloma. *Haematologica* (2014) 99:e81–3. doi: 10.3324/haematol.2013.103085
28. Benson DM Jr, Cohen AD, Jagannath S, Munshi NC, Spitzer G, Hofmeister CC, et al. A Phase I trial of the Anti-KIR antibody IPH2101 and lenalidomide in patients with relapsed/refractory multiple myeloma. *Clin Cancer Res.* (2015) 21:4055–61. doi: 10.1158/1078-0432.CCR-15-0304
29. Kohrt HE, Thielens A, Marabelle A, Sagiv-Barfi I, Sola C, Chanuc F, et al. Anti-KIR antibody enhancement of anti-lymphoma activity of natural killer cells as monotherapy and in combination with anti-CD20 antibodies. *Blood* (2014) 123:678–86. doi: 10.1182/blood-2013-08-519199
30. Carotta S. Targeting NK Cells for Anticancer Immunotherapy: Clinical and Preclinical Approaches. *Front Immunol.* (2016) 7:152. doi: 10.3389/fimmu.2016.00152
31. Burugu S, Dancsok AR, Nielsen TO. Emerging targets in cancer immunotherapy. *Semin Cancer Biol.* (2017). doi: 10.1016/j.semcancer.2017.10.001. [Epub ahead of print].
32. van de Donk NW, Moreau P, Plesner T, Palumbo A, Gay F, Laubach JP, et al. Clinical efficacy and management of monoclonal antibodies targeting CD38 and SLAMF7 in multiple myeloma. *Blood* (2016) 127:681–95. doi: 10.1182/blood-2015-10-646810
33. McWilliams EM, Mele JM, Cheney C, Timmerman EA, Fiazuddin F, Strattan EJ, et al. Therapeutic CD94/NKG2A blockade improves natural killer cell dysfunction in chronic lymphocytic leukemia. *Oncoimmunology* (2016) 5:e1226720. doi: 10.1080/2162402X.2016.1226720
34. Platonova S, Cherfils-Vicini J, Damotte D, Crozet L, Vieillard V, Validire P, et al. Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma. *Cancer Res.* (2011) 71:5412–22. doi: 10.1158/0008-5472.CAN-10-4179
35. Chang WC, Li CH, Chu LH, Huang PS, Sheu BC, Huang SC. Regulatory T cells suppress natural killer cell immunity in patients with human cervical carcinoma. *Int J Gynecol Cancer* (2016) 26:156–62. doi: 10.1097/IGC.0000000000000578
36. Gillard-Bocquet M, Caer C, Cagnard N, Crozet L, Perez M, Fridman WH, et al. Lung tumor microenvironment induces specific gene expression signature in intratumoral NK cells. *Front Immunol.* (2013) 4:19. doi: 10.3389/fimmu.2013.00019
37. Sun C, Xu J, Huang Q, Huang M, Wen H, Zhang C, et al. High NKG2A expression contributes to NK cell exhaustion and predicts a poor prognosis of patients with liver cancer. *Oncoimmunology* (2017) 6:e1264562. doi: 10.1080/2162402X.2016.1264562
38. Gooden M, Lampen M, Jordanova ES, Leffers N, Trimbos JB, van der BurgSH, et al. HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8(+) T lymphocytes. *Proc Natl Acad Sci USA.* (2011) 108:10656–61. doi: 10.1073/pnas.1100354108
39. Bossard C, Bezieau S, Matysiak-Budnik T, Volteau C, Laboisse CL, Jotereau F, Mosnier JF. HLA-E/beta2 microglobulin overexpression in colorectal cancer is associated with recruitment of inhibitory immune cells and tumor progression. *Int J Cancer* (2012) 131:855–63. doi: 10.1002/ijc.26453
40. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science* (2018) 359:1350–5. doi: 10.1126/science.aar4060
41. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med.* (2012) 366:2443–54. doi: 10.1056/NEJMoa1200690
42. Beaver JA, Theoret MR, Mushti S, He K, Libeg M, Goldberg K, et al. FDA Approval of nivolumab for the first-line treatment of patients with BRAF(V600) Wild-type unresectable or metastatic melanoma. *Clin Cancer Res.* (2017) 23:3479–83. doi: 10.1158/1078-0432.CCR-16-0714
43. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med.* (2010) 363:711–23. doi: 10.1056/NEJMoa1003466
44. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med.* (2013) 369:122–33. doi: 10.1056/NEJMoa1302369
45. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, Daud A, et al. Pembrolizumab versus ipilimumab in advanced melanoma. *N Engl J Med.* (2015) 372:2521–32. doi: 10.1056/NEJMoa1503093
46. Schildberg FA, Klein SR, Freeman GJ, Sharpe AH. Coinhibitory pathways in the B7-CD28 ligand-receptor family. *Immunity* (2016) 44:955–72. doi: 10.1016/j.immuni.2016.05.002
47. Stojanovic A, Fiegler N, Brunner-Weinzierl M, Cerwenka A. CTLA-4 is expressed by activated mouse NK cells and inhibits NK Cell IFN-gamma production in response to mature dendritic cells. *J Immunol.* (2014) 192:4184–91. doi: 10.4049/jimmunol.1302091
48. Jie HB, Schuler PJ, Lee SC, Srivastava RM, Argiris A, Ferrone S, et al. CTLA-4(+) Regulatory T cells increased in cetuximab-treated head and neck cancer patients suppress NK cell cytotoxicity and correlate with poor prognosis. *Cancer Res.* (2015) 75:2200–10. doi: 10.1158/0008-5472.CAN-14-2788
49. Romano E, Kusio-Kobialka M, Foukas PG, Baumgaertner P, Meyer C, Ballabeni P, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells *ex vivo* by nonclassical monocytes in melanoma patients. *Proc Natl Acad Sci USA.* (2015) 112:6140–5. doi: 10.1073/pnas.1417320112
50. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med.* (2013) 210:1695–710. doi: 10.1084/jem.20130579

51. Tallerico R, Cristiani CM, Staaf E, Garofalo C, Sottile R, Capone MY et al. IL-15, TIM-3 and NK cells subsets predict responsiveness to anti-CTLA-4 treatment in melanoma patients. *Oncoimmunology* (2017) 6:e1261242. doi: 10.1080/2162402X.2016.1261242
52. Lang S, Vujanovic NL, Wollenberg B, Whiteside TL. Absence of B7.1-CD28/CTLA-4-mediated co-stimulation in human NK cells. *Eur J Immunol.* (1998) 28:780–6.
53. Cook CH, Chen L, Wen J, Zimmerman P, Zhang Y, Trgovcich J, et al. CD28/B7-mediated co-stimulation is critical for early control of murine cytomegalovirus infection. *Viral Immunol.* (2009) 22:91–103. doi: 10.1089/vim.2008.0080
54. Hannani D, Vetizou M, Enot D, Rusakiewicz S, Chaput N, Klatzmann D, et al. Anticancer immunotherapy by CTLA-4 blockade: obligatory contribution of IL-2 receptors and negative prognostic impact of soluble CD25. *Cell Res.* (2015) 25:208–24. doi: 10.1038/cr.2015.3
55. Kerdiles Y, Ugolini S, Vivier E. T cell regulation of natural killer cells. *J Exp Med.* (2013) 210:1065–8. doi: 10.1084/jem.20130960
56. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol.* (2008) 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
57. Boyerinas B, Jochems C, Fantini M, Heery CR, Gulley JL, Tsang KY, et al. Antibody-dependent cellular cytotoxicity activity of a novel Anti-PD-L1 antibody avelumab (MSB0010718C) on human tumor cells. *Cancer Immunol Res.* (2015) 3:1148–57. doi: 10.1158/2326-6066.CIR-15-0059
58. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med.* (2012) 4:127ra137. doi: 10.1126/scitranslmed.3003689
59. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med.* (2002) 8:793–800. doi: 10.1038/nm730
60. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U.S.A.* (2002) 99:12293–7. doi: 10.1073/pnas.192461099
61. Bardhan K, Anagnostou T, Boussiotis VA. The PD1:PD-L1/2 pathway from discovery to clinical implementation. *Front Immunol.* (2016) 7:550. doi: 10.3389/fimmu.2016.00550
62. Boussiotis VA, Chatterjee P, Li L. Biochemical signaling of PD-1 on T cells and its functional implications. *Cancer J.* (2014) 20:265–71. doi: 10.1097/PPO.0000000000000059
63. Pesce S, Greppi M, Tabellini G, Rampinelli F, Parolini S, Olive D, et al. Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization. *J Allergy Clin Immunol.* (2017) 139:335–46.e3. doi: 10.1016/j.jaci.2016.04.025
64. Della Chiesa M, Pesce S, Muccio L, Carlomagno S, Sivori S, Moretta A, et al. Features of memory-Like and PD-1(+) human nk cell subsets. *Front Immunol.* (2016) 7:351. doi: 10.3389/fimmu.2016.00351
65. Seo H, Jeon I, Kim BS, Park M, Bae EA, Song B, et al. IL-21-mediated reversal of NK cell exhaustion facilitates anti-tumour immunity in MHC class I-deficient tumours. *Nat Commun.* (2017) 8:15776. doi: 10.1038/ncomms15776
66. Beldi-Ferchiou A, Lambert M, Dogniaux S, Vely F, Vivier E, Olive D, et al. Caillat-Zucman. PD-1 mediates functional exhaustion of activated NK cells in patients with Kaposi sarcoma. *Oncotarget* (2016) 7:72961–77. doi: 10.18632/oncotarget.12150
67. Tabellini G, Benassi M, Marcenaro E, Coltrini D, Patrizi O, Ricotta D, et al. Primitive neuroectodermal tumor in an ovarian cystic teratoma: natural killer and neuroblastoma cell analysis. *Case Rep Oncol.* (2014) 7:70–8. doi: 10.1159/000357802
68. Benson DM Jr, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* (2010) 116:2286–94. doi: 10.1182/blood-2010-02-271874
69. Lanuza PM, Vigueras A, Olivan S, Prats AC, Costas S, Llamazares G, et al. Activated human primary NK cells efficiently kill colorectal cancer cells in 3D spheroid cultures irrespectively of the level of PD-L1 expression. *Oncoimmunology* (2018) 7:e1395123. doi: 10.1080/2162402X.2017.1395123
70. Vari F, Arpon D, Keane C, Hertzberg MS, Talaulikar D, Jain S, et al. Immune evasion via PD-1/PD-L1 on NK cells and monocyte/macrophages is more prominent in Hodgkin lymphoma than DLBCL. *Blood* (2018) 131:1809–19. doi: 10.1182/blood-2017-07-796342
71. Das R, Verma R, Sznol M, Boddupalli CS, Gettinger S, N, Kluger H, et al. Combination therapy with anti-CTLA-4 and anti-PD-1 leads to distinct immunologic changes in vivo. *J Immunol.* (2015) 194:950–9. doi: 10.4049/jimmunol.1401686
72. Das M, Zhu C, Kuchroo VK. Tim-3 and its role in regulating anti-tumor immunity. *Immunol Rev.* (2017) 276:97–111. doi: 10.1111/imr.12520
73. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* (2002) 415:536–41. doi: 10.1038/415536a
74. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med.* (2010) 207:2175–86. doi: 10.1084/jem.20100637
75. Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA.* (2010) 107:14733–8. doi: 10.1073/pnas.1009731107
76. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med.* (2010) 207:2187–94. doi: 10.1084/jem.20100643
77. Ngiow SF, von Scheidt B, Akiba H, Yagita H, Teng M, W, Smyth MJ. Anti-TIM3 antibody promotes T cell IFN-gamma-mediated antitumor immunity and suppresses established tumors. *Cancer Res.* (2011) 71:3540–51. doi: 10.1158/0008-5472.CAN-11-0096
78. Khademi M, Illes Z, Gielen AW, Marta M, Takazawa N, Baecher-Allan C, et al. T Cell Ig- and mucin-domain-containing molecule-3 (TIM-3) and TIM-1 molecules are differentially expressed on human Th1 and Th2 cells and in cerebrospinal fluid-derived mononuclear cells in multiple sclerosis. *J Immunol.* (2004) 172:7169–76. doi: 10.4049/jimmunol.172.11.7169
79. Ndhlovu LC, Lopez-Verges S, Barbour JD, Jones RB, Jha AR, Long BR, et al. Tim-3 marks human natural killer cell maturation and suppresses cell-mediated cytotoxicity. *Blood* (2012) 119:3734–43. doi: 10.1182/blood-2011-11-392951
80. Gleason MK, Lenvik TR, McCullar V, Felices M, O'Brien MS, Cooley SA, et al. Tim-3 is an inducible human natural killer cell receptor that enhances interferon gamma production in response to galectin-9. *Blood* (2012) 119:3064–72. doi: 10.1182/blood-2011-06-360321
81. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol.* (2005) 6:1245–52. doi: 10.1038/ni1271
82. Nakayama M, Akiba H, Takeda K, Kojima Y, Hashiguchi M, Azuma M, et al. Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. *Blood* (2009) 113:3821–30. doi: 10.1182/blood-2008-10-185884
83. Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, et al. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. *Nat Immunol.* (2012) 13:832–42. doi: 10.1038/ni.2376
84. Huang YH, Zhu C, Kondo Y, Anderson AC, Gandhi A, Russell A, et al. CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. *Nature* (2015) 517:386–90. doi: 10.1038/nature13848
85. Rangachari M, Zhu C, Sakuishi K, Xiao S, Karman J, Chen A, et al. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3-mediated cell death and exhaustion. *Nat Med.* (2012) 18:1394–400. doi: 10.1038/nm.2871
86. Davidson D, Schraven B, Veillette A. PAG-associated FynT regulates calcium signaling and promotes anergy in T lymphocytes. *Mol Cell Biol.* (2007) 27:1960–73. doi: 10.1128/MCB.01983-06

87. Golden-Mason L, McMahan RH, Strong M, Reisdorph R, Mahaffey S, Palmer BE, et al. Galectin-9 functionally impairs natural killer cells in humans and mice. *J Virol.* (2013) 87:4835–45. doi: 10.1128/JVI.01085-12
88. Wang Z, Zhu J, Gu H, Yuan Y, Zhang B, Zhu D, et al. The clinical significance of abnormal Tim-3 expression on NK cells from patients with gastric cancer. *Immunol Invest.* (2015) 44:578–589. doi: 10.3109/08820139.2015.1052145
89. Xu L, Huang Y, Tan L, Yu W, Chen D, Lu C, et al. Increased Tim-3 expression in peripheral NK cells predicts a poorer prognosis and Tim-3 blockade improves NK cell-mediated cytotoxicity in human lung adenocarcinoma. *Int Immunopharmacol.* (2015) 29:635–41. doi: 10.1016/j.intimp.2015.09.017
90. da Silva IP, Gallois A, Jimenez-Baranda S, Khan S, Anderson AC, Kuchroo VK, et al. Reversal of NK-cell exhaustion in advanced melanoma by Tim-3 blockade. *Cancer Immunol Res.* (2014) 2:410–22. doi: 10.1158/2326-6066.CIR-13-0171
91. Komita H, Koido S, Hayashi K, Kan S, Ito M, Kamata Y, et al. Expression of immune checkpoint molecules of T cell immunoglobulin and mucin protein 3/galectin-9 for NK cell suppression in human gastrointestinal stromal tumors. *Oncol Rep.* (2015) 34:2099–105. doi: 10.3892/or.2015.4149
92. Gallois A, Silva I, Osman I, Bhardwaj N. Reversal of natural killer cell exhaustion by TIM-3 blockade. *Oncoimmunology* (2014) 3:e946365. doi: 10.4161/21624011.2014.946365
93. Van Audenaerde JRM, De Waele J, Marcq E, Van Loenhout J, Lion EJ, Van den Bergh MJ, et al. Interleukin-15 stimulates natural killer cell-mediated killing of both human pancreatic cancer and stellate cells. *Oncotarget* (2017) 8:56968–79. doi: 10.18632/oncotarget.18185
94. Folgiero V, Cifaldi L, Li Pira G, Goffredo BM, Vinti L, Locatelli F. TIM-3/Gal-9 interaction induces IFNgamma-dependent IDO1 expression in acute myeloid leukemia blast cells. *J Hematol Oncol.* (2015) 8:36. doi: 10.1186/s13045-015-0134-4
95. Sloan KE, Eustace BK, Stewart JK, Zehetmeier C, Torella C, Simeone M, et al. CD155/PVR plays a key role in cell motility during tumor cell invasion and migration. *BMC Cancer* (2004) 4:73. doi: 10.1186/1471-2407-4-73
96. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med.* (2003) 198:557–67. doi: 10.1084/jem.20030788
97. Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S, et al. Accelerated tumor growth in mice deficient in DNAM-1 receptor. *J Exp Med.* (2008) 205:2959–64. doi: 10.1084/jem.20081611
98. Liu S, Zhang H, Li M, Hu D, Li C, Ge B, et al. Recruitment of Grb2 and SHIP1 by the ITT-like motif of TIGIT suppresses granule polarization and cytotoxicity of NK cells. *Cell Death Differ.* (2013) 20:456–64. doi: 10.1038/cdd.2012.141
99. Li M, Xia P, Du Y, Liu S, Huang G, Chen J, et al. T-cell immunoglobulin and ITIM domain (TIGIT) receptor/poliovirus receptor (PVR) ligand engagement suppresses interferon-gamma production of natural killer cells via beta-arrestin 2-mediated negative signaling. *J Biol Chem.* (2014) 289:17647–57. doi: 10.1074/jbc.M114.572420
100. Stanietsky N, Rovis TL, Glasner A, Seidel E, Tsukerman P, Yamin R, et al. Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *Eur J Immunol.* (2013) 43:2138–50. doi: 10.1002/eji.201243072
101. Wang PL, O'Farrell S, Clayberger C, Krensky AM. Identification and molecular cloning of tactile. A novel human T cell activation antigen that is a member of the Ig gene superfamily. *J Immunol.* (1992) 148:2600–8.
102. Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A, et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc Natl Acad Sci USA.* (2009) 106:17858–63. doi: 10.1073/pnas.0903474106
103. Sarhan D, Cichocki F, Zhang B, Yingst A, Spellman SR, Cooley S, et al. Adaptive NK cells with low TIGIT expression are inherently resistant to myeloid-derived suppressor Cells. *Cancer Res.* (2016) 76:5696–706. doi: 10.1158/0008-5472.CAN-16-0839
104. Chan CJ, Martinet L, Gilfillan SF. Souza-Fonseca-Guimaraes, Chow MT, Town L, et al. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. *Nat Immunol.* (2014) 15:431–8. doi: 10.1038/ni.2850
105. Blake SJ, Dougall WC, Miles JJ, Teng MW, Smyth MJ. molecular pathways, targeting CD96 and TIGIT for cancer immunotherapy. *Clin Cancer Res.* (2016) 22:5183–8. doi: 10.1158/1078-0432.CCR-16-0933
106. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell* (2014) 26:923–37. doi: 10.1016/j.ccell.2014.10.018
107. Blake SJ, Stannard K, Liu J, Allen S, Yong MC, Mittal D, et al. Suppression of metastases using a new lymphocyte checkpoint target for cancer immunotherapy. *Cancer Disc.* (2016) 6:446–59. doi: 10.1158/2159-8290.CD-15-0944
108. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med.* (1990) 171:1393–405. doi: 10.1084/jem.171.5.1393
109. Baixeras E, Huard B, Miossec C, Jitsukawa S, Martin M, Hercend T, et al. Piatier-Tonneau. Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. *J Exp Med.* (1992) 176:327–37. doi: 10.1084/jem.176.2.327
110. Xu F, Liu J, Liu D, Liu B, Wang M, Hu Z, et al. LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. *Cancer Res.* (2014) 74:3418–28. doi: 10.1158/0008-5472.CAN-13-2690
111. Workman CJ, Dugger KJ, Vignali DA. Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. *J Immunol.* (2002) 169:5392–5. doi: 10.4049/jimmunol.169.10.5392
112. Workman CJ, Vignali DA. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *J Immunol.* (2005) 174:688–95. doi: 10.4049/jimmunol.174.2.688
113. Macon-Lemaitre L, Triebel F. The negative regulatory function of the lymphocyte-activation gene-3 co-receptor (CD223) on human T cells. *Immunology* (2005) 115:170–8. doi: 10.1111/j.1365-2567.2005.02145.x
114. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol.* (2009) 10:29–37. doi: 10.1038/ni.1679
115. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res.* (2012) 72:917–27. doi: 10.1158/0008-5472.CAN-11-1620
116. Miyazaki T, Dierich A, Benoist C, Mathis D. Independent modes of natural killing distinguished in mice lacking Lag3. *Science* (1996) 272:405–8. doi: 10.1126/science.272.5260.405
117. Huard B, Tournier M, Triebel F. LAG-3 does not define a specific mode of natural killing in human. *Immunol Lett.* (1998) 61:109–12. doi: 10.1016/S0165-2478(97)00170-3
118. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol.* (2008) 9:495–502. doi: 10.1038/ni1581
119. Huang F, Gu H. Negative regulation of lymphocyte development and function by the Cbl family of proteins. *Immunol Rev.* (2008) 224:229–38. doi: 10.1111/j.1600-065X.2008.00655.x
120. Paolino M, Choidas A, Wallner S, Pranjic B, Uribesalgo I, Loeser S, et al. The E3 ligase Cbl-b and TAM receptors regulate cancer metastasis via natural killer cells. *Nature* (2014) 507:508–12. doi: 10.1038/nature12998
121. Kwon HJ, Kwon SJ, Lee H, Park HR, Choi GE, Kang SW, et al. NK cell function triggered by multiple activating receptors is negatively regulated by glycogen synthase kinase-3beta. *Cell Signal* (2015) 27:1731–41. doi: 10.1016/j.cellsig.2015.05.012
122. Parameswaran R, Ramakrishnan P, Moreton SA, Xia Z, Hou Y, Lee DA, et al. Repression of GSK3 restores NK cell cytotoxicity in AML patients. *Nat Commun.* (2016) 7:11154. doi: 10.1038/ncomms11154
123. Cichocki F, Valamehr B, Bjordahl R, Zhang B, Rezner B, Rogers P, et al. GSK3 inhibition drives maturation of NK Cells and enhances their antitumor activity. *Cancer Res.* (2017) 77:5664–75. doi: 10.1158/0008-5472.CAN-17-0799
124. Yang E, Singh BK, Paustian AM, Kambayashi T. Diacylglycerol Kinase zeta Is a Target To Enhance NK Cell Function. *J Immunol.* (2016) 197:934–41. doi: 10.4049/jimmunol.1600581

125. Liu CH, Machado FS, Guo R, Nichols KE, Burks AW, Aliberti JC, et al. Diacylglycerol kinase zeta regulates microbial recognition and host resistance to Toxoplasma gondii. *J Exp Med*. (2007) 204:781–92. doi: 10.1084/jem.20061856
126. Joshi RP, Schmidt AM, Das J, Pytel D, Riese MJ, Lester M, et al. The zeta isoform of diacylglycerol kinase plays a predominant role in regulatory T cell development and TCR-mediated ras signaling. *Sci Signal* (2013) 6:ra102. doi: 10.1126/scisignal.2004373
127. Schmidt AM, Zou T, Joshi RP, Leichner TM, Pimentel MA, Sommers CL, et al. Diacylglycerol kinase zeta limits the generation of natural regulatory T cells. *Sci Signal* (2013) 6:ra101. doi: 10.1126/scisignal.2004411
128. Keating N, Nicholson SE. SOCS-mediated immunomodulation of natural killer cells. *Cytokine* (2018). doi: 10.1016/j.cyto.2018.03.033. [Epub ahead of print].
129. Delconte RB, Kolesnik TB, Dagley LF, Rautela J, Shi W, Putz EM, SOCS-mediated immunomodulation of natural killer cells. CIS is a potent checkpoint in NK cell-mediated tumor immunity. *Nat Immunol*. (2016) 17:816–24. doi: 10.1038/ni.3470

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 25 September 2018
doi: 10.3389/fimmu.2018.02148

Control of Immunoregulatory Molecules by miRNAs in T Cell Activation

Ana Rodríguez-Galán[1,2], *Lola Fernández-Messina*[1,2] *and Francisco Sánchez-Madrid*[1,2,3]*

[1] *Servicio de Inmunología, Instituto de Investigación Sanitaria Princesa (IP), Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, Madrid, Spain,* [2] *Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain,* [3] *Centro de Investigación Biomédica en Red de Enfermedades Cardiovasculares, Madrid, Spain*

MiRNA targeting of key immunoregulatory molecules fine-tunes the immune response. This mechanism boosts or dampens immune functions to preserve homeostasis while supporting the full development of effector functions. MiRNA expression changes during T cell activation, highlighting that their function is constrained by a specific spatiotemporal frame related to the signals that induce T cell-based effector functions. Here, we update the state of the art regarding the miRNAs that are differentially expressed during T cell stimulation. We also revisit the existing data on miRNA function in T cell activation, with a special focus on the modulation of the most relevant immunoregulatory molecules.

Keywords: T cell activation, microRNAs (miRNAs), immunoregulatory molecules, miRNA signature, CD4, CD8, T lymphocyte

OPEN ACCESS

Edited by:
Alexandre M. Carmo, i3S, Instituto de Investigação e Inovação em Saúde, Portugal

Reviewed by:
Isabel Castro, i3S, Instituto de Investigação e Inovação em Saúde, Portugal
Cosima T. Baldari, Università degli Studi di Siena, Italy

***Correspondence:**
Francisco Sánchez-Madrid
fsmadrid@salud.madrid.org

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *18 July 2018*
Accepted: *30 August 2018*
Published: *25 September 2018*

Citation:
Rodríguez-Galán A, Fernández-Messina L and Sánchez-Madrid F (2018) Control of Immunoregulatory Molecules by miRNAs in T Cell Activation. Front. Immunol. 9:2148. doi: 10.3389/fimmu.2018.02148

INTRODUCTION

MiRNAs are small (∼19–24 nucleotides) single-stranded non-coding RNA species that act as post-transcriptional modulators; they control gene expression, either by promoting mRNAs degradation or repressing their translation (1). More than 2,500 human mature miRNA sequences have been already listed in MirBase (2) although the total amount of miRNAs is likely up to 10 times higher (3). Friedman et al. (4) estimated that miRNAs could modulate around 60% of protein-coding genes, indicating the relevance of these regulatory pathways in gene expression.

The miRNA repertoire changes upon T cell activation (5–11). **Figure 1** summarizes miRNA species described to be either upregulated or downregulated upon T cell stimulation. Different studies have yielded data that may appear contradictory, likely due to T cell subset differences, the origin of the sample (murine or human) and the strategy of stimulation. Additional differences stem from the strategy used to evaluate miRNA expression, being arrays the most commonly employed technique, together with RT-qPCR and Northern Blot.

Despite variability, some trends are very consistent, including downregulation of miR-26a, miR-26b, miR-150, miR-181a, miR-223, and miR-342-3p; and upregulation of miR-155 and the miR-17∼92 cluster (particularly miR-17-5p, miR-18a-5p, and miR-19b). MiR-146a was downregulated in mouse T cells, but upregulated in human upon activation, while miR-31 behaved in the opposite way, suggesting the existence of species-specific regulatory mechanisms.

In addition to variations in miRNA expression, it would be essential to consider the total abundance of each miRNA in the cell. Interestingly, only 7 miRNAs accounted for around 60% of the total sequencing reads in $CD8^+$ T cells (8).

Beyond individual miRNA changes, it is important to highlight that miRNAs undergo a global downregulation upon stimulation. In this regard, almost three times higher total miRNA array

hybridization signal has been detected in mouse $CD8^+$ naive T cells compared to activated cells (8); similarly, an independent study found a significant downregulation of the total amount of miRNA in stimulated mouse and human $CD4^+$ T cells compared to non-stimulated controls (5).

LESSONS FROM MIRNA-DEFICIENT MODELS

Dicer is an RNase III endonuclease that controls miRNA biogenesis. It processes precursor miRNA (pre-miRNA) into mature miRNA forms (12–14). Constitutive Dicer KO mice display embryonic lethality (15), indicating the relevance of this enzyme in development. Lineage-specific Dicer-deficient models were therefore required to study the consequences of reduced miRNA function in a tissue-specific manner.

Dicer-deficient $CD4^+$ T cells were hyper-responsive to TCR stimulation and produced IL-2 in the absence of co-stimulation (16). After activation, $CD4^+$ Dicer-deficient mice showed reduced proliferation, higher levels of apoptosis and a bias towards Th1 differentiation and IFN-γ release (17). In Th1 differentiation, IFN-γ production and a decline in IL-2 secretion occurred earlier in Dicer-deficient than in wild-type $CD4^+$ T cells (17). Th2 cells presented reduced levels of GATA3 mRNA and failed to suppress IFN-γ expression (17). Consistently, similar phenotypes were observed in T cells lacking Drosha or its RNA-binding cofactor DGCR8, which form a complex responsible for primary miRNA transcript processing. Drosha-deficient naïve $CD4^+$ T cells differentiated into Th1 and Th2, but expressed higher levels of IFN-γ than control cells (18). Similarly, DGCR8-deficient T lymphocytes showed reduced proliferation and an increase in IFN-γ secretion (19). A number of very comprehensive reports have addressed the role of miRNAs in T cell differentiation (20–24). In this review, immunoregulatory molecules responsible for differentiation have been discussed when closely related to T cell activation events.

CD4-specific Dicer deficiency also affects the regulatory T cell compartment, impairing Tregs development in the thymus and reducing their numbers in peripheral lymphoid organs (25). In addition, deficient naïve $CD4^+$ T cells activated in the presence of TGF-β expressed significantly less FOXP3 than control cells (25). Besides, several studies have demonstrated that miRNA disruption in Treg cells leads to autoimmune diseases (18, 26, 27).

Dicer-deficient $CD8^+$ T lymphocytes responded more rapidly to activation *in vitro*, as indicated by faster CD69 up-regulation and an earlier proliferative response, although their survival was reduced after 2 days (28). $CD8^+$ Dicer KO cells also showed a delay in CD69 down-regulation after removal of the TCR-activating stimulus, suggesting a sustained activation of cytotoxic lymphocytes in the absence of miRNAs (28). Furthermore, $CD8^+$ Dicer-deficient cells failed to produce an efficient *in vivo* effector response, including lower proliferation and impaired cytokine production (IFN-γ and TNF-α) (28).

Models with impaired miRNA synthesis machinery highlight the importance of miRNAs as positive (booster) and/or negative (brake) regulators of T cell development and function, which is a major focus of this review (**Figure 2**).

MiR-146a mainly acts as a "brake" miRNA, as miR-146a-deficient mice develop chronic inflammation and autoimmunity (29). $CD4^+$ and $CD8^+$ T cells from miR-146a deficient mice display less apoptosis and increased proliferation, expression of activation markers (CD25 and CD69) and effector cytokines (IL2, IFN-γ, and IL-17A) (30). Likewise, miR-125b is another negative regulator of T cell function, contributing to the maintenance of the naïve state in human $CD4^+$ T cells, in which it appears at high levels (31). This effect is at least partly achieved via targeting key molecules for T cell activation, e.g., BLIMP-1, IL-2Rβ, IL-10Rα, and IFN-γ (31). Conversely, other miRNAs boost the immune response. For instance, miR-142-deficient mouse T cells showed reduced proliferation, deregulated cytokine expression and decreased secretion of pro-inflammatory cytokines such as IFN-γ, IL-17, and IL2 in response to activation (32, 33). Other examples of enhancer miRNAs are miR-155 and miR-17∼92; miR-155-depleted mice are immunodeficient (34), whereas miR-17∼92-deficient T cells exhibited reduced antitumoral responses (35).

IMMUNOREGULATORY MOLECULES AS MIRNA TARGETS

T cell activation requires that the TCR recognizes a specific antigen bound to the MHC on the surface of an APC in the presence of co-stimulation. PI3K, AKT and mTOR are crucial mediators of T cell activation. Their positive signaling, downstream the TCR, is counter-balanced by negative regulators such as PTEN and BIM. Costimulatory signals are provided by surface receptors expressed on T lymphocytes that interact with specific ligands on APCs, and can be either activating (such as CD28 and ICOS) or inhibitory (like CTLA-4 and PD-1). These activating and inhibitory events are integrated into a net response that triggers the activation and/or repression of transcription factors (NFAT, AP-1, NF-κB, and others). Their nuclear localization promotes the synthesis of immune effector molecules, e.g., cytokines. MiRNAs also control the activation and integration of these pathways to support T cell effector functions while maintaining immune homeostasis. Herein, we review the miRNA-mediated regulation of key molecules involved in T cell activation.

Cell Survival and Signaling Molecules

BIM

The balance between BIM and BCL-2 molecules is essential for the fate of T lymphocytes, and their expression is tightly regulated by miRNAs, promoting either apoptosis or survival. BIM is a pro-apoptotic regulator and tumor suppressor downstream

Abbreviations: AKT3, v-akt murine thymoma viral oncogene homolog 3; APC, antigen-presenting cell; BIM, B-cell lymphoma 2 (Bcl-2) interacting mediator of cell death; CTLA-4, Cytotoxic T lymphocyte-associated antigen 4; GVHD, Graft versus host disease; IL, Interleukin; PD-1, Programmed Death 1; PI(3,4,5)P3, phosphatidylinositol-(3,4,5)-triphosphate; PI(4,5)P2, phosphatidylinositol-(4,5)-biphosphate; PTEN, phosphatase and tensin homolog; TCR, T-cell receptor; Tfh, T follicular helper; TGF-β, Transforming Growth Factor- β; Treg, regulatory T cell; tTreg, Thymic-derived regulatory T cells; UTR, untranslated region.

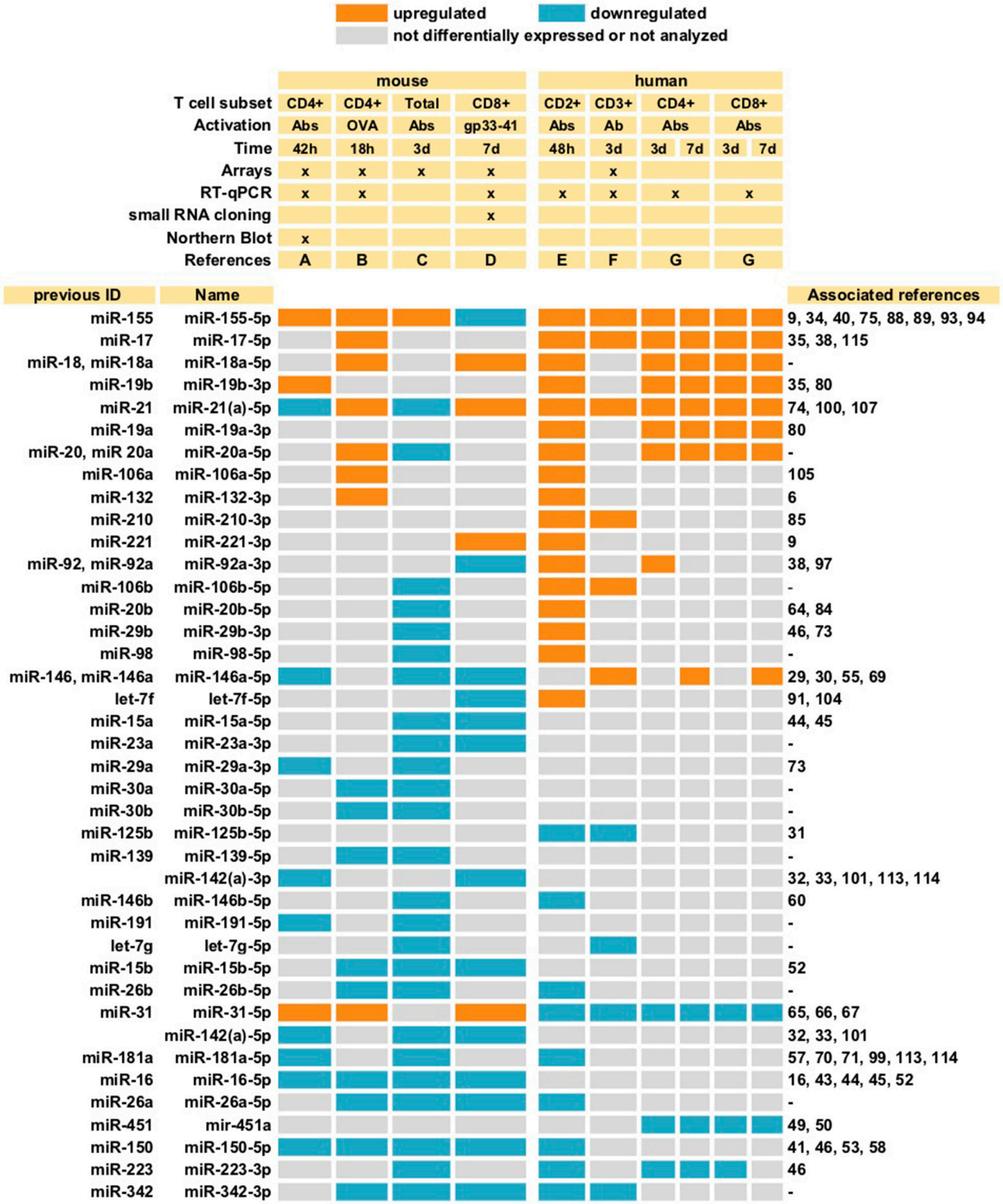


FIGURE 1 | MiRNAs differentially expressed upon T cell stimulation. MiRNAs described in at least two different studies are summarized. Different subsets of T cells (both mouse and human) were activated with either antibodies against CD3 alone (Ab), or together with antibody against CD28 (Abs), or with specific peptides (OVA or gp33-41). Cells were stimulated during different lengths of time ranging from 18 h (18 h) to 7 days (7 d). The studies included in the table are: A (5), B (6), C (7), D (8), E (9), F (10), G (11). Whenever more than one detection method was used, only consistent data obtained with at least two techniques was selected (8). Most studies evaluated miRNA expression with miRNAs arrays, some together with RT-qPCR and Northern Blot, as indicated (x).

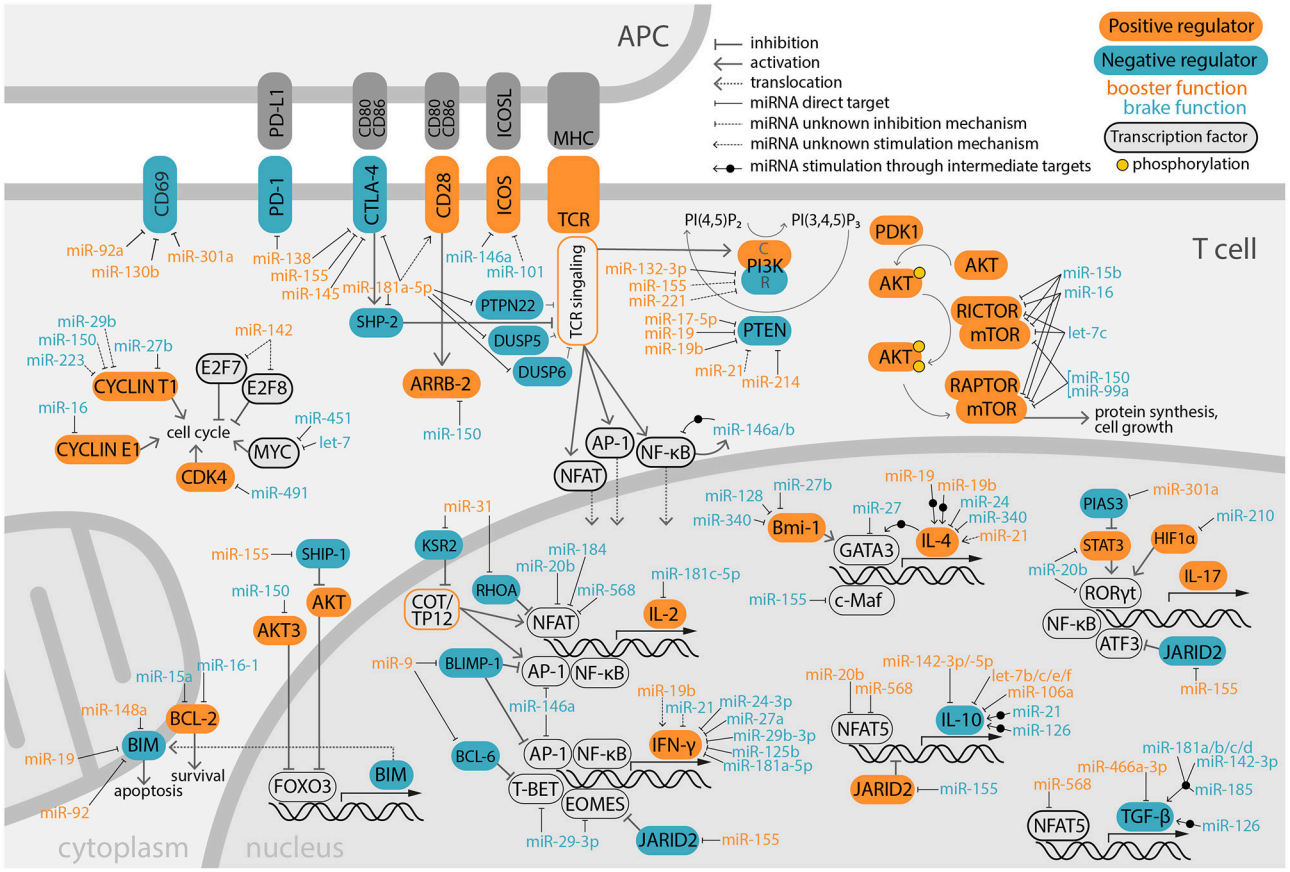


FIGURE 2 | Overview of miRNA modulation on positive and negative immune-regulator molecules. Signaling coming from TCR and costimulatory molecules is integrated by the T lymphocyte promoting cell survival, proliferation and production of effector molecules, such as cytokines. This complex network is fine-tuned by miRNAs that target key immunoregulatory molecules, supporting either T cell activation (booster) or inhibition (brake). MiRNAs exert their function by targeting the mRNA 3′UTR in the cytoplasm, although for simplicity sake some have been depicted in the nucleus, close to their targeted immunoregulators. In PI3K, C and R designated the catalytic and regulatory subunits, respectively.

of AKT3, an important mediator of TCR signaling (36, 37). It destabilizes mitochondrial membrane, inducing CASPASE-9 activation and apoptosis. Within the miR-17∼92 cluster, miR-19 and miR-92 target BIM 3′UTR mRNA (38). MiR-148a is upregulated in mouse Th1 cells after sustained activation (39). It also targets BIM, promoting cell survival (39). MiR-155 indirectly regulates BIM by targeting SHIP-1, which is a phosphatase that reduces AKT activity (40). In turn, AKT represses FOXO3, which is a transcription factor that promotes BIM expression, thus miR-155 limits BIM expression (40). Conversely, miR-150 promotes apoptosis by downregulating AKT3, which induces the accumulation of BIM (41). Human $CD4^+$ T cells with high levels of miR-150 display reduced proliferation, increased apoptosis and lower T cell activation (41).

BCL-2

BCL-2 is an anti-apoptotic protein that antagonizes BIM, stabilizing the mitochondrial membrane and preventing its permeabilization (42). Treatment of mice with experimental autoimmune encephalomyelitis with 3,3′-Diindolylmethane (a plant-derived anti-inflammatory compound), induced the upregulation of miR-16 in brain $CD4^+$ T cells and suppressed BCL-2; consistently, miR-16 overexpression in mouse $CD4^+$ T cells downregulated BCL-2 (43). Interestingly, $CD4^+$ T cells from relapsing-remitting multiple sclerosis patients (an autoimmune disease elicited by activated autoreactive T lymphocytes) displayed lower levels of miR-15a and miR-16, correlating with higher levels of their validated target BCL-2 mRNA (44, 45).

Cell Cycle Regulators

Molecules involved in cell cycle progression are essential mediators of T cell proliferation. miR-142-null T cells displayed gross cell cycle alterations, with cells differentially arrested in S and G_2/M phases (32). Cell-cycle defects were associated to the transcription factors E2F7 and E2F8, which are putative targets for miR-142. MiR-142 is likely responsible of maintaining low levels of both molecules in resting T-cells and limiting their increase upon activation. Treatment of mice with miR-142 antagomir markedly increased survival and reduced clinical

symptoms in a murine GVHD model, suggesting a potential new therapeutic strategy (32).

Cyclins are also directly targeted by miRNAs. Several miRNAs (miR-27b, miR-29b, miR-150, and miR-223) promote CYCLIN T1 downregulation in human resting $CD4^+$ T cells. The levels of these miRNAs decrease upon activation, correlating with an upregulation of CYCLIN T1 (46). MiR-16 downregulates CYCLIN E1 in mouse $CD4^+$ T cells (43). Another molecule involved in cell cycle progression is CDK4, a target of miR-491 in mouse $CD8^+$ T cells (47). MYC is a transcription factor involved in cell cycle and proliferation, is targeted by let-7 in mouse $CD8^+$ T cells (48) and by miR-451 in both mouse (49) and human (50) $CD4^+$ T cells.

mTOR

Mammalian Target Of Rapamycin (mTOR) is a metabolic regulator that promotes protein synthesis and cell growth during the onset of T lymphocyte function (51). mTOR kinase and Raptor are part of the complex mTORC1, while mTORC2 includes mTOR and Rictor. Both miR-16 and let-7c target the 3′UTR of mTOR and RICTOR (16). Elevated mTOR activity in Dicer-deficient $CD4^+$ T cells and the subsequently increased AKT phosphorylation is associated with a lower activation threshold, overcoming the need of co-stimulation. MiRNA-mediated mTOR down-regulation contributes to the correct discrimination of activating and anergic stimuli and prevents co-stimulation independent IL-2, IFN-γ and TNF-α overproduction (16). mTOR signaling suppression is relevant for Treg induction. In this regard, miR-16 and miR-15b, which are abundantly expressed in Tregs, target RICTOR and mTOR mRNAs (52). Furthermore, miR-150 and miR-99a cooperatively target mTOR, promoting Treg induction (53).

Co-stimulatory Molecules

Membrane Receptors: ICOS and CD28

Inducible co-stimulatory (ICOS) molecule and CD28 are surface receptors expressed on T cells that recognize specific ligands on APCs, acting as TCR signaling positive regulators (54). In germinal center responses, miR-146a upregulation in Tfh cells downregulates ICOS by interacting with its ligand on germinal center B cells, facilitating the termination of the immune response (55). MiR-101 is highly represented in human naïve $CD4^+$ T cells and its transfection into the EL4 murine T cell line downregulates ICOS (56). Regarding CD28, miR-181a-5p overexpression in mouse T cells increases its levels (57), whereas miR-150 limits CD28 co-stimulation by targeting the arrestin β-2 protein (ARRB-2), with a subsequent increase in cAMP levels and inhibition of LCK, PI3K and AKT (58).

Cytokines

MiRNA regulation of cytokine expression can be due to direct cytokine mRNA targeting or targeting of transcription factors such as NF-κB, NFAT, or AP-1 or their regulators, often affecting multiple cytokines. For example, miR-146a is induced in mouse $CD4^+$ and $CD8^+$ T cells upon TCR engagement through NF-κB (30). This miRNA provides negative feedback regulation, downregulating NF-κB by targeting TRAF6 and IRAK1 (30, 59). Compared to wild-type cells, both $CD4^+$ and $CD8^+$ mouse T cells lacking miR-146a exhibited a higher induction of genes regulated by NF-κB, e.g., BCL-2, CD25, CD69, IL-2, IFN-γ, and IL-17A (30). TRAF6 is also targeted by miR-146b in mouse Tregs (60).

IL-2

IL-2 is one of the main signatures of T cell activation. MiRNA-based IL-2 regulation relies on the inhibition of translation by miR-181c-5p (downregulated during T cell activation), which binds to the 3′UTR of IL-2 mRNA (61). It also depends on the miRNA-based downregulation of transcription factors such as NFAT or BLIMP-1. MiR-184 inhibits NFAT1 translation in human $CD4^+$ T cells. This is particularly relevant in cells isolated from umbilical cord blood (62). MiR-568 transfection into human $CD4^+$ T cells inhibited IL-2 expression after activation, through NFAT5 downregulation (63). MiR-20b also downregulated IL-2 through NFAT5 targeting (64). MiR-31 upregulates IL-2 by inhibiting RHOA, a small GTPase which suppresses NFAT (65, 66). It also targets the kinase suppressor of RAS2 (KSR2), which inhibits the COT/TPI2 signaling pathway (enhancer of IL-2 expression through NFAT and AP-1) (67). MiR-9 (upregulated in activated human $CD4^+$ T cells) targets BLIMP-1, de-repressing IL-2 transcription (68). MiR-146a is upregulated around 8 days after stimulation in human $CD4^+$ and $CD8^+$ T cells, impairing IL-2 production, by targeting AP-1 (69).

IFN-γ

IFN-γ release orchestrates Th1 immune responses by activating different cell lineages, e.g., dendritic cells, macrophages or NK cells. MiR-125b maintains T cell naïve state by targeting *IFN-γ* among other genes (31). Several miRNAs repress IFN-γ: miR-24-3p (70) and miR-181a-5p in human $CD4^+$ T cells (70, 71); miR-24 and miR-27a in activated human $CD8^+$ T cells (72); and miR-29 directly (73) and indirectly, by downregulating T-BET and EOMES, in mouse $CD4^+$ T cells (19). On the other hand, miR-19b is required for normal IFN-γ production, restoring IFN-γ expression in miR-17∼92-deficient mouse Th1 cells (35). MiR-9 suppresses BLIMP-1 and BCL-6 (repressors of AP-1 and T-BET, respectively), increasing IFN-γ secretion in activated human $CD4^+$ T cells (68). Murine miR-21 KO $CD4^+$ T cells re-stimulated *in vitro* produced more IFN-γ (74). Moreover, IFN-γ responsiveness is regulated by miR-155, which targets IFN-γRα in activated mouse $CD4^+$ T cells, contributing to Th1 differentiation (75).

IL-4

T cell activation stimulates the production of IL-4, leading to Th2 responses (76, 77). Its release is controlled directly by miR-24 [78] and miR-340 (78), or through the targeting of specific transcription factors and kinases/phosphatases. IL-4 triggers the upregulation of GATA3 dependent STAT6, repressing Th1 differentiation and inducing IL-4 production in a positive feedback loop. Conversely, MiR-27 targets the transcription factor GATA3 (79). BMI1 binds to GATA3, preventing its degradation. $CD4^+$ T cells from MS patients display increased expression of miR-27b, miR-128 and miR-340 (78). These

miRNAs inhibited Th2 development by targeting BMI1 (78). MiR-155 targets the 3′UTR of c-MAF mRNA, which is another transcription factor involved in IL-4 expression (34). MiR-21 contributes to IL-4 expression, since *in vitro* re-stimulated miR-21-null mouse $CD4^+$ T cells produced less IL-4 than wild-type cells (74). Both miR-19a and miR-19b rescued IL-4 production in miR-17∼92 cluster-deficient cells by targeting PTEN, SOCS1 and A20 (80).

IL-17

TCR signaling promotes expression of the proinflammatory cytokine IL-17 (81–83). IL-17 expression depends on the transcription factor RORγt downstream of STAT3. miR-20b targets both molecules in mouse $CD4^+$ T cells (84). RORγt transcription is promoted by HIF-1α, which is targeted by miR-210 (85). In turn, STAT3 is inhibited by the E3 SUMO-protein ligase PIAS3, a target of miR-301a that increases IL-17 secretion (86). MiR-212 targets BCL-6 3′UTR, which is a repressor of Th17 differentiation (87). JARID2, a chromatin-binding protein, recruits the polycomb repressive complex 2 (PRC2) and silences transcription of IL22, IL10, ATF3, TBX21, or EOMES through histone methylation (88). MiR-155 inhibits JARID2, releasing the repression of ATF3, which promotes IL-17 (88). ETS-1, a transcription factor that inhibits Th17 differentiation, is a target of miR-155 (89) and miR-326 (90). Li et al. (91) reported IL-17 downregulation due to IL-23R inhibition by let-7f.

Inhibitory Molecules

Membrane Receptors: CTLA-4, PD-1, CD69

CTLA-4 and PD-1 are both co-inhibitory receptors that repress TCR signaling via binding to co-stimulators expressed by APCs (54). CTLA-4 (a target of miR-145) is very abundant in human peripheral blood Tregs, in which miR-145 is downregulated (92). MiR-155 also targeted CTLA-4 in mouse (93) and human (94) $CD4^+$ T cells. MiR-155 overexpression in human $CD4^+$ T cells promoted proliferation, and could underlie chronic inflammation in atopic dermatitis, in which it is highly expressed also by $CD4^+$ T cells present in skin lesions (94). MiR-138 targets CTLA-4 and PD-1, promoting tumor-regression by inhibiting tumor-infiltrating Tregs (95). MiR-181a-5p overexpression in mouse T cells decreased CTLA-4 expression, while increasing CD28 levels (57).

CD69 is an early surface marker of lymphocyte activation (96). Dicer KO $CD8^+$ T cells up-regulated CD69 more rapidly upon stimulation and retained the expression longer after stimuli removal (28), indicating a potential miRNA-based repression of CD69 in naïve stages that restrains activation. MiR-130b and miR-301a increased their levels during $CD8^+$ T cell activation and downregulated CD69 (28). MiR-92, which is downregulated in lamina propria leukocytes from rhesus macaques with chronic simian immunodeficiency virus infection, also targets the 3′UTR of CD69 mRNA (97).

Kinases and Phosphatases

TCR signaling is mediated by downstream kinases and phosphatases, which undergo a tight regulation that ensures functional activation while avoiding hyperreactivity.

PI3K regulatory subunits

Upon TCR and co-receptors engagement, PI3K phosphorylates $PI(4,5)P_2$. *PIK3R1* gene encodes the regulatory subunits p85, p50, and p55 (98). MiRNAs upregulated in $CD4^+$ activated human T cells, e.g., miR-155 and miR-221 downregulate PIK3R1 (9). MiR-132-3p is upregulated in mouse dendritic cell-activated $CD4^+$ T lymphocytes, targeting PIK3R1 mRNA (6).

TCR Inhibitory phosphatases

Phosphatases downstream the TCR pathway counteract signaling by dephosphorylation. Downregulation of some of these phosphatases by miR-181a-5p generates high levels of phosphorylated intermediates in steady-state (57). MiR-181a-5p targets the phosphatases PTPN22, DUSP5 and DUSP6, which dephosphorylate LCK, ZAP70, and ERK1/2; and SHP-2, which mediates negative costimulatory signals from CTLA-4 (57). Therefore, the expression of this miRNA contributes to reduce the activation threshold, increasing the strength and sensitivity of the T cell to peptides with lower affinity (57). In elderly individuals, reduced expression of miR-181a in $CD4^+$ naïve T cells is a cause of the declined T cell responsiveness associated with age (99).

PTEN

PTEN dephosphorylates $PI(3,4,5)P_3$, antagonizing PI3K. As such, PTEN curbs T cell activation, preserving self-tolerance. Transgenic mice overexpressing miR-17∼92 cluster developed lymphoproliferative and autoimmune pathologies associated to the reduced expression of PTEN and BIM (38). PTEN is downregulated by several miRNAs that are increased upon T cell activation: miR-21 (100), miR-214 (7) and the miR-17∼92 cluster [miR-17-5p (38), miR-19 (38), and miR-19b (35)]. Consistently, miR-21 and miR-214 expression increased T cell proliferation (7, 100).

Cytokines

IL-10

IL-10 is an important anti-inflammatory cytokine mainly produced by Th2 and Tregs. It counteracts CD28 signaling and suppresses the expression of IFN-γ and IL-2. IL-10 is directly targeted by miR-142-3p, miR-142-5p (101), miR-let-7e (102), let-7c (103, 104), let-7b (104), let-7f (104), and miR-106a (105). MiRNAs further regulate IL-10 post-transcriptionally by modulating JARID2, NFAT5, p85-β or the programmed cell death protein 4 (PDCD4). JARID2 silences IL-10 and is a target of miR-155, which thus promotes IL-10 expression (88). MiR-568 (downregulated upon human $CD4^+$ T cell activation) reduced IL-10 by targeting NFAT5 (63). NFAT5 was also targeted by miR-20b (64). MiR-126 is highly increased after Treg stimulation and promotes IL-10 expression (106), and miR-126 targeting of p85-β and PI3K/AKT pathway modulation is responsible of IL-10 release (106). MiR-21 is upregulated in $CD4^+$ T cells from systemic lupus erythematosus patients, and its inhibition led to a decrease in IL-10 production (107). MiR-21 positive regulation of IL-10 secretion likely depends on its targeting of PDCD4, a translation inhibitor (107).

TGF-β

TGF-β is expressed in naïve T cells preventing T cell activation until sufficient TCR stimulation downregulates the TGF-β type 1 receptor (108–110). TGF-β induces FOXP3, a key transcription factor that promotes Treg differentiation (111). In addition to IL-10 modulation, miR-568 (63) and miR-126 (106) also regulate TGF-β release. In $CD4^+$ mouse T cells from draining lymph nodes, miR-466a-3p (upregulated in mice after skin allograft) targets TGF-β2, limiting Treg generation (112). MiRNAs also regulate TGF-β function at different levels by targeting upstream molecules involved in cytokine production, TGF-β receptors and effector molecules of the TGF-β signaling pathway. GARP is a transmembrane protein specifically expressed in Tregs that cleaves the precursor form of TGF-β1 (113). GARP is targeted by miRNAs which are less abundant in human Tregs than in T helper subsets, e.g., miR-142-3p, miR-185, and miR-181a/b/c/d (113, 114). MiR-17 targets TGFBR2 (TGF-β receptor II) in mouse and human $CD4^+$ T cells (35, 115). In addition, it has been found that a set of miRNAs upregulated in naïve $CD4^+$ T cells from multiple sclerosis patients target TGFBR1 and/or SMAD4 (both involved in the TGF-β signaling pathway) limiting differentiation into Tregs (116).

CONCLUDING REMARKS

MiRNA-mediated modulation of molecules involved in T cell activation remains far from being fully understood, although strides have been made in recent years. There is a need to advance towards a "network study" of miRNA function. Considering more than one miRNA in experimental designs increases its technical complication, but also enables models that simulate the complexity of the physiological scenarios, in which individual miRNAs interact with a set of targets and each target in turn can be regulated by several miRNAs, at different levels, either directly targeting the molecule or indirectly regulating its expression via targeting its receptor and/or transcription factors.

Finally, integrating basic and clinical research (e.g., cancer, autoimmunity, and GVHD) could help to achieve a better understanding of T cell immune-regulation to design new strategies for therapy in T cell related malignancies.

AUTHOR CONTRIBUTIONS

AR-G wrote the draft manuscript and designed the Figures. LF-M corrected and edited the manuscript. FS-M edited the manuscript. AR-G, LF-M, and FS-M discussed all the items in the manuscript.

ACKNOWLEDGMENTS

We thank Dr M. Vicente-Manzanares for critical reading of the manuscript and for assistance with English editing. This study was supported by the following grants from the Spanish Ministry of Economy and Competitiveness, (grant SAF2017-82886-R to FSM), CIBER CARDIOVASCULAR and PIE 13.0004-BIOIMID from the Instituto de Salud Carlos III (Fondo de Investigación Sanitaria del Instituto de Salud Carlos III with co-funding from the Fondo Europeo de Desarrollo Regional; FEDER), Programa de Actividades en Biomedicina de la Comunidad de Madrid-B2017/BMD-3671-INFLAMUNE to FS-M, and ERC-2011-AdG294340-GENTRIS to FS-M. The Centro Nacional de Investigaciones Cardiovasculares (CNIC) is supported by the Spanish Ministry of Economy and Competitiveness (MINECO) and the Pro-CNIC Foundation and is a Severo Ochoa Center of Excellence (MINECO award SEV-2015-0505). AR-G is supported by the FPU program (Spanish Ministry of Education). LF-M is funded by the CIBER CARDIOVASCULAR.

REFERENCES

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* (2004) 116:281–97. doi: 10.1016/S0092-8674(04)00045-5
2. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. *Nucleic Acids Res.* (2008) 36:D154–8. doi: 10.1093/nar/gkm952
3. Londin E, Loher P, Telonis AG, Quann K, Clark P, Jing Y, et al. Analysis of 13 cell types reveals evidence for the expression of numerous novel primate- and tissue-specific microRNAs. *Proc Natl Acad Sci USA*. (2015) 112:E1106–15. doi: 10.1073/pnas.1420955112
4. Friedman RC, Farh KKH, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* (2009) 19:92–105. doi: 10.1101/gr.082701.108
5. Bronevetsky Y, Villarino AV, Eisley CJ, Barbeau R, Barczak AJ, Heinz GA, et al. T cell activation induces proteasomal degradation of Argonaute and rapid remodeling of the microRNA repertoire. *J Exp Med*. (2013) 210:417–32. doi: 10.1084/jem.20111717
6. Gutiérrez-Vázquez C, Rodríguez-Galán A, Fernández-Alfara M, Mittelbrunn M, Sánchez-Cabo F, Martínez-Herrera DJ, et al. miRNA profiling during antigen-dependent T cell activation: a role for miR-132-3p. *Sci Rep*. (2017) 7:3508. doi: 10.1038/s41598-017-03689-7
7. Jindra PT, Bagley J, Godwin JG, Iacomini J. Costimulation-dependent expression of MicroRNA-214 increases the ability of T cells to proliferate by targeting Pten. *J Immunol*. (2010) 185:990–7. doi: 10.4049/jimmunol.1000793
8. Wu H, Neilson JR, Kumar P, Manocha M, Shankar P, Sharp PA, et al. miRNA Profiling of Naïve, Effector and Memory CD8 T Cells. *PLoS ONE* (2007) 2:e1020. doi: 10.1371/journal.pone.0001020
9. Grigoryev YA, Kurian SM, Hart T, Nakorchevsky AA, Chen C, Campbell D, et al. MicroRNA regulation of molecular networks mapped by global MicroRNA, mRNA, and Protein Expression in Activated T Lymphocytes. *J Immunol*. (2011) 187:2233–43. doi: 10.4049/jimmunol.1101233
10. Sousa IG, do Almo MM, Simi KCR, Bezerra MAG, Andrade RV, Maranhão AQ, et al. MicroRNA expression profiles in human CD3+ T cells following stimulation with anti-human CD3 antibodies. *BMC Res Notes* (2017) 10:124. doi: 10.1186/s13104-017-2442-y
11. Teteloshvili N, Smigielska-Czepiel K, Kroesen B-J, Brouwer E, Kluiver J, Boots A, et al. T-cell Activation Induces Dynamic Changes in miRNA Expression Patterns in CD4 and CD8 T-cell Subsets. *MicroRNA* (2015) 4:117–22. doi: 10.2174/2211536604666150819194636
12. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for bidentate ribnuclease in the initiation site of RNA interference. *Nature* (2001) 409:363–6. doi: 10.1038/35053110
13. Hutvagner G. A cellular function for the RNA-interference enzyme dicer in the maturation of the let-7 small temporal RNA. *Science (80-)* (2001) 293:834–8. doi: 10.1126/science.1062961

14. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, et al. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell*. (2001) 106:23–34. doi: 10.1016/S0092-8674(01)00431-7
15. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, et al. Dicer is essential for mouse development. *Nat Genet*. (2003) 35:215–7. doi: 10.1038/ng1253
16. Marcais A, Blevins R, Graumann J, Feytout A, Dharmalingam G, Carroll T, et al. microRNA-mediated regulation of mTOR complex components facilitates discrimination between activation and anergy in CD4 T cells. *J Exp Med*. (2014) 211:2281–95. doi: 10.1084/jem.20132059
17. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K. Aberrant T cell differentiation in the absence of Dicer. *J Exp Med*. (2005) 202:261–9. doi: 10.1084/jem.20050678
18. Chong MMW, Rasmussen JP, Rudensky AY, Littman DR. The RNAseIII enzyme drosha is critical in T cells for preventing lethal inflammatory disease. *J Exp Med*. (2008) 205:2005–17. doi: 10.1084/jem.20071219090508c
19. Steiner DF, Thomas MF, Hu JK, Yang Z, Babiarz JE, Allen CDC, et al. MicroRNA-29 Regulates T-Box transcription factors and interferon-γ production in helper T cells. *Immunity* (2011) 35:169–81. doi: 10.1016/j.immuni.2011.07.009
20. Kroesen B-J, Teteloshvili N, Smigielska-Czepiel K, Brouwer E, Boots AMH, van den Berg A, et al. Immuno-miRs: critical regulators of T-cell development, function and ageing. *Immunology* (2015) 144:1–10. doi: 10.1111/imm.12367
21. Jeker LT, Bluestone JA. MicroRNA regulation of T-cell differentiation and function. *Immunol Rev*. (2013) 253:65–81. doi: 10.1111/imr.12061
22. Liu J, Wu C-P, Lu B-F, Jiang J-T. Mechanism of T cell regulation by microRNAs. Cancer *Biol Med*. (2013) 10:131–7. doi: 10.7497/j.issn.2095-3941.2013.03.002
23. Baumjohann D, Ansel KM. MicroRNA-mediated regulation of T helper cell differentiation and plasticity. *Nat Rev Immunol*. (2013) 13:666–78. doi: 10.1038/nri3494
24. Podshivalova K, Salomon DR. MicroRNA regulation of T-lymphocyte immunity: modulation of molecular networks responsible for T-cell activation, differentiation, and development. *Crit Rev Immunol*. (2013) 33:435–76. doi: 10.1615/CritRevImmunol.2013006858
25. Cobb BS, Hertweck A, Smith J, O'Connor E, Graf D, Cook T, et al. A role for Dicer in immune regulation. *J Exp Med*. (2006) 203:2519–27. doi: 10.1084/jem.20061692
26. Liston A, Lu L-F, O'Carroll D, Tarakhovsky A, Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. *J Exp Med*. (2008) 205:1993–2004. doi: 10.1084/jem.20081062
27. Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, et al. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med*. (2008) 205:1983–91. doi: 10.1084/jem.20080707
28. Zhang N, Bevan MJ. Dicer controls CD8+ T-cell activation, migration, and survival. *Proc Natl Acad Sci USA*. (2010) 107:21629–34. doi: 10.1073/pnas.1016299107
29. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med*. (2011) 208:1189–201. doi: 10.1084/jem.20101823
30. Yang L, Boldin MP, Yu Y, Liu CS, Ea C-K, Ramakrishnan P, et al. miR-146a controls the resolution of T cell responses in mice. *J Exp Med*. (2012) 209:1655–70. doi: 10.1084/jem.20112218
31. Rossi RL, Rossetti G, Wenandy L, Curti S, Ripamonti A, Bonnal RJP, et al. Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b. *Nat Immunol*. (2011) 12:796–803. doi: 10.1038/ni.2057
32. Sun Y, Oravecz-Wilson K, Mathewson N, Wang Y, McEachin R, Liu C, et al. Mature T cell responses are controlled by microRNA-142. *J Clin Invest*. (2015) 125:2825–40. doi: 10.1172/JCI78753
33. Mildner A, Chapnik E, Varol D, Aychek T, Lampl N, Rivkin N, et al. MicroRNA-142 controls thymocyte proliferation. *Eur J Immunol*. (2017) 47:1142–52. doi: 10.1002/eji.201746987
34. Rodriguez A, Vigorito E, Clare S, Warren M V, Couttet P, Soond DR, et al. Requirement of bic/microRNA-155 for normal immune function. *Science (80-)*. (2007) 316:608–11. doi: 10.1126/science.1139253
35. Jiang S, Li C, Olive V, Lykken E, Feng F, Sevilla J, et al. Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. *Blood* (2011) 118:5487–97. doi: 10.1182/blood-2011-05-355644
36. Hildeman DA, Zhu Y, Mitchell TC, Bouillet P, Strasser A, Kappler J, et al. Activated T cell death *in vivo* mediated by proapoptotic Bcl-2 family member Bim. *Immunity* (2002) 16:759–67. doi: 10.1016/S1074-7613(02)00322-9
37. Reynolds C, Roderick JE, LaBelle JL, Bird G, Mathieu R, Bodaar K, et al. Repression of BIM mediates survival signaling by MYC and AKT in high-risk T-cell acute lymphoblastic leukemia. *Leukemia* (2014) 28:1819–27. doi: 10.1038/leu.2014.78
38. Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat Immunol*. (2008) 9:405–14. doi: 10.1038/ni1575
39. Haftmann C, Stittrich AB, Zimmermann J, Fang Z, Hradilkova K, Bardua M, et al. MiR-148a is upregulated by Twist1 and T-bet and promotes Th1-cell survival by regulating the proapoptotic gene Bim. *Eur J Immunol*. (2015) 45:1192–205. doi: 10.1002/eji.201444633
40. Rouquette-Jazdanian AK, Kortum RL, Li W, Merrill RK, Nguyen PH, Samelson LE, et al. miR-155 controls lymphoproliferation in LAT mutant mice by restraining T-cell apoptosis via SHIP-1/mTOR and PAK1/FOXO3/BIM pathways. *PLoS ONE* (2015) 10:1–27. doi: 10.1371/journal.pone.0131823
41. Sang W, Sun C, Zhang C, Zhang D, Wang Y, Xu L, et al. MicroRNA-150 negatively regulates the function of CD4+T cells through AKT3/Bim signaling pathway. *Cell Immunol*. (2016) 306–307:35–40. doi: 10.1016/j.cellimm.2016.05.007
42. Guerrero AD, Welschhans RL, Chen M, Wang J. Cleavage of Anti-Apoptotic Bcl-2 Family Members after TCR Stimulation Contributes to the Decision between T Cell Activation and Apoptosis. *J Immunol*. (2013) 190:168–73. doi: 10.4049/jimmunol.1201610
43. Rouse M, Rao R, Nagarkatti M, Nagarkatti PS. 3,3'-Diindolylmethane ameliorates experimental autoimmune encephalomyelitis by promoting cell cycle arrest and apoptosis in activated T cells through MicroRNA signaling pathways. *J Pharmacol Exp Ther*. (2014) 350:341–52. doi: 10.1124/jpet.114.214742
44. Lorenzi JCC, Brum DG, Zanette DL, de Paula Alves Souza A, Barbuzano FG, dos Santos AC, et al. miR-15a and 16-1 Are Downregulated in $CD4^+$ T Cells of Multiple Sclerosis Relapsing Patients. *Int J Neurosci*. (2012) 122:466–71. doi: 10.3109/00207454.2012.678444
45. Cimmino A, Calin GA, Fabbri M, Iorio M V., Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA*. (2005) 102:13944–9. doi: 10.1073/pnas.0506654102
46. Chiang K, Sung T-L, Rice AP. Regulation of Cyclin T1 and HIV-1 Replication by MicroRNAs in Resting $CD4^+$ T Lymphocytes. *J Virol*. (2012) 86:3244–52. doi: 10.1128/JVI.05065-11
47. Yu T, Zuo Q-F, Gong L, Wang L-N, Zou Q-M, Xiao B. MicroRNA-491 regulates the proliferation and apoptosis of $CD8^+$ T cells. *Sci Rep*. (2016) 6:30923. doi: 10.1038/srep30923
48. Wells AC, Daniels KA, Angelou CC, Fagerberg E, Burnside AS, Markstein M, et al. Modulation of let-7 miRNAs controls the differentiation of effector CD8 T cells. *Elife*. (2017) 6:e26398. doi: 10.7554/eLife.26398
49. Chapman LM, Ture SK, Field DJ, Morrell CN. miR-451 limits $CD4^+$ T cell proliferative responses to infection in mice. *Immunol Res*. (2017) 65:828–40. doi: 10.1007/s12026-017-8919-x
50. Zeng Z, Wang K, Li Y, Xia N, Nie S, Lv B, et al. Down-regulation of microRNA-451a facilitates the activation and proliferation of $CD4^+$ T cells by targeting Myc in patients with dilated cardiomyopathy. *J Biol Chem*. (2017) 292:6004–13. doi: 10.1074/jbc.M116.765107
51. Liu Y, Zhang D, Liu X. mTOR Signaling in T Cell Immunity and Autoimmunity. *Int Rev Immunol*. (2015) 34:50–66. doi: 10.3109/08830185.2014.933957
52. Singh Y, Garden OA, Lang F, Cobb BS. MicroRNA-15b/16 enhances the induction of regulatory T cells by regulating the expression of rictor and mTOR. *J Immunol*. (2015) 195:5667–77. doi: 10.4049/jimmunol.1401875

53. Warth SC, Hoefig KP, Hiekel A, Schallenberg S, Jovanovic K, Klein L, et al. Induced miR-99a expression represses Mtor cooperatively with miR-150 to promote regulatory T-cell differentiation. *Embo J*. (2015) 34:1195–213. doi: 10.15252/embj.201489589
54. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol*. (2013) 13:227–42. doi: 10.1038/nri3405
55. Pratama A, Srivastava M, Williams NJ, Papa I, Lee SK, Dinh XT, et al. MicroRNA-146a regulates ICOS-ICOSL signalling to limit accumulation of T follicular helper cells and germinal centres. *Nat Commun.* (2015) 6:1–14. doi: 10.1038/ncomms7436
56. Di Yu, Tan AHM, Hu X, Athanasopoulos V, Simpson N, Silva DG, et al. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* (2007) 450:299–303. doi: 10.1038/nature06253
57. Li Q-J, Chau J, Ebert PJR, Sylvester G, Min H, Liu G, et al. miR-181a Is an Intrinsic Modulator of T Cell Sensitivity and Selection. *Cell* (2007) 129:147–61. doi: 10.1016/j.cell.2007.03.008
58. Sang W, Wang Y, Zhang C, Zhang D, Sun C, Niu M, et al. MiR-150 impairs inflammatory cytokine production by targeting ARRB-2 after blocking CD28/B7 costimulatory pathway. *Immunol Lett*. (2016) 172:1–10. doi: 10.1016/j.imlet.2015.11.001
59. Taganov KD, Boldin MP, Chang K-J, Baltimore D. NF- B-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci. USA* (2006) 103:12481–6. doi: 10.1073/pnas.0605298103
60. Lu Y, Hippen KL, Lemire AL, Gu J, Wang W, Ni X, et al. miR-146b antagomir-treated human Tregs acquire increased GVHD inhibitory potency. *Blood*. (2016) 128:1424–35. doi: 10.1182/blood-2016-05-714535
61. Xue Q, Guo ZY, Li W, Wen WH, Meng YL, Jia LT, et al. Human activated $CD4^+$ T lymphocytes increase IL-2 expression by downregulating microRNA-181c. *Mol Immunol.* (2011) 48:592–9. doi: 10.1016/j.molimm.2010.10.021
62. Weitzel RP, Lesniewski ML, Haviernik P, Kadereit S, Leahy P, Greco NJ, et al. microRNA 184 regulates expression of NFAT1 in umbilical cord blood $CD4^+$ T cells. *Blood*. (2009) 113:6648–57. doi: 10.1182/blood-2008-09-181156
63. Li W, Kong L, Li J-T, Guo Z-Y, Xue Q, Yang T, et al. MiR-568 inhibits the activation and function of $CD4^+$ T cells and Treg cells by targeting NFAT5. *Int Immunol.* (2014) 26:269–81. doi: 10.1093/intimm/dxt065
64. Xin Y, Cai H, Lu T, Zhang Y, Yang Y, Cui Y. miR-20b Inhibits T cell proliferation and activation via NFAT Signaling Pathway in Thymoma-Associated Myasthenia Gravis. *Biomed Res Int.* (2016) 2016:9595718. doi: 10.1155/2016/9595718
65. Helms WS, Jeffrey JL, Holmes DA, Townsend MB, Clipstone NA, Su L. Modulation of NFAT-dependent gene expression by the RhoA signaling pathway in T cells. *J Leukoc Biol.* (2007) 82:361–9. doi: 10.1189/jlb.0206120
66. Fan W, Liang D, Tang Y, Qu B, Cui H, Luo X, et al. Identification of microRNA-31 as a novel regulator contributing to impaired interleukin-2 production in T cells from patients with systemic lupus erythematosus. *Arthritis Rheum*. (2012) 64:3715–25. doi: 10.1002/art.34596
67. Xue F, Li H, Zhang J, Lu J, Xia Y, Xia Q. MiR-31 regulates interleukin 2 and kinase suppressor of ras 2 during T cell activation. *Genes Immun*. (2013) 14:127–31. doi: 10.1038/gene.2012.58
68. Thiele S, Wittmann J, Jäck H-M, Pahl A. miR-9 enhances IL-2 production in activated human $CD4^+$ T cells by repressing Blimp-1. *Eur J Immunol.* (2012) 42:2100–8. doi: 10.1002/eji.201142203
69. Curtale G, Citarella F, Carissimi C, Goldoni M, Carucci N, Fulci V, et al. An emerging player in the adaptive immune response: microRNA-146a is a modulator of IL-2 expression and activation-induced cell death in T lymphocytes. *Blood* (2010) 115:265–73. doi: 10.1182/blood-2009-06-225987
70. Fayyad-Kazan H, Hamade E, Rouas R, Najar M, Fayyad-Kazan M, El Zein N, et al. Downregulation of microRNA-24 and−181 parallels the upregulation of IFN-γ secreted by activated human CD4 lymphocytes. *Hum Immunol.* (2014) 75:677–85. doi: 10.1016/j.humimm.2014.01.007
71. Sang W, Zhang C, Zhang D, Wang Y, Sun C, Niu M, et al. MicroRNA-181a, a potential diagnosis marker, alleviates acute graft versus host disease by regulating IFN-γ production. *Am J Hematol.* (2015) 90:998–1007. doi: 10.1002/ajh.24136
72. Chandran PA, Keller A, Weinmann L, Adel Seida A, Braun M, Andreev K, et al. The TGF-β-inducible miR-23a cluster attenuates IFN- levels and antigen-specific cytotoxicity in human $CD8^+$ T cells. *J Leukoc Biol.* (2014) 96:633–45. doi: 10.1189/jlb.3A0114-025R
73. Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, et al. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-γ. *Nat Immunol.* (2011) 12:861–9. doi: 10.1038/ni.2073
74. Lu TX, Hartner J, Lim E-J, Fabry V, Mingler MK, Cole ET, et al. MicroRNA-21 limits *in vivo* immune response-mediated activation of the IL-12/IFN- Pathway, Th1 Polarization, and the severity of delayed-type hypersensitivity. *J Immunol*. (2011) 187:3362–73. doi: 10.4049/jimmunol.1101235
75. Banerjee A, Schambach F, DeJong CS, Hammond SM, Reiner SL. Micro-RNA-155 inhibits IFN-γ signaling in $CD4^+$ T cells. *Eur J Immunol.* (2009) 40:225–31. doi: 10.1002/eji.200939381
76. McAdam AJ, Chang TT, Lumelsky AE, Greenfield EA, Boussiotis VA, Duke-Cohan JS, et al. Mouse Inducible Costimulatory Molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of $CD4^+$ T cells. *J Immunol.* (2000) 165:5035–40. doi: 10.4049/jimmunol.165.9.5035
77. Milner JD. TCR signaling abnormalities in human Th2-associated atopic disease. *Front Immunol.* (2018) 9:2–6. doi: 10.3389/fimmu.2018.00719
78. Guerau-De-Arellano M, Smith KM, Godlewski J, Liu Y, Winger R, Lawler SE, et al. Micro-RNA dysregulation in multiple sclerosis favours pro-inflammatory T-cell-mediated autoimmunity. *Brain* (2011) 134:3575–86. doi: 10.1093/brain/awr262
79. Cho S, Wu C-J, Yasuda T, Cruz LO, Khan AA, Lin L-L, et al. miR-23∼27∼24 clusters control effector T cell differentiation and function. *J Exp Med.* (2016) 213:235–49. doi: 10.1084/jem.20150990
80. Simpson LJ, Patel S, Bhakta NR, Choy DF, Brightbill HD, Ren X, et al. A microRNA upregulated in asthma airway T cells promotes TH2 cytokine production. *Nat Immunol*. (2014) 15:1162–70. doi: 10.1038/ni.3026
81. Chen Z, Tato CM, Muul L, Laurence A, O'Shea JJ. Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis Rheum*. (2007) 56:2936–46. doi: 10.1002/art.22866
82. Gomez-Rodriguez J, Sahu N, Handon R, Davidson TS, Anderson SM, Kirby MR, et al. Differential Expression of Interleukin-17A and−17F is coupled to T Cell Receptor Signaling via Inducible T Cell Kinase. *Immunity* (2009) 31:587–97. doi: 10.1016/j.immuni.2009.07.009
83. Purvis HA, Stoop JN, Mann J, Woods S, Kozijn AE, Hambleton S, et al. Low-strength T-cell activation promotes Th17 responses. *Blood* (2016) 116:4829–38. doi: 10.1182/blood-2010-03-272153
84. Zhu E, Wang X, Zheng B, Wang Q, Hao J, Chen S, et al. miR-20b Suppresses Th17 differentiation and the pathogenesis of experimental autoimmune encephalomyelitis by targeting ROR t and STAT3. *J Immunol.* (2014) 192:5599–609. doi: 10.4049/jimmunol.1303488
85. Wang H, Flach H, Onizawa M, Wei L, Mcmanus MT, Weiss A. Negative regulation of Hif1a expression and TH 17 differentiation by the hypoxia-regulated microRNA miR-210. *Nat Immunol.* (2014) 15:393–401. doi: 10.1038/ni.2846
86. Mycko MP, Cichalewska M, Machlanska A, Cwiklinska H, Mariasiewicz M, Selmaj KW. MicroRNA-301a regulation of a T-helper 17 immune response controls autoimmune demyelination. *Proc Natl Acad Sci USA*. (2012) 109:E1248–57. doi: 10.1073/pnas.1114325109
87. Nakahama T, Hanieh H, Nguyen NT, Chinen I, Ripley B, Millrine D, et al. Aryl hydrocarbon receptor-mediated induction of the microRNA-132/212 cluster promotes interleukin-17-producing T-helper cell differentiation. *Proc Natl Acad Sci USA*. (2013) 110:11964–9. doi: 10.1073/pnas.1311087110
88. Escobar TM, Kanellopoulou C, Kugler DG, Kilaru G, Nguyen CK, Nagarajan V, et al. miR-155 Activates Cytokine Gene Expression in Th17 Cells by Regulating the DNA-Binding Protein Jarid2 to Relieve Polycomb-Mediated Repression. *Immunity* (2014) 40:865–79. doi: 10.1016/j.immuni.2014.03.014
89. Hu R, Huffaker TB, Kagele DA, Runtsch MC, Bake E, Chaudhuri AA, et al. MicroRNA-155 confers encephalogenic potential to Th17 cells by promoting effector gene expression. *J Immunol.* (2013) 190:5972–80. doi: 10.4049/jimmunol.1300351
90. Du C, Liu C, Kang J, Zhao G, Ye Z, Huang S, et al. MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. *Nat Immunol.* (2009) 10:1252–9. doi: 10.1038/ni.1798

91. Li Z, Wu F, Brant SR, Kwon JH. IL-23 receptor regulation by Let-7f in human $CD4^+$ memory T cells. *J Immunol.* (2011) 186:6182–90. doi: 10.4049/jimmunol.1000917
92. Fayyad-Kazan H, Rouas R, Fayyad-Kazan M, Badran R, El Zein N, Lewalle P, et al. MicroRNA profile of circulating CD4-positive regulatory T cells in human adults and impact of differentially expressed microRNAs on expression of two genes essential to their function. *J Biol Chem.* (2012) 287:9910–22. doi: 10.1074/jbc.M111.337154
93. Zhang Y, Sun E, Li X, Zhang M, Tang Z, He L, et al. miR-155 contributes to Df1-induced asthma by increasing the proliferative response of Th cells via CTLA-4 downregulation. *Cell Immunol.* (2017) 314:1–9. doi: 10.1016/j.cellimm.2017.01.005
94. Sonkoly E, Janson P, Majuri M-L, Savinko T, Fyhrquist N, Eidsmo L, et al. MiR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte–associated antigen 4. *J Allergy Clin Immunol.* (2010) 126:581–589.e20. doi: 10.1016/j.jaci.2010.05.045
95. Wei J, Nduom EK, Kong LY, Hashimoto Y, Xu S, Gabrusiewicz K, et al. MiR-138 exerts anti-glioma efficacy by targeting immune checkpoints. *Neuro Oncol.* (2016) 18:639–48. doi: 10.1093/neuonc/nov292
96. Cibrián D, Sánchez-Madrid F. CD69: from activation marker to metabolic gatekeeper. *Eur J Immunol.* (2017) 47:946–53. doi: 10.1002/eji.201646837
97. Kumar V, Torben W, Kenway CS, Schiro FR, Mohan M. Longitudinal examination of the intestinal lamina propria cellular compartment of simian immunodeficiency virus-infected rhesus macaques provides broader and deeper insights into the link between aberrant MicroRNA expression and persistent immune activati. *J Virol.* (2016) 90:5003–19. doi: 10.1128/JVI.00189-16
98. Jean S, Kiger AA. Classes of phosphoinositide 3-kinases at a glance. *J Cell Sci.* (2014) 127:923–8. doi: 10.1242/jcs.093773
99. Li G, Yu M, Lee WW, Tsang M, Krishnan E, Weyand CM, et al. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. *Nat Med.* (2012) 18:1518–24. doi: 10.1038/nm.2963
100. He W, Wang C, Mu R, Liang P, Huang Z, Zhang J, et al. MiR-21 is required for anti-tumor immune response in mice: an implication for its bi-directional roles. *Oncogene* (2017) 36:4212–23. doi: 10.1038/onc.2017.62
101. Ding S, Liang Y, Zhao M, Liang G, Long H, Zhao S, et al. Decreased microRNA-142-3p/5p expression causes $CD4^+$ T cell activation and B cell hyperstimulation in systemic lupus erythematosus. *Arthritis Rheum.* (2012) 64:2953–63. doi: 10.1002/art.34505
102. Guan H, Fan D, Mrelashvili D, Hao H, Singh NP, Singh UP, et al. MicroRNA let-7e is associated with the pathogenesis of experimental autoimmune encephalomyelitis. *Eur J Immunol.* (2013) 43:104–14. doi: 10.1002/eji.201242702
103. Jiang L, Cheng Z, Qiu S, Que Z, Bao W, Jiang C, et al. Altered let-7 expression in Myasthenia gravis and let-7c mediated regulation of IL-10 by directly targeting IL-10 in Jurkat cells. *Int Immunopharmacol.* (2012) 14:217–23. doi: 10.1016/j.intimp.2012.07.003
104. Swaminathan S, Suzuki K, Seddiki N, Kaplan W, Cowley MJ, Hood CL, et al. Differential regulation of the Let-7 family of MicroRNAs in $CD4^+$ T cells alters IL-10 expression. *J Immunol.* (2012) 188:6238–46. doi: 10.4049/jimmunol.1101196
105. Sharma A, Kumar M, Aich J, Hariharan M, Brahmachari SK, Agrawal A, et al. Posttranscriptional regulation of interleukin-10 expression by hsa-miR-106a. *Proc Natl Acad Sci USA.* (2009) 106:5761–6. doi: 10.1073/pnas.0808743106
106. Qin A, Wen Z, Zhou Y, Li Y, Li Y, Luo J, et al. MicroRNA-126 regulates the induction and function of $CD4^+$ $Foxp3^+$ regulatory T cells through PI3K/AKT pathway. *J Cell Mol Med.* (2013) 17:252–64. doi: 10.1111/jcmm.12003
107. Stagakis E, Bertsias G, Verginis P, Nakou M, Hatziapostolou M, Kritikos H, et al. Identification of novel microRNA signatures linked to human lupus disease activity and pathogenesis: MiR-21 regulates aberrant T cell responses through regulation of PDCD4 expression. *Ann Rheum Dis.* (2011) 70:1496–506. doi: 10.1136/ard.2010.139857
108. Chen CH, Seguin-Devaux C, Burke NA, Oriss TB, Watkins SC, Clipstone N, et al. Transforming growth factor beta blocks Tec kinase phosphorylation, $Ca2^+$ influx, and NFATc translocation causing inhibition of T cell differentiation. *J Exp Med.* (2003) 197:1689–99. doi: 10.1084/jem.20021170
109. Li MO, Sanjabi S, Flavell RA. Transforming growth factor-β controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* (2006) 25:455–71. doi: 10.1016/j.immuni.2006.07.011
110. Tu E, Chia CPZ, Chen W, Zhang D, Park SA, Jin W, et al. T cell receptor-regulated TGF-β Type I receptor expression determines T cell quiescence and activation. *Immunity* (2018) 48:745–759.e6. doi: 10.1016/j.immuni.2018.03.025
111. Li MO, Flavell RA. TGF-β: a master of all T cell trades. *Cell* (2008) 134:392–404. doi: 10.1016/j.cell.2008.07.025
112. Becker W, Nagarkatti M, Nagarkatti PS. miR-466a targeting of TGF-β2 contributes to $FoxP3^+$regulatory T cell differentiation in a murine model of allogeneic transplantation. *Front Immunol.* (2018) 9:688. doi: 10.3389/fimmu.2018.00688
113. Gauthy E, Cuende J, Stockis J, Huygens C, Lethé B, Collet JF, et al. GARP is regulated by miRNAs and controls latent TGF-β1 production by human regulatory T cells. *PLoS ONE* (2013) 8:e76186. doi: 10.1371/journal.pone.0076186
114. Zhou Q, Haupt S, Prots I, Thummler K, Kremmer E, Lipsky PE, et al. miR-142-3p Is involved in CD25+ CD4 T cell proliferation by targeting the expression of glycoprotein A repetitions predominant. *J Immunol.* (2013) 190:6579–88. doi: 10.4049/jimmunol.1202993
115. Meira M, Sievers C, Hoffmann F, Rasenack M, Kuhle J, Derfuss T, et al. Unraveling natalizumab effects on deregulated miR-17 expression in $CD4^+$ T cells of patients with relapsing-remitting multiple sclerosis. *J Immunol Res.* (2014) 2014:897249. doi: 10.1155/2014/897249
116. Severin ME, Lee PW, Liu Y, Selhorst AJ, Gormley MG, Pei W, et al. MicroRNAs targeting TGFb signalling underlie the regulatory T cell defect in multiple sclerosis. *Brain* (2016) 139:1747–61. doi: 10.1093/brain/aww084

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 28 September 2018
doi: 10.3389/fimmu.2018.02235

CD22: A Regulator of Innate and Adaptive B Cell Responses and Autoimmunity

Edward A. Clark[1,2]* *and Natalia V. Giltiay*[2]

[1] Department of Immunology, University of Washington, Seattle, WA, United States, [2] Division of Rheumatology, Department of Medicine, University of Washington, Seattle, WA, United States

CD22 (Siglec 2) is a receptor predominantly restricted to B cells. It was initially characterized over 30 years ago and named "CD22" in 1984 at the 2nd International workshop in Boston (1). Several excellent reviews have detailed CD22 functions, CD22-regulated signaling pathways and B cell subsets regulated by CD22 or Siglec G (2–4). This review is an attempt to highlight recent and possibly forgotten findings. We also describe the role of CD22 in autoimmunity and the great potential for CD22-based immunotherapeutics for the treatment of autoimmune diseases such as systemic lupus erythematosus (SLE).

Keywords: CD22, B cells, autoimmunity, antigens, TLR7, T cell dependent, T cell independent

INTRODUCTION

CD22 is classified as an "inhibitory receptor" because it contains four ITIMs within its cytoplasmic tail. Yet to classify it simply as a receptor that inhibits B cell functions would mean ignoring data that reveal a more nuanced story. For instance, besides the two distal ITIMs in the cytoplasmic tail of CD22 that recruit the protein tyrosine phosphatase (PTP), SHP-1, another motif, Y828 (or mouse Y807), when tyrosine phosphorylated, binds Grb2 and Shc and, forms a complex with SHIP and activation of a MAP kinase pathway that can regulate cell survival and proliferation (5–7). Just how and when these two CD22 cytoplasmic domains are utilized are still not well understood. One possibility is that B cell responses to T cell independent (TI) antigens (Ags) may utilize one or both binding domains, while other receptor responses use a different domain. In support of this model, Fujimoto et al. (8) reported that BCR ligation leads to rapid tyrosine phosphorylation of both the classic ITIMs and the Grb2 recruitment motif, while CD40 ligation only induces tyrosine phosphorylation of the ITIM domains.

Within the group of B cell-associated surface molecules, CD22 stands out not only because it can physically associate with the B cell receptor (BCR), but also because crosslinking the BCR on CD22-deficient B cells induces elevated responses such as mobilization of intracellular calcium (9–11). Hence, it has been emphasized that CD22's main function is to inhibit BCR signaling (2). Yet several initial studies of CD22-deficient mice showed that CD22 regulates TLR signaling and the survival of B cells and not just BCR signaling (see below). In our initial study, we reported that CD22 KO B cells proliferated less well than WT B cells after anti-IgM treatment but better after treatment with LPS (10).

The extracellular domain of CD22 binds to α 2,6-linked sialic acid ligands linked to galactose, which are expressed on a number of cell types including hematopoetic cells, certain endothelial cells and T and B cells. The enzyme α 2,6 sialyltransferase 1 (ST6Gal1) synthesizes this ligand, and ST6Gal1$^{-/-}$ mice phenocopy many but not all of the defects seen in CD22$^{-/-}$ mice (12, 13). CD22 itself expresses its ligand as does surface IgM (sIgM) and CD45, so CD22 can associate with itself or other cell surface molecules on B cells in a "cis" configuration or with ligands on other cells in a "trans" configuration. Endogenous CD22-CD22 *cis*-interactions

OPEN ACCESS

Edited by:
Aaron James Marshall, University of Manitoba, Canada

Reviewed by:
Jean-Philippe Julien, Hospital for Sick Children, Canada
James Paulson, The Scripps Research Institute, United States

***Correspondence:**
Edward A. Clark
eaclark@uw.edu

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *10 July 2018*
Accepted: *07 September 2018*
Published: *28 September 2018*

Citation:
Clark EA and Giltiay NV (2018) CD22: A Regulator of Innate and Adaptive B Cell Responses and Autoimmunity. Front. Immunol. 9:2235. doi: 10.3389/fimmu.2018.02235

can "mask" CD22, limiting its ability for binding to ligands in *trans*. Not all CD22 expresses its ligand, so CD22 also is found on B cells in a ligand-free, "un-masked" form. The relative roles of "masked" and "unmasked" forms of CD22 working in "cis" or "trans" are presented in detail elsewhere (3, 13–15).

REGULATION OF BCR SIGNALING BY CD22

The model that others and we helped to develop is as follows: After BCR ligation the protein tyrosine kinase (PTK) Lyn is activated to phosphorylate two distal ITIM motifs of CD22, which in effect then recruit the PTP, SHP-1, to come to the plasma membrane and get tyrosine-phosphorylated and activated (2–4, 13–15). Both genetic and biochemical data support the importance of this pathway. Mice with a combination of half doses of Lyn, CD22 and SHP-1 have a defective phenotype found in homozygous parents (16). Recruitment of SHP-1 (PTP.1C) to the plasma membrane may increase its enzymatic activity more than a 1,000 fold (17). Thus, there is no question that the SHP-1 associating with CD22 is ready and able to dephosphorylate its substrates.

Just what all those substrates might be in B cells still is not entirely clear. Yes, phosphorylated ITIMs can be dephosphorylated by SHP-1 *in vitro* (18, 19), but *in vitro* data do not necessarily reflect *in vivo* substrates. A phosphopeptide of the cytoplasmic tail of CD22 is not a particularly good substrate for SHP-1, unlike phosphopeptides from some other ITIM-containing receptors (20). Using a catalytically inactive trapping mutant of SHP-1, the Hozumi group showed that after BCR ligation both myosin and CD72 are substrates for SHP-1 (21, 22). SLP-76 and BLNK may also be SHP-1 substrates in B cells (23, 24).

Several studies have emphasized functions of CD22 that do not rely entirely on SHP-1. Chen et al. (25) found that CD22 can associate with plasma membrane calcium ATPase (PMCA) to enhance calcium efflux after BCR ligation; this association only occurs if CD22 is tyrosine phosphorylated. The non-ITIM Y828 site in CD22 that associates with Grb2 must be tyrosine phosphorylated for PMCA to interact with CD22, and Grb2 is required for this association (26). Chen et al. (25, 26) propose that PMCA regulates Ca^{2+} in B cells through its interaction with CD22 via a SHP-1-independent pathway. Grb2 has been previously implicated in the negative regulation of Ca2+ in B cells through its localization by the adaptor protein Dok-3 to the plasma membrane and subsequent inhibition of Btk (27). CD22, which like Dok-3 is a substrate for Lyn, may help to facilitate this process.

Most studies examining the role of CD22 in BCR signaling have used biochemical assays. Han et al. in a different approach used *in situ* photoaffnity crosslinking of glycan ligands to CD22 (28). Their results showed recognition of formation glycans of neighboring CD22 molecules, forming homomultimeric complexes, suggesting that CD22 is distributed in membrane microdomains, which the authors suggested restricts CD22 interactions with other glycoproteins. More recently, Gasparrini et al. (29) used super-resolution microscopy to examine the interactions of CD22 with the actin cytoskeleton. They found that CD22 works within the "cortical cytoskeleton" to regulate BCR signaling including tonic signaling and that it is organized into nanodomains. Simple inhibition of actin polymerization with latrunculin A led to rapid tyrosine phosphorylation of both CD22 and SHP-1. Using advanced microscopic methods such as dual-color structured illumination microscopy, they found that IgM, IgD, CD19, and CD22 exist on the cell surface of resting B cells in "preformed but distinct islands," with some co-localization. CD22 was not randomly distributed but rather more likely to be found in clusters about 100 nm in radius. *In silico* modeling showed that a high lateral mobility of CD22 nanoclusters would enable CD22 to come in contact with many BCR nanoclusters and thereby regulate tonic or Ag-induced signaling. Indeed, CD22, when tracked, turned out to be highly mobile, able to diffuse about four to five times faster than either sIgD or CD19 and nearly twice as fast as sIgM. The authors suggested that this would enable CD22 to mediate "global BCR surveillance."

Interestingly, Gasparrini et al. (29) also found that the extent of CD22 nanoclustering is regulated by the PTP, CD45; the less CD45 on B cells, the larger the CD22 nanoclusters were and the slower CD22 diffused. CD45 expresses α-2,6 sialic acid and, like CD22, is a CD22 ligand (30, 31). A reduction or absence of CD45 most likely leads to more CD22-CD22 homotypic interactions and thus larger clusters. Couglin et al. (32) also implicated extracellular CD45 in the regulation of CD22. They found that expression of transgenes encoding either extracellular CD45 without its cytoplasmic domain or CD45 with a catalytically inactive form of CD45 in $CD45^{-/-}$ mice rescued B cell defects seen in these mice such as elevated basal Ca^{2+} levels but not T cell defects. This effect required CD22.

Recently, the crystal structure of the first three extracellular domains (ECD) of human CD22 was deduced at a 2.1 A resolution (33). Strands of domain 1 elongate and extend into a ß-hairpin that shapes a preformed binding site for the sialic acid ligand. Analysis of CD22 molecules including a full length CD22 ECD revealed that CD22 is relatively inflexible and behaves as a tilted "elongated rod," which does not change its conformation much after ligand binding (33). The authors propose that "the elongated, tilted CD22 structure—and the location of its binding site at the N-terminus—is ideal for inter-molecular interactions with flexible bi-, tri-, and/or tetra-antennary glycans" that terminate in sialic acid. Because the bent-in CD22 molecules have relatively weak interactions within the *cis* nanoclusters, contact with other cells could lead CD22 to redistribute to sites of cell contact and via its elongated rod bind to ligands in *trans*.

ROLE OF CD22 IN RESPONSE TO ANTIGENS AND PATHOGENIC PRODUCTS

CD22 has been implicated in the regulation of B cell responses to T cell-independent (TI) type 2 antigens (Ags), TLR agonists and T cell-dependent (TD) Ags.

Antibody (Ab) responses to TI-2 Ags are impaired in $CD22^{-/-}$ mice (9–11), perhaps because they are deficient in

marginal zone (MZ) B cells and MZ B cell precursors (34, 35). Just why MZ B cells require CD22 is unclear. One possibility is that they are more sensitive to dysregulated signaling in the absence of CD22 (34); but it is also noteworthy that MZ precursors express the highest levels of CD22 of any B cell subset (35), implying that CD22 may be more or less required during stages in B cell development. Mice expressing all of CD22 except the extracellular domains 1 and 2 (CD22Δ1-2 mice) have reduced MZ B cells but normal TI-2 Ab responses (15, 36, 37), so a MZ B cell deficiency alone is not sufficient to lead to impaired TI-2 Ab responses. Recently, Haas et al. (36) reported that B-1b cells from $CD22^{-/-}$ mice have impaired proliferative responses and elevated Ca^{2+} responses to anti-IgM ligation and that $CD22^{-/-}$ mice have reduced expansion of splenic B-1b B cells after immunization with TNP-Ficoll. There results suggest that whether or not $CD22^{-/-}$ mice have defective TI-2 Ab responses depends on the Ag complexity and route of administration used.

Ab responses to LPS are elevated in $CD22^{-/-}$ mice (9–11), and $CD22^{-/-}$ B cells proliferate *in vitro* more strongly than WT B cells to TLR7 (R848) and TLR9 (CpG) agonists (38, 39). Kawasaki et al. (39) showed that $CD22^{-/-}$ B cells also are hyperproliferative to the TLR3 agonist poly I:C and that some of this hyperproliferation, unlike the hyperproliferation to TLR4 and TLR9 agonists, is MyD88-independent. TLR agonists also induced larger increases in MHC class II and CD86 in $CD22^{-/-}$ B cells than WT B cells, suggesting that B cells with dysregulated CD22 may more readily become effective Ag presenting cells, possibly to autoAgs. Kawasaki et al. concluded that this hyperresponsiveness to TLR agonists was not due to $CD22^{-/-}$ B cells expressing higher levels of TLRs; rather their results suggest that CD22 may normally function during TLR signaling of B cells to activate suppressors of cytokine signaling (SOCS) SOCS1 and SOCS3 proteins that are known to blunt responses to TLR ligands. Thus, CD22 normally may play a role in the direct inhibition of TLR signaling in B cells. The natural ligands for CD22 apparently do not play a direct role in regulating proliferative responses to TLR agonists since CD22 ligand-deficient $ST6Gal1^{-/-}$ B cells have normal responses to LPS and CpG (40). CD22 is an endocytic receptor that recycles between the cell surface and the endosomes, where endosomal TLRs resides (41). A model proposed by Paulson et al. (42) suggests that sequestration of CD22 and/or other changes in the CD22 microdomain organization may affect CD22 concentrations in the endosomes and further affect endosomal TLR signaling.

The role for CD22 in TD Ab responses is controversial. Initial studies reported that $CD22^{-/-}$ mice have normal responses to TD Ags (9–11); however, mice were evaluated for short times following immunization, and Ag boosts were administered before primary immune responses had subsided. Ligands for CD22 have been identified on CD22 itself and on T cells (28, 30, 43). Thus, CD22 may engage CD22 ligands in *trans* on T cells and affect T cell activation (14, 44). Furthermore, $ST6GalI^{-/-}$ mice unable to express CD22 ligands have normal T cells but defective TD Ab responses to Ag + adjuvant or influenza infection (12, 45). B cell proliferation induced via the "T cell-help" CD40 receptor is elevated in $CD22^{-/-}$ B cells (37). CD22 also affects intracellular free calcium released by IgG^+ B cells (46, 47), again implying that CD22-CD22L interactions may influence TD B cell responses.

A recent study suggested that CD22 plays a role in the generation of memory B cells in response to a TD Ag. $CD22^{-/-}$ B1-8^{hi} B cells with a BCR specific for the hapten, 4(hydroxy-3-nitrophenyl)-acetyl (NP) were able to respond to immunization with a TD Ag (NP-CGG in alum) and develop into germinal center (GC) B cells; however, they did not differentiate efficiently into memory B cells or long-lived plasma cells (LLPCs) or sustain Ab levels over time (48). The lack of GC B cell output was associated with a failure of $CD22^{-/-}$ B cells to develop a subset of $CXCR4^+CD38^+$ GC B cells, which may be GC-derived precursors of memory B cells and LLPCs.

In contrast, Onodera et al. (49) reported that after immunization $CD22^{-/-}$ B cells, including GC B cells, rapidly expand and generate short-lived AFCs and antibodies. Unlike in Chappell et al. the recipient mice were previously immunized with CGG ("carrier primed"). Thus, both CGG-specific Tfh cells and CGG-anti-CGG immune complexes that can be taken up by $Fc\gamma R^+$ cells may have contributed to the rapid hyperproliferative and extrafollicular B cell responses observed, as has been reported (50). Such hyperproliferation was not evident in the bona fide primary immune responses (48). Nevertheless, both studies suggest that $CD22^{-/-}$ B cells do not efficiently generate memory B cells.

SHP-1, which can be recruited to bind CD22, plays a role in GC maintenance and memory cell development (51, 52). Thus, it is possible that the absence of CD22 leads to decreased SHP-1 recruitment required for efficient memory B cell development. Unlike SHP-1 deletion, however, GCs are not completely ablated in the absence of CD22; rather, a small subset of $CXCR4^+CD38^+$ PNA^+ GC B cells fail to develop that normally appear early in the immune response (48). Cognate interactions between B and T cells are critical for GC initiation and maintenance, and CD22 ligands (CD22Ls) are expressed on T cells as well as B cells (30, 53). Interestingly, a recent study showed that CD22 on naïve and memory B cells is masked through interactions with "high affinity" ligand (Neu5Ac2– 6Gal1– 4(6S)GlcNAc); however, loss of the 6S sulfate modification on GC B-cells results in the appearance of Neu5Ac2– 6Gal1– 4GlcNAc glycans with a lower affinity for CD22 (54). Thus, it is possible that once CD22 is unmasked on GC B cells, CD22L-CD22 interactions may then occur *in trans* between $CD22L^+$ CD4 T_{FH} cells and $CD22^+$ GC B cells to promote further B cell survival and maturation. $CD22^{-/-}$ GC B cells that are not capable of receiving this type of "help" from T_{FH} cells may not be as competent as WT B cells for memory B cell formation. Thus, in addition to altered BCR signaling, defective interactions between B and T cells may also contribute to the lack of memory formation by $CD22^{-/-}$ B cells.

Hass et al. found $CD22^{-/-}$ mice have elevated IgM and IgG Ab primary and secondary responses to DNP-KLH, while Jellusova et al. found that $CD22^{-/-}$ mice have reduced primary Ab responses to NP-OVA in alum (36, 38). How can these differences be explained? Given that CD22 clearly regulates innate immune and TI signaling, one possibility is that role CD22 plays depends of the nature of the Ag or adjuvant used with the Ag. CD22 may be an attenuator of Ab

responses, which does not simply function along a TD vs. TI dichotomy.

ROLE OF CD22 IN MIGRATION AND OTHER TRANS INTERACTIONS

CD22 has long been known to be an adhesion molecule (1). But recent studies from Eugene Butcher's group at Stanford have uncovered a surprising and new role for CD22 in B cell homing to gut associated lymphoid tissues. The Peyer's patches (PP) in the gut are major site for B cell responses to intestinal Ags and attract large numbers of circulating B cells. TheSt6Gal1 ligand for CD22 is selectively expressed on mouse PP high endothelial venules (HEVs) and not on peripheral lymph node (LN) HEVs or on endothelial cells in capillaries (55). Homing to PP is dramatically reduced in both $CD22^{-/-}$ and $ST6Gal1^{-/-}$ mice. An Ab specific for human St6Gal1 binds to mucosal lymphoid organs (56), suggesting that homing to human GALT may be regulated by CD22 as well.

$CD22^{-/-}$ mice are highly susceptible to infection by West Nile virus (WNV) and die within 10 days post-infection (57). Humoral immune responses are normal in WNV-infected $CD22^{-/-}$ mice; however, homing to draining LNs in infected mice is defective. Fewer $CD22^{-/-}$ NK cells, CD4 T cells and CD8 T cells enter LNs than WT counterparts, while migration of $CD22^{-/-}$ B cell and dendritic cells (DCs) is normal. These results suggest that CD22 may regulate cell migration not simply by CD22L-CD22 interactions, but also indirectly, perhaps via regulation of chemokine or chemokine receptor expression. Indeed, the draining LNs of WNV-infected $CD22^{-/-}$ mice had reduced expression of both *Ccl3* and *Ccl5* genes (57).

CD22 also plays a role in the migration of recirculating B cells to the bone marrow (BM). Although B cell development in the BM is not affected in $CD22^{-/-}$ mice, numbers of recirculating $B220^{hi}sIgM^{lo}$ B cells (or IgD^{hi} B cells) are reduced in $CD22^{-/-}$ mice (9, 10) as well as CD22L deficient $ST6Gal1^{-/-}$ mice (58). The endothelial cells in BM sinusoids express the α2,6-linked sialic acid ligand for CD22 (59). WT B cells adoptively transferred into $ST6Gal1^{-/-}$ mice have reduced migration to the BM but not to the spleen (58).

DCs can directly regulate and activate B cells (60), and CD22 can bind to ligands expressed on DCs. Immature DCs but not mature DCs can inhibit B cell proliferation in a contact-dependent manner that requires CD22 expression on B cells (35, 61). Immature DCs can also inhibit TLR2- or TLR4-induced proliferation of mouse B cells via a contact and CD22-dependent mechanism (61). Surprisingly, $ST6Gal1^{-/-}$ DCs were just as efficient as wildtype DCs in inhibiting B cell responses to either BCR-ligation or LPS (35, 61), suggesting that CD22 may mediate inhibition of B cells through an interaction not dependent on ST6Gal1. Two groups found that murine CD22 is expressed on a subset of splenic $CD8\alpha^{-}$ DCs (57, 62). CD22 has also been reported to be expressed on human plasmacytoid DC tumors and follicular dendritic cells (63, 64). It is not clear how non-B cell CD22 might function.

CD22 AND INFECTIONS

Although CD22 regulates multiple B cell functions, the role of CD22 in protection against viral pathogens is unclear. For example, $CD22^{-/-}$ mice infected with lymphocytic choriomeningitis virus, vesicular stomatitis virus (65), or *Staphylococcus aureus* (66) have no differences in survival compared to wild-type (WT) mice. CD22-deficiency not only leads to increased susceptibility to WNV (55), but also accelerates murine AIDS MAIDS induced by a murine leukemia virus (67), $CD22^{-/-}$ mice had a more rapid onset of splenomegaly and lymphadenopathy 4 weeks after infection.

CD22 AND AUTOIMMUNITY

While B cells are critical for protection against pathogens, they can also contribute to harmful immune responses in many autoimmune diseases by producing Ab directed toward self-Ags, by presenting self-Ags and producing pro-inflammatory cytokines. A number of studies in human and mouse SLE have shown that hyper-responsiveness of B cells due to defects in the regulation of BCR signaling or increased signaling thought the nuclear-sensing TLRs can alter the selection of autoreactive B cells and promote the production of pathogenic auto-Abs (68). CD22 contributes to the regulation of autoimmunity. Some recent data suggest that targeting CD22 can suppress pathogenic B cell responses.

CD22 ALLELES AND CD22 DEFICIENCY IN MICE

Several studies in autoimmune-prone mice have identified *Cd22* as a candidate gene associated with susceptibility to lupus-like disease (69, 70). Mapping of autoimmune loci in B6.NZW (New Zealand White) x B6.Yaa (Y-linked autoimmune accelerator) F1 backcross males revealed the presence of a major autoimmune locus on chromosome 7 in the vicinity of $Cd22^{a}$. This allele was associated with the production of IgG anti-DNA autoantibodies and the development of glomerulonephritis (69).

This brings us back to the original isolation of genomic clones of mouse *Cd22* which demonstrated the presence of at least two (or more) *Cd22* alleles (71). The $Cd22^{a}$ allele expressed in DBA/2J, DBNl, NZB, and NZC mice has a distinct polypeptide coding sequences, as compared to the $Cd22^{b}$ allele, expressed in BALB/c, B10, C3H, and C57BL mice (71). This is due to the presence of a restriction fragment length polymorphism (RFLP) within the *Cd22* gene. The two allelic forms of *Cd22* ($Cd22^{a}$ and $Cd22^{b}$) differ in the exons encoding the distal extracellular region of mCD22, suggestive of functional differences between the two CD22 isoforms. Others studies confirmed that lupus-prone NZB and NZW mice carry the $Cd22^{a}$ allele (70, 72) and later, the expression of a third *Cd22* allele, $Cd22^{c}$, was described in autoimmune prone BXSB mice and the parental SB/Le strain. Similar to the "autoimmune" $Cd22^{a}$ allele, the $Cd22^{c}$ showed differences in the distal extracellular regions constituting the ligand-binding domains of CD22. Mary et al. (70) found that, in

addition to the wild-type *Cd22* transcripts, $Cd22^a$ and $Cd22^c$-alle bearing autoimmune mice express abnormally processed *Cd22* mRNA transcripts; this was due to the presence of interspersed nucleotide element (B1-, B4-, and ID) insertions, a class of retrotransposons, found in intron 2 of the $Cd22^a$ and $Cd22^c$ alleles that are not present in the non-autoimmune ($Cd22^b$) allele. Sequence analysis of aberrant *Cd22* mRNA $Cd22^a$ revealed that some of the mRNAs produce truncated forms of CD22 and others might not be expressed at all due the presence of premature stop-codons. These data suggest that the expression of $Cd22^a$ and other alleles can result in lower CD22 expression. The presence of the defective mRNA transcript was further associated with a reduced ability of LPS-activated B cells to up-regulate CD22 (70). Studies by Nitschke et al. (72) showed that CD22 encoded by the $Cd22^a$ allele expressed on B cells in lupus-prone mice is less efficient in binding to CD22L as compared to the $Cd22^b$ counterparts. A significant portion of CD22 in $Cd22^a$ mice was constitutively unmasked and did not bind surface *cis*-ligands. Similar to Mary et al., the Nitschke group showed that CD22 expression on $Cd22^a$ B cells is lower both in a steady-state condition and upon B cell stimulation. As a result, $Cd22^a$ B cells display a constitutively active phenotype, similar to the phenotype of B cells expressing a mutant CD22 missing its ligand-binding domain (72).

An initial study showed that aged $CD22^{-/-}$ mice have increases in auto-Ab production (73). However, since the $CD22^{-/-}$ mice used were generated using 129/Sv embryonic stem (ES) cells, it is possible that 129-derived loci may have contributed to the autoimmune phenotype (74). Other studies show that $CD22^{-/-}$ mice generated using C57BL/6 ES cells do not develop an autoimmune phenotype spontaneously (9). CD22 deficiency however does accelerate the development of autoimmunity in autoimmune-susceptible mice, Mary at al. showed that crossing $CD22^{-/-}$ mice onto mice carrying the *Yaa* locus, which predisposes mice to develop lupus-like disease due to duplication of *TLR7* and other genes, significantly increased auto-Ab production (70). This study also demonstrated a *Cd22* "gene dosage" effect, since even a partial reduction of CD22 expression (i.e., in heterozygous $CD22^{+/-}$ mice) increased auto-Ab production. Another interesting study showed that deletion of *Cd22* in anti-DNA transgenic (D42HTg) mice rescued autoreactive cells from peripheral toleralization and further promoted the production of high-affinity, class-switched anti-DNA Auto-Abs (75).

The fact that deletion of *Cd22* alone might not be sufficient to drive autoimmune disease in some mice can be explained by some functional redundancy between CD22 and Siglec-G, another Siglec family member, expressed on B cells also implicated in the regulation of BCR signaling (2, 76, 77). Unlike other autoimmune models, *Cd22* deficiency does not promote significant changes in B cell development, except for a decrease in MZ B cells (34). One alternative explanation for the decrease in MZ B cells is that $CD22^{-/-}$ MZ B cells might be partially activated. Similar egress of MZ B cells can be found in other autoimmune models, particularly those associated with TLR7 overexpression (78, 79). The role of CD22 in regulating MZ B cells and a possible link between MZ B cell decrease and increased autoimmunity needs further elucidation.

CD22 GENE VARIANTS IN HUMAN AUTOIMMUNE DISEASES

Genetic variants of CD22, or enzymes involved in the glycosylation of ligands of CD22 have been linked to susceptibility in human autoimmune diseases (80, 81). One example is the loss-of-function mutations in the enzyme sialic acid esterase (SIAE), which mediates the deacetylation of N-glycan sialic acids of CD22 ligands, a modification that enables ligand binding to CD22. These rare mutations were found more frequently in patients with autoimmune diseases, such as rheumatoid arthritis (RA), type 1 diabetes (T1D), and SLE (82–84). Furthermore, *Siae* mutant mice display defects in B cell tolerance and spontaneously develop autoantibodies, further supporting the link to autoimmunity (85).

Polymorphisms in the *CD22* gene itself have also been linked to autoimmunity. Hatta et al. (81) performed a systematic variation screening of the human *CD22* gene and studied possible associations between *CD22* polymorphisms and susceptibility to RA and SLE. They identified more than 13 SNPs within the *CD22* locus, the majority of which fell within the coding sequence, and some within introns flanking the exon-intron junctions. Seven of the SNPs resulted in amino acid substitutions within the extracellular domains of CD22. Among these variations, the Q152E substitution was more frequently found in SLE patients, particularly, those with central nervous system (CNS) involvement. Although the association of the Q152E variant with SLE was only marginally significant (81), of interest is that the Q152E substitution is located within the CD22 extracellular domain (at the interface between Ig domains 2 and 3) and introduces a charge difference; since it is located far from the SA-binding pocket, it is unlikely to directly affect CD22 binding to α2-6 sialylated ligands; however, this polymorphism might affect other aspects of CD22 biology such as stability, adhesion and trafficking. Another CD22 polymorphism, identified by Hatta et al. is a non-conservative amino acid substitution (G745D) within the cytoplasmic domain, proximal to a YXXM motif that is a binding site for PI3K (7). While no associations with this polymorphism and SLE or RA disease susceptibility were found, the amino acid change within the CD22 cytoplasmic tail nonetheless might interfere its binding to PI3K, Lyn or SHP-1 and thus, affect CD22 downstream signaling.

A study of patients with cutaneous systemic sclerosis (SSc), an autoimmune disease associated with B cell hyperactivation and the production of autoantibodies, showed a significant association between SSc disease susceptibility and synonymous SNP *c.2304C > A (P768P, rs34826052)* located within exon 13 of the *CD22* gene (86). The A/A genotype was present exclusively in patients with limited cutaneous SSc; furthermore, this genotype was associated with a decreased surface expression of CD22 in B cells compared to the A/C and C/C genotypes (86). Studies in a European population, however, found no significant association between *CD22* gene variations, including the *rs34826052* SNP, and susceptibility of SSc (87); this most likely reflects differences in the allele distributions in different populations. In fact data from the 1,000 Genomes project, showed that the *A* allele is found in only 1–3% in Africans, Americans or Europeans, but is more

frequent (9–15%) in East Asians and South Asians, which could explain the difference between these studies.

Genome-wide association studies (GWAS) do not support CD22 as disease susceptibility locus in SLE or other autoimmune diseases; however it seems that polymorphisms in *CD22* are relatively rare and variable between populations. More studies are needed to assess the functional significance of different CD22 SNPs and their possible contribution to autoimmune disease.

REGULATION OF CD22 EXPRESSION WITH IMPLICATIONS IN AUTOIMMUNITY

How the expression of CD22 is regulated in B cells is still not well understood. CD22 on murine B2 cells is down-regulated after BCR cross-linking with anti-IgM mAb, but it is up-regulated after stimulation with other stimuli such as LPS, anti-CD40 mAb, or IL-4. In contrast, BCR crosslinking of $CD5^+$ B1 B cells did not change the expression levels of CD22, and B1 cells downregulated CD22 in response to LPS or CpG (88). Thus, CD22 expression is differentially regulated in B1 and B2 cells. CD22 expression can be regulated at the mRNA level (88) or by CD22 endocytosis and recycling. John et al. (89) reported a clathrin-mediated internalization of CD22 and CD22 association with AP50, one of the subunits of the clathrin-associated AP-2 protein adapter complex. Furthermore, BCR crosslinking and CD22 phosphorylation can transiently inhibit CD22 endocytosis. It is not known if upon inflammatory/autoimmune conditions, CD22 mRNA expression and endocytosis is altered. As mentioned above, the presence of $Cd22^a$ allele in mice has been associated with a decrease of CD22 expression.

Relatively few studies have examined the expression of CD22 on B cells from SLE patients; one study reported a decrease in CD22 levels on B cells from SLE patients with active disease; another study showed an association between disease improvement and increased CD22 expression after treatment (90, 91). SSc patients may also have decreases in CD22 expression and reduced CD22 phosphorylation (92). Interestingly, anti-CD22 Abs capable of inhibiting tyrosine phosphorylation of CD22 have been found in both SSc and SLE patients, which might be another, yet-to-be-explored, mechanism for regulation of CD22 function (93).

Another important question is how CD22 expression is regulated during different stages of B cell development and its possible impact on the selection of autoreactive B cells. CD22 is most highly expressed on MZ B cell precursors (35) and remains at high levels on mature B cells; some studies suggest that developing B cells in the BM express low levels of CD22, starting at the Pre-B stage (9). Whereas the numbers of B cell precursors are normal in the BM of CD22-deficient mice, the effects of CD22 on the selection of B cell progenitors have not been studied in detail. Given its role in the regulation of BCR and TLR signaling, it is possible that CD22 may also control the signaling thresholds on developing B cells, and therefore, play a role in the central selection and tolerance induction.

We found that in healthy conditions newly-formed transitional (TR) B cells in both human and mice express relatively high levels of CD22 (Giltiay NV, unpublished data), which might function to prevent unwanted activation, as a number of studies have shown that TR B cells express BCRs that are polyreactive and can bind endogenous antigens (94, 95). Danzer at al. found that the proportion of murine B cells with unmasked CD22 is increased in splenic TR and MZ B cells and peritoneal B1 cells when compared to other mature B cells (96). They proposed that unmasking of CD22 could be functionally involved in lowering the signaling threshold at "developmental checkpoints," or might be a consequence of cell activation. A combination of predisposing factors such as TLR signals along with the unmasking of CD22 at the TR stage would favor the activation of poly/autoreactive TR B cells and thus contribute to the development of autoimmunity. Of future interest would be to compare the expression (and unmasking) of CD22 on different human B cell subsets in healthy or autoimmune conditions. Studies using auto-Ag-specific BCR transgenic mice lacking *Cd22* might be also useful to study the contribution of CD22 in regulating the selection and activation of autoreactive B cells at different stages of B cell development.

A ROLE OF CD22 IN SELF- AND NON-SELF-DISCRIMINATION

An important question is how CD22-CD22L *cis*- or *trans*-interactions affect the association of CD22 with sIgM and BCR signaling. A model proposed by Cyster and Goodnow (97) suggested that lower levels of sialylated proteins in non-lymphoid tissues promote CD22-sIg associations that "dampen the BCR signaling"; however, when B cells enter a lymphoid environment which is richer in α2,6-sialylated proteins, CD22 might be "drawn away" from sIgM through *trans*-interactions, thus promoting BCR signaling and B cell activation. Such "release" of the BCR from control by CD22 might be necessary when Ag-engaged B cells migrate into the B-cell follicles and interact with T_{FH} cells and form GCs. This model fits well with a study that showed changes in the glycosylation patterns due to altered enzyme activity in the GC, leading to unmasking of CD22 on GC B cells compared naive and memory B-cells, (54).

However, what happens when a B cell binds self-Ag? Some studies proposed that engagement of CD22 may provide a signal to distinguish between self-Ags and non-self, foreign Ags and to prevent self-reactivity (43, 98, 99). It is important to point out that sialyated glycans are abundant in vertebrates' cells/tissues and are usually absent in bacteria. Thus, they can be regarded as "self-structures" (100). Lanoue et al. (98), showed that expression of αST6Gall on self-Ags diminishes the activation of self-Ag-specific B cells, supporting the idea that CD22-2,6-sialoglycoconjugate interactions could bias against B cells being selected by self-Ags arrayed on such cells. More recently, Duong et al. reported that "decorating" TI-2 Ags with native sialylated ligands for CD22 and Siglec-G strongly suppresses antibody responses and promotes a sustained immune tolerance (99). These findings suggest CD22 influences how B cells maintain self-tolerance to cell surface proteins, or secreted high-molecular weight self-Ags.

CD22 also binds soluble self-molecules present in serum. For example, soluble IgM has been proposed as a α2,6-sialylated ligand for CD22 (43). Thus, CD22 might be recruited to the BCR when B cells bind IgM-antigen complexes, or IgM alone and thereby inhibit cell activation by acting as a kind of an inhibitory "IgM-Fc" receptor (97). This might be relevant to therapies using immunomodulatory IVIg. Séïté et al. recently showed that SA-positive IgG, but not SA-negative IgG from IVIg binds to CD22 and can inhibit B cell activation (101).

A ROLE FOR CD22 IN TLR REGULATION OF B CELL RESPONSES TO AUTO-Ags

Many studies suggest that B cells that recognize self-Ags, especially nuclear Ags, receive second signals from TLRs that recognize DNA or RNA motifs and drive their activation (68). TLR7 and TLR9 in particular have been implicated in the activation of autoreactive cells and antinuclear auto-Ab production in mouse models of lupus (78, 79, 102, 103). Signaling though TLRs can promote multiple functions of B cells, including cytokine production, cell differentiation, class-switch and Ab production. CD22-null mouse B cells have increased proliferative responses to TLR4, TLR7 or TLR9 ligands (38, 39); CD22 as noted above may inhibit TLR signaling in part by reducing the expression of SOCS1 and SOCS3 (39).

We found that engagement of CD22 on human B cells by anti-CD22 Ab inhibits the expression of *PRDM1* in response to TLR7 ligand or a combination of anti-IgM plus TLR7 ligand stimulation (104). *PRDM1* encodes Blimp1, a key transcription factor required for B cells to mature into antibody-secreting plasma cells. CD22 ligation limited B cell differentiation into plasmablasts in response to TLR7 ligation, suggesting that *in vivo* CD22 may function to inhibit TLR7-driven B cell activation of autoreactive B cells. Engagement of CD22 also affects cytokine production by human B cells in response to TLR7 or TLR9 stimulation. CD22 ligation inhibited IL-6 production and increased IL-10 production (104, 105), which might further inhibit pathogenic B cell responses. Mechanistically, CD22 engagement by antibody can induce MAPK/ERK phosphorylation, which can turn on the production of IL-10 (106, 107).

A balance between TLR7 signaling and CD22/CD22L interactions might be important for maintaining self-tolerance and keeping autoreactive B cells "in check" (**Figure 1**). Since a large proportion of peripheral B cells are poly/autoreactive, and presumably can encounter nuclear Ags in the form of nuclear debris of dying cells, CD22 ligation might be an important mechanism for limiting BCR and TLR signaling. Some studies suggest that cells undergoing apoptosis have a reduced surface expression of α2,3- and α2,6-linked sialic acids, which could affect CD22-*trans* binding and possibly-limit the ability of CD22 to regulate BCR and/or TLR7 responses (108). This might occur in SLE where the accumulation of necrotic/apoptotic cells and improper clearance play a major role in promoting immune cell activation (109). Genetic factors affecting CD22 or TLR7 expression may affect the ability of CD22 to regulate TLR7-mediated B cell activation and contribute to autoimmunity. Inflammatory conditions such as viral infections associated with type I IFN production, increase *TLR7* expression and can promote changes in protein glycosylation, and further affect CD22/TLR7 crosstalk. Increased expression of TLR7 promotes the expansion and activation of newly-formed TR B cells, which, may contribute to the production of anti-RNA/RNP auto-Abs (79, 95). Increased TLR7 signaling and dysregulation of CD22/CD22L interactions may further affect the activation of TR B cells.

It will be important to further explore the mechanisms for CD22-mediated inhibition of TLR7-indiced B cell activation. For example, it is possible that a crosstalk between TLR7 and CD22 might occur after CD22 internalization. Interaction between CD22 and TLR7 might depend on the CD22 "cargo" in the endosomal compartment and may be affected by changes in CD22 microdomain localization. CD22 ligation by self-Ags possibly could add to co-localization with TLR7 in the endosomes and promote inhibition on TLR7 signaling. A recent study showed that CD72 binds to endogenous TLR7 ligand Sm/RNP and inhibits TLR7-driven B cell responses (110). This finding is in line with previous studies which showed that CD72 deficiency in mice causes lupus-like disease, and associations between CD72 polymorphisms with SLE (111). CD72 and CD22 share similar signaling molecules; furthermore, the ITIM motif of CD72 can be a substrate for SHP-1, possibly downstream of CD22. In addition to that, CD22 signaling might interfere with B cell survival and cell-proliferation induced downstream of TLR7 by affecting the activation of NF-κB and pro-survival molecules, such as Bcl_{XL} and Mcl1 (106). Siglec-G/10 has been shown to suppress TLR4 signaling and NFkB activation by forming a complex with CD24, which binds endogenous TLR ligands such as HSP and HMGB1, but not LPS, providing selective repression of the inflammatory responses to Danger-Associated Molecular Patterns (DAMPs), but not pathogen-associated molecular patterns (PAMS) (42, 112).

CD22: A TARGET FOR IMMUNOTHERAPY

CD22 is expressed on the surface of most B-cell leukemias and lymphomas and therefore has been explored as a target for Ab-based therapies [reviewed by (113, 114)]. The first fully-humanized anti-CD22 IgG1 antibody, Epratuzumab (Emab) has been evaluated in clinical trials of B-cell NHL and ALL (113). Unlike Rituximab, which depletes circulating B cells, Emab does not induce complement-dependent cytotoxicity or Ab-dependent cellular cytotoxicity (115). While Emab is not very potent in killing malignant B cells as a single agent, positive results were reported when it was used in a combination with Rituximab and different types of chemotherapy (114, 116). Another anti-CD22Ab (HB22.7), which binds the ligand-binding domains of CD22, is under investigation (114). Upon antibody binding, CD22 is internalized, and because of that, it has been utilized for targeting Ab-drug conjugates (ADCs) or immunotoxins. The use of inotuzumab ozogamicin, which combines an anti-CD22 mAb with calicheamicin, an enediyne antibiotic, which, binds DNA and causes DNA breakage is now approved for use in relapsed or refractory B-cell precursor ALL.

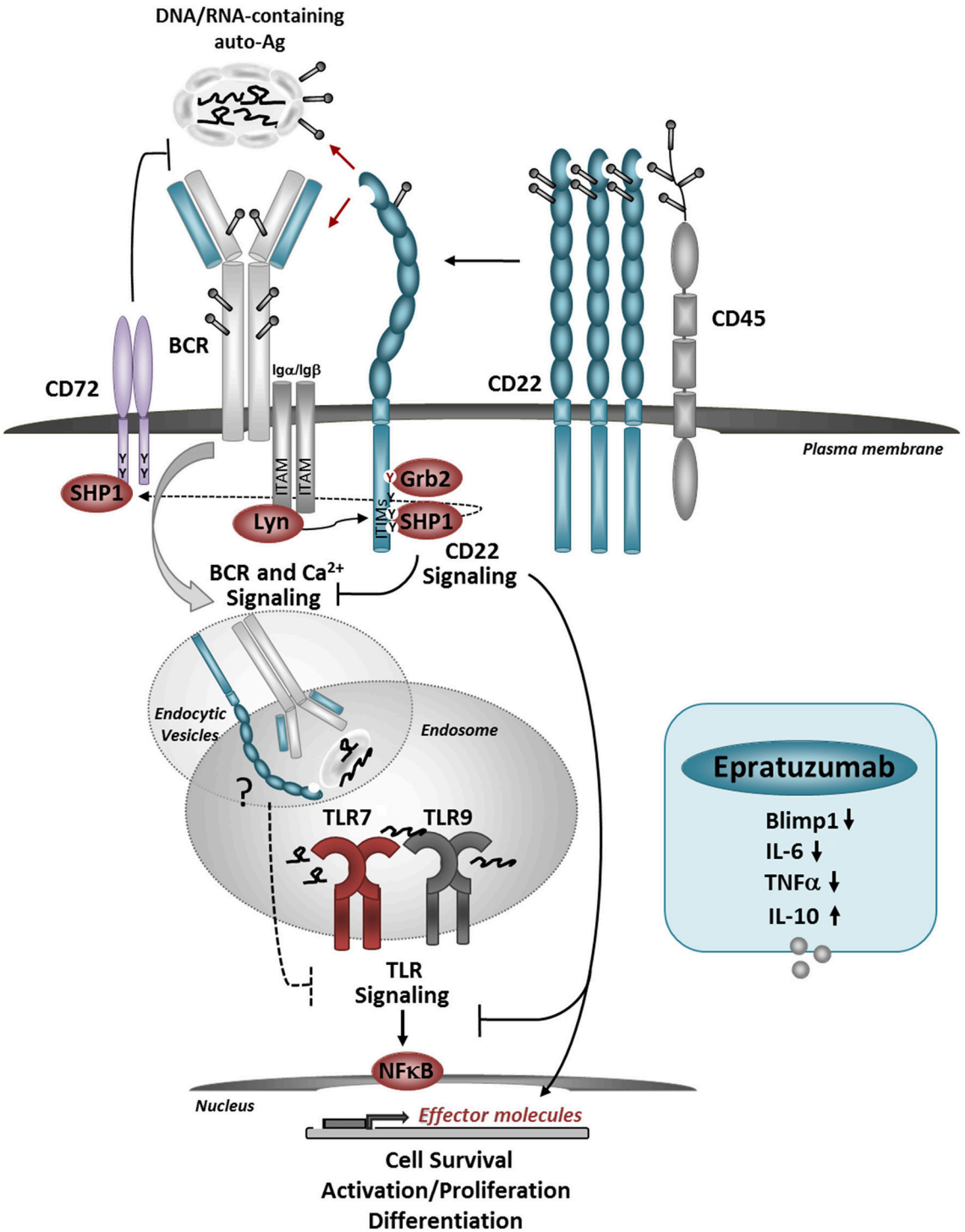


FIGURE 1 | Model for the role of CD22 in regulating BCR/TLR-mediated B cell responses to autoantigens. CD22 molecules are organized in nanodomains, regulated by interactions with CD45. Self-Ags, decorated with sialylated ligands may recruit CD22 molecules close to the BCR, upon which CD22-SHP-1 activation inhibits downstream signaling. The uptake up of nuclear-containing Ags triggers TLR7 and TLR9 activation in the endosomes. CD22 may inhibit TLRs activation *via* several mechanisms: promoting the activation of CD72, direct inhibition of TLR signaling after internalization, and/or affecting the expression and activation of NF-κB and pro-survival pathways. Crosslinking of CD22 with a therapeutic antibody Epratuzumab, inhibits the expression of Blimp1 and pro-inflammatory cytokines in response to TLRs stimulation. Antibody-mediated CD22 ligation induces internalization of CD22, SHP-1, and Grb2 activation and may also promote co-localization with TLRs in the endosomes.

Another drug, Moxetumomab Pasudotox, which combines anti-CD22 with PE38, a fragment of Pseudomonas exotoxin A, has shown efficacy in patients with hairy cell leukemia (HCL) (114). Other approaches for targeting CD22 in B cell malignancies have utilized high-affinity CD22 ligands (117, 118). Recently, the use of chimeric antigen receptor (CAR) T-cell therapy, specific for CD22 was reported to provide high response rates for patients with B-cell acute lymphoblastic leukemia (B-ALL) who had failed chemotherapy and/or a CD19-targeted CAR T-cell treatment (119).

There has been a significant interest in adopting CD22-targeted agents, such as Emab as therapies for autoimmune diseases, and in particular, for SLE (120, 121). The safety and efficacy of Emab in SLE have been evaluated in several clinical trials (120). Since Emab potentiates reduced BCR signaling and B cell activation, it was predicted to have potent immunomodulatory effects and a good safety profile. Indeed, Phase I and II clinical trials demonstrated clinically relevant, sustained improvements in patients with moderate-to-severe SLE and with no significant side effects (121). However, Emab did not reach its primary clinical endpoint at phase III clinical trial in SLE (122). A very high placebo response and early rescue of non-responders with increased doses of glucocorticoids might have confounded the results of this particular trail. A post-hoc analysis of the Phase III trial showed improved SLE disease activity in response to Emab in a subgroup of SLE patients with associated Sjogren's Syndrome (SjS), suggesting a future use of Emab (123) or other CD22-based drugs.

The mode-of-action of Emab in SLE is not fully understood. CD22 ligation by Emab induces rapid internalization and phosphorylation of CD22, inhibition of Syk and PLCγ2, and reduces intracellular Ca^{2+} mobilization after BCR stimulation in *vitro* (124). Emab-induced CD22 phosphorylation also enhances its co-localization with SHP-1 and Grb2 (125). Epratuzumab induces a partial reduction of circulating B cells in SLE patients, which might be associated with the effects of Emab on the expression of the adhesion molecules such as CD62L, β7 integrin, and β1 integrin and changes of B-cell migration (124).

Emab affects the production of cytokines in response to BCR/TLR stimulation, by skewing B cells to produce immunoregulatory cytokines such as IL-10 while inhibiting IL-6 and TNF alpha production (104, 105). Thus, targeting CD22 may restore IL-10 production by regulatory B cells, reported to be impaired in SLE patients (126). Emab inhibits the activation and the expression of PRDM1/Blimp1 in response to BCR and TLR7 stimulation in a subset of $CD27^{-}IgD^{-}$ double-negative (DN) memory B-cells (104), known to be elevated in SLE patients with more active disease (127).

More work needs to be done to understand the possible therapeutic effects of CD22-based drugs in SLE and potentially to predict which patients respond to CD22-mediated therapies; genetic factors, including defects in CD22 and CD22L, might play a role in responsiveness to CD22 targeting. Recently, Ereño-Orbea et al. delineated the CD22 site targeted by Emab and showed that glycosylation of CD22, which might be altered in B-cell malignancies and autoimmune conditions such as SLE, can affect the ability of Emab to bind its epitope on CD22 (33).

Macauley et al. (128) used liposomal nanoparticles bearing a synthetic high-affinity ligand for CD22, which contained optimized ratios of Ag that can deliver Ag to B cell while engaging CD22. The administration of these SIGLEC-engaging Ag-liposomes (STALs) in mice decreased Ab responses upon a second challenge with the same particles without CD22L, suggesting the induction of Ag-specific immunogenic tolerance (128, 129). The authors showed that STAL-induced B cell tolerance was associated with CD22-mediated inhibition of BCR signaling and recruitment of SHP-1. The potential of STALs was also demonstrated in a mouse model of hemophilia A, which showed a sustained inhibition of anti-VIII Ab responses after mice were administered recombinant FVIII replacement therapy. Another study in MRL/lpr mice has demonstrated the use of chimeric antibody constructed by coupling copies of a DNA mimotope peptide and CD22-binding STN peptide to a mouse IgG backbone. This triple chimera targeted selectively autoreactive B cells and the simultaneous engagement of the BCR, CD22 and, FcgRIIb inhibited anti-DNA Ab production and delayed the development of disease (130). Targeting anti-CD22 was also shown to partly deplete and reprogram B-cells in autoimmune NOD mice, thereby reversing the development of autoimmune diabetes (131). Recently STALS targeting hCD22 ligand were reported to induce to immunological tolerance in humanized CD22 Tg mice. This new model may provide a valuable tool to study the function of human CD22 *in vivo* and for future preclinical studies (132).

CONCLUSIONS

CD22 plays a key role in affecting B cell responses to Ags and innate immune signals, and CD22-CD22L interactions are essential for maintaining self-tolerance. Despite the evidence implicating CD22 in murine lupus, human genetic studies do not support *CD22* as a major disease susceptibility locus in SLE. However, it is likely that defects in *CD22* combined with other genetic factors have additive or synergistic effects on disease susceptibility. The ability of CD22 to regulate both BCR and TLRs represents an attractive therapeutic strategy for manipulating B cell responses in autoimmunity. A challenge for the future would be to fully understand the mode-of action of different CD22-tarageting agents. New methods for CD22-mediated targeting of pathogenic autoreactive B cells without compromising the host's ability to respond to foreign pathogens are a potential new exciting avenue for immunotherapies.

AUTHOR CONTRIBUTIONS

EC wrote sections of the review and edited sections written by NG, completed final manuscript. NG wrote section of the review and edited sections written by EC, prepared Figure.

FUNDING

This work was funded in part by NIH grants AI44257 and AI52203 to EC.

REFERENCES

1. Clark EA. CD22, a B cell-specific receptor, mediates adhesion and signal transduction. *J Immunol.* (1993) 150:4715–8.
2. Jellusova J, Nitschke L. Regulation of B cell functions by the sialic acid-binding receptors siglec-G and CD22. *Front Immunol.* (2011) 2:96. doi: 10.3389/fimmu.2011.00096
3. Tedder TF, Poe JC, Haas KM. CD22: a multifunctional receptor that regulates B lymphocyte survival and signal transduction. *Adv Immunol.* (2005) 88:1–50. doi: 10.1016/S0065-2776(05)88001-0
4. Walker JA, Smith KG. CD22: an inhibitory enigma. *Immunology* (2008) 123:314–25. doi: 10.1111/j.1365-2567.2007.02752.x
5. Otipoby KL, Draves KE, Clark EA. CD22 regulates B cell receptor-mediated signals via two domains that independently recruit Grb2 and SHP-1. *J Biol Chem.* (2001) 276:44315–22. doi: 10.1074/jbc.M105446200
6. Poe JC, Fujimoto M, Jansen PJ, Miller AS, Tedder TF. CD22 forms a quaternary complex with SHIP, Grb2, and Shc. A pathway for regulation of B lymphocyte antigen receptor-induced calcium flux. *J Biol Chem.* (2000) 275:17420–7. doi: 10.1074/jbc.M001892200
7. Yohannan J, Wienands J, Coggeshall KM, Justement LB. Analysis of tyrosine phosphorylation-dependent interactions between stimulatory effector proteins and the B cell co-receptor CD22. *J Biol Chem.* (1999) 274:18769–76. doi: 10.1074/jbc.274.26.18769
8. Fujimoto M, Kuwano Y, Watanabe R, Asashima N, Nakashima H, Yoshitake S, et al. B cell antigen receptor and CD40 differentially regulate CD22 tyrosine phosphorylation. *J Immunol.* (2006) 176:873–9. doi: 10.4049/jimmunol.176.2.873
9. Nitschke L, Carsetti R, Ocker B, Kohler G, Lamers MC. CD22 is a negative regulator of B-cell receptor signalling. *Curr Biol.* (1997) 7:133–43. doi: 10.1016/S0960-9822(06)00057-1
10. Otipoby KL, Andersson KB, Draves KE, Klaus SJ, Farr AG, Kerner JD, et al. CD22 regulates thymus-independent responses and the lifespan of B cells. *Nature* (1996) 384:634–7. doi: 10.1038/384634a0
11. Sato S, Miller AS, Inaoki M, Bock CB, Jansen PJ, Tang ML, et al. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity* (1996) 5:551–62. doi: 10.1016/S1074-7613(00)80270-8
12. Hennet T, Chui D, Paulson JC, Marth JD. Immune regulation by the ST6Gal sialyltransferase. *Proc Natl Acad Sci USA.* (1998) 95:4504–9. doi: 10.1073/pnas.95.8.4504
13. Macauley MS, Crocker PR, Paulson JC. Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol.* (2014) 14:653–66. doi: 10.1038/nri3737
14. Collins BE, Blixt O, DeSieno AR, Bovin N, Marth JD, Paulson JC. Masking of CD22 by cis ligands does not prevent redistribution of CD22 to sites of cell contact. *Proc Natl Acad Sci USA.* (2004) 101:6104–9. doi: 10.1073/pnas.0400851101
15. Poe JC, Fujimoto Y, Hasegawa M, Haas KM, Miller AS, Sanford IG, et al. CD22 regulates B lymphocyte function *in vivo* through both ligand-dependent and ligand-independent mechanisms. *Nat Immunol.* (2004) 5:1078–87. doi: 10.1038/ni1121
16. Cornall RJ, Cyster JG, Hibbs ML, Dunn AR, Otipoby KL, Clark EA, et al. Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection. *Immunity* (1998) 8:497–508. doi: 10.1016/S1074-7613(00)80554-3
17. Zhao Z, Shen SH, Fischer EH. Stimulation by phospholipids of a protein-tyrosine-phosphatase containing two src homology 2 domains. *Proc Natl Acad Sci USA.* (1993) 90:4251–5. doi: 10.1073/pnas.90.9.4251
18. Bone H, Dechert U, Jirik F, Schrader JW, Welham MJ. SHP1 and SHP2 protein-tyrosine phosphatases associate with betac after interleukin-3-induced receptor tyrosine phosphorylation. Identification of potential binding sites and substrates. *J Biol Chem.* (1997) 272:14470–6.
19. Wheadon H, Paling NR, Welham MJ. Molecular interactions of SHP1 and SHP2 in IL-3-signalling. *Cell Signal.* (2002) 14:219–29. doi: 10.1016/S0898-6568(01)00241-8
20. Burshtyn DN, Yang W, Yi T, Long EO. A novel phosphotyrosine motif with a critical amino acid at position−2 for the SH2 domain-mediated activation of the tyrosine phosphatase SHP-1. *J Biol Chem.* (1997) 272:13066–72. doi: 10.1074/jbc.272.20.13066
21. Baba T, Fusaki N, Shinya N, Iwamatsu A, Hozumi N. Myosin is an *in vivo* substrate of the protein tyrosine phosphatase (SHP-1) after mIgM cross-linking. *Biochem Biophys Res Commun.* (2003) 304:67–72. doi: 10.1016/S0006-291X(03)00542-4
22. Wu Y, Nadler MJ, Brennan LA, Gish GD, Timms JF, Fusaki N, et al. The B-cell transmembrane protein CD72 binds to and is an *in vivo* substrate of the protein tyrosine phosphatase SHP-1. *Curr Biol.* (1998) 8:1009–17. doi: 10.1016/S0960-9822(07)00421-6
23. Gerlach J, Ghosh S, Jumaa H, Reth M, Wienands J, Chan AC, et al. B cell defects in SLP65/BLNK-deficient mice can be partially corrected by the absence of CD22, an inhibitory coreceptor for BCR signaling. *Eur J Immunol.* (2003) 33:3418–26. doi: 10.1002/eji.200324290
24. Mizuno K, Tagawa Y, Watanabe N, Ogimoto M, Yakura H. SLP-76 is recruited to CD22 and dephosphorylated by SHP-1, thereby regulating B cell receptor-induced c-Jun N-terminal kinase activation. *Eur J Immunol.* (2005) 35:644–54. doi: 10.1002/eji.200425465
25. Chen J, McLean PA, Neel BG, Okunade G, Shull GE, Wortis HH. CD22 attenuates calcium signaling by potentiating plasma membrane calcium-ATPase activity. *Nat Immunol.* (2004) 5:651–7. doi: 10.1038/ni1072
26. Chen J, Wang H, Xu WP, Wei SS, Li HJ, Mei YQ, et al. Besides an ITIM/SHP-1-dependent pathway, CD22 collaborates with Grb2 and plasma membrane calcium-ATPase in an ITIM/SHP-1-independent pathway of attenuation of Ca^{2+}i signal in B cells. *Oncotarget* (2016) 7:56129–46. doi: 10.18632/oncotarget.9794
27. Stork B, Neumann K, Goldbeck I, Alers S, Kahne T, Naumann M, et al. Subcellular localization of Grb2 by the adaptor protein Dok-3 restricts the intensity of Ca^{2+} signaling in B cells. *EMBO J.* (2007) 26:1140–9. doi: 10.1038/sj.emboj.7601557
28. Han S, Collins BE, Bengtson P, Paulson JC. Homomultimeric complexes of CD22 in B cells revealed by protein-glycan cross-linking. *Nat Chem Biol.* (2005) 1:93–7. doi: 10.1038/nchembio713
29. Gasparrini F, Feest C, Bruckbauer A, Mattila PK, Muller J, Nitschke L, et al. Nanoscale organization and dynamics of the siglec CD22 cooperate with the cytoskeleton in restraining BCR signalling. *EMBO J.* (2016) 35:258–80. doi: 10.15252/embj.201593027
30. Law CL, Aruffo A, Chandran KA, Doty RT, Clark EA. Ig domains 1 and 2 of murine CD22 constitute the ligand-binding domain and bind multiple sialylated ligands expressed on B and T cells. *J Immunol.* (1995) 155:3368–76.
31. Stamenkovic I, Sgroi D, Aruffo A, Sy MS, Anderson T. The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and alpha 2–6 sialyltransferase, CD75, on B cells. *Cell* (1991) 66:1133–44. doi: 10.1016/0092-8674(91)90036-X
32. Coughlin S, Noviski M, Mueller JL, Chuwonpad A, Raschke WC, Weiss A, et al. An extracatalytic function of CD45 in B cells is mediated by CD22. *Proc Natl Acad Sci USA.* (2015) 112:E6515–24. doi: 10.1073/pnas.1519925112
33. Ereno-Orbea J, Sicard T, Cui H, Mazhab-Jafari MT, Benlekbir S, Guarne A, et al. Molecular basis of human CD22 function and therapeutic targeting. *Nat Commun.* (2017) 8:764. doi: 10.1038/s41467-017-00836-6
34. Samardzic T, Marinkovic D, Danzer CP, Gerlach J, Nitschke L, Wirth T. Reduction of marginal zone B cells in CD22-deficient mice. *Eur J Immunol.* (2002) 32:561–7. doi: 10.1002/1521-4141(200202)32:2<561::AID-IMMU561>3.0.CO;2-H
35. Santos L, Draves KE, Boton M, Grewal PK, Marth JD, Clark EA. Dendritic cell-dependent inhibition of B cell proliferation requires CD22. *J Immunol.* (2008) 180:4561–9. doi: 10.4049/jimmunol.180.7.4561
36. Haas KM, Johnson KL, Phipps JP, Do C. CD22 Promotes B-1b cell responses to T cell-independent type 2 antigens. *J Immunol.* (2018) 200:1671–1681. doi: 10.4049/jimmunol.1701578
37. Poe JC, Haas KM, Uchida J, Lee Y, Fujimoto M, Tedder TF. Severely impaired B lymphocyte proliferation, survival, and induction of the c-Myc:Cullin 1 ubiquitin ligase pathway resulting from CD22 deficiency on the C57BL/6 genetic background. *J Immunol.* (2004) 172:2100–10. doi: 10.4049/jimmunol.172.4.2100
38. Jellusova J, Wellmann U, Amann K, Winkler TH, Nitschke L. CD22 x Siglec-G double-deficient mice have massively increased B1 cell numbers

and develop systemic autoimmunity. *J Immunol.* (2010) 184:3618–27. doi: 10.4049/jimmunol.0902711
39. Kawasaki N, Rademacher C, Paulson JC. CD22 regulates adaptive and innate immune responses of B cells. *J Innate Immun.* (2011) 3:411–9. doi: 10.1159/000322375
40. Matsubara N, Imamura A, Yonemizu T, Akatsu C, Yang H, Ueki A, et al. CD22-binding synthetic sialosides regulate b lymphocyte proliferation through CD22 ligand-dependent and independent pathways, and enhance antibody production in mice. *Front Immunol.* (2018) 9:820. doi: 10.3389/fimmu.2018.00820
41. O'Reilly MK, Tian H, Paulson JC. CD22 is a recycling receptor that can shuttle cargo between the cell surface and endosomal compartments of B cells. *J Immunol.* (2011) 186:1554–63. doi: 10.4049/jimmunol.1003005
42. Paulson JC, Macauley MS, Kawasaki N. Siglecs as sensors of self in innate and adaptive immune responses. *Ann N Y Acad Sci.* (2012) 1253:37–48. doi: 10.1111/j.1749-6632.2011.06362.x
43. Hanasaki K, Powell LD, Varki A. Binding of human plasma sialoglycoproteins by the B cell-specific lectin CD22. Selective recognition of immunoglobulin M and haptoglobin. *J Biol Chem.* (1995) 270:7543–50.
44. Tuscano J, Engel P, Tedder TF, Kehrl JH. Engagement of the adhesion receptor CD22 triggers a potent stimulatory signal for B cells and blocking CD22/CD22L interactions impairs T-cell proliferation. *Blood* (1996) 87:4723–30.
45. Zeng J, Joo HM, Rajini B, Wrammert JP, Sangster MY, Onami TM. The generation of influenza-specific humoral responses is impaired in ST6Gal I-deficient mice. *J Immunol.* (2009) 182:4721–7. doi: 10.4049/jimmunol.0802833
46. Horikawa K, Martin SW, Pogue SL, Silver K, Peng K, Takatsu K, et al. Enhancement and suppression of signaling by the conserved tail of IgG memory-type B cell antigen receptors. *J Exp Med.* (2007) 204:759–69. doi: 10.1084/jem.20061923
47. Waisman A, Kraus M, Seagal J, Ghosh S, Melamed D, Song J, et al. IgG1 B cell receptor signaling is inhibited by CD22 and promotes the development of B cells whose survival is less dependent on Ig alpha/beta. *J Exp Med.* (2007) 204:747–58. doi: 10.1084/jem.20062024
48. Chappell CP, Draves KE, Clark EA. CD22 is required for formation of memory B cell precursors within germinal centers. *PLoS ONE* (2017) 12:e0174661. doi: 10.1371/journal.pone.0174661
49. Onodera T, Poe JC, Tedder TF, Tsubata T. CD22 regulates time course of both B cell division and antibody response. *J Immunol.* (2008) 180:907–13. doi: 10.4049/jimmunol.180.2.907
50. Goins CL, Chappell CP, Shashidharamurthy R, Selvaraj P, Jacob J. Immune complex-mediated enhancement of secondary antibody responses. *J Immunol.* (2010) 184:6293–8. doi: 10.4049/jimmunol.0902530
51. Khalil AM, Cambier JC, Shlomchik MJ. B cell receptor signal transduction in the GC is short-circuited by high phosphatase activity. *Science* (2012) 336:1178–81. doi: 10.1126/science.1213368
52. Li YF, Xu S, Ou X, Lam KP. Shp1 signalling is required to establish the long-lived bone marrow plasma cell pool. *Nat Commun.* (2014) 5:4273. doi: 10.1038/ncomms5273
53. Lajaunias F, Ida A, Kikuchi S, Fossati-Jimack L, Martinez-Soria E, Moll T, et al. Differential control of CD22 ligand expression on B and T lymphocytes, and enhanced expression in murine systemic lupus. *Arthritis Rheum.* (2003) 48:1612–21. doi: 10.1002/art.11021
54. Macauley MS, Kawasaki N, Peng W, Wang SH, He Y, Arlian BM, et al. Unmasking of CD22 Co-receptor on germinal center b-cells occurs by alternative mechanisms in mouse and man. *J Biol Chem.* (2015) 290:30066–77. doi: 10.1074/jbc.M115.691337
55. Lee M, Kiefel H, LaJevic MD, Macauley MS, Kawashima H, O'Hara E, et al. Transcriptional programs of lymphoid tissue capillary and high endothelium reveal control mechanisms for lymphocyte homing. *Nat Immunol.* (2014) 15:982–95. doi: 10.1038/ni.2983
56. Kimura N, Ohmori K, Miyazaki K, Izawa M, Matsuzaki Y, Yasuda Y, et al. Human B-lymphocytes express alpha2–6-sialylated 6-sulfo-N-acetyllactosamine serving as a preferred ligand for CD22/Siglec-2. *J Biol Chem.* (2007) 282:32200–7. doi: 10.1074/jbc.M702341200
57. Ma DY, Suthar MS, Kasahara S, Gale M Jr, Clark EA. CD22 is required for protection against West Nile virus Infection. *J Virol.* (2013) 87:3361–75. doi: 10.1128/JVI.02368-12
58. Ghosh S, Bandulet C, Nitschke L. Regulation of B cell development and B cell signalling by CD22 and its ligands alpha2,6-linked sialic acids. *Int Immunol.* (2006) 18:603–11. doi: 10.1093/intimm/dxh402
59. Nitschke L, Floyd H, Ferguson DJ, Crocker PR. Identification of CD22 ligands on bone marrow sinusoidal endothelium implicated in CD22-dependent homing of recirculating B cells. *J Exp Med.* (1999) 189:1513–8. doi: 10.1084/jem.189.9.1513
60. Chappell CP, Draves KE, Giltiay NV, Clark EA. Extrafollicular B cell activation by marginal zone dendritic cells drives T cell-dependent antibody responses. *J Exp Med.* (2012) 209:1825–40. doi: 10.1084/jem.20120774
61. Sindhava VJ, Tuna H, Gachuki BW, DiLillo DJ, Avdiushko MG, Onami TM, et al. Bone marrow dendritic cell-mediated regulation of TLR and B cell receptor signaling in B cells. *J Immunol.* (2012) 189:3355–67. doi: 10.4049/jimmunol.1101352
62. Edwards AD, Chaussabel D, Tomlinson S, Schulz O, Sher A, Reis e Sousa, C. Relationships among murine CD11c(high) dendritic cell subsets as revealed by baseline gene expression patterns. *J Immunol.* (2003) 171:47–60. doi: 10.4049/jimmunol.171.1.47
63. Ogata T, Yamakawa M, Imai Y, Takahashi T. Follicular dendritic cells adhere to fibronectin and laminin fibers via their respective receptors. *Blood* (1996) 88:2995–3003.
64. Reineks EZ, Osei ES, Rosenberg A, Auletta J, Meyerson HJ. CD22 expression on blastic plasmacytoid dendritic cell neoplasms and reactivity of anti-CD22 antibodies to peripheral blood dendritic cells. *Cytometry B Clin Cytom.* (2009) 76:237–48. doi: 10.1002/cyto.b.20469
65. Fehr T, Lopez-Macias C, Odermatt B, Torres RM, Schubart DB, O'Keefe TL, et al. Correlation of anti-viral B cell responses and splenic morphology with expression of B cell-specific molecules. *Int Immunol.* (2000) 12:1275–84. doi: 10.1093/intimm/12.9.1275
66. Gjertsson I, Nitschke L, Tarkowski A. The role of B cell CD22 expression in *Staphylococcus aureus* arthritis and sepsis. *Microbes Infect.* (2004) 6:377–82. doi: 10.1016/j.micinf.2003.12.013
67. Knoetig SM, Torrey TA, Naghashfar Z, McCarty T, Morse HC III. CD19 signaling pathways play a major role for murine AIDS induction and progression. *J Immunol.* (2002) 169:5607–14. doi: 10.4049/jimmunol.169.10.5607
68. Green NM, Marshak-Rothstein A. Toll-like receptor driven B cell activation in the induction of systemic autoimmunity. *Semin Immunol.* (2011) 23:106–12. . doi: 10.1016/j.smim.2011.01.016
69. Santiago ML, Mary C, Parzy D, Jacquet C, Montagutelli X, Parkhouse RM et al. Linkage of a major quantitative trait locus to Yaa gene-induced lupus-like nephritis in (NZW x C57BL/6)F1 mice. *Eur J Immunol.* (1998) 28:4257–67.
70. Mary C, Laporte C, Parzy D, Santiago ML, Stefani F, Lajaunias F, et al. Dysregulated expression of the Cd22 gene as a result of a short interspersed nucleotide element insertion in Cd22a lupus-prone mice. *J Immunol.* (2000) 165:2987–96. doi: 10.4049/jimmunol.165.6.2987
71. Law CL, Torres RM, Sundberg HA, Parkhouse RM, Brannan CI, Copeland NG, et al. Organization of the murine Cd22 locus. Mapping to chromosome 7 and characterization of two alleles. *J Immunol.* (1993) 151:175–87.
72. Nitschke L, Lajaunias F, Moll T, Ho L, Martinez-Soria E, Kikuchi S, et al. Expression of aberrant forms of CD22 on B lymphocytes in Cd22a lupus-prone mice affects ligand binding. *Int Immunol.* (2006) 18:59–68. doi: 10.1093/intimm/dxh349
73. O'Keefe TL, Williams GT, Batista FD, Neuberger MS. Deficiency in CD22, a B cell-specific inhibitory receptor, is sufficient to predispose to development of high affinity autoantibodies. *J Exp Med.* (1999) 189:1307–13. doi: 10.1084/jem.189.8.1307
74. Bygrave AE, Rose KL, Cortes-Hernandez J, Warren J, Rigby RJ, Cook HT, et al. Spontaneous autoimmunity in 129 and C57BL/6 mice-implications for autoimmunity described in gene-targeted mice. *PLoS Biol.* (2004) 2:E243. doi: 10.1371/journal.pbio.0020243
75. Yarkoni Y, Fischel R, Kat I, Yachimovich-Cohen N, Eilat D. Peripheral B cell receptor editing may promote the production of high-affinity

autoantibodies in CD22-deficient mice. *Eur J Immunol.* (2006) 36:2755–67. doi: 10.1002/eji.200636190
76. Bokers S, Urbat A, Daniel C, Amann K, Smith KG, Espeli M, et al. Siglec-G deficiency leads to more severe collagen-induced arthritis and earlier onset of lupus-like symptoms in MRL/lpr mice. *J Immunol.* (2014) 192:2994–3002. doi: 10.4049/jimmunol.1303367
77. Muller J, Nitschke L. The role of CD22 and Siglec-G in B-cell tolerance and autoimmune disease. *Nat Rev Rheumatol.* (2014) 10:422–8. doi: 10.1038/nrrheum.2014.54
78. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science* (2006) 312:1669–72. doi: 10.1126/science.1124978
79. Giltiay NV, Chappell CP, Sun X, Kolhatkar N, Teal TH, Wiedeman AE, et al. Overexpression of TLR7 promotes cell-intrinsic expansion and autoantibody production by transitional T1 B cells. *J Exp Med.* (2013) 210:2773–89. doi: 10.1084/jem.20122798
80. Angata T. Associations of genetic polymorphisms of Siglecs with human diseases. *Glycobiology* (2014) 24:785–93. doi: 10.1093/glycob/cwu043
81. Hatta Y, Tsuchiya N, Matsushita M, Shiota M, Hagiwara K, Tokunaga K. Identification of the gene variations in human CD22. *Immunogenetics* (1999) 49:280–6. doi: 10.1007/s002510050494
82. Pillai S, Cariappa A, Pirnie SP. Esterases and autoimmunity: the sialic acid acetylesterase pathway and the regulation of peripheral B cell tolerance. *Trends Immunol.* (2009) 30:488–93. doi: 10.1016/j.it.2009.07.006
83. Surolia I, Pirnie SP, Chellappa V, Taylor KN, Cariappa A, Moya J, et al. Functionally defective germline variants of sialic acid acetylesterase in autoimmunity. *Nature* (2010) 466:243–7. doi: 10.1038/nature09115
84. Chellappa V, Taylor KN, Pedrick K, Donado C, Netravali IA, Haider K, et al. M89V Sialic acid Acetyl Esterase (SIAE) and all other non-synonymous common variants of this gene are catalytically normal. *PLoS ONE* (2013) 8:e53453. doi: 10.1371/journal.pone.0053453
85. Cariappa A, Takematsu H, Liu H, Diaz S, Haider K, Boboila C, et al. B cell antigen receptor signal strength and peripheral B cell development are regulated by a 9-O-acetyl sialic acid esterase. *J Exp Med.* (2009) 206:125–38. doi: 10.1084/jem.20081399
86. Hitomi Y, Tsuchiya N, Hasegawa M, Fujimoto M, Takehara K, Tokunaga K, et al. Association of CD22 gene polymorphism with susceptibility to limited cutaneous systemic sclerosis. *Tissue Antigens* (2007) 69:242–9. doi: 10.1111/j.1399-0039.2007.00801.x
87. Dawidowicz K, Dieudé P, Avouac J, Wipff J, Hachulla E, Diot E, et al. Association study of B-cell marker gene polymorphisms in European Caucasian patients with systemic sclerosis. *Clin Exp Rheumatol.* (2011) 29:839–42.
88. Lajaunias F, Nitschke L, Moll T, Martinez-Soria E, Semac I, Chicheportiche Y, et al. Differentially regulated expression and function of CD22 in activated B-1 and B-2 lymphocytes. *J Immunol.* (2002) 168:6078–83. doi: 10.4049/jimmunol.168.12.6078
89. John B, Herrin BR, Raman C, Wang YN, Bobbitt KR, Brody BA, et al.The B cell coreceptor CD22 associates with AP50, a clathrin-coated pit adapter protein, via tyrosine-dependent interaction. *J Immunol.* (2003) 170:3534–43. doi: 10.4049/jimmunol.170.7.3534
90. El-Sayed ZA, Ragab SM, Khalifa KA, El Ashmawy RA. Altered CD19/CD22 balance in Egyptian children and adolescents with systemic lupus erythematosus. *Egypt J Immunol.* (2009) 16:27–38.
91. Suzuki J, Nakano S, Nakairi Y, Mitsuo A, Amano H, Morimoto S, et al. CD19/22 balance relates to improvement of disease activity in systemic lupus erythematosus. *Mod Rheumatol.* (2006) 16:235–8. doi: 10.3109/s10165-006-0497-z
92. Melissaropoulos K, Liossis SN. Decreased CD22 expression and intracellular signaling aberrations in B cells of patients with systemic sclerosis. *Rheumatol Int.* (2018) 38:1225–34. doi: 10.1007/s00296-018-4076-3
93. Odaka M, Hasegawa M, Hamaguchi Y, Ishiura N, Kumada S, Matsushita T, (2012) Autoantibody-mediated regulation of B cell responses by functional anti-CD22 autoantibodies in patients with systemic sclerosis. *Clin Exp Immunol.* (2010) 159:176–84. doi: 10.1111/j.1365-2249.2009.04059.x
94. Giltiay NV, Chappell CP, Clark EA. B-cell selection and the development of autoantibodies. *Arthritis Res Ther.* (2012) 14(Suppl. 4):S1. doi: 10.1186/ar3918
95. Meffre E, Wardemann H. B-cell tolerance checkpoints in health and autoimmunity. *Curr Opin Immunol.* (2008) 20:632–8. doi: 10.1016/j.coi.2008.09.001
96. Danzer CP, Collins BE, Blixt O, Paulson JC, Nitschke L. Transitional and marginal zone B cells have a high proportion of unmasked CD22: implications for BCR signaling. *Int Immunol.* (2003) 15:1137–47. doi: 10.1093/intimm/dxg114
97. Cyster JG, Goodnow CC. Tuning antigen receptor signaling by CD22: integrating cues from antigens and the microenvironment. *Immunity* (1997) 6:509–17. doi: 10.1016/S1074-7613(00)80339-8
98. Lanoue A, Batista FD, Stewart M, Neuberger MS. Interaction of CD22 with alpha2,6-linked sialoglycoconjugates: innate recognition of self to dampen B cell autoreactivity? *Eur J Immunol.* (2002) 32:348–55. doi: 10.1002/1521-4141(200202)32:2<348::AID-IMMU348>3.0.CO;2-5
99. Duong BH, Tian H, Ota T, Completo G, Han S, Vela JL, et al. Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance *in vivo*. *J Exp Med.* (2010) 207:173–87. doi: 10.1084/jem.20091873
100. Gagneux P, Varki A. Evolutionary considerations in relating oligosaccharide diversity to biological function. *Glycobiology* (1999) 9:747–55. doi: 10.1093/glycob/9.8.747
101. Seite JF, Cornec D, Renaudineau Y, Youinou P, Mageed RA, Hillion S. IVIg modulates BCR signaling through CD22 and promotes apoptosis in mature human B lymphocytes. *Blood* (2010) 116:1698–704. doi: 10.1182/blood-2009-12-261461
102. Berland R, Fernandez L, Kari E, Han JH, Lomakin I, Akira S, et al. Toll-like receptor 7-dependent loss of B cell tolerance in pathogenic autoantibody knockin mice. *Immunity* (2006) 25:429–40.
103. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* (2006) 25:417–28. doi: 10.1016/j.immuni.2006.07.013
104. Giltiay NV, Shu GL, Shock A, Clark EA. Targeting CD22 with the monoclonal antibody epratuzumab modulates human B-cell maturation and cytokine production in response to Toll-like receptor 7 (TLR7) and B-cell receptor (BCR) signaling. *Arthritis Res Ther.* (2017) 19:91. doi: 10.1186/s13075-017-1284-2
105. Fleischer V, Sieber J, Fleischer SJ, Shock A, Heine G, Daridon C, et al. pratuzumab inhibits the production of the proinflammatory cytokines IL-6 and TNF-alpha, but not the regulatory cytokine IL-10, by B cells from healthy donors and SLE patients. *Arthritis Res. Ther* (2015) 17:185. doi: 10.1186/s13075-015-0686-2
106. Tuscano JM, Riva A, Toscano SN, Tedder TF, Kehrl JH. CD22 cross-linking generates B-cell antigen receptor-independent signals that activate the JNK/SAPK signaling cascade. *Blood* (1999) 94:1382–92.
107. Liu BS, Cao Y, Huizinga TW, Hafler DA, Toes RE. TLR-mediated STAT3 and ERK activation controls IL-10 secretion by human B cells. *Eur J Immunol.* (2014) 44:2121–9. doi: 10.1002/eji.201344341
108. Meesmann HM, Fehr EM, Kierschke S, Herrmann M, Bilyy R, Heyder P, et al. Decrease of sialic acid residues as an eat-me signal on the surface of apoptotic lymphocytes. *J Cell Sci.* (2010) 123:3347–56. doi: 10.1242/jcs.066696
109. Colonna L, Lood C, Elkon KB. Beyond apoptosis in lupus. *Curr Opin Rheumatol.* (2014) 26:459–66. doi: 10.1097/BOR.0000000000000083
110. Akatsu C, Shinagawa K, Numoto N, Liu Z, Ucar AK, Aslam M, et al. CD72 negatively regulates B lymphocyte responses to the lupus-related endogenous toll-like receptor 7 ligand Sm/RNP. *J Exp Med.* (2016) 213:2691–2706. doi: 10.1084/jem.20160560
111. Xu M, Hou R, Sato-Hayashizaki A, Man R, Zhu C, Wakabayashi C, et al. Cd72(c) is a modifier gene that regulates Fas(lpr)-induced autoimmune disease. *J Immunol.* (2013) 190:5436–45. doi: 10.4049/jimmunol.1203576
112. Liu Y, Chen GY, Zheng P. CD24-Siglec G/10 discriminates danger- from pathogen-associated molecular patterns. *Trends Immunol.* (2009) 30:557–61. doi: 10.1016/j.it.2009.09.006

113. Leonard JP, Goldenberg DM. Preclinical and clinical evaluation of epratuzumab (anti-CD22 IgG) in B-cell malignancies. *Oncogene* (2007) 26:3704–13. doi: 10.1038/sj.onc.1210370
114. Sullivan-Chang L, O'Donnell RT, Tuscano JM. Targeting CD22 in B-cell malignancies: current status and clinical outlook. *BioDrugs* (2013) 27:293–304. doi: 10.1007/s40259-013-0016-7
115. Carnahan J, Stein R, Qu Z, Hess K, Cesano A, Hansen HJ, et al. Epratuzumab, a CD22-targeting recombinant humanized antibody with a different mode of action from rituximab. *Mol Immunol.* (2007) 44:1331–41. doi: 10.1016/j.molimm.2006.05.007
116. Leonard JP, Coleman M, Ketas JC, Chadburn A, Ely S, Furman RR, et al. Phase I/II trial of epratuzumab (humanized anti-CD22 antibody) in indolent non-Hodgkin's lymphoma. *J Clin Oncol.* (2003) 21:3051–9. doi: 10.1200/JCO.2003.01.082
117. Chen WC, Sigal DS, Saven A, Paulson JC. Targeting B lymphoma with nanoparticles bearing glycan ligands of CD22. *Leuk Lymphoma* (2012) 53:208–10. doi: 10.3109/10428194.2011.604755
118. Collins BE, Blixt O, Han S, Duong B, Li H, Nathan JK, et al. High-affinity ligand probes of CD22 overcome the threshold set by cis ligands to allow for binding, endocytosis, and killing of B cells. *J Immunol.* (2006) 177:2994–3003. doi: 10.4049/jimmunol.177.5.2994
119. Fry TJ, Shah NN, Orentas RJ, Stetler-Stevenson M, Yuan CM, Ramakrishna S, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nat Med.* (2018) 24:20–8. doi: 10.1038/nm.4441
120. Geh D, Gordon C. Epratuzumab for the treatment of systemic lupus erythematosus. *Expert Rev Clin Immunol.* (2018) 14:245–58. doi: 10.1080/1744666X.2018.1450141
121. Wallace DJ, Goldenberg DM. Epratuzumab for systemic lupus erythematosus. *Lupus* (2013) 22:400–5. doi: 10.1177/0961203312469692
122. Clowse ME, Wallace DJ, Furie RA, Petri MA, Pike MC, Leszczynski P, et al. Efficacy and safety of epratuzumab in moderately to severely active systemic lupus erythematosus: results from two phase III randomized, double-blind, placebo-controlled trials. *Arthritis Rheumatol.* (2017) 69:362–75. doi: 10.1002/art.39856
123. Gottenberg JE, Dorner T, Bootsma H, Devauchelle-Pensec V, Bowman SJ, Mariette X, et al. Efficacy of epratuzumab, an anti-CD22 monoclonal IgG antibody, in systemic lupus erythematosus patients with associated sjogren's syndrome: *post hoc* analyses from the EMBODY trials. *Arthritis Rheumatol.* (2018) 70:763–73. doi: 10.1002/art.40425
124. Dorner T, Shock A, Goldenberg DM, Lipsky PE. The mechanistic impact of CD22 engagement with epratuzumab on B cell function: implications for the treatment of systemic lupus erythematosus. *Autoimmun Rev.* (2015) 14:1079–86. doi: 10.1016/j.autrev.2015.07.013
125. Lumb S, Fleischer SJ, Wiedemann A, Daridon C, Maloney A, Shock A, et al. Engagement of CD22 on B cells with the monoclonal antibody epratuzumab stimulates the phosphorylation of upstream inhibitory signals of the B cell receptor. *J Cell Commun Signal.* (2016) 10:143–51. doi: 10.1007/s12079-016-0322-1
126. Sim JH, Kim HR, Chang SH, Kim IJ, Lipsky PE, Lee J. Autoregulatory function of interleukin-10-producing pre-naive B cells is defective in systemic lupus erythematosus. *Arthritis Res Ther.* (2015) 17:190. doi: 10.1186/s13075-015-0687-1
127. Jacobi AM, Reiter K, Mackay M, Aranow C, Hiepe F, Radbruch A, et al. Activated memory B cell subsets correlate with disease activity in systemic lupus erythematosus: delineation by expression of CD27, IgD, and CD95. *Arthritis Rheum.* (2008) 58:1762–73. doi: 10.1002/art.23498
128. Macauley MS, Pfrengle F, Rademacher C, Nycholat CM, Gale AJ, von Drygalski A, et al. Antigenic liposomes displaying CD22 ligands induce antigen-specific B cell apoptosis. *J Clin Invest.* (2013) 123:3074–83. doi: 10.1172/JCI69187
129. Chappell CP, Clark EA. STALing B cell responses with CD22. *J Clin Invest.* (2013) 123:2778–80. doi: 10.1172/JCI69670
130. Mihaylova V, Salih MA, Mukhtar MM, Abuzeid HA, El-Sadig SM, von der Hagen, Huebner AM, et al. Refinement of the clinical phenotype in musk-related congenital myasthenic syndromes. *Neurology* (2009) 73:1926–8. doi: 10.1212/WNL.0b013e3181c3fce9
131. Fiorina P, Vergani A, Dada S, Jurewicz M, Wong M, Law K, et al. Targeting CD22 reprograms B-cells and reverses autoimmune diabetes. *Diabetes* (2008) 57:3013–24. doi: 10.2337/db08-0420
132. Bednar KJ, Shanina E, Ballet R, Connors EP, Duan S, Juan J, et al. Human CD22 inhibits murine B cell receptor activation in a human CD22 transgenic mouse model. *J Immunol.* (2017) 199:3116–28. doi: 10.4049/jimmunol.1700898

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 02 October 2018
doi: 10.3389/fimmu.2018.02276

Ligand Recognition Determines the Role of Inhibitory B Cell Co-receptors in the Regulation of B Cell Homeostasis and Autoimmunity

*Takeshi Tsubata**

Department of Immunology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

B cells express various inhibitory co-receptors including CD22, CD72, and Siglec-G. These receptors contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the cytoplasmic region. Although many of the inhibitory co-receptors negatively regulate BCR signaling by activating SH2-containing protein tyrosine phosphatase 1 (SHP-1), different inhibitory co-receptors have distinct functional properties. CD22, Siglec-G, and CD72 preferentially regulate tonic signaling in conventional B cells, B-1 cell homeostasis, and development of lupus-like disease, respectively. CD72 recognizes RNA-related lupus self-antigen Sm/RNP as a ligand. This ligand recognition recruits CD72 to BCR in Sm/RNP-reactive B cells thereby suppressing production of anti-Sm/RNP autoantibody involved in the pathogenesis of lupus. In contrast, Siglec-G recognizes α2,3 as well as α2,6 sialic acids whereas CD22 recognizes α2,6 sialic acid alone. Because glycoproteins including BCR are dominantly glycosylated with α2,3 sialic acids in B-1 cells, Siglec-G but not CD22 recruits BCR as a ligand specifically in B-1 cells, and regulates B-1 cell homeostasis by suppressing BCR signaling in B-1 cells. Thus, recognition of distinct ligands determines functional properties of different inhibitory B cell co-receptors.

Keywords: inhibitory B cell co-receptor, CD72, CD22, siglec-G, systemic lupus erythematosus, B-1 cells, Sm/RNP, sialic acid

OPEN ACCESS

Edited by:
Aaron James Marshall,
University of Manitoba, Canada

Reviewed by:
Pablo Engel,
University of Barcelona, Spain
Louis Justement,
University of Alabama at Birmingham, United States

***Correspondence:**
Takeshi Tsubata
tsubata.imm@mri.tmd.ac.jp

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *02 July 2018*
Accepted: *13 September 2018*
Published: *02 October 2018*

Citation:
Tsubata T (2018) Ligand Recognition Determines the Role of Inhibitory B Cell Co-receptors in the Regulation of B Cell Homeostasis and Autoimmunity. Front. Immunol. 9:2276. doi: 10.3389/fimmu.2018.02276

INTRODUCTION

Antigen-induced signaling through B cell receptor (BCR) plays a central role in B cell responses to antigens (1). BCR also transmits constitutive low level signaling called tonic signaling in the absence of antigen stimulation (2). Tonic signaling regulates B cell survival and development. BCR ligation activates protein tyrosine kinases such as Lyn and Syk, which phosphorylate and activate various down-stream signaling molecules (1). BCR signaling is negatively regulated by various inhibitory co-receptors such as FcγRIIB, Sialic acid-binding Ig-like lectin (Siglec)-10/G (human/mouse ortholog), CD22 (also known as Siglec-2), CD72, PECAM1 (also known as CD31), CEACAM-1, and LILRB/PIR-B (human/mouse ortholog) (3, 4). These inhibitory co-receptors contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the cytoplasmic region. ITIMs in FcγRIIB and CD22 are shown to be phosphorylated by Lyn when BCR is ligated. Lyn may also be responsible for phosphorylation of the ITIMs in the other inhibitory co-receptors. Upon phosphorylation, these ITIMs recruit and activate SH2-containing phosphatases such as SH2-containing protein tyrosine phosphatase (SHP)-1, SHP-2, and SH2-containing inositol 5′-phosphatase (SHIP)-1, thereby down-modulating BCR signaling by dephosphorylating signaling

molecules activated by BCR ligation (**Figure 1**). SHIP-1 negatively regulates phosphatidyl inositol 3-kinase (PI-3K)-Akt pathway by dephosphorylating PIP3 generated by PI-3K (5). Studies on B cells deficient in SHP-1 or inhibitory co-receptors demonstrated that proximal signaling molecules of BCR including Lyn, Syk, Igα/Igβ, BLNK/SLP-65 are hyperphosphorylated (6, 7). Because SHP-1 associates with Lyn (8) and Syk (9), these kinases appear to be substrates of SHP-1. The other BCR signaling molecules may be directly or indirectly dephosphorylated by SHP-1. It may be unlikely that SHP-1 activated by different co-receptors dephosphorylate distinct substrates though there is no evidence. CD22 was reported to recruit stimulatory signaling molecules including Syk and phospholipase Cγ (10). However, SHP-1 appears to be the dominant effector of CD22 because CD22 negatively regulates BCR signaling.

FcγRIIB recruits SHIP-1 but not SHP-1 or SHP-2 at phosphorylated ITIMs whereas the other inhibitory B cell co-receptors recruit SHP-1, SHP-2 or both (3). Although the sequence of ITIMs may determine which phosphatase is recruited, the precise mechanism is not yet clear. Although the roles of SHP-2 in B cells is not yet clear, SHP-1 is shown to play crucial roles in the maintenance of B cell homeostasis. B cell-specific conditional SHP-1$^{-/-}$ mice show alterations in the development of conventional B cells, expansion of B-1 cells and development of lupus-like autoimmune disease (11) (**Table 1**). B cell-specific SHIP-1-deficient mice show similar phenotypes (12). However, FcγRIIB$^{-/-}$ mice show none of these phenotypes (13) although FcγRIIB down-regulates antibody responses and is associated with autoimmune diseases (14). How SHIP-1 is activated to regulate development and homeostasis of B cells is not yet clear. In contrast, deficiency in SHP-1-activating co-receptors CD22, Siglec-G and CD72 causes alterations in the development of conventional B cells (11, 15–18), expansion of B-1 cells (19), and development of lupus-like disease (20, 21), respectively. Thus, SHP-1 activated by different co-receptors regulates distinct B cell phenotypes. Because the roles of the ligands are extensively studied in CD22, Siglec-G, and CD72 among SHP-1-activating B cell co-receptors, I would like to discuss distinct functional properties of different inhibitory co-receptors and the role of ligand recognition in determining their functional properties by focusing on CD22, Siglec-G and CD72.

DISTINCT FUNCTIONAL PROPERTIES OF CD22, SIGLEC-G, AND CD72

In Siglec-G$^{-/-}$ mice, the number of B-1 cells in the peritoneal cavity is increased by around 10-folds (19), which is almost equivalent to B-1 cell expansion observed in B cell-specific SHP-1$^{-/-}$ mice (11). In contrast, CD22$^{-/-}$ mice (15, 16), PECAM1$^{-/-}$ mice (22), and PIR-B$^{-/-}$ mice (23) show only modest increase in the number of B-1 cells. Thus, Siglec-G plays a central role in SHP-1-mediated regulation of B-1 cells, whereas other inhibitory co-receptors play an auxiliary or no role in the regulation of B-1 cell homeostasis.

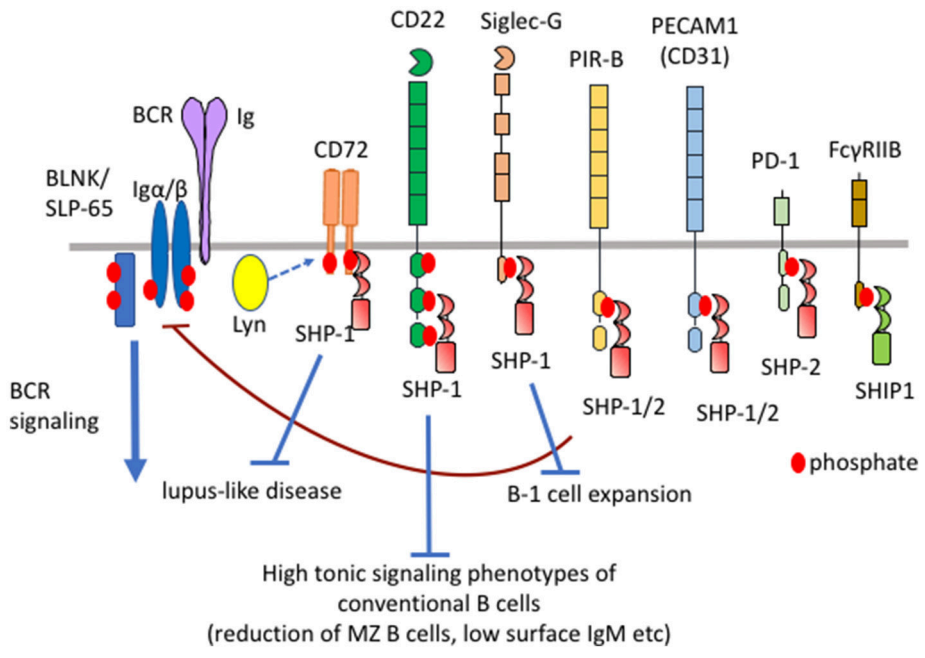


FIGURE 1 | Differential functional properties of inhibitory B cell co-receptors. Mouse B cells express various inhibitory co-receptors such as CD72, CD22, Siglec-G, PIR-B, PEACAM1, PD-1, and FcγRIIB. These receptors contain ITIMs in the cytoplasmic region and recruit SH2-containing phosphatases such as SHP-1, SHP-2, and SHIP-1 upon phosphorylation by Lyn, leading to down-modulation of BCR signaling. Although many of these inhibitory receptors activate SHP-1, CD72, CD22, and Siglec-G inhibits development of lupus-like disease, high tonic signaling phenotypes of conventional B cells, and B-1 cell expansion, respectively.

TABLE 1 | B cell phenotypes of mice deficient in inhibitory B cell co-receptors, ligands, and effector phosphatases.

Phenotype[a]	Mice						
	SHP-1$^{-/-}$	SHIP-1$^{-/-}$	CD22$^{-/-}$	CD72$^{-/-}$	Siglec-G$^{-/-}$	Siglec-G R120E[b]	FcγRIIB
High tonic signaling in conventional B cells[c]	++	++	++	−	−	−	−
B-1 cell expansion	++	++	±	−	++	++	−
Lupus-like disease	++	++	−	++	−	NA[d]	−

[a] *Both ST6Gall$^{-/-}$ mice deficient in α2,6 sialic acid and CD22 R130E mice expressing CD22 deficient in ligand binding show reduction in BCR signaling in conventional B cells upon BCR ligation.*
[b] *Deficient in ligand binding.*
[c] *Reduction in marginal zone B cell population and reduction in the level of cell surface IgM.*
[d] *Not available.*

CD22$^{-/-}$ mice as well as B cell-specific SHP1$^{-/-}$ or Lyn$^{-/-}$ mice show various alterations in conventional B cells such as reduction in the number of marginal zone (MZ) B cells and reduction in the level of IgM on the surface of follicular B cells (11, 15–18). Recently, Yasuda et al. demonstrated that IgM^{hi} cells show higher phosphorylation levels of signaling molecules such as Erk and Akt, and better *in vitro* survival compared to IgM^{lo} cells (24), suggesting that the total tonic signaling level required for B cell survival depends on the expression level of BCR. If BCR carries high tonic signaling activity, total tonic signaling level in IgM^{lo} cells may be sufficient for survival. Thus, the reduction in the level of surface IgM in CD22$^{-/-}$ B cells suggests increased tonic signaling activity in the absence of CD22. This notion is also supported by the reduction in MZ B cells in CD22$^{-/-}$ mice because B cells with low tonic signaling are suggested to preferentially differentiate to MZ B cells (25). In contrast, these alterations in conventional B cells are not observed in mice deficient in other inhibitory co-receptors such as CD72.

Almost all CD72$^{-/-}$ mice spontaneously develop lupus-like glomerulonephritis by 6 months of age (21). CD72$^{-/-}$Fas$^{lpr/lpr}$ mice on the C57BL/6 background develop severe lupus-like disease comparable to MRL.Fas^{lpr} mice. Both CD72$^{-/-}$Fas$^{lpr/lpr}$ mice and MRL.Fas^{lpr} mice produce large amounts of autoantibodies such as anti-DNA antibody and develop glomerulonephritis with severe histological changes at 6 months of age. In contrast, mice deficient in other inhibitory co-receptors such as CD22$^{-/-}$ mice and PIR-B$^{-/-}$ mice do not develop autoimmune disease (26, 27). Even by introduction of Fas^{lpr}, only a fraction of PIR-B$^{-/-}$Fas$^{lpr/lpr}$ mice develop lupus-like disease at 12 months of age (27). Only a fraction of PECAM1$^{-/-}$ mice and Siglec-G$^{-/-}$ mice develop mild lupus-line disease after 12 months of age (22, 26). Because development of autoimmune disease partly depends on the cleanness of the animal facility, it is not possible to discuss small differences in the disease severity among the different mice housed in different facilities. Nonetheless, CD72$^{-/-}$ mice develop lupus-like disease that is clearly more severe than that developed in mice deficient in other inhibitory co-receptors. Thus, CD72 appears to be a dominant inhibitory B cell co-receptor in the regulation of autoimmune disease.

Taken together, Siglec-G, CD22, and CD72 regulate B-1 cell homeostasis, tonic signaling of conventional B cells, and development of lupus-like disease, respectively (**Figure 1**; **Table 1**), suggesting that different inhibitory B cell co-receptors regulate distinct B cell phenotypes.

ROLE OF LIGANDS IN DETERMINING FUNCTIONAL PROPERTIES OF INHIBITORY B CELL CO-RECEPTORS

Most of the inhibitory co-receptors recognize endogenous ligands (**Table 2**). Role of the endogenous ligands in determining the functional properties of inhibitory co-receptors was first demonstrated in FcγRIIB already in 1990s. FcγRIIB inhibits BCR signaling when co-ligated with BCR. Binding of immune complexes composed of antigens and IgG with BCR induces co-ligation of FcγRIIB and BCR, thereby down-regulating BCR signaling and antibody responses to the antigens (28, 29). In contrast, roles of endogenous ligands of SHP-1-activating inhibitory B cell co-receptors were not clear until a few years ago.

CD72 is a type II membrane molecule containing a C-type lectin-like domain (CTLD) in the extracellular region. The ligand of CD72 was initially reported to be CD5, although this result has not been reproduced (30). Later, CD100 (also known as Semaphorin-4D) was shown to be an inhibitory ligand of CD72 (31). The functional significance of this inhibitory ligand is not yet clear. We demonstrated that the extracellular CTLD of CD72 specifically recognizes the lupus self-antigen Sm/RNP as a ligand (32). Sm/RNP is a major RNA-containing lupus self-antigen, and a ligand of the endosomal RNA sensor TLR7 (33). Because TLR7 but not the DNA sensor TLR9 is essential for development of lupus-like disease in multiple mouse models (34), autoimmune response to RNA-related self-antigens such as Sm/RNP appears to be crucial in development of SLE.

When BCR is ligated by Sm/RNP, CD72$^{-/-}$ B cells show augmented Ca^{2+} and proliferative responses compared to CD72$^{+/+}$ B cells (32). In contrast, Ca^{2+} and proliferative responses to a control antigen in CD72$^{-/-}$ B cells are comparable to that in CD72$^{+/+}$ B cells. This result suggests that CD72 specifically down-regulates BCR signaling when BCR is ligated by Sm/RNP. When Sm/RNP binds to BCR expressed on the surface of Sm/RNP-reactive B cells, CD72 appears to be recruited to BCR because of its binding to Sm/RNP (**Figure 2A**). Antigen-mediated recruitment of CD72 to Sm/RNP-reactive BCR may

TABLE 2 | Inhibitory co-receptors and their ligands.

Inhibitory co-receptors	Expression	Ligands	Role of ligands[a]	Expression of ligands
CD22	Constitutive	α2,6 sialic acid	Inhibitory	Ubiquitous
CD72	Constitutive	Sm/RNP	Stimulatory	Released from dead cells
		CD100 (Sema4D)	Inhibitory	Various hematopoietic and non-hematopoietic cells
Siglec-G	Constitutive	α2,3 sialic acid	Stimulatory	Ubiquitous, B1 cells >> conventional B cells
		α2,6 sialic acid	Stimulatory	Ubiquitous
PIA-B	Constitutive	MHCI	Stimulatory	Ubiquitous
PECAM1	Constitutive	PECAM1	?	Endothelial cells, hematopoietic cells
		α2,6 sialic acid	?	Ubiquitous
PD-1	Inducible	PD-L1	Stimulatory	Hematopoietic cells, various non-hematopoietic cells
		PD-L2	Stimulatory	Macrophages, DCs, mast cells, B-1 cells
FcγRIIB	Constitutive	IgG	Stimulatory	

[a] *Inhibitory or stimulatory role in co-receptor-mediated signal inhibition.*

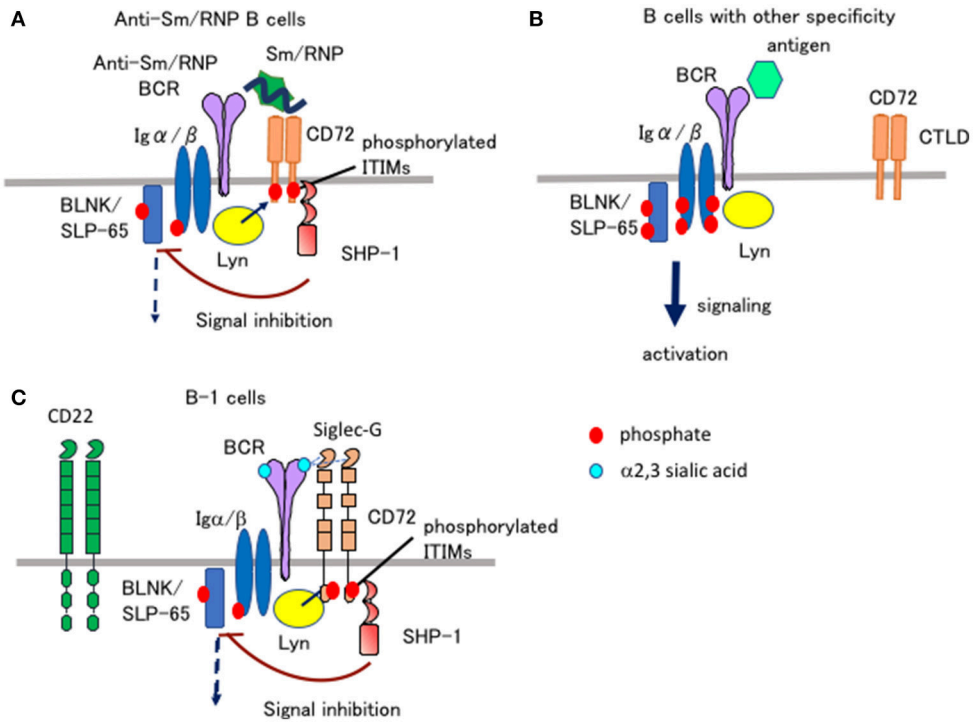


FIGURE 2 | Ligand recognition determines the functional properties of CD72 and Siglec-G. **(A,B)** CD72 specifically inhibits BCR signaling in Sm/RNP-reactive B cells by recognizing Sm/RNP as a ligand. When Sm/RNP interacts with Sm/RNP-reactive BCR, CD72 is recruited to BCR by recognition of Sm/RNP **(A)**. CD72 ITIM is then phosphorylated by Lyn and recruits SHP-1. This leads to suppression of BCR signaling in Sm/RNP-reactive B cells thereby inhibiting production of anti-Sm/RNP antibody crucial for development of lupus. When other antigens that do not bind to CD72 interact with BCR, CD72 is kept away from BCR and does not inhibit BCR signaling **(B)**. **(C)** Siglec-G inhibits BCR signaling in B-1 cells by recognizing α2,3 sialic acid as a ligand. Because glycoproteins in B-1 cells are dominantly glycosylated with α2,3 sialic acid, Siglec-G constitutively associates with BCR by recognizing α2,3 sialic acid expressed on BCR in B-1 cells, thereby down-modulating BCR signaling (35). CD22 does not regulate BCR signaling in B-1 cells because CD22 recognizes α2,6 sialic acid but not α2,3 sialic acid.

induce phosphorylation of the CD72 ITIM by BCR-associated Lyn, leading to SHP-1-mediated suppression of BCR signaling. In contrast, CD72 may not be recruited to BCR when BCR interacts with the other antigens that do not bind to CD72 (**Figure 2B**). Thus, CD72 negatively regulates BCR signaling induced by Sm/RNP but not the other antigens, thereby specifically inhibits activation of B cells reactive to Sm/RNP. In $CD72^{-/-}$ mice, Sm/RNP activates B cells reactive to Sm/RNP probably by inducing both BCR signaling and TLR7 signaling, leading to the production of anti-Sm/RNP antibody crucial for development

of lupus. CD72 appears to inhibit development of lupus by inhibiting activation of Sm/RNP-reactive B cells.

Both CD22 and Siglec-G are members of the Siglec family, and recognize sialic acids as a ligand (36). CD22 specifically recognizes α2,6 sialic acid, whereas Siglec-G broadly recognizes both α2,3 and α2,6 sialic acids. Previously, Nitschke and his collaborators addressed how Siglec-G but not CD22 strongly regulates BCR signaling in B-1 cells and B-1 cell homeostasis (35), although both Siglec-G and CD22 are expressed by both B-1 cells. They demonstrated that the Siglec-G mutant deficient in ligand binding no longer associates with BCR nor down-regulates BCR signaling, suggesting that Siglec-G associates with BCR by recognizing sialic acid located in BCR thereby inhibiting BCR signaling. They further demonstrated that B-1 cells express α2,3 sialic acid at much higher level than conventional B cells. Recognition of α2,3 sialic acid by Siglec-G induces association of Siglec-G and BCR specifically in B-1 cells, which may induce phosphorylation of the Siglec-G ITIM by Lyn and activation of SHP-1 required for inhibition of BCR signaling (**Figure 2C**).

Although CD22 regulates tonic signaling, how ligand recognition of CD22 is involved in this function is not yet clear. As is the case for Siglec-G in B-1 cells, CD22 is shown to be associated with BCR by recognizing a sialylated ligand in conventional B cells (37). However, studies with mice deficient in ST6GalI, the sialyl transferase required for the synthesis of α2,6 sialic acid, and those with mice expressing a mutant CD22 that do not recognize α2,6 sialic acid showed that endogenous ligands rather down-modulate suppressive activity of CD22 (38–40). These findings are contradictory to the model in which ligand recognition induces CD22-mediated signal inhibition. Whether ligand recognition is involved in the functional properties of CD22 needs to be further studied. Other inhibitory co-receptors also recognize endogenous ligands (3). PIR-B is known to interact with MHC I (41). Because PIR-B phosphorylation is modestly reduced in $\beta 2m^{-/-}$ splenocytes (42), interaction of PIR-B with MHC-I may facilitate PIR-B-mediated signal inhibition. PECAM1 and CEACAM-1 (4) show homotypic interaction with trans-ligands, and PECAM1 was also shown to recognize sialic acids (43). How ligand recognition regulates the functional activities of these inhibitory co-receptors is not yet clear (**Table 2**).

CONCLUSIONS AND FUTURE PERSPECTIVE

The inhibitory B cell co-receptors CD22, CD72, and Siglec-G regulate distinct B cell functions: CD22 regulates tonic signaling in conventional B cells, Siglec-G regulates B-1 cell homeostasis and CD72 regulates autoimmunity. Recognition of Sm/RNP induces association of CD72 with BCR in B cells reactive to Sm/RNP whereas recognition of α2,3 sialic acid induces association of Siglec-G with BCR in B-1 cells. Thus, different inhibitory co-receptors associate with BCR in distinct B cell populations depending on the ligand recognition of inhibitory co-receptors, thereby regulating distinct B cell functions, i.e., development of lupus-like disease by CD72 and B-1 cell homeostasis by Siglec-G. Recognition of endogenous ligands thus determines the B cell phenotypes regulated by CD72 and Siglec-G. Further determination of ligands of inhibitory co-receptors and elucidation of the roles of ligand recognition may advance our understandings on how inhibitory co-receptors regulate development and differentiation of B cells and suppress activation of pathological B cells. These studies may provide clues in understanding pathogenesis of immunological diseases.

AUTHOR CONTRIBUTIONS

TT conceived of this mini review and wrote the manuscript.

ACKNOWLEDGMENTS

I thank Dr. J.-Y. Wang (Fudan University) for critically reading the manuscript. The author's work was supported by JPSP Grant-in-Aid for Scientific Research 26293062, 17H05790, 18H02610.

REFERENCES

1. Kurosaki T, Shinohara H, Baba Y. B cell signaling and fate decision. *Annu Rev Immunol.* (2010) 28:21–55. doi: 10.1146/annurev.immunol.021908.132541
2. Myers DR, Zikherman J, Roose JP. Tonic signals: why do lymphocytes bother? *Trends Immunol.* (2017) 38:844–57. doi: 10.1016/j.it.2017.06.010
3. Tsubata T. Role of inhibitory BCR co-receptors in immunity. *Infect Disord Drug Targets* (2012) 12:181–90. doi: 10.2174/187152612800564455
4. Lobo EO, Zhang Z, Shively JE. Pivotal advance: CEACAM1 is a negative coreceptor for the B cell receptor and promotes CD19-mediated adhesion of B cells in a PI3K-dependent manner. *J Leukoc Biol.* (2009) 86:205–18. doi: 10.1189/jlb.0109037
5. Conde C, Gloire G, Piette J. Enzymatic and non-enzymatic activities of SHIP-1 in signal transduction and cancer. *Biochem Pharmacol.* (2011) 82:1320–34. doi: 10.1016/j.bcp.2011.05.031
6. Zhang J, Somani AK, Siminovitch KA. Roles of the SHP-1 tyrosine phosphatase in the negative regulation of cell signalling. *Semin Immunol.* (2000) 12:361–78. doi: 10.1006/smim.2000.0223
7. Adachi T, Wienands J, Wakabayashi C, Yakura H, Reth M, Tsubata T. SHP-1 requires inhibitory co-receptors to down-modulate B cell antigen receptor-mediated phosphorylation of cellular substrates. *J Biol Chem.* (2001) 276:26648–55. doi: 10.1074/jbc.M100997200
8. Somani AK, Yuen K, Xu F, Zhang J, Branch DR, Siminovitch KA. The SH2 domain containing tyrosine phosphatase-1 down-regulates activation of Lyn and Lyn-induced tyrosine phosphorylation of the CD19 receptor in B cells. *J Biol Chem.* (2001) 276:1938–44. doi: 10.1074/jbc.M006820200
9. Dustin LB, Plas DR, Wong J, Hu YT, Soto C, Chan AC, et al. Expression of dominant-negative src-homology domain 2-containing protein tyrosine phosphatase-1 results in increased syk tyrosine kinase activity and B cell activation. *J Immunol.* (1999) 162:2717–24.
10. Nitschke L. CD22 and Siglec-G: B-cell inhibitory receptors with distinct functions. *Immunol Rev.* (2009) 230:128–43. doi: 10.1111/j.1600-065X.2009.00801.x
11. Pao LI, Lam KP, Henderson JM, Kutok JL, Alimzhanov M, Nitschke L, et al. B cell-specific deletion of protein-tyrosine phosphatase Shp1 promotes B-1a cell

development and causes systemic autoimmunity. *Immunity* (2007) 27:35–48. doi: 10.1016/j.immuni.2007.04.016
12. Maxwell MJ, Duan M, Armes JE, Anderson GP, Tarlinton DM, Hibbs ML. Genetic segregation of inflammatory lung disease and autoimmune disease severity in SHIP-1$^{-/-}$ mice. *J Immunol.* (2011) 186:7164–75. doi: 10.4049/jimmunol.1004185
13. Yajima K, Nakamura A, Sugahara A, Takai T. FcgammaRIIB deficiency with Fas mutation is sufficient for the development of systemic autoimmune disease. *Eur J Immunol.* (2003) 33:1020–9. doi: 10.1002/eji.200323794
14. Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol.* (2008) 8:34–47. doi: 10.1038/nri2206
15. Sato S, Miller AS, Inaoki M, Bock CB, Jansen PJ, Tang ML, et al. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity* (1996) 5:551–62. doi: 10.1016/s1074-7613(00)80270-8
16. Nitschke L, Carsetti R, Ocker B, Kohler G, Lamers MC. CD22 is a negative regulator of B-cell receptor signalling. *Curr Biol.* (1997) 7:133–43. doi: 10.1016/s0960-9822(06)00057-1
17. Otipoby KL, Andersson KB, Draves KE, Klaus SJ, Farr AG, Kerner JD, et al. CD22 regulates thymus-independent responses and the lifespan of B cells. *Nature* (1996) 384:634–7. doi: 10.1038/384634a0
18. Lamagna C, Hu Y, DeFranco AL, Lowell CA. B cell-specific loss of Lyn kinase leads to autoimmunity. *J Immunol.* (2014) 192:919–28. doi: 10.4049/jimmunol.1301979
19. Hoffmann A, Kerr S, Jellusova J, Zhang J, Weisel F, Wellmann U, et al. Siglec-G is a B1 cell-inhibitory receptor that controls expansion and calcium signaling of the B1 cell population. *Nat Immunol.* (2007) 8:695–704. doi: 10.1038/ni1480
20. Li DH, Winslow MM, Cao TM, Chen AH, Davis CR, Mellins ED, et al. Modulation of peripheral B cell tolerance by CD72 in a murine model. *Arthritis Rheum.* (2008) 58:3192–204. doi: 10.1002/art.23812
21. Xu M, Hou R, Sato-Hayashizaki A, Man R, Zhu C, Wakabayashi C, et al. Cd72(c) is a modifier gene that regulates Fas(lpr)-induced autoimmune disease. *J Immunol.* (2013) 190:5436–45. doi: 10.4049/jimmunol.1203576
22. Wilkinson R, Lyons AB, Roberts D, Wong MX, Bartley PA, Jackson DE. Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) acts as a regulator of B-cell development, B-cell antigen receptor (BCR)-mediated activation, and autoimmune disease. *Blood* (2002) 100:184–93. doi: 10.1182/blood-2002-01-0027
23. Ujike A, Takeda K, Nakamura A, Ebihara S, Akiyama K, Takai T. Impaired dendritic cell maturation and increased T(H)2 responses in PIR-B$^{-/-}$ mice. *Nat Immunol.* (2002) 3:542–8. doi: 10.1038/ni801
24. Yasuda S, Zhou Y, Wang Y, Yamamura M, Wang JY. A model integrating tonic and antigen-triggered BCR signals to predict the survival of primary B cells. *Sci Rep.* (2017) 7:14888. doi: 10.1038/s41598-017-13993-x
25. Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol.* (2009) 9:767–77. doi: 10.1038/nri2656
26. Jellusova J, Wellmann U, Amann K, Winkler TH, Nitschke L. CD22 x Siglec-G double-deficient mice have massively increased B1 cell numbers and develop systemic autoimmunity. *J Immunol.* (2010) 184:3618–27. doi: 10.4049/jimmunol.0902711
27. Takai T, Nakamura A, Endo S. Role of PIR-B in autoimmune glomerulonephritis. *J Biomed Biotechnol.* (2011) 2011:275302. doi: 10.1155/2011/275302
28. Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch JV. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. *Nature* (1994) 368:70–3. doi: 10.1038/368070a0
29. Wernersson S, Karlsson MC, Dahlstrom J, Mattsson R, Verbeek JS, Heyman B. IgG-mediated enhancement of antibody responses is low in Fc receptor gamma chain-deficient mice and increased in Fc gamma RII-deficient mice. *J Immunol.* (1999) 163:618–22.
30. Brown MH, Lacey E. A ligand for CD5 is CD5. *J Immunol.* (2010) 185:6068–74. doi: 10.4049/jimmunol.0903823
31. Kumanogoh A, Watanabe C, Lee I, Wang X, Shi W, Araki H, et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* (2000) 13:621–31. doi: 10.1016/s1074-7613(00)00062-5
32. Akatsu C, Shinagawa K, Numoto N, Liu Z, Ucar AK, Aslam M, et al. CD72 negatively regulates B lymphocyte responses to the lupus-related endogenous toll-like receptor 7 ligand Sm/RNP. *J Exp Med.* (2016) 213:2691–706. doi: 10.1084/jem.20160560
33. Lau CM, Broughton C, Tabor AS, Akira S, Flavell RA, Mamula MJ, et al. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/toll-like receptor 7 engagement. *J Exp Med.* (2005) 202:1171–7. doi: 10.1084/jem.20050630
34. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* (2006) 25:417–28. doi: 10.1016/j.immuni.2006.07.013
35. Hutzler S, Ozgor L, Naito-Matsui Y, Klasener K, Winkler TH, Reth M, et al. The ligand-binding domain of Siglec-G is crucial for its selective inhibitory function on B1 cells. *J Immunol.* (2014) 192:5406–14. doi: 10.4049/jimmunol.1302875
36. Macauley MS, Crocker PR, Paulson JC. Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol.* (2014) 14:653–66. doi: 10.1038/nri3737
37. Alborzian Deh Sheikh A, Akatsu C, Imamura A, Abdu-Allah HHM, Takematsu H, Ando H, et al. Proximity labeling of cis-ligands of CD22/Siglec-2 reveals stepwise alpha2,6 sialic acid-dependent and -independent interactions. *Biochem Biophys Res Commun.* (2018) 495:854–9. doi: 10.1016/j.bbrc.2017.11.086
38. Hennet T, Chui D, Paulson JC, Marth JD. Immune regulation by the ST6Gal sialyltransferase. *Proc Natl Acad Sci USA.* (1998) 95:4504–9. doi: 10.1073/pnas.95.8.4504
39. Collins BE, Smith BA, Bengtson P, Paulson JC. Ablation of CD22 in ligand-deficient mice restores B cell receptor signaling. *Nat Immunol.* (2006) 7:199–206. doi: 10.1038/ni1283
40. Muller J, Obermeier I, Wohner M, Brandl C, Mrotzek S, Angermuller S, et al. CD22 ligand-binding and signaling domains reciprocally regulate B-cell Ca^{2+} signaling. *Proc Natl Acad Sci USA.* (2013) 110:12402–7. doi: 10.1073/pnas.1304888110
41. Takai T. Paired immunoglobulin-like receptors and their MHC class I recognition. *Immunology* (2005) 115:433–40. doi: 10.1111/j.1365-2567.2005.02177.x
42. Ho LH, Uehara T, Chen CC, Kubagawa H, Cooper MD. Constitutive tyrosine phosphorylation of the inhibitory paired Ig-like receptor PIR-B. *Proc Natl Acad Sci USA.* (1999) 96:15086–90. doi: 10.1073/pnas.96.26.15086
43. Kitazume S, Imamaki R, Ogawa K, Komi Y, Futakawa S, Kojima S, et al. Alpha2,6-sialic acid on platelet endothelial cell adhesion molecule (PECAM) regulates its homophilic interactions and downstream antiapoptotic signaling. *J Biol Chem.* (2010) 285:6515–21. doi: 10.1074/jbc.M109.073106

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 08 October 2018
doi: 10.3389/fimmu.2018.02306

Immune Checkpoints as Therapeutic Targets in Autoimmunity

Christopher Paluch[1,2], *Ana Mafalda Santos*[1,3], *Consuelo Anzilotti*[1,3], *Richard J. Cornall*[1,2]* *and Simon J. Davis*[1,3]*

[1] MRC Human Immunology Unit, University of Oxford, Oxford, United Kingdom, [2] Nuffield Department of Clinical Medicine, University of Oxford, Oxford, United Kingdom, [3] Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom

Antibodies that block the immune checkpoint receptors PD1 and CTLA4 have revolutionized the treatment of melanoma and several other cancers, but in the process, a new class of drug side effect has emerged—immune related adverse events. The observation that therapeutic blockade of these inhibitory receptors is sufficient to break self-tolerance, highlights their crucial role in the physiological modulation of immune responses. Here, we discuss the rationale for targeting immune checkpoint receptors with agonistic agents in autoimmunity, to restore tolerance when it is lost. We review progress that has been made to date, using Fc-fusion proteins, monoclonal antibodies or other novel constructs to induce immunosuppressive signaling through these pathways. Finally, we explore potential mechanisms by which these receptors trigger and modulate immune cell function, and how understanding these processes might shape the design of more effective therapeutic agents in future.

Keywords: immune checkpoint, inhibitory receptor, agonist, antibody, autoimmunity, immunosuppression

OPEN ACCESS

Edited by:
Aaron James Marshall, University of Manitoba, Canada

Reviewed by:
Christopher E. Rudd, Université de Montréal, Canada
Mark S. Cragg, University of Southampton, United Kingdom
Lekh Dahal, University of Southampton, United Kingdom in collaboration with reviewer MC

***Correspondence:**
Richard J. Cornall
richard.cornall@ndm.ox.ac.uk
Simon J. Davis
simon.davis@imm.ox.ac.uk

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *08 June 2018*
Accepted: *17 September 2018*
Published: *08 October 2018*

Citation:
Paluch C, Santos AM, Anzilotti C, Cornall RJ and Davis SJ (2018) Immune Checkpoints as Therapeutic Targets in Autoimmunity. Front. Immunol. 9:2306. doi: 10.3389/fimmu.2018.02306

INTRODUCTION

Immune Checkpoint Receptors

The immune system comprises a powerful arsenal of effector mechanisms capable of inflicting devastating damage on invading pathogens, but also with the capacity to do great harm to the body itself. In order to prevent such destruction of host tissues and to restore quiescence after an inflammatory response, careful immune regulation is required. In the periphery, immune cell responses are controlled by a balance between positive and negative signals, which attune effector cells to their environment. For a T cell these signals are delivered by a myriad of co-stimulatory and co-inhibitory surface receptors, whose inputs are integrated alongside T cell receptor (TCR) signaling to determine the cell's fate. The co-inhibitory receptors such as programmed cell death protein 1 (PD1) and cytotoxic T lymphocyte associated protein 4 (CTLA4), also known as immune checkpoints, recognize surface-expressed ligands on self-tissues and act to dampen unwanted immune activation. In theory, a T cell which has escaped central tolerance, with a potentially autoreactive TCR, will be prevented from causing harm as it encounters its antigen in the context of healthy self-tissue expressing co-inhibitory ligands and no danger signals. Similar mechanisms control the response of innate immune cells to other inflammatory signals.

Immune Checkpoint Receptors as Targets in Cancer

In recent years it has become clear that cancers can co-opt these immune checkpoint pathways to evade the immune system, and therapeutic antibodies that block these receptors can take the brakes off the anti-tumor immune response, with astonishing results. An antibody blocking the receptor CTLA4 was the first to show efficacy in treating malignant melanoma (1), followed by antibodies blocking PD1

or its ligand PDL1 (2). These new immunotherapies, known as checkpoint inhibitors, have revolutionized the treatment of metastatic melanoma. They offer a subset of patients a durable remission from a disease that was previously invariably terminal. Since these initial trials checkpoint inhibitors have gone on to show efficacy in a wide range of other cancers (3) and whilst the list of indications for CTLA4 and PD1 blockade is growing, other immune inhibitory receptors are being investigated as potential targets in cancer therapy (4).

One of the limitations of checkpoint inhibitors has been the new genre of side effect they have led to, referred to as immune related adverse events (IRAEs). Treated patients can develop a wide range of autoimmune phenomena affecting almost any organ, including the gut, skin, pituitary, thyroid, lung, liver, joints, kidneys, pancreas, or haematopoietic system (5). These adverse events highlight the importance of immune checkpoint receptors in maintaining self-tolerance and raise the question of to what extent defects in these pathways could be contributing to spontaneous autoimmune disease.

Immune Checkpoint Defects in Autoimmunity

In both humans and mice immune checkpoint receptors have been shown to play a crucial role in preserving peripheral tolerance. CTLA4 knock out mice develop massive lymphoproliferation and die of multiorgan tissue destruction early in life (6), whilst human patients with heterozygous loss of function mutations in CTLA4 also develop widespread immune dysregulation (7). PD1 knockout mice on a BALB/c background develop autoimmune cardiomyopathy (8) whilst on a C57BL/6 background they develop a late onset lupus-like disease (9). In humans regulatory polymorphisms in the *PDCD1* gene are associated with susceptibility to a variety of autoimmune conditions including systemic lupus erythematosus (10), atopy and rheumatoid arthritis (11, 12), and progression in multiple sclerosis (MS) (13). It is in fact possible that the therapeutic benefit of interferon-beta in MS may be due to it upregulating PDL1 expression on myeloid cells (14). Furthermore, autoantibodies against PDL1 have been found in patients with rheumatoid arthritis and correlate with disease activity (15).

In addition to PD1 and CTLA4 there are numerous other immune checkpoint receptors that have been shown to have important immune regulatory function. B- and T-lymphocyte attenuator (BTLA) knock-out mice gradually develop multi-organ inflammatory infiltrates and a hepatitis-like disease (16), whilst a gene polymorphism in humans is associated with rheumatoid arthritis (17). Mice lacking T cell Immunoreceptor with Ig and ITIM domains (TIGIT) do not develop spontaneous autoimmunity but have increased susceptibility to experimental autoimmune encephalitis (EAE) (18). Similarly, mice without Lymphocyte-activation gene 3 (LAG3) do not develop spontaneous disease but have accelerated diabetes onset when bred onto a NOD background. Polymorphisms of the T cell immunoglobulin and mucin domain 3 (TIM-3) receptor in humans have been associated with MS (19), rheumatoid arthritis (20) and ankylosing spondylitis (21).

Rationale for Targeting Immune Checkpoints in Autoimmunity

The association of immune checkpoint receptors with autoimmunity in humans and the autoimmune phenomena seen when these receptors are knocked out in experimental mice or blocked therapeutically in patients all offer evidence of the crucial role these pathways play in regulating immune responses. It also raises the possibility that inducing signaling through these receptors could switch off detrimental immune responses and drive the immune system back toward a state of tolerance after control has been lost in autoimmune disease. This idea has been explored for a range of different targets and in multiple mouse models of autoimmunity (summarized in **Table 1**). Below we will review attempts that have been made to date to create agonistic compounds capable of delivering inhibitory signals to T cells through checkpoint receptors. Such inhibitory agonists, if they could be translated into human disease, would comprise a new, broadly useful class of immunosuppressive drug (see **Table 2**: Summary of key points).

INHIBITORY AGONISTS TARGETING IMMUNE CHECKPOINTS IN MOUSE MODELS OF AUTOIMMUNITY

Agonistic Agents Based on Natural Ligands

One therapeutic approach to induce signaling through co-inhibitory receptors has been to make use of their naturally occurring ligands. Ligand expression is normally confined to specific tissues and cell types, but by systemic administration of recombinantly produced ligand it is possible to induce inhibitory signaling through a receptor in tissues where this pathway is not normally functioning, thereby supplementing the body's natural tolerance checkpoints. The simplest application of this is demonstrated by the TIM-3 ligand Galectin-9 which, when administered as a soluble protein to mice, ameliorated EAE (35), prolonged skin and cardiac allograft survival (33, 34), and reduced inflammation in collagen induced arthritis (CIA) (35). However, the promiscuous nature of galectins, binding to sugars on multiple different glycoproteins, makes it difficult to definitively attribute these effects to TIM-3 signaling rather than the manipulation of another galectin-9 binding partner (48).

Galectin-9 is a rare example of a ligand that has been successfully employed as a standalone protein. A more widespread approach is to express the ligand as an Fc fusion, linked to the hinge and constant domains (CH2 and CH3) of an immunoglobulin heavy chain. The potential advantages of an added Fc region include easier protein expression and purification, and extended serum half-life. Furthermore, expression as an Fc fusion dimerises the ligand, turning relatively low receptor affinities into substantially higher avidities, as well as enabling receptor crosslinking. The ability of the Fc portion to be captured by Fc receptors on antigen presenting cells also

TABLE 1 | Checkpoint agonists that have shown efficacy in treating mouse models of autoimmunity.

Target receptor	Agonist compound	Mouse disease model	References
PD-1	mPDL1-mIgG2a^{mut} Fc fusion*	CIA CIA DSS/T cell colitis	(22) (23) (24)
	hPDL1-hIgG4 Fc fusion	Islet transplant	(25)
	PDL1 transfected dendritic cells	EAE	(26)
BTLA	mHVEM-mIgG1 Fc fusion	GVHD	(27)
	mHVEM-hIgG1 Fc fusion	Cardiac allograft	(28)
	Hamster IgG antibody (clone 6A6)	GVHD	(29)
	Rat IgG antibody (clone Byk-1)	GVHD	(30)
TIGIT	Armenian hamster IgG antibody (4D4)	EAE	(31)
TIM-3	Galectin 9	EAE, Cardiac allograft, Skin allograft, CIA	(32) (33) (34) (35)
CD200 Receptor	mCD200-mIgG2a^{mut}	CIA CIA Rat islet xenograft	(36) (37) (38)
	mCD200-mIgG2a	EAE	(39)
	Rat IgG1 antibody (clone OX110)	CIA Influenza infection	(40) (41)
	Rat IgG1 antibody (clone DX109)	Autoimmune uveoretinitis	(42)
	DNA aptamers	Skin graft	(43)
CD200R/ TGFβR	CD200—TGFβ fusion protein	Skin graft	(44)
VISTA	Armenian hamster antibody (MH5A)	GVHD	(45)
	Mouse IgG1 antibody (mam82)	Concanavalin A hepatitis	(46)
Unknown	Pentameric VISTA-COMP fusion protein	Skin allograft	(47)

**mIgG2a^{mut} contains the mutations E318A, K320A, K322A to inactivate the C1q binding site and L235E to reduce FcγR1 binding.*

TABLE 2 | Summary of key points.

- Checkpoint receptors deliver inhibitory signals to immune cells to prevent inappropriate or excessive activation
- The absence or blockade of these receptors leads to autoimmunity
- Conversely, inducing signaling through these pathways could help to switch off unwanted immune responses for the treatment of autoimmune disease
- Agonist antibodies, Fc-fusion proteins and other novel compounds that trigger these receptors have demonstrated promise in treating animal models of autoimmunity, but this has not yet been translated to human disease
- The epitope position, along with an ability to bind to Fc receptors, and to cause receptor aggregation, all play a role in determining the potency of an agonist compound
- Better understanding the mechanisms by which agonists induce signaling could direct the design of more effective therapeutic agents

effectively turns the ligand into an immobilized cell surface receptor rather than a soluble protein.

Several attempts have been made to target the potent inhibitory receptor PD1 with Fc fusions. A construct comprising murine PDL1 with mIgG2a^{mut} Fc (mutated to inactivate the C1q and FcγR1 binding sites) dampened collagen-specific T cell responses and improved clinical scores in CIA (22, 23). An adenovirus vector expressing the same construct ameliorated dextran sodium sulfate-induced experimental colitis, whilst the recombinant form reduced the severity of T-cell induced colitis (24). A human PDL1 hIgG4 fusion protein delayed rejection of islet cell transplants in mice but only when used in conjunction with CD40L blockade (25).

The CD200 receptor (CD200R), predominantly expressed on myeloid cells, has also had success as a target for ligand-Fc inhibitory agonists. A mCD200-mIgG2a^{mut} fusion protein prevented CIA when given alongside collagen immunization (36) and significantly delayed rejection of rat-to-mouse islet xenografts (38). Separately, mCD200-mIgG2a was able to reduce disease severity in established arthritis (37) and, via suppression of microglia and astrocyte activity, attenuated disease in EAE (39). The latter two studies did not specify whether the Fc construct used contained the same mutations removing high affinity FcR and complement binding, so cytotoxic depletion of CD200R1 expressing cells may have been a contributing factor.

Fc fusions of HVEM, the ligand for the inhibitory receptor BTLA have also displayed promise as immunosuppressants. Mouse or human HVEM-IgG1 fusion proteins inhibited T cell responses *in vitro*, but only when crosslinked by a secondary antibody or when high molecular weight aggregates were present (49). *In vivo*, mHVEM-hIgG1 prolonged survival of cardiac allografts when used in combination with cyclosporine (28) and mHVEM-mIgG1 ameliorated a model of graft vs. host disease (GVHD) (27). Conversely mHVEM-hIgG1 exacerbated CIA (50) which may have been due to inducing inflammatory signaling through the activating co-receptor LIGHT which also binds to HVEM. As highlighted here many inhibitory receptors such as BTLA act in paired systems, sharing their ligands with activating receptors (**Table 3**), which presents a challenge to utilizing the natural ligands as immunosuppressive agents. For example, CD80-Fc and CD86-Fc fusion proteins which may be expected to have an inhibitory effect on T cells via CTLA4 signaling, in fact have a net activating effect due to also binding CD28, and have been shown to enhance anti-tumor immune responses (51).

Agonist Antibodies

In contrast to natural ligands, therapeutic antibodies can be produced which have specificity for only the inhibitory partner in paired receptor systems, avoiding the risk of inducing counterproductive signaling through activating receptors. Antibodies can also be selected with many-fold higher affinity

TABLE 3 | Selected immune checkpoint receptors alongside their ligands and paired receptors.

Checkpoint receptor	Ligands	Paired receptors (sharing the same ligand)
CTLA4 (cytotoxic T lymphocyte associated protein 4)	CD80, CD86	Activating: CD28
PD1 (Programmed cell death protein 1)	PDL1, PDL2	–
BTLA (B- and T-Lymphocyte attenuator)	HVEM (Herpesvirus entry mediator)	Activating: LIGHT, LTα Inhibitory: CD160
TIGIT (T cell Immunoreceptor with Ig and ITIM domains)	CD155, CD112	Activating: CD226 Inhibitory: CD96
CD200 Receptor (CD200R1)	CD200	Activating: CD200R2-5 (mice only, not expressed in humans)
TIM-3 (T cell immunoglobulin and mucin domain 3)	Galectin 9, HMGB1, Phosphatidylserine, CEACAM-1	Numerous
LAG-3 (Lymphocyte-activation gene 3)	MHC Class II	Activating: T cell receptor, CD4
VISTA (V-domain Ig suppressor of T cell activation)	Unknown (VISTA may also serve as a co-inhibitory ligand for an, as yet, unidentified receptor)	–

for their cognate receptor than the affinity of the endogenous receptor-ligand interaction. Furthermore, the significant precedent for monoclonal antibodies to be used as therapeutics in humans, could mean that translation to the clinic will face fewer challenges than would be encountered by novel Fc-fusions or other innovative constructs.

It was demonstrated long ago in the context of the activating co-stimulatory receptor CD28, that antibodies could substitute for natural ligands, and in fact could deliver a far more potent signal (52). This was confirmed in an unfortunate way in the clinical trial of the CD28 superagonist antibody TGN1412 in which widespread T cell activation caused a cytokine storm in the participating healthy volunteers (53). Conversely, agonistic antibodies against inhibitory receptors have shown promise in mouse models of autoimmunity, although there are currently very few registered clinical trials of agonists against these targets in humans.

Krieg et al. screened eight rat anti-mouse BTLA antibodies and found one with significant agonistic activity, which was able to inhibit CD4 T cell activation when immobilized, even if delivered 24 h after the initial anti-CD3 activation signal (54). Separately, a hamster IgG targeting BTLA abrogated disease in a model of GVHD in wildtype but not $BTLA^{-/-}$ C57BL/6 mice (29). Of note, this antibody had previously been shown to block binding of the natural ligand HVEM (55), but as it was capable of ameliorating disease even in $HVEM^{-/-}$ mice, and was shown to be non-depleting, Albring et al concluded the effect must be due to direct signaling through BTLA.

An IgG1 rat anti-mouse CD200R1 antibody (OX110) reduced disease severity in overtly arthritic mice (40) and alleviated influenza-induced illness by dampening excessive innate cell activation (41). Another rat IgG1 antibody against mouse CD200R1 (DX109) suppressed macrophage activation and prevented tissue damage in experimental autoimmune uveitis (42). *In vitro* DX109 was able to inhibit degranulation of CD200R1 overexpressing mast cells, whilst a rat anti-human CD200R antibody (DX183) suppressed primary human mast cells (56).

Targeting the receptor VISTA (PD-1H), an Armenian hamster IgG prevented GVHD by tolerising effector T cells and selectively promoting regulatory T cell (Treg) expansion (57), whilst a mIgG1 VISTA agonist antibody suppressed acute inflammation in a model of Concanavalin-A induced hepatitis (46). Dixon et al recently described a mIgG1 antibody targeting the receptor TIGIT which suppressed T cell responses to immunization with myelin oligodendrocyte glycoprotein (MOG) peptide and modulated disease severity in EAE (31).

The success of CTLA4 and PD1 as targets of checkpoint blockade in cancer highlights these two receptors as particularly crucial regulators of tolerance. So it is conspicuous that no successful attempts to utilize agonist antibodies against these receptors in treating autoimmunity has been published. In the case of CTLA4 this may add weight to the suggestion that the receptor does not have an important intrinsic signaling capability but instead acts predominantly by sequestering the ligands CD86 and CD80, preventing their interaction with CD28 (58). This is supported by the clinical success of the CTLA4-Fc fusion protein Abatacept, which is used in the treatment of rheumatoid arthritis amongst other indications (59). Rather than acting as an agonist, like the Fc-fusion proteins described above, Abatacept acts as a blocking agent, binding to CD80 and CD86 on antigen presenting cells and preventing their co-stimulatory interaction with CD28 on T cells. The fact that soluble CTLA4-Fc is a potent immunosuppressive and can compensate for CTLA4 haploinsufficiency (60) suggests that competition with CD28 for ligand binding is the predominant mode of action of this inhibitory receptor. However, there is also substantial evidence for an intrinsic signaling function of CTLA4 and a membrane bound single chain antibody (ScFv) recognizing CTLA-4 has been reported as having a T cell suppressive effect if expressed on the same cell as the TCR antigen, suggesting that it may be possible to develop agonist antibodies against this receptor (61, 62).

The immune checkpoint PD1 does have a potent intrinsic signaling function so the reason for a lack of successful agonists targeting this receptor is unclear. There are reports of a PD1 antibody ameliorating autoimmunity in a lupus-like disease model in mice (63–65) but, as this antibody had previously been shown to act as a PD1 blocking agent, the authors attributed the effect to either cytotoxic depletion of PD1 expressing cells or enhanced suppressive activity of Tregs following PD1 blockade. Based on the efficacy of PDL1/Fc fusion proteins in murine models of autoimmunity described above, whether antibody agonists targeting PD1 can be developed is an area that certainly merits wider exploration.

Novel Approaches to Checkpoint Agonism

Aside from agonist antibodies and ligand/Fc fusions a variety of other constructs have been employed to induce immunosuppressive signaling through inhibitory receptors. Cheung et al. exploited the cytomegalovirus protein UL144, which binds to BTLA and is presumably used by the virus as an immune evasion strategy, and showed that immobilized UL144-Fc more potently suppressed CD4 T cells *in vitro* than HVEM-Fc. Šedý et al. studied the structure of UL144 to guide their design of a mutated HVEM-Fc protein capable of binding BTLA with 10 fold higher affinity than wildtype HVEM and with no binding to the receptors LIGHT or CD160. *In vitro* this construct regulated B, T, and NK cell cytokine production (66). There are numerous other viral proteins that have evolved to mimic inhibitory ligands, which presents an opportunity to further explore these compounds as therapeutic agents and once again highlights the potential merits of exploiting signaling through inhibitory receptors to switch off unwanted immune responses.

In another innovative approach to inhibitory agonism, a bivalent construct of CD200Fc linked to TGF-β1 displayed more potent T cell suppression *in vitro* than either protein alone, and prolonged survival of allogeneic skin grafts *in vivo* (44). In mixed leucocyte reactions (MLRs), binding to CD200R on antigen presenting cells and TGF-β receptor on responder T cells was shown to be necessary for maximal suppressive effect. Separately, Prodeus et al. developed short single-stranded DNA aptamers with binding specificity for CD200R1 and demonstrated that they were capable of suppressing T cell function in MLRs, whilst a PEGylated DNA aptamer prolonged skin graft survival with equal efficacy to CD200-Fc (43). Finally, a pentameric construct of VISTA fused to the pentamerization domain from cartilage oligomeric matrix protein (COMP) prolonged skin allograft survival and rescued mice from acute concanavalin-A-induced hepatitis, although, assuming that this construct functions as an inhibitory agonist, it is not known what receptor it is targeting (47).

The idea of overexpressing an inhibitory ligand on dendritic cells to produce a tolerogenic cell that can be used as a therapeutic agent has also been investigated. Dendritic cells transfected with both PDL1 and MOG peptide and injected intraperitoneally were able to induce tolerance and reduce severity of MOG-induced EAE (26). Similarly, splenocytes from Balb/c mice primed with allogeneic dendritic cells overexpressing PDL1 and loaded with GAD65 had impaired responses when subsequently stimulated with the same antigens *ex vivo* (67). However, whether transfected dendritic cells could ever be translated into an acceptable therapeutic for use in human autoimmune disease is uncertain.

RATIONAL DEVELOPMENT OF CHECKPOINT AGONISTS

Defining the Necessary Characteristics for a Checkpoint Agonist

For a compound to act as an immune checkpoint agonist it not only has to bind to the receptor but must also be capable of delivering a signal through it. Very little has been done to establish the criteria that determine this function. Despite the development of the numerous agonists described above there is still little clarity as to what characteristics are necessary in an agent to confer upon it this agonistic ability.

Agonists to TNFR Family Receptors

In the context of activating TNFR family immune cell receptors, such as CD40, it has been demonstrated that antibody agonism results from receptor aggregation, which in turn is dependent on capture of the antibody, via its Fc portion, by a scaffold of FcγRIIB on the surface of adjacent cells (68). As such, agonist activity can be augmented by increasing affinity for FcγRIIB (69). Furthermore, FcγRIIB independent agonism can be conferred by an isoform of human IgG2 in which the CH1 domain is linked via a disulfide bond to the hinge, which holds the antibody in a more compact and rigid structure and presumably aids tighter packing or more efficient aggregation of bound receptors (70).

Mechanism of Triggering of Checkpoint Receptors

However, it is important to remember that TNFR family receptors fall into a different family from the inhibitory immune receptors we have discussed here, with different signaling mechanisms and, presumably, different attributes necessary for agents acting as agonists. TNFR family receptors are normally engaged by multivalent ligands and signal after receptor trimerization leads to the recruitment of downstream adapter proteins. Immune checkpoint receptors on the other hand predominantly fall into a category of receptors that have been referred to as non-catalytic tyrosine phosphorylated receptors or NTRs (71). These receptors have tyrosine containing motifs in their cytoplasmic tail that become phosphorylated by extrinsic kinases following ligand binding, which in turn leads to recruitment of SH2 domain-containing downstream signaling proteins or adapters. Understanding the mechanism by which ligand engagement leads to phosphorylation of these intracellular motifs (referred to as receptor triggering) is clearly crucial to understanding how artificial agonists might operate. There are several different, but not necessarily mutually exclusive, models for how this process can occur based on the aggregation, conformational change or segregation of membrane proteins (71).

Receptor aggregation models dictate that ligand binding leads to clustering of receptors that, at rest, are loosely associated with intracellular kinases, leading to cross-phosphorylation of tyrosine containing motifs on adjacent receptors. Conformational change models require ligand binding to lead to structural changes in the receptor which either expose previously buried signaling motifs or allow subsequent receptor aggregation. In contrast, the kinetic-segregation model proposes that binding to ligand on an apposing cell holds the receptor in a close contact formed between the two cell surfaces from which bulky receptor-type phosphatases are excluded, which in turn leads to net phosphorylation by kinases that are not excluded because they are associated with the inner leaflet of the membrane (**Figure 1A**) (72).

Aggregation of Checkpoint Receptors

As with TNFR family receptor agonists there is some evidence that aggregation plays a role in the action of checkpoint agonists. Many of the agonists described above have been shown to inhibit immune cells more potently *in vitro* when crosslinked by a secondary antibody. Also, most of the agonists described above are at least dimeric, and therefore capable of bringing together two of their cognate receptors (and clustering multiple receptors if their cognate receptors themselves oligomerise). The mere fact that soluble natural ligands function as agonists only when dimerised in the form of an Fc fusion protein lends some support to the idea that aggregation is important. Galectin 9, whilst not dimeric, has 2 separate carbohydrate recognition domains capable of binding TIM-3, and can cluster receptors into glycoconjugates which may either induce signaling directly or alter the half-life of the receptor on the cell surface (73). For the checkpoint receptor CD200R it has been shown that agonist antibody isotype is also key, with the compact isoform of human IgG2, which aids receptor clustering, serving to enhance agonism, as seen for TNFR family receptors (74). As inhibitory receptors are thought generally to associate with phosphatases rather than kinases it might seem paradoxical that aggregating them would lead to receptor phosphorylation. However, along the lines of the kinetic-segregation model described above, it may be that clustering receptors into a tightly packed group creates an area of densely occupied membrane from which bulky phosphatases are excluded, allowing for net phosphorylation of signaling motifs by smaller membrane-associated or intracellular kinases (**Figure 1B**).

Requirement for Fc Receptor Binding

There is also evidence that Fc receptor binding is important to the action of agonists against NTRs. The superagonistic activity of the antibody TGN1412 which targeted the costimulatory receptor CD28 was found to depend on binding to FcγRIIB *in vitro* (75). Similarly, agonistic antibodies targeting the murine inhibitory receptor FcγRIIB, which is itself an NTR, require that both their variable and Fc portions are able to bind Fc receptors (76). As in the case of TNFR agonists, the requirement for Fc receptor binding may be because it aids receptor clustering. Alternatively, if the kinetic-segregation mechanism of receptor triggering plays a role, then Fc receptor binding would be expected to be necessary as the agonistic agent would need to be immobilized on an opposing surface (such as an FcR expressing cell) in order to create the close contact zone which excludes phosphatases (**Figure 1C**).

Of course the requirement for Fc receptor binding also raises the possibility that the immunosuppressive effects of checkpoint "agonists" could be due to inadvertent depletion of checkpoint expressing effector T cells. Very few of the publications cited above, which showed inhibitory effects on the overall immune response, used assays (such as Phospho-Flow or western blotting) to look at the downstream signaling of these receptors and confirm that the agents were truly agonistic. Furthermore, few convincingly demonstrated that there was no cytotoxic depletion of effector T cells. Recent data suggesting that the immune enhancing effects of CTLA4 "blocking" antibodies may in fact be due to the FcR-dependent depletion of T-regs (77) highlights that we should remain open minded about the potential mechanism of action of novel therapeutics.

Epitope Position

A number of studies suggest that epitope position may influence the agonistic activity of monoclonal antibodies. From a panel of anti-BTLA antibodies Zhang et al. demonstrated that all those with agonistic effects mapped to the same epitope whilst non-agonists bound elsewhere (78). Interestingly, it does not seem to matter if the antibody competes with binding of the natural ligand. Agonists targeting TIGIT (31) and BTLA (29) were both shown to inhibit ligand binding and to be capable of treating disease in models of autoimmunity.

Clues to how epitope position may be influencing agonist activity come from studies of the activating receptor CD28. It has been demonstrated that superagonist antibodies targeting this receptor bind to a shared epitope on a laterally exposed loop of the receptor (79) and that this results in a relatively compact structure with the antibody lying close to and parallel to the membrane (80). This means that when the antibody Fc portion is immobilized by Fc receptors on an opposing cell, the receptor may be held in a very close contact between the two membranes. In the kinetic-segregation mechanism of receptor triggering, a narrower contact zone would more effectively exclude phosphatases to initiate signaling. Of note, Evans et al. have shown that superagonistic and non-superagonist antibodies are equally capable of binding CD28 bivalently, and so a differential ability to cause receptor aggregation is unlikely to account for the difference in activity. Furthermore, they saw insufficient structural rearrangements of CD28 following antibody binding for a conformational change-based mechanism to readily explain triggering. The idea that the epitope influences agonism because of the resulting width of the gap between cells is supported by the fact that cytotoxic antibodies used clinically tend to target molecules with small extracellular domains such as CD20 (rituximab) and CD52 (campath-1). These antibodies mediate antibody dependent cell mediated cytotoxicity (ADCC) by binding to activatory Fc receptors, which fall into the same NTR family, so may also be dependent on the small dimensions of the interaction. Chimeric antigen receptors (CARs) and

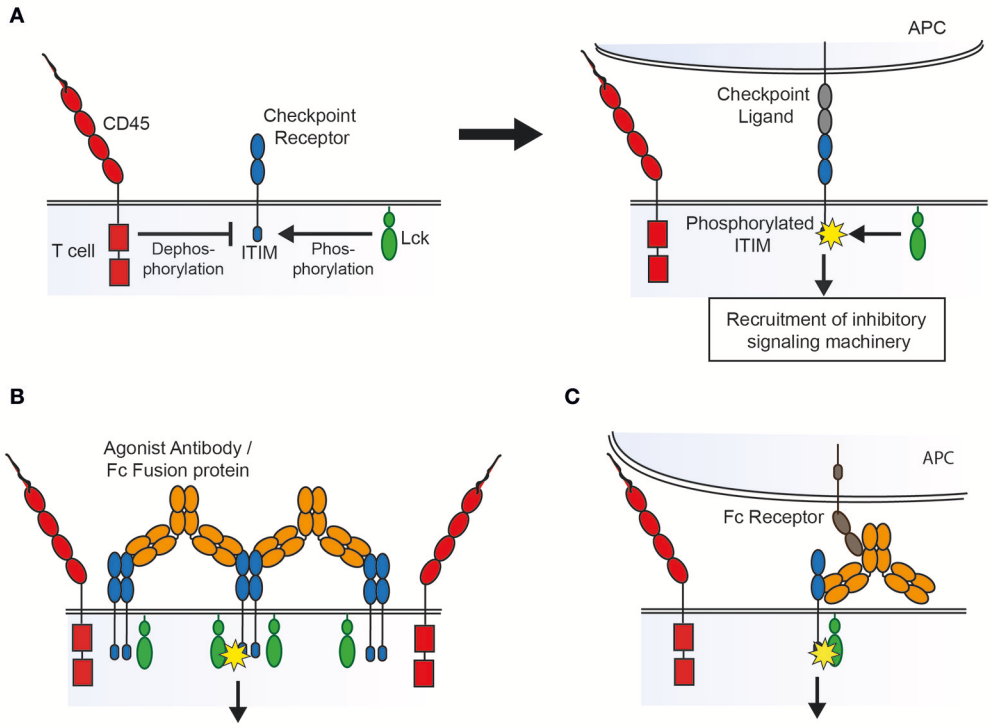


FIGURE 1 | Possible mechanisms of action of agonist agents, based on the kinetic-segregation model of receptor signaling. **(A)** The kinetic-segregation model. (Left) Checkpoint receptors contain intracellular motifs such as the ITIM which are phosphorylated by small membrane associated kinases (e.g., Lck) but rapidly dephosphorylated by abundant bulky phosphatases (e.g., CD45), with no net signaling. (Right) When the receptor encounters its ligand on an apposing cell the balance of kinase and phosphatase activity is tipped in favor of kinases, for example by steric exclusion of phosphatases from the contact zone, resulting in net phosphorylation of the ITIM and subsequent recruitment of signaling machinery which inhibits cellular activation. **(B)** Triggering by aggregation. An agonist compound may cause receptor triggering by densely clustering kinase-associated receptors so that bulky phosphatases are again sterically excluded. **(C)** Triggering by an Fc receptor immobilized compound. An agonist that binds to Fc receptors on an apposing cell could lead to triggering by holding the receptor in a close contact zone that phosphatases cannot enter.

bispecific T cell engagers (BiTEs), which both act as artificial NTRs, targeting larger proteins such as CD22 and FcRH5 are most effective if they bind a membrane proximal epitope (81, 82).

Co-localization of Inhibitory and Activating Signals

Finally, there is evidence to suggest that the function of inhibitory agonists depends on the co-incidence of inhibitory and activating signals within the cell. For example, a LAG-3 agonistic antibody was able to inhibit T cell proliferation *in vitro* only when co-crosslinked with the TCR by a secondary antibody (83). In addition, the effect of CD200R agonists on mast cell degranulation is enhanced by co-crosslinking to the FcεR (56), and BTLA agonists are effective *in vitro* only when presented alongside the activating anti-CD3 antibody (78). This fits with a mechanism of signaling in which inhibitory receptors recruit phosphatases capable of dephosphorylating the signaling motifs of neighboring activatory receptors. In the context of therapeutic inhibitory agonists, it suggests that a useful agonist will need to be capable of accessing the immune synapse where T cell activation is occurring.

Choice of Mouse Model

A variety of murine autoimmune models have been used to assess the effects of inhibitory agonists *in vivo*. It may be that disease associations seen with human checkpoint polymorphisms can give clues to which tissues are more dependent on these pathways for maintaining tolerance and guide the selection of disease model. Similarly, the prevalence of different autoimmune manifestations in checkpoint blockade-treated patients may aid this process. For example, involvement of the pituitary is a relatively common adverse event with CTLA4 blockade, occurring in 10% of patients, but is very rare following PD1 blockade suggesting that different pathways can have tissue specific importance (84). Whether this is due to tissue specific differences in ligand expression or some other factor is unclear but as new blocking antibodies targeting different checkpoint receptors make their way into clinical trials, more information about the organ specific relevance of different pathways will become available. The specific diseases seen in knockout mice may also direct the selection of disease models.

However, it does not necessarily follow that the parts of the body worst affected by blockade or absence of a particular checkpoint receptor would serve to benefit most from agonist

agents targeting this receptor. It may be that tissues which develop disease following checkpoint blockade are those where these inhibitory pathways are constitutively active, and that other tissues which don't normally have functional signaling through these receptors are more prone to spontaneous autoimmunity, and more likely to benefit from artificially-induced inhibitory signaling.

There are also many difficulties in extrapolating findings from mouse models back to human disease. For example, whilst PD1 blockade in man leads most often to autoimmunity affecting the gut, liver, and skin, in PD1 knockout mice autoimmune manifestations include cardiomyopathy in BALB/c mice and lupus like disease in C57BL/6 mice, suggesting that knock out models do not always phenocopy the effect of blocking antibodies in man. As seen with the CD28 superagonist TGN1412, not even primate studies can always accurately predict the effects of therapeutic antibodies in man (85).

Rationale for Agonist Combinations

With checkpoint inhibitors in cancer we have seen that combination blockade of both CTLA4 and PD1 is superior to either alone (86), and similarly we may expect that combining agonists against multiple pathways may enhance immunosuppression. The choice of combinations to use may be guided by the effects seen in double knockout mice. For example, LAG-3 or VISTA deficiency alone does not lead to spontaneous autoimmunity, but does exacerbate disease in the absence of PD1 (87, 88). Further clues toward synergistic combinations may be gathered from more in-depth understanding of the different downstream signaling pathways of these unique and non-redundant receptors, as well as the expression pattern on different cells of the immune system (89).

Risk of Cancer

The success of checkpoint blockade has highlighted the key role the immune system can play in cancer surveillance and raises the issue of whether inhibitory agonists could aid developing tumors to escape the immune response. There is no suggestion so far from animal models that inhibitory agonists might increase cancer risk, but the timescale of such experiments might be insufficient for this to be clear and longer-term observation of treated mice could be useful. The long experience to date with other clinically used immunosuppressives, however, has been that the increased cancer risk is likely very small, if it is increased at all, and outweighed by the clinical benefit of immune suppression in the context of debilitating autoimmune disease.

CONCLUSION

Previous reviews that have discussed immune cell co-receptors as potential targets in autoimmunity have focused primarily on agents that block the action of activating receptors (90–92). Here instead we have concentrated on attempts that have been made to enhance the signaling of inhibitory receptors. Whilst this approach has displayed significant promise in animal models of autoimmunity there is a need for more thorough investigation of the mechanisms underlying artificial agonism of checkpoint receptors, to guide more rational design of the most potent agonists. This, alongside reasoned approaches to selecting the most appropriate combinations of agents and the best models to test them in, could help to unveil the true potential of this previously untapped class of therapeutic antibodies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust [109029/Z/15/Z], [207547/Z/17/Z]; the Medical Research Council [MC_UU_12010].

REFERENCES

1. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. (2010) 363:711–23. doi: 10.1056/NEJMoa1003466
2. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, et al. Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med*. (2015) 372:320–30. doi: 10.1056/NEJMoa1412082
3. Gong J, Chehrazi-Raffle A, Reddi S, and Salgia R. Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations. *J Immunother Cancer* (2018) 6:8. doi: 10.1186/s40425-018-0316-z
4. Le Mercier I, Lines JL, Noelle RJ. Beyond CTLA-4 and PD-1, the generation Z of negative checkpoint regulators. *Front Immunol*. (2015) 6:418. doi: 10.3389/fimmu.2015.00418
5. Michot JM, Bigenwald C, Champiat S, Collins M, Carbonnel F, Postel-Vinay S, et al. Immune-related adverse events with immune checkpoint blockade: a comprehensive review. *Eur J Cancer* (2016) 54:139–48. doi: 10.1016/j.ejca.2015.11.016
6. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* (1995) 3:541–7. doi: 10.1016/1074-7613(95)90125-6
7. Kuehn HS, Ouyang W, Lo B, Deenick EK, Niemela JE, Avery DT, et al. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science* (2014) 345:1623–7. doi: 10.1126/science.1255904
8. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* (2001) 291:319–22. doi: 10.1126/science.291.5502.319
9. Nishimura H, Nose M, Hiai H, Minato N, and Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* (1999) 11:141–51. doi: 10.1016/S1074-7613(00)80089-8
10. Prokunina L, Castillejo-López C, Oberg F, Gunnarsson I, Berg L, Magnusson V, et al. A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. *Nat Genet*. (2002) 32:666–9. doi: 10.1038/ng1020
11. James ES, Harney S, Wordsworth BP, Cookson WO, Davis SJ, Moffatt MF. PDCD1: a tissue-specific susceptibility locus for inherited inflammatory disorders. *Genes Immun*. (2005) 6:430–7. doi: 10.1038/sj.gene.6364223

12. Lee YH, Bae SC, Kim JH, Song GG. Meta-analysis of genetic polymorphisms in programmed cell death 1. Associations with rheumatoid arthritis, ankylosing spondylitis, and type 1 diabetes susceptibility. *Z Rheumatol*. (2015) 74:230–9. doi: 10.1007/s00393-014-1415-y
13. Kroner A, Mehling M, Hemmer B, Rieckmann P, Toyka KV, Mäurer M, et al. A PD-1 polymorphism is associated with disease progression in multiple sclerosis. *Ann Neurol*. (2005) 58:50–7. doi: 10.1002/ana.20514
14. Schreiner B, Mitsdoerffer M, Kieseier BC, Chen L, Hartung HP, Weller M, et al. Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. *J Neuroimmunol*. (2004) 155:172–82. doi: 10.1016/j.jneuroim.2004.06.013
15. Dong H, Strome SE, Matteson EL, Moder KG, Flies DB, Zhu G, et al. Costimulating aberrant T cell responses by B7-H1 autoantibodies in rheumatoid arthritis. *J Clin Invest*. (2003) 111:363–70. doi: 10.1172/JCI16015
16. Oya Y, Watanabe N, Owada T, Oki M, Hirose K, Suto A, et al. Development of autoimmune hepatitis-like disease and production of autoantibodies to nuclear antigens in mice lacking B and T lymphocyte attenuator. *Arthritis Rheum*. (2008) 58:2498–510. doi: 10.1002/art.23674
17. Oki M, Watanabe N, Owada T, Oya Y, Ikeda K, Saito Y, et al. A functional polymorphism in B and T lymphocyte attenuator is associated with susceptibility to rheumatoid arthritis. *Clin Dev Immunol*. (2011) 2011:305656. doi: 10.1155/2011/305656
18. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol*. (2011) 186:1338–42. doi: 10.4049/jimmunol.1003081
19. Yaghoobi E, Abedian S, Babani O, Izad M. TIM-3 Rs10515746 (A/C) and Rs10053538 (C/A) Gene polymorphisms and risk of multiple sclerosis. *Iran J Public Health* (2016) 45:644–49.
20. Chae SC, Park YR, Shim SC, Yoon KS, Chung HT. The polymorphisms of Th1 cell surface gene Tim-3 are associated in a Korean population with rheumatoid arthritis. *Immunol Lett*. (2004) 95:91–5. doi: 10.1016/j.imlet.2004.06.008
21. Wang M, Ji B, Wang J, Cheng X, Zhou Q, Zhou J, et al. Tim-3 polymorphism downregulates gene expression and is involved in the susceptibility to ankylosing spondylitis. *DNA Cell Biol*. (2014) 33:723–8. doi: 10.1089/dna.2014.2456
22. Raptopoulou AP, Bertsias G, Makrygiannakis D, Verginis P, Kritikos I, Tzardi M, et al. The programmed death 1/programmed death ligand 1 inhibitory pathway is up-regulated in rheumatoid synovium and regulates peripheral T cell responses in human and murine arthritis. *Arthritis Rheum*. (2010). 62:1870–80. doi: 10.1002/art.27500
23. Wang G, Hu P, Yang J, Shen G, Wu X. The effects of PDL-Ig on collagen-induced arthritis. *Rheumatol Int*. (2011) 31:513–9. doi: 10.1007/s00296-009-1249-0
24. Song MY, Hong CP, Park SJ, Kim JH, Yang BG, Park Y, et al. Protective effects of Fc-fused PD-L1 on two different animal models of colitis. *Gut* (2015) 64:260–71. doi: 10.1136/gutjnl-2014-307311
25. Gao W, Demirci G, Strom TB, Li XC. Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. *Transplantation* (2003) 76:994–9. doi: 10.1097/01.TP.0000085010.39567.FB
26. Hirata S, Senju S, Matsuyoshi H, Fukuma D, Uemura Y, and Nishimura Y. Prevention of experimental autoimmune encephalomyelitis by transfer of embryonic stem cell-derived dendritic cells expressing myelin oligodendrocyte glycoprotein peptide along with TRAIL or programmed death-1 ligand. *J Immunol*. (2005) 174:1888–97. doi: 10.4049/jimmunol.174.4.1888
27. Brown GR, Lee EL, El-Hayek J, Kintner K, Luck C. IL-12-independent LIGHT signaling enhances MHC class II disparate $CD4^+$ T cell alloproliferation, IFN-γ responses, and intestinal graft-versus-host disease. *J Immunol*. (2005) 174:4688–95. doi: 10.4049/jimmunol.174.8.4688
28. Ye Q, Fraser CC, Gao W, Wang L, Busfield SJ, Wang C, et al. Modulation of LIGHT-HVEM costimulation prolongs cardiac allograft survival. *J Exp Med*. (2002) 195:795–800. doi: 10.1084/jem.20012088
29. Albring JC, Sandau MM, Rapaport AS, Edelson BT, Satpathy A, Mashayekhi M, et al. Targeting of B and T lymphocyte associated (BTLA) prevents graft-versus-host disease without global immunosuppression. *J Exp Med*. (2010) 207:2551–59. doi: 10.1084/jem.20102017
30. Sakoda Y, Park JJ, Zhao Y, Kuramasu A, Geng D, Liu Y, et al. Dichotomous regulation of GVHD through bidirectional functions of the BTLA-HVEM pathway. *Blood* (2011) 117:2506–14. doi: 10.1182/blood-2010-08-301325
31. Dixon KO, Schorer M, Nevin J, Etminan Y, Amoozgar Z, Kondo T, et al. Functional Anti-TIGIT antibodies regulate development of autoimmunity and antitumor immunity. *J Immunol*. (2018) 200:3000–7 doi: 10.4049/jimmunol.1700407
32. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khourys SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol*. (2005) 6:1245–52. doi: 10.1038/ni1271
33. He W, Fang Z, Wang F, Wu K, Xu Y, Zhou H, et al. Galectin-9 significantly prolongs the survival of fully mismatched cardiac allografts in mice. *Transplantation* (2009) 88:782–90. doi: 10.1097/TP.0b013e3181b47f25
34. Wang F, He W, Zhou H, Yuan J, Wu K, Xu L, et al. The Tim-3 ligand galectin-9 negatively regulates CD8+ alloreactive T cell and prolongs survival of skin graft. *Cell Immunol*. (2007) 250:68–74. doi: 10.1016/j.cellimm.2008.01.006
35. Seki M, Oomizu S, Sakata KM, Sakata A, Arikawa T, Watanabe K, et al. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin Immunol*. (2008) 127:78–88. doi: 10.1016/j.clim.2008.01.006
36. Gorczynski RM, Chen Z, Yu K, Hu J. CD200 immunoadhesin suppresses collagen-induced arthritis in mice. *Clin Immunol*. (2001) 101:328–34. doi: 10.1006/clim.2001.5117
37. Simelyte E, Criado G, Essex D, Uger RA, Feldmann M, Williams RO. CD200-Fc, a novel antiarthritic biologic agent that targets proinflammatory cytokine expression in the joints of mice with collagen-induced arthritis. *Arthritis Rheum*. (2008) 58:1038–43. doi: 10.1002/art.23378
38. Gorczynski RM, Hu J, Chen Z, Kai Y, Lei J. A CD200FC immunoadhesin prolongs rat islet xenograft survival in mice. *Transplantation* (2002) 73:1948–53. doi: 10.1097/00007890-200206270-00018
39. Liu Y, Bando Y, Vargas-Lowy D, Elyaman W, Khoury SJ, Huang T, et al. CD200R1 agonist attenuates mechanisms of chronic disease in a murine model of multiple sclerosis. *J Neurosci*. (2010) 30:2025–38. doi: 10.1523/JNEUROSCI.4272-09.2010
40. Gorczynski RM, Chen Z, Lee L, Yu K, Hu J. Anti-CD200R ameliorates collagen-induced arthritis in mice. *Clin Immunol*. (2002) 104:256–64. doi: 10.1006/clim.2002.5232
41. Snelgrove RJ, Goulding J, Didierlaurent AM, Lyonga D, Vekaria S, Edwards L, et al. A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. *Nat Immunol*. (2008) 9:1074–83. doi: 10.1038/ni.1637
42. Copland DA, Calder CJ, Raveney BJ, Nicholson LB, Phillips J, Cherwinski H, et al. Monoclonal antibody-mediated CD200 receptor signaling suppresses macrophage\ activation and tissue damage in experimental autoimmune uveoretinitis. *Am J Pathol*. (2007) 171:580–88. doi: 10.2353/ajpath.2007.070272
43. Prodeus A, Cydzik M, Abdul-Wahid A, Huang E, Khatri I, Gorczynski R, et al. Agonistic CD200R1 DNA aptamers are potent immunosuppressants that prolong allogeneic skin graft survival. *Mol Ther Nucleic Acids* (2014) 3:e190. doi: 10.1038/mtna.2014.41
44. Gorczynski RM, Chen Z, Shivagnahnam S, Taseva A, Wong K, Yu K, et al. Potent immunosuppression by a bivalent molecule binding to CD200R and TGF-betaR. *Transplantation* (2010) 90:150–9. doi: 10.1097/TP.0b013e3181e2d6a1
45. Flies DB, Wang S, Xu H, Chen L. Cutting edge: a monoclonal antibody specific for the programmed death-1 homolog prevents graft-versus-host disease in mouse models. *J Immunol*. (2011) 187:1537–41. doi: 10.4049/jimmunol.1100660
46. Flies DB, Han X, Higuchi T, Zheng L, Sun J, Ye J, et al. Coinhibitory receptor PD-1H preferentially suppresses $CD4^+$ T cell-mediated immunity. *J Clin Invest*. (2014) 124:1966–75. doi: 10.1172/JCI74589
47. Prodeus A, Abdul-Wahid A, Sparkes A, Fischer NW, Cydzik M, Chiang N, et al. VISTA.COMP - an engineered checkpoint receptor agonist that potently suppresses T cell-mediated immune responses. *JCI Insight* (2017) 2:e94308. doi: 10.1172/jci.insight.94308
48. Cooper DN, Barondes S H. God must love galectins; he made so many of them. *Glycobiology* (1999) 9:979–84. doi: 10.1093/glycob/9.10.979

49. Gonzalez LC, Loyet KM, Calemine-Fenaux J, Chauhan V, Wranik B, Ouyang W, et al. A coreceptor interaction between the CD28 and TNF receptor family members B and T\ lymphocyte attenuator and herpesvirus entry mediator. *Proc Natl Acad Sci USA*. (2005) 102:1116–21. doi: 10.1073/pnas.0409071102
50. Pierer M, Schulz A, Rossol M, Kendzia E, Kyburz D, Haentzschel H, et al. Herpesvirus entry mediator-Ig treatment during immunization aggravates rheumatoid arthritis in the collagen-induced arthritis model. *J Immunol*. (2009) 182:3139–45. doi: 10.4049/jimmunol.0713715
51. Sturmhoefel K, Lee K, Gray GS, Thomas J, Zollner R, O'Toole M, et al. Potent activity of soluble B7-IgG fusion proteins in therapy of established tumors and as vaccine adjuvant. *Cancer Res*. (1999) 59:4964–72.
52. Tacke M, Hanke G, Hanke T, Hünig T. CD28-mediated induction of proliferation in resting T cells *in vitro* and *in vivo* without engagement of the T cell receptor: evidence for functionally distinct forms of CD28. *Eur J Immunol*. (1997) 27:239–47. doi: 10.1002/eji.1830270136
53. Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med*. (2006). 355:1018–28. doi: 10.1056/NEJMoa063842
54. Krieg C, Han P, Stone R, Goularte OD, Kaye J. Functional analysis of B and T lymphocyte attenuator engagement on $CD4^+$ and $CD8^+$ T cells. *J Immunol*. (2005) 175:6420–7. doi: 10.4049/jimmunol.175.10.6420
55. Hurchla MA, Sedy JR, Gavrieli M, Gavrielli M, Drake CG, Murphy TL, et al. B and T lymphocyte attenuator exhibits structural and expression polymorphisms and is highly Induced in anergic $CD4^+$ T cells. *J Immunol*. (2005) 174:3377–85. doi: 10.4049/jimmunol.174.6.3377
56. Cherwinski HM, Murphy CA, Joyce BL, Bigler ME, Song YS, Zurawski SM, et al. The CD200 receptor is a novel and potent regulator of murine and human mast cell \ function. *J Immunol*. (2005) 174:1348–56. doi: 10.4049/jimmunol.174.3.1348
57. Flies DB, Higuchi T, Chen L. Mechanistic assessment of PD-1H coinhibitory receptor-induced T cell tolerance to allogeneic antigens. *J Immunol*. (2015) 194:5294–304. doi: 10.4049/jimmunol.1402648
58. Walker LS, Sansom DM. Confusing signals: recent progress in CTLA-4 biology. *Trends Immunol*. (2015) 36:63–70. doi: 10.1016/j.it.2014.12.001
59. Moreland L, Bate G, Kirkpatrick P. Abatacept. *Nat Rev Drug Discov*. (2006) 5:185–6. doi: 10.1038/nrd1989
60. Lee S, Moon JS, Lee CR, Kim HE, Baek SM, Hwang S, et al. Abatacept alleviates severe autoimmune symptoms in a patient carrying a de novo variant in CTLA-4. *J Allergy Clin Immunol*. (2016) 137:327–30. doi: 10.1016/j.jaci.2015.08.036
61. Griffin MD, Hong DK, Holman PO, Lee KM, Whitters MJ, O'Herrin SM, et al. Blockade of T cell activation using a surface-linked single-chain antibody to CTLA-4 (CD152). *J Immunol*. (2000) 164:4433–42. doi: 10.4049/jimmunol.164.9.4433
62. Fife BT, Griffin MD, Abbas AK, Locksley RM, Bluestone JA. Inhibition of T cell activation and autoimmune diabetes using a B cell surface-linked CTLA-4 agonist. *J Clin Invest*. (2006) 116:2252–61. doi: 10.1172/JCI27856
63. Kasagi S, Kawano S, Okazaki T, Honjo T, Morinobu A, Hatachi S, et al. Anti-programmed cell death 1 antibody reduces $CD4^+PD\text{-}1^+$ T cells and relieves the lupus-like nephritis of NZB/W F1 mice. *J Immunol*. (2010) 184:2337–47. doi: 10.4049/jimmunol.0901652
64. Wong M, La Cava A, Singh RP, Hahn B H. Blockade of programmed death-1 in young (New Zealand black x New Zealand white)F1 mice promotes the activity of suppressive $CD8^+$ T cells that protect from lupus-like disease. *J Immunol*. (2010) 185:6563–71. doi: 10.4049/jimmunol.0903401
65. Wong M, La Cava A, Hahn BH. Blockade of programmed death-1 in young (New Zealand Black x New Zealand White)F1 mice promotes the suppressive capacity of $CD4^+$ regulatory T cells protecting from lupus-like disease. *J Immunol*. (2013) 190:5402–10. doi: 10.4049/jimmunol.1202382
66. Šedý JR, Balmert MO, Ware BC, Smith W, Nemčovičova I, Norris PS, et al. A herpesvirus entry mediator mutein with selective agonist action for the inhibitory receptor B and T lymphocyte attenuator. *J Biol Chem*. (2017) 292:21060–70. doi: 10.1074/jbc.M117.813295
67. He FR, Zhu HF, Huang H, Dai YD, Shen X, Wang M, et al. Programmed death-1 ligands-transfected dendritic cells loaded with glutamic acid decarboxylase 65 (GAD65) inhibit both the alloresponse and the GAD65-reactive lymphocyte response. *Clin Exp Immunol*. (2008) 151:86–93. doi: 10.1111/j.1365-2249.2007.03546.x
68. Li F, Ravetch JV. A general requirement for FcγRIIB co-engagement of agonistic anti-TNFR antibodies. *Cell Cycle* (2012) 11:3343–44. doi: 10.4161/cc.21842
69. Dahan R, Barnhart BC, Li F, Yamniuk AP, Korman AJ, Ravetch JV. Therapeutic activity of agonistic, human anti-CD40 monoclonal antibodies requires selective FcγR engagement. *Cancer Cell* (2016) 29:820–31. doi: 10.1016/j.ccell.2016.05.001
70. White AL, Chan HT, French RR, Willoughby J, Mockridge CI, Roghanian A, et al. Conformation of the human immunoglobulin G2 hinge imparts superagonistic properties to immunostimulatory anticancer antibodies. *Cancer Cell* (2015) 27:138–48. doi: 10.1016/j.ccell.2014.11.001
71. Dushek O, Goyette J, van der Merwe PA. Non-catalytic tyrosine-phosphorylated receptors. *Immunol Rev*. (2012) 250:258–76. doi: 10.1111/imr.12008
72. Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol*. (2006) 7:803–809. doi: 10.1038/ni1369
73. Belardi B, O'Donoghue GP, Smith AW, Groves JT, Bertozzi CR. Investigating cell surface galectin-mediated cross-linking on glycoengineered cells. *J Am Chem Soc*. (2012) 134:9549–52. doi: 10.1021/ja301694s
74. Grujic O, Stevens J, Chou RY, Weiszmann JV, Sekirov L, Thomson C, et al. Impact of antibody subclass and disulfide isoform differences on the biological activity of CD200R and βklotho agonist antibodies. *Biochem Biophys Res Commun*. (2017) 486:985–91. doi: 10.1016/j.bbrc.2017.03.145
75. Bartholomaeus P, Semmler LY, Bukur T, Boisguerin V, Römer PS, Tabares P, et al. Cell contact-dependent priming and Fc interaction with $CD32^+$ immune cells contribute to the TGN1412-triggered cytokine response. *J Immunol*. (2014) 192:2091–2098. doi: 10.4049/jimmunol.1302461
76. Williams EL, Tutt AL, French RR, Chan HT, Lau B, Penfold CA, et al. Development and characterisation of monoclonal antibodies specific for the murine inhibitory FcγRIIB (CD32B). *Eur J Immunol*. (2012) 42:2109–2120. doi: 10.1002/eji.201142302
77. Du X, Tang F, Liu M, Su J, Zhang Y, Wu W, et al. A reappraisal of CTLA-4 checkpoint blockade in cancer immunotherapy. *Cell Res*. (2018) 28:416–32. doi: 10.1038/s41422-018-0011-0
78. Zhang M, Howard K, Winters A, Steavenson S, Anderson S, Smelt S, et al. Monoclonal antibodies to B and T lymphocyte attenuator (BTLA) have no effect on *in vitro* B cell proliferation and act to inhibit *in vitro* T cell proliferation when presented in a cis, but not trans, format relative to the activating stimulus. *Clin Exp Immunol*. (2011) 163:77–87. doi: 10.1111/j.1365-2249.2010.04259.x
79. Lühder F, Huang Y, Dennehy KM, Guntermann C, Müller I, Winkler E., et al. Topological requirements and signaling properties of T cell-activating, anti-CD28 antibody superagonists. *J Exp Med*. (2003) 197:955–66. doi: 10.1084/jem.20021024
80. Evans EJ, Esnouf RM, Manso-Sancho R, Gilbert RJ, James JR, Yu C, et al. Crystal structure of a soluble CD28-Fab complex. *Nat Immunol*. (2005) 6:271–9. doi: 10.1038/ni1170
81. James SE, Greenberg PD, Jensen MC, Lin Y, Wang J, Till BG, et al. Antigen sensitivity of CD22-specific chimeric TCR is modulated by target epitope distance from the cell membrane. *J Immunol*. (2008) 180:7028–38. doi: 10.4049/jimmunol.180.10.7028
82. Li J, Stagg NJ, Johnston J, Harris MJ, Menzies SA, DiCara D, et al. Membrane-proximal epitope facilitates efficient T cell synapse formation by anti-FcRH5/CD3 and is a requirement for myeloma cell killing. *Cancer Cell* (2017) 31:383–95. doi: 10.1016/j.ccell.2017.02.001
83. Hannier S, Tournier M, Bismuth G, Triebel F. CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. *J Immunol*. (1998) 161:4058–65.
84. Faje A. Immunotherapy and hypophysitis: clinical presentation, treatment, and biologic insights. *Pituitary* (2016) 19:82–92. doi: 10.1007/s11102-015-0671-4
85. Pallardy M, Hünig T. Primate testing of TGN1412: right target, wrong cell. *Br J Pharmacol*. (2010) 161:509–11. doi: 10.1111/j.1476-5381.2010.00925.x

86. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med*. (2013) 369:122–33. doi: 10.1056/NEJMoa1302369
87. Okazaki T, Okazaki IM, Wang J, Sugiura D, Nakaki F, Yoshida T, et al. PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. *J Exp Med*. (2011) 208:395–407. doi: 10.1084/jem.20100466
88. Liu J, Yuan Y, Chen W, Putra J, Suriawinata AA, Schenk AD, et al. Immune-checkpoint proteins VISTA and PD-1 nonredundantly regulate murine T-cell responses. *Proc Natl Acad Sci USA*. (2015) 112:6682–7. doi: 10.1073/pnas.1420370112
89. Nirschl CJ, Drake CG. Molecular pathways: coexpression of immune checkpoint molecules: signaling pathways and implications for cancer immunotherapy. *Clin Cancer Res*. (2013) 19:4917–24. doi: 10.1158/1078-0432.CCR-12-1972
90. Ford ML, Adams AB, Pearson TC. Targeting co-stimulatory pathways: transplantation and autoimmunity. *Nat Rev Nephrol*. (2014) 10:14–24. doi: 10.1038/nrneph.2013.183
91. Murakami N, Riella LV. Co-inhibitory pathways and their importance in immune regulation. *Transplantation* (2014) 98:3–14. doi: 10.1097/TP.0000000000000169
92. Zhang Q, Vignali DA. Co-stimulatory and co-inhibitory pathways in autoimmunity. *Immunity* (2016) 44:1034–51. doi: 10.1016/j.immuni.2016.04.017

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 17 October 2018
doi: 10.3389/fimmu.2018.02374

Inhibitory Receptors and Pathways of Lymphocytes: The Role of PD-1 in Treg Development and Their Involvement in Autoimmunity Onset and Cancer Progression

Elena Gianchecchi[1,2] *and Alessandra Fierabracci*[1]*

[1] Infectivology and Clinical Trials Research Department, Children's Hospital Bambino Gesù, Rome, Italy, [2] VisMederi S.r.l., Siena, Italy

Regulatory T (Treg) cells represent a subpopulation of suppressor $CD4^+$ T cells critically involved in the establishment of peripheral tolerance through the inhibition of effector T (Teff) cells and the suppression of the immune-mediated tissue destruction toward self-antigens. Treg generation, their suppressive properties and also Treg-Teff cell interactions could be modulated at least in part by programmed cell death-1 (PD-1) expression on their surface and through binding between PD-1 and programmed cell death ligand-1 (PD-L1). Defects involving PD-1 and Tregs can lead to the development of pathological conditions, including autoimmune disorders or promote cancer progression by favoring tumor evasion from the host immune response. At the same time, PD-1 and Tregs could represent attractive targets for treatment, as demonstrated by the therapeutic blockade of PD-L1 applied for the management of different cancer conditions in humans. In the present Review, we focus specifically the role of PD-1/PD-L1 on Treg development and activity.

Keywords: PD-1, Tregs, autoimmunity, T1D, cancer

OPEN ACCESS

Edited by:
Alexandre M. Carmo, i3S, Instituto de Investigação e Inovação em Saúde, Portugal

Reviewed by:
Christopher E. Rudd, Université de Montréal, Canada
Lawrence Kane, University of Pittsburgh, United States

***Correspondence:**
Alessandra Fierabracci
alessandra.fierabracci@opbg.net

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *28 May 2018*
Accepted: *24 September 2018*
Published: *17 October 2018*

Citation:
Gianchecchi E and Fierabracci A (2018) Inhibitory Receptors and Pathways of Lymphocytes: The Role of PD-1 in Treg Development and Their Involvement in Autoimmunity Onset and Cancer Progression. Front. Immunol. 9:2374. doi: 10.3389/fimmu.2018.02374

INTRODUCTION

The programmed cell death 1 (PD-1, CD80) molecule is a 55kDa type I transmembrane protein (1) belonging to the immunoglobulin superfamily. PD-1 bears the immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic region (2), which is present also in several immunological negative receptors such as killer cell immunoglobulin-like receptors (KIRs) on natural killer (NK) cells and cluster of differentiation (CD) 22 and FcγRIIB on B cells. PD-1 was isolated for the first time in 1992 by the group of Ishida from a murine T cell hybridoma undergoing programmed cell death (2). Although murine PD-1 (mPD-1) mRNA expression is associated with activation-induced apoptosis in murine T cell hybridomas, PD-1 binding does not lead to cell death, instead it causes cell cycle blockade. Merely 10 years later from PD-1 discovery, the physiological role of this pathway and wherein is involved remain to be elucidated.

In more detail, the study involving PD-1 deficient mice has revealed a key role for PD-1 as a negative regulator of immune responses (3). Murine models with different genetic background showed the development of different autoimmune conditions characterized by delayed onset, organ-specific effects and incomplete penetrance. In particular, PD-1-deficient C57BL/6 mice

spontaneously developed lupus like arthritis, splenomegaly, and glomerulonephritis; furthermore, these animals showed an increased number of B and myeloid cells, and enhanced IgA, IgG2b, and IgG3 levels in the serum (4). In Balb/c mice, PD-1 deletion caused a peculiar autoimmune phenotype already at 5 weeks of age, characterized by dilated cardiomyopathy, gastritis, and elevated circulating levels of troponin reactive IgG1 (5). In non-obese diabetic (NOD) mice, PD-1 deficiency accelerated subacute Type I diabetes (T1D) development, but it did not cause the onset of other autoimmune conditions. Hence it promoted the inherent autoimmune susceptibility in this background without modifying its specificity. Finally, lethal myocarditis developed in mice with Murphy Roths Large (MRL) background (6, 7). It is supposed that PD-1 deficiency could promote tissue-specific autoimmunity inherent in the strain by favoring the activation of those T cells that in *Pdcd1*$^{+/+}$ mice were found anergic (8).

The homolog of murine PD-1 (mPD-1) is PD-1 (CD279) in humans, which is characterized by 60% identity with mPD-1 (9–11). PD-1 expression is identified on a limited population of $CD4^{-}CD8^{-}$ double negative (DN) thymocytes and is present on several cell types, such as activated T and B lymphocytes (12, 13), NK T cells, regulatory T cells (Tregs) (14), activated monocytes, and dendritic cells (DCs) in both humans and mice (15) and on human germinal center-associated T cells (16). The expression of PD-1 on the surface of activated T cells occurs during the initial activation phase. However, PD-1 regulates the immune response at a later stage during the peripheral tissue infiltration by effector T cells (Teffs). This is different in respect to Cytotoxic T Lymphocyte Antigen-4 (CTLA4) which represents another key immune checkpoint and is mainly involved in the modulation of the magnitude during the initial stages of T cell activation (priming) in the regional lymph node.

Two ligands, programmed cell death ligand-1 (PD-L1) (B7-H1, CD274), and PD-L2 (B7-DC, CD273), are recognized by PD-1, however PD-L1 expression is wider than PD-L2 expression. Among the cells that constitutively express PD-L1 there are T and B lymphocytes, DCs, macrophages, mesenchymal stem cells, bone marrow-derived mast cells (17) and activated Tregs. Furthermore, it has been detected also at sites of immune privilege, such as the eye, placenta, and testes (15). PD-L1 expression has been described also on tumor cells (18). Conversely, the expression of PD-L2 is restricted mainly on macrophages and DCs. The engagement of PD-1 by its ligands provides inhibitory signals involved in the regulation of central and peripheral tolerance through the inhibition of cytokine synthesis (19), T cell proliferation and cytotoxic activity. Mazanet et al. (19) observed that the negative regulation of T lymphocytes activated by endothelial cells (EC) and involving PD-1 signaling pathway did not involve activation markers, but affected selectively the production of cytokines. Furthermore, this inhibitory effect was directly correlated with the strength of the primary stimulus.

During the process of central tolerance, PD-1 and PD-L1 expression has been detected on the surface of maturing thymocytes. The thymus shows a wide expression of PD-L1, whereas PD-L2 expression has been observed on thymic medulla. The finding that thymocyte transition from DN to the $CD4^{+}CD8^{+}$ double positive (DP) stage was considerably promoted by PD-1 deficiency has allowed to speculate that PD-1 pathway could modulate the repertoire of mature T lymphocytes; the phenomenon was observed in both T cell receptor (TCR) transgenic lines and in recombination activating gene (RAG)-$2^{-/-}$ mice receiving anti-CD3 mAb. This could occur by negatively modulating the threshold for β selection and regulating the positive selection (20).

The pathway PD-1/PD-L1 has been recognized to modulate and maintain peripheral CD4, including $CD4^{+}$ Tregs and $CD8^{+}$ T cell tolerance at several levels, in particular both T lymphocyte stability and integrity. More specifically, it can down-regulate self-reactive T cells during the presentation of self-antigen by DCs (21, 22). PD-1 is also able to directly promote interleukin-10 (IL-10) secretion by T cells (23) and inhibit the maturation of DCs (24). PD-L1/PD-1 interaction shows a critical role also for the establishment of fetus tolerance (25, 26).

Nishimura et al. (4) first highlighted a correlation between PD-1 pathway and the onset of autoimmunity (4). More specifically, PD-1 disruption resulted in the spontaneous development of lupus-like autoimmune disease associated with glomerulonephritis and predominant IgG3 deposition in aged C57BL/6(B6)-PD-$1^{-/-}$congenic mice. This phenomenon is putatively due to the chronic breakdown of peripheral self-tolerance. Conversely, B6-PD-$1^{+/+}$ mice at the same age did not show arthritis and presented only marginal and probably age-associated glomerular lesions (4).

Recent investigations have moreover supported the presence of an association between defects affecting this pathway and the onset and progression of several autoimmune conditions (**Figure 1**) (27, 28). The PD-1-PD-L1/L2 pathway has a protecting effect for the host toward hyper-activated Teff cells in case of microbial infections inhibiting both Teff proliferation and capacity which could otherwise lead to chronic infection; conversely, in case of cancer, this signaling pathway can favor cancer progression through strong inhibitory mediators (**Figure 1**) (29, 30). In this respect, PD-1 immuno-checkpoint blockade exerted significant antitumor effects in several malignancies, especially in melanoma patients, attracting much attention in oncotherapy in the last years (31). The pharmacological treatment based on PD-1-PD-L1/PD-L2 immune checkpoint inhibitors (ICIs) is able to restore the number of Teff cells and promote their cytotoxic immune responses directed against chemotherapy-refractory tumors and restore the activity of exhausted $CD8^{+}$ T cells in chronic viral infections (32). The therapy promotes also the synthesis of pro-inflammatory cytokines restoring the ongoing tumor immunity (33–36). On one side, if treatment with anti-PD-1/PDL-1 agents was responsible for better survival in several different cancers, on the other, after such treatments, ICIs can cause the onset of inflammatory side effects affecting any organ system, conditions defined as immune-related adverse events (IrAEs) (37). In addition to organ specific IrAEs, more general AEs related to immune activation, such as fatigue, rash and diarrhea, as well as AEs potentially attributable to systemic inflammation, especially musculoskeletal manifestations, have been reported in patients

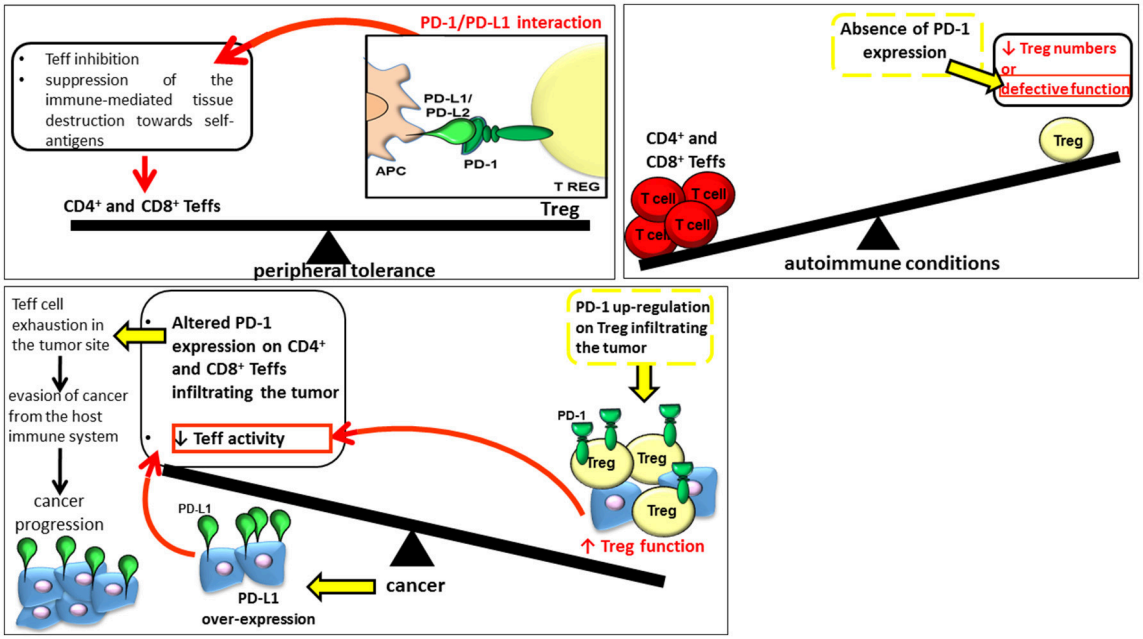


FIGURE 1 | The role of PD-1 and Tregs in peripheral tolerance, onset of autoimmune conditions and cancer progression.

receiving anti-PD-1 treatments. ICI therapy boosts the body's natural defense against tumor by promoting the T cell specific immune response, and although it shows a lower toxicity respect to standard chemotherapy, it can lead to previously described AEs. AEs are in fact a consequence of an altered immunologic tolerance due to immune checkpoint disruption. The misdirected stimulation of the immune system toward a normal tissue due to a prolonged immune activation can lead to autoimmune-like/inflammatory side-effects. Delayed autoimmune toxicity can even emerge over time after discontinuing anti-PD-1 antibody treatment. Thus, in light of rapid increase in the number of patients receiving anti-PD-1 agents, a longer term follow-up of patients treated with ICIs would be recommended. To this aim, it would be suitable to comprise the period after cessation of therapy.

It has been recently demonstrated that pre-existing active rheumatic diseases heightened in patients receiving anti-PD-1 treatments (38). However, PD-1 inhibitors were responsible for lower toxicity compared to other immunotherapies, such as IL-2 and CTLA-4 blockade. In more detail, the majority of patients receiving monotherapy with PD-1 antagonists presented modest side effects respect to other immunotherapies, such as those involving IL-2. The severity, in particular of colitis, was lower for PD-1 antagonists respect to patients treated with anti–CTLA-4 mAb. Even though the combined treatment with anti-PD-1 plus anti–CTLA-4 mAbs resulted in increased response rates in patients less responsive to monotherapy, it resulted in a higher severity of side effects (39).

The analysis recently conducted by Le Burel et al. (40) through the investigation of the Registre des Effets Indésirables Sévères des Anticorps Monoclonaux Immunomodulateurs en Cancérologie (REISAMIC) registry, reported for the first time the onset of connective tissue diseases (CTD) in 4 out of a total of 448 patients treated with anti-PD-1/anti-PD-L1 agents. More specifically, two cases of Sjögren's syndrome and one case of cryoglobulinemic vasculitis as a complication of suspected Sjögren's syndrome, and one case of myositis positive for antinuclear antibodies^{+} (ANA^{+}) were observed. Three of the patients were females and all had metastatic cancer. Two subjects had received anti-PD-1 agents and two anti-PD-L1 agents and no apparent symptom of CTD was detected before the treatment with ICIs. The correlation between anti-PD-1/PD-L1 cancer immunotherapy and CTD onset revealed the necessity to identify asymptomatic patients at risk of IrAEs (40). The participation of subjects presenting autoimmune disorders has been mostly excluded by immunotherapy clinical trials.

In this Review, we discuss specifically the role of PD-1/PD-L1 on Tregs in view of future therapeutic perspectives targeting this specific immunotype. Although these molecules are at high expression, the involvement of the pathway in the expansion and function of this cell population remains indeed to be fully elucidated (41).

TREGS AND PD-1 EXPRESSION

The group of Sakaguchi et al. (42) discovered Tregs in 1995. Since their discovery, our knowledge regarding this population has widely increased. They represent a developmentally distinct subset of suppressor CD4^{+} T cells critically involved in the quality and magnitude of immune responses, in the establishment of peripheral tolerance through the inhibition of Teff cells and the suppression of the immune-mediated tissue destruction toward self-antigens. Treg activity occurs

primarily at the site of inflammation where they are attracted by inflammatory signals. Several suppressor mechanisms are used by Tregs (**Figure 1**) (43, 44) depending on the physiological and inflammatory underlining condition (45, 46).

Tregs are able to modulate the immune response in an antigen-dependent and independent manner (47). The suppression of $CD4^+$ T cell activities by Tregs is mediated by inhibitory cytokines including transforming growth factor β (TGF-β) and IL-10, the latter being important for its immunosuppressive activity at environmental interfaces (48). The release of IL-10 from Th1 cells is triggered by TGF-β1 (49), which inhibits the further cytokine synthesis and directly reduces the activity of Teffs (50). In addition, Cottrez et al. (49) found that IL-10 potentiates the response of activated T cells to TGF-β1 by modulating TGF receptor expression. TGF-β and IL-10 exert limited effect on Teff expansion.

This local qualitative cytokine composition in the inflammatory microenvironment is able to modulate the magnitude of the immune response that halts antigen presenting cell (APC) functions. In addition, the inhibitory activity of Tregs can occur also by cell–cell contact with pathogenic immunotypes at the sites of inflammation through CTLA-4, lymphocyte-activation gene 3 (LAG-3) (51) and PD-1 (52). CTLA-4, LAG-3, PD-1 as well as PD-L1 are indeed highly expressed on Tregs (44).

Tregs can be distinguished into two subpopulations: naturally occurring Tregs (nTregs) and adaptive or induced Tregs (iTregs). The development of nTregs occurs in the thymus, and in basal conditions they are mitotically quiescent (53). They necessitate antigenic stimulation to expand *in vivo* (53), but they do not need TCR engagement to execute their inhibitory tasks (54). Conversely, iTregs develop from $CD4^+$ forkhead box protein 3 $(Foxp3)^-$ naive T cells in the periphery following antigenic stimulation. Chen et al. (55) demonstrated the generation of iTregs from peripheral $CD4^+CD25^-$ naive T cells through TGF-β induction of transcription factor *Foxp3*. Foxp3 belongs to the forkhead/winged-helix transcription factor family and plays a key role in Treg cell development and immunosuppressive activity. Mice presenting a genetic defect in Foxp3 are characterized by dysfunctional Tregs and develop systemic autoimmune features resembling lupus-like disease.

The inhibition of CTLA4 signaling using anti-CTLA4 blocking antibody considerably altered Treg frequency leading to an increase in this cell population as demonstrated by Tang et al. (56) and highlighting a new role for CTLA4 in the modulation of Treg turnover.

In addition, nTregs show elevated levels of CD25, the expression of Foxp3 (57) and a TCR repertoire recognizing self-antigens.

Treg development in the thymus is fundamental for the stable *Foxp3* expression, which represents the principal transcription factor involved in the regulation and maintenance of Treg phenotype and function. Tregs in the thymus can indeed recognize self-antigens (57, 58). Treg population represents a heterogeneous cell population which complicates Treg isolation based on the markers CD4/CD25/Foxp3. Indeed, different microRNAs, transcription factors, chemokine receptors, cytokines, inhibitor molecules, and other immune-related proteins can be expressed on different Treg subpopulations depending on the pathological and environmental situation. Recently, different subpopulations within the Treg population have been recognized through the identification of many novel additional markers (59), such as CD45RA which allows to distinguish $CD45RA^+Foxp3^{lo}$ resting Tregs (rTregs), $CD45RA^-Foxp3^{hi}$ activated Tregs (aTregs), and cytokine-secreting CD45RA-$Foxp3^{lo}$ non-suppressive Tregs (60). In addition to Tregs, other regulatory $CD4^+$ T cells are present, such as Type 1 regulatory T cells (Tr1) and Th3 cells, characterized by suppressive activities but do not express Foxp3 [*rev. in* (61)].

In addition, the critical role played by Tregs during pregnancy has also been demonstrated (62). In more detail, during normal pregnancy circulating maternal Tregs specific for fetal antigens increase their number already in the early stage of pregnancy allowing the maintenance of tolerance toward foreign paternal alloantigens by the maternal immune system (63). Treg number is maintained high also after delivery, even though their reduction post-partum has been reported by several studies. Moreover, their quick proliferation during the subsequent pregnancies has been reported. Accordingly, a defective number as well as activity of Tregs have been often correlated with unexplained infertility, miscarriage and pre-eclampsia (64–67). A recent study performed by Care et al. (68) also revealed that a reduced Treg number was responsible for uterine artery dysfunction in mice.

Mutations affecting *Foxp3* have been identified in immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX) syndrome characterized by non-functional Tregs (69). Similarly, $Foxp3^-$ mutant scurfy mice and $Foxp3^-$ null mice show the deficiency of $CD4^+CD25^+$ Tregs causing an aggressive lymphoproliferative autoimmune disorder which can disappear with Treg subset restoration. The addition of *Foxp3* transgene can also promote Treg differentiation in immunodeficient mice (56).

However, *Foxp3* expression is not specific to Tregs, but it has been described also on Teff lymphocytes. A reduction in Treg numbers or a defective function of this subpopulation causes the onset of autoimmune conditions in adult mice (46). Accordingly, several conditions in animal models including NOD and inflammatory bowel disease (IBD) mouse models can be reduced upon adoptive transfer of Tregs.

It has been observed that Treg generation as well as suppressive Treg properties and also Treg/Teff-cell interaction could be modulated at least in part by PD-1 expression (33) and by PD-1/PD-L1 binding.

In the presence of TGF-β, *Foxp3* expression is induced on naive $CD4^+$ T cells generating iTregs (55, 70–72) which showed high levels of CD25, CTLA-4, and glucocorticoid-induced TNF receptor (GITR). Activated Tregs show PD-1 expression that has been identified on conventional T cells, even if at a lower level (73). The absence of PD-1 expression promoted autoimmune disorders in animal models and humans (4, 5, 74). PD-1 signaling in $CD4^+$ Tregs is fundamental for the restriction of the number as well as for the suppression of Ag-reactive activity of Teff cells

that accumulate in the periphery in response to an immunogenic stimulus (19).

Accordingly, the progression of many autoimmune disorders, including experimental autoimmune encephalomyelitis (EAE) (75), diabetes, and colitis, was promoted when the interaction between PD-1 and B7-H1 was inhibited (76, 77). Bedke et al. (52) demonstrated a significant increase of immunosuppressive activity of $CD4^+CD25^+Foxp3^+$ Tregs upon EC contact mediated by PD-1 up-regulation on Tregs occurring during the extravasation of these cells from the blood into the inflamed tissue. The change of Treg phenotype was associated also with elevated IL-10 and TGF-β synthesis (52). Furthermore, recent evidences have highlighted the correlation between an altered function of Tregs and the development of an autoimmune condition as well as with skin tumors.

THE ROLE OF PD-1 IN TREG DEVELOPMENT AND ACTIVITY

Tregs are characterized by the expression of both PD-1 and PD-L1, which exert a role in the regulation of T cell tolerance (78). Even though PD-1/PD-L1 signaling pathway has been identified on $Foxp3^+$ Tregs, its role in the regulation of their function and activity has not been fully elucidated (30).

Treg development from naive T cells could be promoted by the interaction occurring between DCs expressing PD-L1 and T lymphocytes (3). In addition, PD-1/PD-L1 binding reduced the generation of T cells, the release of cytokines and survival. Moreover, PD-1 prompts Foxp3 expression and enhanced Treg suppressive activity (79). However, the host environment and PD-1 signaling play a significant role in Treg development as demonstrated by the fact that APCs deficient for PD-L1 caused a diminished generation of Tregs from $CD4^+$ T lymphocytes.

Raimondi et al. (73) observed that the regulated compartmentalization of PD-1 discriminates $CD4^+CD25^+$ resting Tregs from activated T cells. PD-1 signaling pathway is also important for the maintenance of the suppressive capacity of Tregs. Francisco et al. (79) reported indeed that iTreg cell differentiation, maintenance and function was induced by PD-L1 by sustaining and increasing the expression of Foxp3 in iTregs. PD-L1 promotes iTreg conversion through the inhibition of phosphatidylinositol-3-kinase/mammalian target of rapamycin (Akt/mTOR) signaling cascade and the simultaneous phosphatase and tensin homolog (PTEN) up-regulation. Accordingly, the development of iTregs from naive $CD4^+$ T cells was critically lowered in PD-$L1^{-/-}$ antigen-presenting cells, whereas a significant diminishment in iTreg development associated with a lethal immune-mediated pulmonary damage, was observed *in vivo* in PD-$L1^{-/-}$PD-$L2^{-/-}$ $Rag^{-/-}$ recipients of naïve CD4 T cells. The involvement of PD-1 in Treg conversion was further confirmed by naive CD4 T cell transfer to $Rag^{-/-}$ recipients receiving an anti–PD-L1 blocking antibody. The analysis of Treg cell development and immunopathology revealed an important defect in *de novo* iTreg differentiation in $Rag^{-/-}$ mice receiving the anti–PD-L1 mAb treatment in respect to the control group. Moreover, a moderate lung inflammatory phenotype characterized the lungs of $Rag^{-/-}$ mice administered with anti–PD-L1 mAb. Consistently with the results from PD-$L1^{-/-}$PD-$L2^{-/-}$ $Rag^{-/-}$ recipients receiving naive CD4 T cells, defective iTreg differentiation as well as pulmonary inflammation develops in wild type $Rag^{-/-}$ mice treated with anti–PD-L1 mAb. *Foxp3* expression as well as the suppressive Treg function were increased upon activation of T lymphocytes in presence of PD-L1-Ig (79).

In this regard, Amarnath et al. (80) reported the conversion of human Th1 cells into Tregs through the involvement of the PD-L1-PD-1 axis. In detail, conventional T cells or irradiated K562 myeloid tumor cells, characterized by a hyper-expression of PD-L1, were able to induce the conversion of $TBET^+$ Th1 cells into $Foxp3^+$ Tregs *in vivo*, preventing human-into-mouse xenogeneic GvHD (xGvHD) onset. Th1 cells could mediate lethal xGVHD when PD-1 expression on Th1 cells was halted or PD-1 signaling was inhibited. Hence, targeting PD-1 signaling through blockade of PD-1 expression or pharmacological inhibition of PD-1 signaling pathway could represent a potential strategy to increase T cell immunity against infection and cancer (80).

In order to investigate how Tregs inhibit antibody production, Gotot et al. (81) used transgenic mice expressing model antigens in the kidney demonstrating that the establishment of peripheral B-cell tolerance toward glomerular autoantibodies involved PD-1. More specifically, B cell suppression by Tregs do not need intermediate Th cells. In fact, the inhibition of autoreactive B cells by Tregs involved directly the interaction of both PD-1 ligands on Tregs with PD-1 on autoreactive B lymphocytes. The engagement of PD-1 suppressed both the activation and proliferation of self-reactive B cells, and promoted their apoptosis.

The study conducted by Wong et al. (82) investigated whether PD-1 expression could affect the generation of $CD4^+$ Tregs by treating mice with a neutralizing antibody. The anti-PD-1 treatment diminished PD-1 expression on $CD4^+$ Treg ($PD1^{lo}CD4^+$Treg) *in vivo*; the suppressive activity of $CD4^+$ Tregs was indeed affected by PD-1 level. More specifically, the blockade of PD-1 induced the formation of adaptive regulatory $CD4^+CD25^+$ T cells. In fact, $PD1^{low}CD4^+$ Tregs showed a higher capacity to elicit B cell apoptosis and inhibit $CD4^+$ helper T cells (T_h) in respect to $CD4^+$ Tregs presenting an elevated PD-1 expression ($PD1^{hi}CD4^+$Tregs) (82).

EVIDENCES OF PD-1 AND TREG INVOLVEMENT IN AUTOIMMUNITY

An increasing number of studies support the involvement of PD-1 and Treg interactions in the onset of different autoimmune conditions such as insulin-dependent diabetes mellitus (Type 1 diabetes, T1D), psoriasis, vitiligo, systemic lupus erythematosus (SLE) and inflammatory bowel diseases (IBD). Here we summarize recent supporting literature on these conditions.

Type 1 Diabetes

Type 1 diabetes represents a multifactorial autoimmune disorder characterized by the destruction of pancreatic β cells by

autoreactive T lymphocytes (83). Both genetics (84, 85) and environmental factors (86) are involved in T1D pathogenesis. The autoimmune response directed against pancreatic islet cells leads to a slow progressive and selective destruction of these cells (a condition identified as primary autoimmune insulitis) and, over the years, to a clinically manifested disease (87). In NOD mice, PD-1 blockade (88) or deficiency promoted both T1D development and induction of autoreactive T lymphocyte proliferation and their pancreatic infiltration (6). Recent studies have highlighted the correlation between the pharmacological treatment with PD-1 or PDL-1 antibodies (nivolumab or pembrolizumab) and T1D onset (89, 90). Mellati et al. (90) suggested that anti–PD-1, and possibly anti–PDL-1 antibody treatment could be responsible for a quick progression of autoimmune diabetes in human subjects characterized by an elevated underlying genetic predisposition to T1D, similarly to that observed in rodent models.

In T1D patients, Tregs showed a significant increase whereas Teffs were significantly diminished respect to controls. The observation that Treg/Teff ratio was higher in patients than in controls allowed to hypothesize that Tregs were functional in T1D patients (91). Concerning PD-1 expression on Tregs, no difference was found between patients and controls. Upon stimulation with CD3/CD28, Treg proliferation was defective in T1D subjects. In addition, healthy controls showed also a higher Teff proliferation. Concerning the ratio between Treg and Teffs, after 6 days from stimulation, the control group showed a significant increase respect to T1D group suggesting the reduced inhibitory functionality of Tregs in T1D patients respect to healthy subjects. Moreover, in T1D patients these cells presented reduced percentages of total PD-1^{+}, PD-1^{low}, and PD-1^{high} suggesting that reduced PD-1 expression and hence a defective PD-1/PD-L1 signaling pathway could lead to a deficient Treg activation (91).

A recent investigation conducted by Iijima et al. (92) studied for the first time the expression of PD-1 in circulating $CD4^{+}$ and $CD8^{+}$ T cells from fulminant T1D onset to 12 weeks after initiation of treatment. A consistent reduction was observed in circulating $CD4^{+}PD\text{-}1^{+}$ and $CD8^{+}PD\text{-}1^{+}$ T cells at the onset of fulminant T1D in two subjects with diabetic ketoacidosis (DKA) caused by T1D. Their number was restored upon treatment, as opposite to the number of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs.

Psoriasis

Psoriasis constitutes a chronic inflammatory skin disease mediated by multiple molecules and cells belonging both to the innate and adaptive immune arms. Psoriasis is characterized by a defective basal keratinocyte differentiation responsible for an enhanced proliferation and incomplete differentiation of these cells. One of the hallmark histologic features of psoriasis is the presence of neutrophils infiltrating the epidermis, where they are attracted by several chemotactic factors. The altered immune response characterizing psoriasis is due to a pathogenic cross-talk involving keratinocytes, DCs, and T lymphocytes. T cells have a key role in the initiation phase of the disease, especially those residing in the skin as tissue-resident memory T (TRM) cells. The inflammatory process is sustained by IL-17, IL-22, and TNF. Although the etiology of the disease remains to be fully elucidated, a combination of environmental and genetic factors could be responsible for an abnormal immune response. The identification of T cell subsets that are specifically involved in the pathogenic process has not been clarified yet (93). Myeloid-derived suppressor cells (MDSCs) are among the defective cell components with immunosuppressive activity believed to play a role in non-malignant inflammatory diseases, such as asthma, IBD, arthritis, and psoriasis (94). Soler and McCormick (94) demonstrated that PD-1 surface expression was reduced on monocytic MDSCs (Mo-MDSCs) from psoriatic patients; in addition, although the generation of Tregs from naive Teffs was induced both by psoriatic and control Mo-MDSCs, Tregs induced by psoriatic Mo-MDSCs showed a reduced suppressive activity. It is possible that T cell proliferation and hyper-activation is not limited due to alterations in psoriatic Mo-MDSCs (94).

Since keratinocytes express PD-L1 and PD-L2, Kim et al. (95) investigated whether their expression in terms of mRNA and protein levels on keratinocytes obtained through skin biopsies from psoriasis, allergic contact dermatitis (ACD), pityriasis rosea (PR), and lichen planus (LP) were altered respect to normal epidermis. Concerning psoriatic epidermis, PD-L1 and PD-L2 mRNA levels were consistently reduced respect to healthy epidermis. In psoriasis, PD-L1 protein expression was reduced compared ACD, PR, LP and normal epidermis. Psoriatic and normal epidermis showed no expression and minimal expression of PD-L2; conversely, it was enhanced in the other inflammatory skin disorders ACD, PR, and LP. These changes in psoriatic epidermis allow to hypothesize that PD-L1 and PD-L2 could contribute to the chronic dysregulated inflammatory process underlying psoriasis by promoting the continuous T cell activation. The normal expressions of PD-L1 could inhibit the hyper-activated state of T lymphocytes (95). Although merely few data are currently available concerning Tregs in psoriasis, Fujimura et al. (96) reported a reduction in PD-L1 expression on APCs in case of Treg depletion (96). The altered Treg function may lead to reduced PD-L1 and PD-L2 expression.

Vitiligo

Vitiligo is a skin autoimmune disorder characterized by the destruction of melanocytes by antigen-specific T cells, causing a white patchy depigmentation. Vitiligo affects 0.5–2% of the population worldwide (97). Even though the etiology remains to be elucidated, the involvement of both genetic and environmental factors was hypothesized (98).

Miao et al. (99) demonstrated that the treatment of adult pre-melanosomal protein-1 (Pmel-1) vitiligo mice with a PD-L1 fusion protein reversed consistently the progression of depigmentation (99). This occurred through the activation and enhancement of Tregs in the skin. The increase of this cell population was observed also in the spleen and in the circulation. Treatment targeting PD-L1 blocked the immune process and was able to revert the depigmentation. PD-L1 fusion protein exerted even a more prolonged activity (until 8 weeks after the final treatment) in respect to CCL22 DNA and simvastatin treatments (2 weeks and 1 week, respectively). The immune

response involving melanocyte-reactive T cells in vitiligo was inhibited *in vivo* upon PD-L1 protein therapy. By enhancing remarkably Treg abundance in the skin, this treatment was able also to revert depigmentation development in Pmel-1 vitiligo mice. Hence, PD-L1 fusion protein could represent a novel potential therapeutic strategy for patients with vitiligo (99).

Systemic Lupus Erythematosus

SLE represents a severe systemic autoimmune disease characterized by the production of pathogenic autoantibodies directed against several self-antigens. It is responsible for the tissue inflammation and causes damage in several organs, such as the skin and kidneys (100).

The investigation conducted by Mesquita et al. (101) reported several alterations in the expression of critical surface molecules on Treg and Teff cells in 26 SLE patients with active disease compared to 31 with inactive disease and 26 healthy controls, despite healthy controls and SLE patients showed equivalent Treg cell frequency. More specifically, a higher $CD40L^+$ Treg cell frequency, associated with reduced CTLA-4^+ Treg and $CD28^+$ Treg cell frequencies, characterized SLE subjects. However, a further characterization of Tregs based on the expression of regulatory, effector and activation molecules, including PD-1 expression, revealed no difference in terms of PD-1^+ Treg cells as well as Treg/Teff ratio between SLE patients and healthy controls. Conversely, a reduced frequency of CTLA-4^+ and $CD28^+$ Tregs, together with a higher frequency of $CD40L^+$ Tregs and an increased ratio of Treg/Teff $CD40L^+$ cells, was observed in SLE patients. The frequency of $CD40L^+$ Tregs was positively correlated with the SLE disease activity index. These alterations could play an important role in SLE pathogenesis (101).

Inflammatory Bowel Diseases

IBD includes Crohn's disease (CD) and ulcerative colitis (UC), which are chronic inflammatory disorders affecting the gastrointestinal tract. Even though the exact pathogenesis remains to be elucidated, a defective regulation of the host immune response to intestinal flora in genetically susceptible individuals could be involved (102). Studies conducted both in mouse models and human patients support the involvement of Tregs in the IBD etiopathogenesis.

Recently, Alfen et al. (103) for the first time characterized *ex vivo* human intestinal type 1 regulatory T (T_R1) cells. In particular, they observed that human intestinal Treg1 either expressing interferon (IFN)-γ and IL-10, revealed also the presence of C-C chemokine receptor type 5 (CCR5) and PD-1. These cells showed IFN-γ expression and were able to efficiently reduce T-cell proliferation and colitis symptoms. Conversely, intestinal IFN- γ –producing type 1 Tregs that co-expressed CCR5 and PD-1, were obtained from the inflamed guts of IBD patients and mice and showed the downregulation in the synthesis of the anti-inflammatory IL-10 cytokine. The diminished production of IL-10 by T_R1 cells in response to the pro-inflammatory cytokines (IL-1β and IL-23) critically involved in colitis onset, was observed in both UC and CD patients and represent a common characteristic of IBDs. The abnormal intestinal inflammation characterizing IBD patients could be caused by the selective inhibition of IL-10 production by IL-10 and IFN-γ co-expressing IFN-γ^+ T_R1 cells in response to pro-inflammatory cytokines (103).

EVIDENCES FOR PD-1 PATHWAY AND TREG INVOLVEMENT IN CANCER

A common feature of several different tumors is the ability to evade the host immune response (104). This phenomenon occurs through two well-recognized mechanisms: the negative modulation of $CD4^+$ and $CD8^+$ T cells by Foxp3-dependent Tregs and the expression of PD-L1 that can inhibit the antitumor activity of PD-1 positive $CD8^+$ T lymphocytes (**Figure 1**).

Indeed the PD-1/PD-L1 interaction in the tumor microenvironment promotes tumor escape from immune surveillance and favors its growth (**Figure 1**). The anticancer immune response of T lymphocytes is inhibited by PD-L1 overexpression, a phenomenon observed in different tumor types i.e., breast adenocarcinoma, colon adenocarcinoma, and squamous cell carcinoma (105). Tumorigenesis and invasiveness are also enhanced *in vivo* following PD-L1 transgenic expression. Moreover, cancers showing a higher PD-L1 expression are even more resistant to specific $CD8^+$ T cell–mediated lysis *in vitro*. In addition, an altered expression of PD-1 on $CD4^+$ and $CD8^+$ T cells could be responsible for Teff cell exhaustion in the tumor site (106–108). Specifically, PD-L1 could be involved in two mechanisms of evasion in cancer cells: innate and adaptive resistance. The over-expression of PD-L1, promoted by oncogenic and constitutively activated signals, including epidermal growth factor receptor (EGFR), protein kinase B (AKT) and Kirsten rat sarcoma viral oncogene homolog (KRAS) pathways, is responsible for an innate (tumor cell intrinsic) resistance (109–113). PDL-1 can be induced not only in cancer but also in immune cells (myeloid suppressor cells, dendritic cell, macrophage, and lymphocytes) in the tumor microenvironment by inflammatory signals, a mechanism identified as adaptive resistance (114, 115).

Park et al. (116) confirmed PD-1 up-regulation in tumor-infiltrating Teffs and Tregs compared to those at distant site from the tumor. In addition, these authors also reported the over-expression of multiple suppressive receptors, including T cell immunoglobulin mucin 3 (TIM-3), CTLA-4, GITR, and LAG-3, consistently with previous published data; indeed the higher expression of these receptors was reported in infiltrating lymphocytes in an immunosuppressive environment (117–122). These data allow hypothesizing a possible correlation between the up-regulation of these inhibitory receptors and the higher suppressive activity of tumor-infiltrating Tregs (116). Therefore, the host immune response is not able to eliminate cancer cells, which can proliferate and develop metastasis (116). In order to clarify the putative role of PD-1 in melanoma growth, Kleffel et al. (123) produced stable *Pdcd1* knockdown (KD) and *Pdcd1*-overexpressing (OE) B16 melanoma lines observing that melanoma-specific Pdcd1-KD and Pdcd1-OE showed a reduction and an increase in melanoma growth,

respectively, in immunocompetent C57BL/6 mice respect to controls. Tumorigenesis was increased also upon binding of PD-1 by PD-L1. Accordingly, the inhibition of PD-L1 by using RNA interference (RNAi), blocking antibodies, or introducing two single point mutations in the two PD-1 signaling motifs ITIM and the immunoreceptor tyrosine-based switch motif (ITSM) located within the cytoplasmic region of PD-1, abolished the tumor growth in immunocompetent, immunocompromised and PD-1-deficient tumor graft recipient mice. These results support the crucial involvement of PD-1 in the efficient melanoma growth (123).

PD-1-PD-L1 blocking agents can restore tumor immunity targeting the immune alterations evoked by tumor in its microenvironment. In particular, PD-1-PD-L1 inhibitors enhanced the cytolytic activity of tumor-specific T cells, reduced suppressive cytokine IL-10 production, whereas enhanced pro-inflammatory cytokine synthesis, promoted the presence of Teffs and diminished the numbers and suppressive function of Tregs in the tumor site (35–38). Tregs have also been demonstrated to exert a cancer immunosuppressive activity in several murine tumor models (124).

A higher activity of Tregs can cause immunosuppression and lead to Th1 cell number reduction, favoring the onset and the progression of skin cancers. Upon binding between PD-1 and PD-L1, Th1, and Tc1 cells are inhibited and the synthesis of their cytokines (IFN-γ and IL-2) is reduced while T cell migration, proliferation, as well as the secretion of suppressive (IL-10) or cytotoxic mediators are inhibited (125).

The unprecedented and durable response rates described recently in a remarkable percentage of cancer patients, including treatment-refractory patients with advanced cancers, have allowed since 2011, the approval by US Food and Drug Administration (FDA) of several inhibitors of PD-1/PD-L1 axis in many cancers (126). In patients, the therapeutic blockade of PD-L1 contrasts effectively different tumors, such as classic Hodgkin lymphoma (HL), melanoma (127), non–small cell lung cancer (NSCLC), small cell lung cancer (128), urothelial carcinoma, renal cell carcinoma (RCC) (129), gastric carcinomas, and hepatocellular carcinoma (130). The first clinical trial investigating the effects of PD-1 blockade revealed a positive correlation between PD-L1 expression on tumor cells and therapeutic response (131–133).

The inhibition of Treg activity by blocking CTLA4 (134) and PD-1 (132) represents an effective therapeutic target in some cases of melanoma, especially if both proteins are targeted simultaneously (135, 136), despite the risk of dose-dependent toxicity and autoimmune disorders.

For this reason, in human cancer immunotherapy, only partial blockage of CTLA-4 has been recommended. PD-1 targeting led to a mild autoimmune condition, which resulted more severe in the simultaneous block of CTLA4 and PD-1 pathways (5).

CONCLUSIONS

The strictly regulated interaction between inhibitory and activating receptors and their ligands plays a fundamental role in the establishment and maintenance of immune system homeostasis (14). Defects affecting cells involved in immuneregulation such as Tregs or altered expression of molecules on their surface or in their regulatory pathways can lead to the development of pathological conditions including autoimmune disorders or promote cancer progression by favoring the evasion of tumor cells from the host immune response. At the same time, these molecules or subpopulations could represent potential therapeutic targets, as demonstrated by the efficacy of therapeutic blockade of PD-1/PD-L1 pathway used in the management of different cancers in humans. Further investigations are necessary in order to fully comprehend the complex mechanisms underlying the onset of these pathological conditions. This would improve the efficacy of tailored approaches in the personalized treatment of autoimmune and cancer conditions.

AUTHOR CONTRIBUTIONS

EG conducted the literature research, wrote the article. AF supervised the conduction of literature search and wrote the article.

FUNDING

This work was supported by the Italian Ministry of Health Ricerca Corrente 201702P003967.

ACKNOWLEDGMENTS

We acknowledge Dr Marha Pellegrino for linguistic revision.

REFERENCES

1. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol.* (1996) 8:765–72. doi: 10.1093/intimm/8.5.765
2. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* (1992) 11:3887–95. doi: 10.1002/j.1460-2075.1992.tb05481.x
3. Brown JA, Dorfman DM, Ma FR, Sullivan EL, Munoz O, Wood CR, et al. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol.* (2003) 170:1257–66. doi: 10.4049/jimmunol.170.3.1257
4. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* (1999) 11:141–51. doi: 10.1016/S1074-7613(00)80089-8
5. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* (2001) 291:319–22. doi: 10.1126/science.291.5502.319
6. Wang J, Yoshida T, Nakaki F, Hiai H, Okazaki T, Honjo T. Establishment of NOD-Pdcd1-/- mice as an efficient animal model of type I diabetes. *Proc Natl Acad Sci USA.* (2005) 102:11823–8. doi: 10.1073/pnas.0505497102

7. Wang J, Okazaki IM, Yoshida T, Chikuma S, Kato Y, Nakaki F, et al. PD-1 deficiency results in the development of fatal myocarditis in MRL mice. *Int Immunol*. (2010) 22:443–52. doi: 10.1093/intimm/dxq026
8. Tsushima F, Yao S, Shin T, Flies A, Flies S, Xu, H, et al. Interaction between B7–H1 and PD-1 determines initiation and reversal of T-cell anergy. *Blood* (2007) 110:180–5. doi: 10.1182/blood-2006-11-060087
9. Finger LR, Pu J, Wasserman R, Vibhakar R, Louie E, Hardy RR, et al. The human PD-1 gene (complete cDNA, genomic organization, and developmentally regulated expression in B cell progenitors). *Gene* (1997) 197:177–87. doi: 10.1016/S0378-1119(97)00260-6
10. Vibhakar R, Juan G, Traganos F, Darzynkiewicz Z, Finger LR. Activation-induced expression of human programmed death-1 gene in T-lymphocytes. *Exp Cell Res*. (1997) 232:25–8. doi: 10.1006/excr.1997.3493
11. Zhang X, Schwartz JC, Guo X, Bhatia S, Cao E, Lorenz M, et al. Structural and functional analysis of the costimulatory receptor programmed death-1. *Immunity* (2004) 20:337–47. doi: 10.1016/S1074-7613(04)00051-2
12. Linsley P, Ledbetter J. The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol*. (1993) 11:191–212. doi: 10.1146/annurev.iy.11.040193.001203
13. Suh WK, Tafuri A, Berg-Brown NN, Shahinian A, Plyte S, Duncan GS, et al. The inducible costimulator plays the major costimulatory role in humoral immune responses in the absence of CD28. *J Immunol*. (2004) 172:5917–23. doi: 10.4049/jimmunol.172.10.5917
14. Sansom DM, Manzotti CN, Zheng Y. What's the difference between CD80 and CD86? *Trends Immunol*. (2003) 24:313–8. doi: 10.1016/S1471-4906(03)00111-X
15. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. (2008). 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
16. Dorfman DM, Brown JA, Shahsafaei A, Freeman GJ. Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma. *Am J Surg Pathol*. (2006) 30:802–10. doi: 10.1097/01.pas.0000209855.28282.ce
17. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol*. (2002) 169:5538–45. doi: 10.4049/jimmunol.169.10.5538
18. Topalian SL, Taube JM, Anders RA, Pardoll DM. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. *Nat Rev Cancer* (2016) 16:275–87. doi: 10.1038/nrc.2016.36
19. Mazanet MM, Hughes CC. B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis. *J Immunol*. (2002) 169:3581–8. doi: 10.4049/jimmunol.169.7.3581
20. Nishimura H, Honjo T, Minato N. Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice. *J Exp Med*. (2000) 191:891–8. doi: 10.1084/jem.191.5.891
21. Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol*. (2005) 6:280–6. doi: 10.1038/ni1165
22. Keir ME, Freeman GJ, Sharpe A.H. PD-1 regulates selfreactive CD8+ T cell responses to antigen in lymph nodes and tissues. *J Immunol*. (2007) 179:5064–70. doi: 10.4049/jimmunol.179.8.5064
23. Dong H, Strome SE, Matteson EL, Moder KG, Flies DB, Zhu G, et al. Costimulating aberrant T cell responses by B7-H1 autoantibodies in rheumatoid arthritis. *J Clin Invest*. (2003) 111:363–70. doi: 10.1172/JCI16015
24. Kuipers H, Muskens F, Willart M, Hijdra D, van Assema FB, Coyle AJ, et al. Contribution of the PD-1 ligands/PD-1 signaling pathway to dendritic cell-mediated CD41 T cell activation. *Eur J Immunol*. (2006) 36:2472–82. doi: 10.1002/eji.200635978
25. Tripathi S, Guleria I. Role of PD1/PDL1 pathway, and Th17 and Treg cells in maternal tolerance to the fetus. *Biomed J*. (2015) 38:25–31. doi: 10.4103/2319-4170.143511
26. Zhang YH, Tian M, Tang MX, Liu ZZ, Liao AH. Recent insight into the role of the PD-1/PD-L1 pathway in feto-maternal tolerance and pregnancy. *Am J Reprod Immunol*. (2015) 74:201–8. doi: 10.1111/aji.12365
27. Gianchecchi E, Delfino DV, Fierabracci A. Recent insights into the role of the PD-1/PD-L1 pathway in immunological tolerance and autoimmunity. *Autoimmun Rev*. (2013) 12:1091–100. doi: 10.1016/j.autrev.2013.05.003
28. Javan MR, Aslani S, Zamani MR, Rostamnejad J, Asadi M, Farhoodi M, et al. Downregulation of immunosuppressive molecules, PD-1 and PD-L1 but not PD-L2, in the patients with multiple sclerosis. *Iran J Allergy Asthma Immunol*. (2016) 15:296–302.
29. Sharpe AH, Wherry EJ, Ahmed, R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol*. (2007) 8:239–45. doi: 10.1038/ni1443
30. Park HJ, Park JS, Jeong YH, Son J, Ban YH, Lee BH, et al. PD-1 upregulated on regulatory T cells during chronic virus infection enhances the suppression of CD8+ T cell immune response via the interaction with PD-L1 expressed on CD8+ T cells. *J Immunol*. (2015) 194:5801–11. doi: 10.4049/jimmunol.1401936
31. Mahoney KM, Freeman GJ, McDermott DF. The next immune-checkpoint inhibitors: PD-1/PD-L1 blockade in melanoma. *Clin Ther*. (2015) 37:764–82. doi: 10.1016/j.clinthera.2015.02.018
32. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe, A.H. et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* (2006) 439:682–7. doi: 10.1038/nature04444
33. Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* (2010). 116:1757–66. doi: 10.1002/cncr.24899
34. Gatalica Z, Snyder C, Maney T, Ghazalpour A, Holterman DA, Xiao N, et al. Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type. *Cancer Epidemiol Biomarkers Prev*. (2014) 23:2965–70. doi: 10.1158/1055-9965.EPI-14-0654
35. Okiyama N, Katz SI. Programmed cell death 1 (PD-1) regulates the effector function of CD8 T cells via PD-L1 expressed on target keratinocytes. J Autoimmun. (2014) 53:1–9. doi: 10.1016/j.jaut.2014.06.005
36. Spranger S, Koblish HK, Horton B, Scherle PA, Newton R, Gajewski TF. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8(+) T cells directly within the tumor microenvironment. *J Immunother Cancer* (2014) 2:3. doi: 10.1186/2051-1426-2-3
37. Boutros C, Tarhini A, Routier E, Lambotte O, Ladurie FL, Carbonnel F, et al. Safety profiles of anti-CTLA-4 and anti-PD-1 antibodies alone and in combination. *Nat Rev Clin Oncol*. (2016) 13:473–86. doi: 10.1038/nrclinonc.2016.58
38. Menzies AM, Johnson DB, Ramanujam S, Atkinson VG, Wong ANM, Park JJ, et al. Anti-PD-1 therapy in patients with advanced melanoma and preexisting autoimmune disorders or major toxicity with ipilimumab. *Ann Oncol*. (2016) 28:368–76. doi: 10.1093/annonc/mdw443
39. Ma W, Gilligan BM, Yuan J, Li T. Current status and perspectives in translational biomarker research for PD-1/PD-L1 immune checkpoint blockade therapy. *J Hematol Oncol*. (2016) 9:47. doi: 10.1186/s13045-016-0277-y
40. Le Burel S, Champiat S, Routier E, Aspeslagh S, Albiges L, Szwebel T, et al. Onset of connective tissue disease following anti-PD1/PD-L1 cancer immunotherapy. *Ann Rheum Dis*. (2017) 77:468-470. doi: 10.1136/annrheumdis-2016-210820
41. Krupnick AS, Gelman AE, Barchet W, Richardson S, Kreisel FH, Turka LA, et al. Murine vascular endothelium activates and induces the generation of allogeneic CD4+25+Foxp3+ regulatory T cells. *J Immunol*. (2005) 175:6265–70. doi: 10.4049/jimmunol.175.10.6265
42. Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med*. (1985) 161:72–87.
43. Tang Q, Bluestone, JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol*. (2008) 9:239–44. doi: 10.1038/ni1572
44. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev*. (2010) 236:219–42. doi: 10.1111/j.1600-065X.2010.00923.x
45. Yamaguchi T, Wing JB, Sakaguchi S. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. *Semin Immunol*. (2011) 23:424–30. doi: 10.1016/j.smim.2011.10.002

46. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol*. (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106.141623
47. Szymczak-Workman AL, Workman CJ, Vignali DA. Cutting edge: regulatory T cells do not require stimulation through their TCR to suppress. *J Immunol*. (2009) 182:5188–92. doi: 10.4049/jimmunol.0803123
48. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* (2008) 28:546–58. doi: 10.1016/j.immuni.2008.02.017
49. Cottrez F, Groux H. Regulation of TGF-beta response during T cell activation is modulated by IL-10. *J Immunol*. (2001) 167:773–8. doi: 10.4049/jimmunol.167.2.773
50. Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J Immunol*. (2001) 166:3008–18. doi: 10.4049/jimmunol.166.5.3008
51. Vignali DA, Collison LW, Workman C.J. How regulatory T cells work. *Nat Rev Immunol*. (2008) 8:523–32. doi: 10.1038/nri2343
52. Bedke T, Pretsch L, Karakhanova S, Enk AH, Mahnke K. Endothelial cells augment the suppressive function of CD4+ CD25+Foxp3+ regulatory T cells: involvement of programmed death-1 and IL-10. *J Immunol*. (2010) 184:5562–70. doi: 10.4049/jimmunol.0902458
53. Kuniyasu Y, Takahashi T, Itoh M, Shimizu J, Toda G, Sakaguchi S. (2000). Naturally anergic and suppressive CD25+CD4+ T cells as a functionally and phenotypically distinct immunoregulatory T cell subpopulation. *Int Immunol*. (2010) 12:1145–55. doi: 10.1093/intimm/12.8.1145
54. Bienvenu B, Martin B, Auffray C, Cordie C, Becourt C, Lucas B. Peripheral CD8+CD25+ T lymphocytes fromMHCclass II-deficient mice exhibit regulatory activity. *J Immunol*. (2005) 175:246–53. doi: 10.4049/jimmunol.175.1.246
55. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral CD4+CD25? naive T cells to CD4+CD25+ regulatory T cells by TGF-? induction of transcription factor Foxp3. *J Exp Med*. (2003) 198:1875–86. doi: 10.1084/jem.20030152
56. Tang AL, Teijaro JR, Njau MN, Chandran SS, Azimzadeh A, Nadler SG, et al. CTLA4 expression is an indicator and regulator of steady-state CD4+ FoxP3+ T cell homeostasis. *J Immunol*. (2008) 181:1806–13. doi: 10.4049/jimmunol.181.3.1806
57. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* (2003) 299:1057–61. doi: 10.1126/science.1079490
58. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol*. (2003) 4:337–42. doi: 10.1038/ni909
59. Mohr A, Malhotra R, Mayer G, Gorochov G, Miyara M. Human FOXP3+ T regulatory cell heterogeneity. *Clin Transl Immunol*. (2018) 7:e1005. doi: 10.1002/cti2.1005
60. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4 + T cells expressing the FoxP3 transcription factor. *Immunity* (2009) 30:899–911. doi: 10.1016/j.immuni.2009.03.019
61. Chien CH, Chiang BL. Regulatory T cells induced by B cells: a novel subpopulation of regulatory T cells. *J Biomed Sci*. (2017) 24:86. doi: 10.1186/s12929-017-0391-3
62. La Rocca C, Carbone F, Longobardi S, Matarese G. The immunology of pregnancy: Regulatory T cells control maternal immune tolerance toward the fetus. *Immunol Lett*. (2014) 162:41–8. doi: 10.1016/j.imlet.2014.06.013
63. Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT. Normal human pregnancy is associated with an elevation in the immune suppressive CD25+CD4+ regulatory T-cell subset. *Immunology* (2004) 112:38–43. doi: 10.1111/j.1365-2567.2004.01869.x
64. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol*. (2004) 5:266–71. doi: 10.1038/ni1037
65. Zenclussen AC, Gerlof K, Zenclussen ML, Sollwedel A, Bertoja AZ, Ritter T, et al. Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model. *Am J Pathol*. (2005) 166:811–22. doi: 10.1016/S0002-9440(10)62302-4
66. Jasper MJ, Tremellen KP, Robertson SA. Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. *Mol Hum Reprod*. (2006) 12:301–8. doi: 10.1093/molehr/gal032
67. Winger EE, Reed JL. Low circulating CD4(+) CD25(+) Foxp3(+) T regulatory cell levels predict miscarriage risk in newly pregnant women with a history of failure. *Am J Reprod Immunol*. (2011) 66:320–8. doi: 10.1111/j.1600-0897.2011.00992.x
68. Care AS, Bourque SL, Morton JS, Hjartarson EP, Robertson SA, Davidge ST. Reduction in regulatory T cells in early pregnancy causes uterine artery dysfunction in mice. *Hypertension* (2018) 72:177–87. doi: 10.1161/HYPERTENSIONAHA.118.10858
69. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. (2001) 27:20–21. doi: 10.1038/83713
70. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol*. (2004) 172:5149–53. doi: 10.4049/jimmunol.172.9.5149
71. Coombes JL, Siddiqui KR, Arancibia-Cárcamo CV, Hall J, Sun CM, Y. Belkaid, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-?– and retinoic acid–dependent mechanism. *J Exp Med*. (2007) 204:1757–64. doi: 10.1084/jem.20070590
72. Rubtsov YP, Rudensky AY. TGFbeta signalling in control of T-cell-mediated self-reactivity. *Nat Rev Immunol*. (2007) 7:443–53. doi: 10.1038/nri2095
73. Raimondi G, Shufesky WJ, Tokita D, Morelli AE, Thomson AW. Regulated compartmentalization of programmed cell death-1 discriminates CD4+CD25+ resting regulatory T cells from activated T cells. *J Immunol*. (2006) 176:2808–16. doi: 10.4049/jimmunol.176.5.2808
74. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. *Int Immunol*. (2007) 19:813–24. doi: 10.1093/intimm/dxm057
75. Magnus T, Schreiner B, Korn T, Jack C, Guo H, Antel J, et al. Microglial expression of the B7 family member B7 homolog 1 confers strong immune inhibition: implications for immune responses and autoimmunity in the CNS. *J Neurosci*. (2005) 25:2537–46. doi: 10.1523/JNEUROSCI.4794-04.2005
76. Liang SC, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, et al. (2003). Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol*. (2005) 33:2706–16. doi: 10.1002/eji.200324228
77. Totsuka T, Kanai T, Makita S, Fujii R, Nemoto Y, Oshima S, et al. Regulation of murine chronic colitis by CD4+CD25- programmed death-1+ T cells. *Eur J Immunol*. (2005) 35:1773–85. doi: 10.1002/eji.200425109
78. Lohr J, Knoechel B, Abbas AK. Regulatory T cells in the periphery. *Immunol Rev*. (2006) 212:149–62. doi: 10.1111/j.0105-2896.2006.00414.x
79. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. (2009) 206:3015–29. doi: 10.1084/jem.20090847
80. Amarnath S. The PDL1-PD1 axis converts human Th1 cells into regulatory T cells. *Sci Transl Med*. (2011) 3:111ra120. doi: 10.1126/scitranslmed.3003130
81. Gotot J, Gottschalk C, Leopold S, Knolle PA, Yagita H, Kurts C, et al. Regulatory T cells use programmed death 1 ligands to directly suppress autoreactive B cells *in vivo*. *Proc Natl Acad Sci USA*. (2012) 109:10468–73. doi: 10.1073/pnas.1201131109
82. Wong M, La Cava A, Hahn BH. Blockade of programmed death-1 in young (New Zealand Black x New Zealand White)F1 mice promotes the suppressive capacity of CD4+ regulatory T cells protecting from lupus-like disease. *J. Immunol*. (2013) 190:5402–10. doi: 10.4049/jimmunol.1202382
83. Fierabracci A. The potential of multimer technologies in type 1 diabetes prediction strategies. *Diabetes Metab Res Rev*. (2011) 27:216–29. doi: 10.1002/dmrr.1165
84. Krischer JP, Liu X, Lernmark Å, Hagopian WA, Rewers MJ, She JX, et al. The influence of type 1 diabetes genetic susceptibility regions, age, sex, and family history on the progression from multiple autoantibodies to type 1

diabetes: A TEDDY Study Report. *Diabetes* (2017) 66:3122–29. doi: 10.2337/db17-0261
85. Gui Y, Lei X, Huang S. Collective effects of common single nucleotide polymorphisms and genetic risk prediction in type 1 diabetes. *Clin Genet*. (2018) 93:1069–74. doi: 10.1111/cge.13193
86. Jakobsen OAJ, Szereday L. The "Three Amigos" lurking behind type 1 diabetes: hygiene, gut microbiota and viruses. *Acta Microbiol Immunol Hung*. (2018) 28:1–18. doi: 10.1556/030.65.2018.017
87. La Marca V, Gianchecchi E, Fierabracci A. Type 1 diabetes and its multi-factorial pathogenesis: the putative role of NK cells. *Int J Mol Sci*. (2018) 19:E794. doi: 10.3390/ijms19030794
88. Ansari MJ, Salama AD, Chitnis T, Smith RN, Yagita H, Akiba H, et al. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med*. (2003) 198:63–69. doi: 10.1084/jem.20022125
89. Hughes J, Vudattu N, Sznol M, Gettinger S, Kluger H, Lupsa B, et al. Precipitation of autoimmune diabetes with anti-PD-1 immunotherapy *Diabetes Care* (2015) 38:e55–7. doi: 10.2337/dc14-2349
90. Mellati M, Eaton KD, Brooks-Worrell BM, Hagopian WA, Martins R, Palmer JP, et al. Anti-PD-1 and anti-PDL-1 monoclonal antibodies causing type 1 diabetes. *Diabetes Care* . (2015) 38:e137–8. doi: 10.2337/dc15-0889
91. Perri V, Russo B, Crinò A, Schiaffini R, Giorda E, Cappa M, et al. Expression of PD-1 molecule on regulatory T lymphocytes in patients with insulin-dependent diabetes mellitus. *Int J Mol Sci*. (2015) 16:22584–605. doi: 10.3390/ijms160922584
92. Iijima T, Kato K, Jojima T, Tomotsune T, Fukushima M, Suzuki K, et al. Circulating CD4+PD-1+ and CD8+PD-1+ T cells are profoundly decreased at the onset of fulminant type 1 diabetes and are restored by treatment, contrasting with CD4+CD25+FoxP3+ regulatory T cells. *Diabetes Res Clin Pract*. (2017) 133:10–12. doi: 10.1016/j.diabres.2017.07.036
93. Boehncke WH, Schön MP. *Psoriasis Lancet* (2015) 386:983–94. doi: 10.1016/S0140-6736(14)61909-7
94. Soler DC, McCormick TS. Expanding the list of dysregulated immunosuppressive cells in psoriasis. *J Investi Dermatol*. (2016) 136:1749–51. doi: 10.1016/j.jid.2016.04.029
95. Kim DS, Je JH, Kim SH, Shin D, Kim, TG, Kim DY, et al. Programmed death-ligand 1, 2 expressions are decreased in the psoriatic epidermis. *Arch Dermatol Res*. (2015) 307:531–8. doi: 10.1007/s00403-015-1588-5
96. Fujimura T, Ring S, Umansky V, Mahnke K, Enk AH. Regulatory T cells stimulate B7-H1 expression in myeloid derived suppressor cells in ret melanomas. *J Invest Dermatol*. (2012) 132:1239–46. doi: 10.1038/jid.2011.416
97. Alikhan A, Felsten LM, Daly M, Petronic-Rosic V. Vitiligo: a comprehensive overview Part I. Introduction, epidemiology, quality of life, diagnosis, differential diagnosis, associations, histopathology, etiology, and work-up. *J Am Acad Dermatol*. (2011) 65:473–91. doi: 10.1016/j.jaad.2010.11.061
98. Spritz RA. Six decades of vitiligo genetics: genome-wide studies provide insights into autoimmune pathogenesis. *J Invest Dermatol*. (2012) 132:268–73. doi: 10.1038/jid.2011.321
99. Miao X, Xu R, Fan B, Chen J, Li X, Mao W, et al. PD-L1 reverses depigmentation in Pmel-1 vitiligo mice by increasing the abundance of Tregs in the skin. *Sci Rep*. (2018) 8:1605. doi: 10.1038/s41598-018-19407-w
100. Malkiel S, Barlev AN, Atisha-Fregoso Y, Suurmond J, Diamond B. Plasma cell differentiation pathways in systemic lupus erythematosus. *Front Immunol*. (2018) 9:427. doi: 10.3389/fimmu.2018.00427
101. Mesquita D Jr, Cruvinel WM, Araujo JAP, Salmazi KC, Kallas EG, Andrade LEC. Imbalanced expression of functional surface molecules in regulatory and effector T cells in systemic lupus erythematosus. *Brazilian J Med Biol Res*. (2014) 47:662–69. doi: 10.1590/1414-431X20143483
102. Ek WE, D'Amato M, Halfvarson J. The history of genetics in inflammatory bowel disease. *Ann Gastroenterol*. (2014) 27:294–303.
103. Alfen JS, Larghi P, Facciotti F, Gagliani N, Bosotti R, Paroni, M, et al. Intestinal IFN-g–producing type 1 regulatory T cells coexpress CCR5 and programmed cell death protein 1 and downregulate IL-10 in the inflamed guts of patients with inflammatory bowel disease. *J Allergy Clin Immunol*. (2018) doi: 10.1016/j.jaci.2017.12.984. [Epub ahead of print].
104. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol*. (2006) 6:715–27. doi: 10.1038/nri1936
105. Iwai Y, Hamanishi J, Chamoto K, Honjo T. Cancer immunotherapies targeting the PD-1 signaling pathway. *J Biomed Sci*. (2017) 24:26. doi: 10.1186/s12929-017-0329-9
106. Blank C, Kuball J, Voelkl S, Wiendl H, Becker B, Walter B, et al. Blockade of PD-L1 (B7–H1) augments human tumor-specific T cell responses *in vitro*. *Int J Cancer* (2006) 119:317–27. doi: 10.1002/ijc.21775
107. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* (2009) 114:1537–44. doi: 10.1182/blood-2008-12-195792
108. Gehring AJ, Ho ZZ, Tan AT, Aung MO, Lee KH, Tan KC, et al. Profile of tumor antigen-specific CD8 T cells in patients with hepatitis B virus-related hepatocellular carcinoma, *Gastroenterology* (2009) 137:682–90. doi: 10.1053/j.gastro.2009.04.045
109. Azuma K, Ota K, Kawahara A, Hattori S, Iwama E, Harada T, et al. Association of PD-L1 overexpression with activating EGFR mutations in surgically resected non small-cell lung cancer. *Ann Oncol*. (2014) 25:1935–40. doi: 10.1093/annonc/mdu242
110. Chen N, Fang W, Zhan J, Hong S, Tang Y, Kang S, et al. Upregulation of PDL1 by EGFR activation mediates the immune escape in EGFR-driven NSCLC: implication for optional immune targeted therapy for NSCLC patients with EGFR mutation. *J Thorac Oncol*. (2015) 10:910–23. doi: 10.1097/JTO.0000000000000500
111. Ota K, Azuma K, Kawahara A, Hattori S, Iwama E, Tanizaki J, et al. Induction of PD-L1 expression by the EML4-ALK oncoprotein and downstream signaling pathways in non-small cell lung cancer. *Clin Cancer Res*. (2015) 21:4014–21. doi: 10.1158/1078-0432.CCR-15-0016
112. Skoulidis F, Byers LA, Diao L, Papadimitrakopoulou VA, Tong P, Izzo J, et al. Co-occurring genomic alterations define major subsets of KRAS-mutant lung adenocarcinoma with distinct biology, immune profiles, and therapeutic vulnerabilities. Cancer Discov. (2015) 5:860–77. doi: 10.1158/2159-8290.CD-14-1236
113. Lastwika KJ, Wilson W, Li QK, Norris J, Xu H, Ghazarian SR, et al. Control of PD-L1 expression by oncogenic activation of the AKT-mTOR pathway in non-small cell lung cancer. *Cancer Res*. (2016) 76:227–38. doi: 10.1158/0008-5472.CAN-14-3362
114. Ribas A. Adaptive immune resistance: how cancer protects from immune attack. *Cancer Discov*. (2015) 5:915–9. doi: 10.1158/2159-8290.CD-15-0563
115. Lou Y, Diao L, Parra Cuentas ER, Denning WL, Chen L, Fan YH, et al. Epithelial-mesenchymal transition is associated with a distinct tumor microenvironment including elevation of inflammatory signals and multiple immune checkpoints in lung adenocarcinoma. *Clin Cancer Res*. (2016) 22:3630–42. doi: 10.1158/1078-0432.CCR-15-1434
116. Park HJ, Kusnadi A, Lee, EJ, Kim WW, Cho BC, Lee IJ, et al. Tumor-infiltrating regulatory T cells delineated by upregulation of PD-1 and inhibitory receptors. *Cell Immunol*. (2012) 278:76–83. doi: 10.1016/j.cellimm.2012.07.001
117. Grosso JF, Kelleher CC, Harris TJ, Maris CH, Hipkiss EL, De Marzo A, et al. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self and tumor-tolerance systems. *J Clin Investig*. (2007) 117:3383–92. doi: 10.1172/JCI31184
118. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med*. (2010) 207:2175–86. doi: 10.1084/jem.20100637
119. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, Beck A, Miller A, Tsuji T, et al. Tumor-infiltrating NYESO- 1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. *Proc Natl Acad Sci USA*. (2010) 107:7875–80. doi: 10.1073/pnas.1003345107
120. Mitsui J, Nishikawa H, Muraoka D, Wang LA, Noguchi T, Sato E, et al. Two distinct mechanisms of augmented antitumor activity by modulation of immunostimulatory/ inhibitory signals. *Clin Cancer Res*. (2010) 16:2781–91. doi: 10.1158/1078-0432.CCR-09-3243
121. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med*. (2011) 207:2187–94. doi: 10.1084/jem.20100643

122. Zhou Q, Munger ME, Veenstra RG, Weigel BJ, Hirashima M, Munn DH, et al. Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood* (2011) 117:4501–10. doi: 10.1182/blood-2010-10-310425
123. Kleffel S, Posch C, Barthel SR, Mueller H, Schlapbach C, Guenova E, et al. Melanoma cell-intrinsic PD-1 receptor functions promote tumor growth. *Cell* (2015) 162:1242–56. doi: 10.1016/j.cell.2015.08.052
124. Farsam V, Hassan ZM, Zavaran-Hosseini A, Noori S, Mahdavi M, Ranjbar M. Antitumor and immunomodulatory properties of artemether and its ability to reduce CD4+ CD25+ FoxP3+ T reg cells *in vivo*. *Int Immunopharmacol*. (2011) 11:1802–8. doi: 10.1016/j.intimp.2011.07.008
125. Nedoszytko B, Lange M, Sokołowska-Wojdyło M, Renke J, Trzonkowski P, Sobjanek M, et al. The role of regulatory T cells and genes involved in their differentiation in pathogenesis of selected inflammatory and neoplastic skin diseases. Part I: treg properties and functions. *Postepy Dermatol Alergol*. (2017) 34:285–294. doi: 10.5114/ada.2017.69305
126. Hargadon KM, Johnson CE, Williams CJ. Immune checkpoint blockade therapy for cancer: An overview of FDA-approved immune checkpoint inhibitors. *Int Immunopharmacol*. (2018) 62:29–39. doi: 10.1016/j.intimp.2018.06.001
127. Ligtenberg MA, Pico de Coaña Y, Shmushkovich T, Yoshimoto Y, Truxova I, Yang Y, et al. (2018). Self-delivering RNAi targeting PD-1 improves tumor-specific T cell functionality for adoptive cell therapy of malignant melanoma. *Mol Ther*. (2018) 26:1482–93. doi: 10.1016/j.ymthe.2018.04.015
128. Saito M, Shiraishi K, Goto A, Suzuki H, Kohno T, Kono K. Development of targeted therapy and immunotherapy for treatment of small cell lung cancer. *Jpn J Clin Oncol*. (2018) 48:603–8. doi: 10.1093/jjco/hyy068
129. McKay RR, Bossé D, Xie W, Wankowicz SAM, Flaifel A, Brandao R, et al. The clinical activity of PD-1/PD-L1 inhibitors in metastatic non-clear cell renal cell carcinoma. *Cancer Immunol Res*. (2018) 6:758–65. doi: 10.1158/2326-6066.CIR-17-0475
130. Franchina DG, He F, Brenner D. Survival of the fittest: Cancer challenges T cell metabolism. *Cancer Lett*. (2018) 412:216–223. doi: 10.1016/j.canlet.2017.10.014
131. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-PD-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol*. (2010) 28:3167–75. doi: 10.1200/JCO.2009.26.7609
132. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. *N Engl J Med*. (2012) 366:2443–54. doi: 10.1056/NEJMoa1200690
133. Taube JM. Unleashing the immune system: PD-1 and PD-Ls in the pretreatment tumor microenvironment and correlation with response to PD-1/PD-L1 blockade. *Oncoimmunology* (2014). 3:e963413. doi: 10.4161/21624011.2014.963413
134. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. (2011) 364:2517–26. doi: 10.1056/NEJMoa1104621
135. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med*. (2013) 369:122–33. doi: 10.1056/NEJMoa1302369
136. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, et al. Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. *N Engl J Med*. (2015) 372:2006–17. doi: 10.1056/NEJMoa1414428

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 31 October 2018
doi: 10.3389/fimmu.2018.02504

Protein Tyrosine Phosphatases: Regulators of CD4 T Cells in Inflammatory Bowel Disease

Kelly A. Pike[1,2]* *and Michel L. Tremblay*[1,3,4,5]*

[1] Department of Microbiology and Immunology, McGill University, Montréal, QC, Canada, [2] Inception Sciences Canada, Montréal, QC, Canada, [3] Rosalind and Morris Goodman Cancer Centre, McGill University, Montréal, QC, Canada, [4] Division of Experimental Medicine, Department of Medicine, McGill University, Montréal, QC, Canada, [5] Department of Biochemistry, McGill University, Montréal, QC, Canada

Protein tyrosine phosphatases (PTPs) play a critical role in co-ordinating the signaling networks that maintain lymphocyte homeostasis and direct lymphocyte activation. By dephosphorylating tyrosine residues, PTPs have been shown to modulate enzyme activity and both mediate and disrupt protein-protein interactions. Through these molecular mechanisms, PTPs ultimately impact lymphocyte responses to environmental cues such as inflammatory cytokines and chemokines, as well as antigenic stimulation. Mouse models of acute and chronic intestinal inflammation have been shown to be exacerbated in the absence of PTPs such as PTPN2 and PTPN22. This increase in disease severity is due in part to hyper-activation of lymphocytes in the absence of PTP activity. In accordance, human PTPs have been linked to intestinal inflammation. Genome wide association studies (GWAS) identified several PTPs within risk loci for inflammatory bowel disease (IBD). Therapeutically targeting PTP substrates and their associated signaling pathways, such as those implicated in $CD4^+$ T cell responses, has demonstrated clinical efficacy. The current review focuses on the role of PTPs in controlling $CD4^+$ T cell activity in the intestinal mucosa and how disruption of PTP activity in $CD4^+$ T cells can contribute to intestinal inflammation.

Keywords: protein tyrosine phosphatase, CD4 T cells, cytokine, JAK-STAT, inflammatory bowel disease

OPEN ACCESS

Edited by:
Paul E. Love,
National Institutes of Health (NIH), United States

Reviewed by:
Thomas Ciucci,
National Cancer Institute (NCI), United States
Ulrike Lorenz,
University of Virginia, United States

***Correspondence:**
Kelly A. Pike
kelly-anne.pike@mail.mcgill.ca
Michel L. Tremblay
michel.tremblay@mcgill.ca

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *25 July 2018*
Accepted: *10 October 2018*
Published: *31 October 2018*

Citation:
Pike KA and Tremblay ML (2018) Protein Tyrosine Phosphatases: Regulators of CD4 T Cells in Inflammatory Bowel Disease. Front. Immunol. 9:2504. doi: 10.3389/fimmu.2018.02504

INTRODUCTION

The gastrointestinal track is a large mucosal surface at which the host's immune system is juxtaposed with a dense microbial population and a diverse array of dietary antigens. Immune recognition of enteric antigens however, is minimized by physical compartmentalization. Bacteria and dietary products are retained within the gut lumen, while the host immune system is localized in the mucosal tissue. This physical separation is preserved by the intestinal mucosal barrier, which includes a mucin layer, a single epithelial cell lining sealed by tight and adherens junctions and a continuous secretion of anti-inflammatory soluble mediators (**Figure 1**) (1–5).

The mucosal barrier is not absolute however, and a small number of bacteria do translocate from the lumen to the underlying lamina propria. In such instances, a complex network of innate and adaptive immune cells impedes the spread of intestinal bacteria in a manner that limits tissue damage (6, 7). This cellular network includes dendritic cells, resident macrophages and the largest population of T cells in the body.

Physical compartmentalization and mucosal immunity therefore establish and maintain microbiome-host mutualism (8). The loss of mutualism and a hyper-activation of the innate and adaptive immune system can result in intestinal inflammatory diseases such as Crohn's disease (CD) and ulcerative colitis (UC), the two main forms of inflammatory bowel disease (IBD). UC and CD are polygenic diseases characterized by chronic relapsing inflammation that results in intestinal pain, intestinal bleeding and diarrhea (9–11). While UC is restricted to the large intestine where it manifests as a uniform continuous pattern of inflammation, CD can occur anywhere throughout the gastrointestinal track in patches. The thickness of the inflammation also distinguishes the two diseases, UC being confined to the mucosa and CD presenting in both the mucosa and underlying muscle tissue (transmural).

The mechanisms that initiate and sustain IBD are incompletely understood. Current evidence supports a model in which genetic alterations and environmental factors, increase IBD susceptibility by deregulating the interplay between the microbiome, the intestinal epithelial barrier and the immune system. While the nature of pre-disposing environmental factors remains under debate, functional annotation of IBD-associated genes has identified gene variants that impact processes such as intestinal barrier function, anti-microbial activity, and autophagy (12–14). In addition, the disruption of the adaptive immune system has been implicated, with multiple IBD susceptibility genes being shown to contribute to $CD4^+$ T cell development and function. Examples include *IL23R*, *JAK2,* and *STAT3* (12, 15–21).

$CD4^+$ T Cells and IBD

$CD4^+$ T cells direct suitable immune responses, maintain immune tolerance and support the differentiation of endurable immunological memory. However, $CD4^+$ T cell subsets have also been shown to contribute to chronic intestinal inflammation, accumulating in the mucosa of both UC and CD patients (22). Additional evidence supporting a role for $CD4^+$ T cells in IBD, is based on HIV^+ IBD patients who, with a reduced total CD4 T cell count, have a higher incidence of remission as compared to non-HIV IBD patients (23, 24). Therapeutically, $CD4^+$ T cell-depleting and blocking antibodies (cM-T412, MAX.16H5, and B-F5) have been shown to induce remission in both CD and UC patients (25, 26), while alternate therapies that inhibit the differentiation of $CD4^+$ T cell subsets and the cytokines they secrete, have proven to be efficacious in IBD patients, These would include Tofacitinib (oral JAK inhibitor), Ustekinumab (human monoclonal antibody directed against IL-12 and Il-23) and Infliximab (chimeric hiamn/mouse monoclonal antibody directed against TNFα) (27–33). It should be noted, that such therapies also target other immune cell lineages and as such, efficacy may not be solely driven through a $CD4^+$ T cell specific mechanism.

$CD4^+$ T cells are classified into distinct subsets based on their inducing cytokines, transcription factor expression, and effector cytokine secretion. The initial classification of $CD4^+$ T cells as T_H1 IFNγ producers vs. T_H2 IL-4 producers, has been broadened to include multiple additional subsets (34, 35). These subsets, and the cytokines they secrete, include T_H9 (IL-9), T_H17 (IL-17A, IL-17F, and IL-22), T_H22 (IL-22), T follicular helper T_{FH} (IL-21) cells, as well as thymic-derived and peripherally-induced T regulatory cells (IL-10, TGFβ) (36–40) (**Figure 1**).

The contribution of the various $CD4^+$ T cell subsets to CD and UC remains an area of ongoing research. Originally, CD was thought to be driven by T_H1 T cells and UC by T_H2 T cells. The use of such a T_H1/T_H2 paradigm to describe the different T cell responses involved in CD and UC has proven over simplistic however. It did not account for the role of more recently identified subsets such as T_H17 T cells and Tregs. Moreover, the recent discovery of ongoing T cell plasticity in the intestinal mucosa of both CD and UC patients, has added further complexity to the $CD4^+$ T cell response in these diseases (41, 42).

Protein Phosphorylation and $CD4^+$ T Cell Differentiation

Protein tyrosine phosphorylation is required for $CD4^+$ T cell differentiation and activation. Cascades of reversible protein phosphorylation events downstream of cytokine receptors (CytR), co-stimulatory molecules, and the T cell receptor (TCR), converge to induce gene expression profiles that drive $CD4^+$ T cell activation and differentiation into distinct subsets (40).

Naive T cells in peripheral circulation are activated upon TCR recognition of its cognate antigen in the context of major histocompatibility complex (MHC) expressed on antigen presenting cells. Upon TCR engagement, Src-family kinases (Lck, Fyn) are activated and phosphorylate tyrosine residues within the immune-receptor tyrosine-based activation motifs (ITAMs) in the TCR-associated CD3 and zeta chains (43–46). Phosphorylated ITAMs then provide docking sites for the recruitment and activation of the zeta-associated protein kinase (ZAP-70) (47). Cooperatively, Src-family kinases and Zap70 phosphorylate downstream signaling pathways which dictate the cellular response (**Figure 2**).

The strength of TCR signaling has a direct impact on $CD4^+$ T cell differentiation (48). For example, $Foxp3^+$ peripheral T regulatory (Treg) cells are generated primarily from $CD4^+$ $Foxp3^-$ T cells exposed to antigen under tolerogenic conditions or during homeostatic proliferation (49–52). In the presence of TGFβ, IL-2 and co-stimulatory signaling, intermediate TCR signaling induces Foxp3 expression and Treg differentiation (iTreg). By comparison, weak and strong TCR signaling are less potent at inducing Foxp3 expression (53, 54). Regulatory mechanisms are therefore required to establish and maintain the strength of TCR signaling within a given range. Such regulatory mechanisms include the modulation of protein tyrosine phosphorylation.

Critically, engagement of the TCR and co-stimulatory molecules is not sufficient to drive the polarization of $CD4^+$ T cells (40). Rather, the presence of inductive cytokines and the activation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway is also required. Binding of cytokines to their corresponding receptor results in receptor dimerization, allowing for the juxtaposition and subsequent cross-phosphorylation of associated JAK

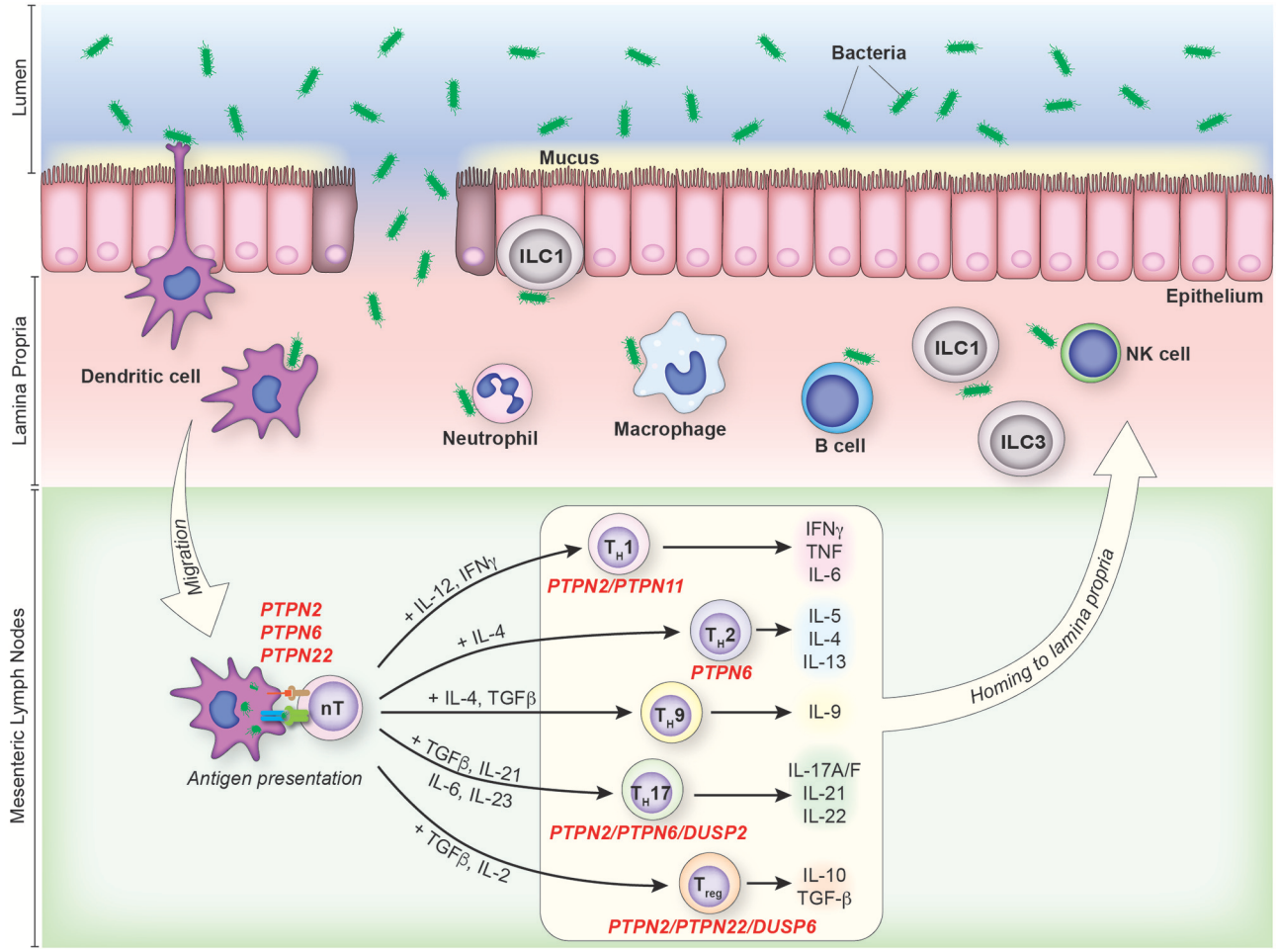


FIGURE 1 | The contribution of PTPs to CD4$^+$ T cell activation and differentiation in colonic inflammation. IBD pathophysiology is associated with the disruption of the intestinal mucosal barrier due to genetic, environmental and/or immunological factors. Under such circumstances, an increase in the uptake and processing of luminal antigens by innate immune cells initiates and maintains the chronic inflammatory response characteristic of IBD. CD4$^+$ T cells are activated in the mesenteric lymph nodes following recognition of their cognate antigen presented in the context of MHC on the surface of antigen presenting cells. Activated CD4$^+$ T cells then enter the lamina propria from circulation and perpetuate inflammation, secreting pathogenic pro-inflammatory mediators and chemokines which recruit additional leukocytes. PTPs involved in the activation and/or differentiation of specific T cell subsets are indicated.

molecules (55). Activated JAK molecules are then responsible for the phosphorylation of STAT family members. Tyrosine phosphorylated STAT molecules dimerize and translocate to the nucleus, where they promote specific gene expression profiles (**Figure 2**) (56–62).

Thus a network of signaling pathways, heavily dependent on tyrosine phosphorylation, directs CD4$^+$ T cell activation and differentiation. The current review will examine the role of protein tyrosine phosphatases (PTP) in safeguarding this network, and how PTP deletion can perturb CD4$^+$ T cell function and consequently contribute to intestinal inflammation.

The Protein Tyrosine Phosphatase Family

The PTP family comprises a heterogeneous set of enzymes that were first defined by Tonks and colleagues by their capacity to dephosphorylate phospho-tyrosine residues and by their structurally related phosphatase catalytic domain (63, 64). PTP1B was the first phosphatase identified. Its sequence homology with a segment of the CD45 receptor protein (65), pointed to the existence of a conserved catalytic domain that became the main feature of the PTP gene family. CD45 also brought an interesting first link between PTPs and the immune system.

The human PTP family members are divided into distinct classes based on their structural and biochemical properties (**Figure 3**). The majority of PTPs have a conserved catalytic domain that contains a cysteine which executes a nucleophilic attack on substrate residues. There are 3 classes of such Cys-based PTPs. Class I is comprised of both classical PTPs that target phosphorylated tyrosine residues, and dual-specific phosphatases (DUSP) that target phosphorylated tyrosine, serine and threonine residues. Class II includes two PTPs, namely low molecular weight PTP and SSU72, whereas

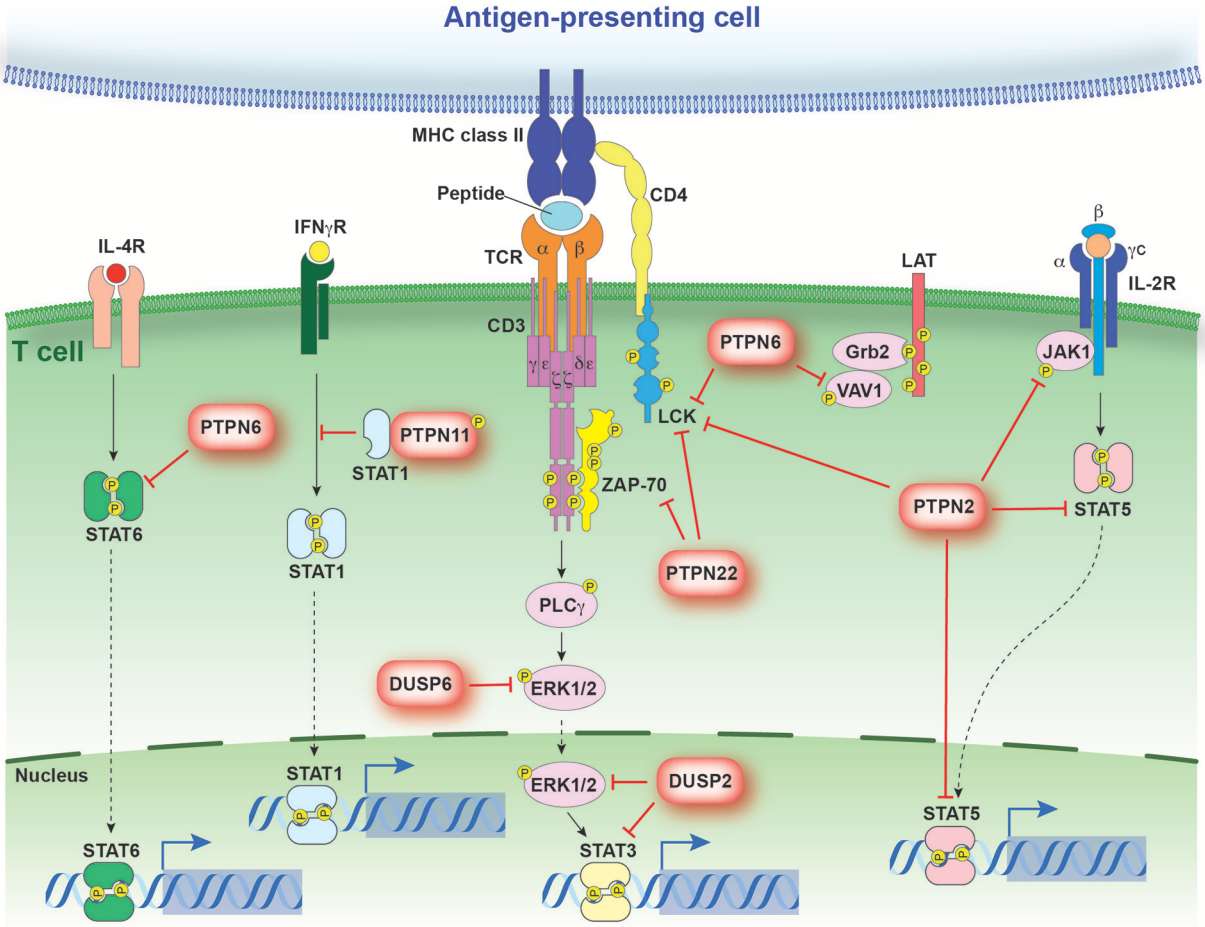


FIGURE 2 | PTP regulation of antigen and cytokine receptor signaling. Schematic representation of signaling events regulated by PTPs discussed in the text. PTPs are linked to their respective substrates by a red bar-headed line. Dotted arrows depict translocation while solid black lines identify molecules linked in a signaling cascade. The direct interaction between STAT1 and PTPN11 models the sequestration of STAT1 from the IFNγR.

Class III comprises the three cell cycle Cdc25 regulatory proteins.

More recent studies characterized enzymes using alternate nucleophilic catalytic residues which were then added to the list of PTP family members. These two additional classes are the aspartic-based and histidine-based acid phosphatase enzymes. Thus, within this classification frame, Alonso and Pulido expanded the PTP gene superfamily to 125 members (66).

Tyrosine phosphorylation is a reversible mechanism that promotes protein–protein interactions and propagates signaling cascades in all cell types. It therefore stands to reason that the PTPs targeting these phosphorylated amino acids play important roles in modulating the strength and duration with which a signaling pathway is activated. Moreover, given that PTPs are a heterogeneous gene family (expression, function, and regulation) it is not surprising that PTPs have been associated with many distinct pathologies in both animal models and in human disease (67). Of importance herein, these include multiple immune-related disorders.

The immune system relies heavily on the use of protein phosphorylation to recognize and transmit intra- and extracellular signals. Signaling cascades initiated at the cell surface by receptor kinases, or from the recruitment of cytoplasmic kinases such as the JAK and Src-like kinase families, translate into multiple immune cell responses like cellular adhesion, division, migration and others. Hence, from the list of over 100 classical Cys-based PTPs, Arimura and Yagi demonstrated that between 58 and 76 of them are expressed in various cell lineages of the immune system (68) and that the vast majority of those (40 to 50) are present in T-cells (69). In T cells, examples of PTP activity mediating both positive and negative regulation of intracellular signaling events have been reported (69).

Multiple genome wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) linked with IBD, in or near PTP coding units (70, 71). Examples include the PTPN22, PTPN2, PTPRC, PTPRK, and MTMR3 loci (72). Murine pre-clinical colitis models and tissue-specific PTP

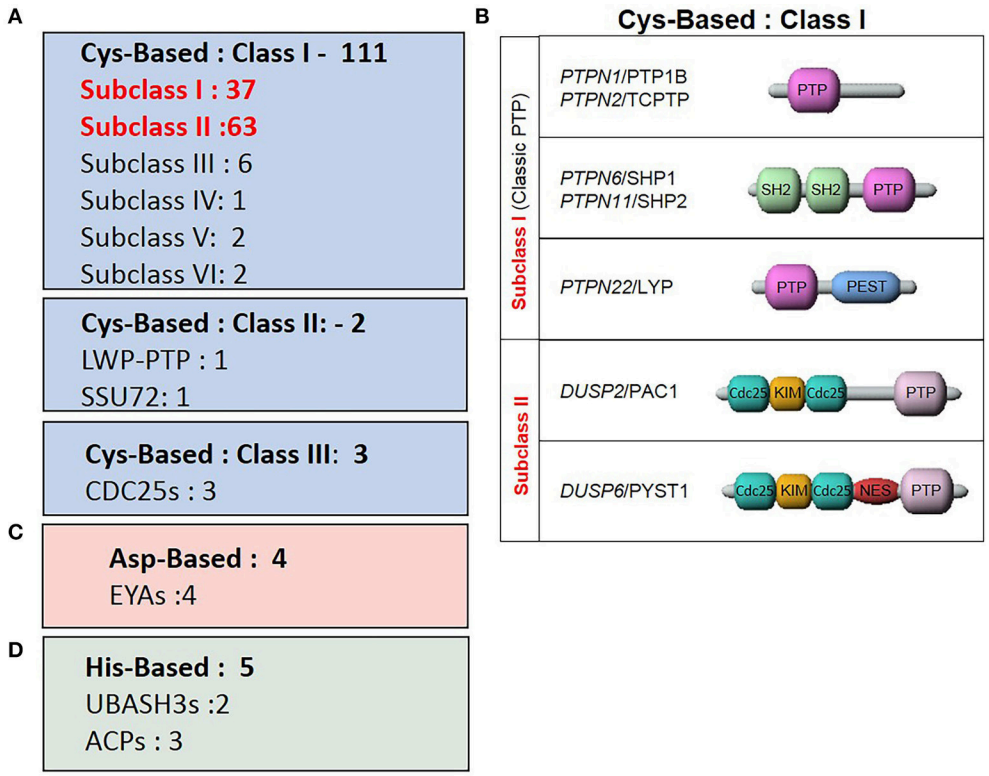


FIGURE 3 | Classes of the tyrosine phosphatase family. **(A)** Sub-classes of Cys-based PTPs and the number of associated genes. **(B)** Representative PTPs from Class I Cys-based enzymes described in the review. **(C)** ASP-based PTPs. **(D)** His-based PTPs.

knockout mouse strains have been used to discern the role of such PTPs in T cells in the inflamed intestine. Such studies, demonstrated that the perturbation of PTPs such as PTP22, PTPN2, PTPN11, DUSP2, and DUSP6 impacts IBD relevant T cell subsets and/or deregulates T-cell function (73–77). Yet only PTPN22 and PTPN2 have been clearly validated in both patients and animal models for their involvement in IBD.

PTP REGULATION OF CD4$^+$ T CELL ACTIVITY IN IBD

PTPN22

PTPN22 is a non-receptor protein tyrosine phosphatase that is expressed primarily in hematopoietic cells (78). It has been linked with multiple inflammatory disorders and in fact, PTPN22 genetic variation is among the strongest genetic risk factors for autoimmunity diseases such as type I diabetes (T1D), rheumatoid arthritis and systemic lupus erythematosus (79–83). One of the most extensively studied SNPs within the PTPN22 locus is SNP rs33996649 (G788>C) which causes a substitution of arginine 263 with a glutamine residue (263Q variant). While this variant increases susceptibility to multiple autoimmune diseases, it is in fact protective against CD (12, 79). The mechanism mediating this differential effect on disease susceptibility remains unclear. It has been hypothesized that it is due to the different role innate vs. adaptive immune cells play in the onset of these distinct inflammatory diseases (77). Further studies examining the cell type specific effects of the G788>C variant will provide clarity, although the continued debate regarding whether the variant encodes a gain-of-function or altered-function protein will need to be resolved (84–87).

PTPN22 deficient mice exhibit a lymphoproliferative disease and accumulate memory-phenotype T cells with age, but do not display signs of spontaneous autoimmunity (88). It is important to note that a PTPN22 deficiency can cooperate with the E613R CD45 mutant to induce autoimmune diseases. This suggests that the loss of PTPN22 can play a role in increasing susceptibility to certain inflammatory disorders (89).

A comprehensive examination of the different T cell compartments in *PTPN22*$^{-/-}$ mice provided an example of how cellular context can change the importance of the regulatory function of a given PTP. During the first 2 days following TCR activation, *PTPN22*$^{+/+}$ and *PTPN22*$^{-/-}$ T cells display comparable proliferation, cytokine production and expression of activation markers. Beyond 2 days however, *PTPN22*$^{-/-}$ T cells exhibit an increased rate of cell cycling. In addition, reactivated knockout effector T cells undergo a more rapid and robust proliferation and secrete higher levels of cytokines compared to controls. Likewise *ex vivo* stimulation of CD44^{HI} CD62L^{LO} *PTPN22*$^{-/-}$ effector/memory cells display an increased proliferative response upon stimulation. This has been attributed to enhanced and prolonged phosphorylation of the Lck

auto-regulatory (Tyr394) tyrosine following TCR ligation and not due to changes to IL-2 or IL-15 receptor signaling (88).

PTP localization also contributes to the regulation of PTP activity. In addition to Lck, Zap70 has been shown to be a substrate of PTPN22 and both Csk and Vav have been shown to be interacting partners. Csk plays a critical role in sequestering PTPN22 from the TCR complex, thereby limiting the capacity of PTPN22 to inhibit TCR signaling (90–93). The kinetics and localization of PTPN22 activity, and its binding partners in antigen experienced vs. inexperienced T cells, remains unclear. Such an understanding would provide insight into why PTPN22 is particularly important beyond the initial stage of T cell activation.

Murine colitis models indicate that PTPN22 does not solely play a role in the function of conventional T cells. Using the T cell transfer colitis model, PTPN22 was shown to contribute to both effector and regulatory T cells in colitis. The transfer of *PTPN22*$^{-/-}$ naïve CD4^{+}CD45RB^{HI} T cells into an immune-deficient recipient resulted in a more severe colitis phenotype, characterized by rapid weight loss and decreased survival. Strikingly, while co-transfer of WT Tregs with *PTPN22*$^{-/-}$ naïve CD4^{+}CD45RB^{HI} T cells did not suppress disease, co-transfer of *PTPN22*$^{-/-}$ Tregs did. Protection was attributed to the increased secretion of anti-inflammatory cytokines by *PTPN22*$^{-/-}$ Tregs (76). It then follows that the lack of spontaneous autoimmunity in the *PTPN22*$^{-/-}$ mice may be due to a coincident increase in both effector and regulatory T cell activity.

Further investigation is required to determine if human PTPN22 variants contribute to IBD pathology through their effects on T cell homeostasis in the gut. Although two independent studies confirmed the role of PTPN22 in acute colitis using the dextran-sodium sulfate (DSS) induced model, these studies attributed the increase in disease severity to changes in TLR4 signaling, type I interferon production and macrophage polarization (94, 95).

PTPN2

PTPN2 is a classical cytoplasmic phosphatase expressed ubiquitously, with the highest levels of expression being detected in lymphoid cells. Its critical role in the homeostasis of the immune system is evident by the progressive systemic inflammatory disease that develops in PTPN2 deficient mice within 1–2 weeks of birth (96). Pro-inflammatory mediators IFNγ, TNFα, and inducible nitric oxide synthase (iNOS) are readily detectable as early as 3 days after birth, followed by the infiltration of mononuclear cells into lymphoid and non-lymphoid organs (97). In comparison, T-cell specific PTPN2 deficient mice do not develop systemic inflammation but rather develop spontaneous autoimmunity at older ages. A reduction in the TCR threshold in both *PTPN2*$^{-/-}$ CD4 and CD8 was identified as causing increased T cell activation due to hyper-phosphorylation of the activating tyrosine residue of Lck (98). It was then proposed that through its negative regulation of TCR signaling, PTPN2 contributes to the maintenance of immune homeostasis.

The loss of PTPN2 has been shown to alter CD4^{+} T cell differentiation, impacting disease severity in multiple murine colitis models. In the T cell transfer colitis model, the transfer of naïve *PTPN2*$^{-/-}$ CD4^{+} T cells resulted in an earlier onset of disease, as well as an increased in weight loss, spleen weight and macroscopic signs of colitis. These clinical signs correlated with more pronounced intestinal inflammation as detected by histological scoring of immune infiltration and epithelial damage. The more pronounced disease severity was confirmed in both acute and chronic DSS colitis models, which both demonstrated a more dramatic weight loss and colonic shortening (99).

Mechanistically, an increase in STAT1 and STAT3 phosphorylation was detected in whole colon lysates. An increase in STAT1/3 phosphorylation was also detected in *PTPN2*$^{-/-}$ CD4^{+} T cells cultured under polarization conditions. The effects of PTPN2 loss on T_H differentiation *in vivo* were also examined. In the T cell transfer colitis model, the introduction of PTPN2 deficient CD4^{+} T cells resulted in an almost 3-fold increase in the frequency of IFNγ producers and a 2-fold increase in the frequency of IFNγ^{+} IL17^{+} double producers. In contrast, the frequency of Tregs was reduced over 3-fold. These findings were also recapitulated in the DSS model, although with a less pronounced effect that most likely reflected the fact that pathogenic T cells are not induced robustly in this model (99). One point for further investigation is whether the observed increase in STAT1/3 phosphorylation alters the propensity to commit to a given subset, or if it heightens proliferation rates following commitment.

Importantly, the consequences of PTPN2 loss in murine CD4^{+} T cell differentiation are in line with the sequencing of human PTPN2 variants that have been to be associated with CD. Specifically, transcriptional profiling of CD patients expressing the PTPN2 loss-of-function variant (rs1893217) presented higher expression of T_H1 and T_H17-related transcription factors and cytokines (serum and intestinal biopsies) in comparison to PTPN2 expressing CD patients. Such human studies solidify the important role of PTPN2 CD4^{+} T cell responses in the context of human IBD (99).

PTPN6

The Src homology region 2 domain-containing tyrosine phosphatase-1 (SHP-1, *PTPN6*) is a cytoplasmic phosphatase expressed in all hematopoietic cell lineages throughout development and activation (100–103). Functionally, PTPN6 has been shown to negatively regulate signaling downstream of multiple receptors which co-ordinate immune cell homeostasis and function. These include antigen, cytokine, chemokine and integrin receptors (104–115). As such, it is not surprising that systemic inflammation is the dominant phenotype of the motheaten (*me*) and motheaten viable (*me*^{v}) mice. These mice harbor mutations that result in undetectable and reduced PTPN6 expression respectively. Similar to PTPN2 deficient mice, *me/me* mice succumb to disease 2–3 weeks following birth (116, 117).

Due to the complex inflammatory pathology of the *me/me* mice, it has proven difficult to dissect the T cell intrinsic effects of a *PTPN6* deficiency. Extensive characterization of T cells derived from *me/me* mice suggests PTPN6 plays a role in regulating the threshold for TCR activation in thymic and peripheral T cells. *Ex vivo* stimulation of *me/me*-derived T cells, as well as

T cells expressing a PTPN6 dominant-negative allele, results in a hyper-proliferative response at low antigen concentrations (104, 118–121). Mechanistically, PTPN6 can dephosphorylate multiple proteins downstream of the TCR complex (104, 122–125). Whether one substrate is the physiological substrate, critical to setting the threshold of TCR activation remains a matter of debate.

In vivo, the generation of T cell specific PTPN6 deficient mice by different groups produced conflicting findings regarding the role of PTPN6 in T cell polarization. Johnson et al. reported that the loss of PTPN6 does not alter thymocyte or peripheral T cell sensitivity to TCR activation. Rather, the authors note a higher frequency of memory phenotype T cells in the peripheral T cell pool. Given that memory-phenotype T cells respond more robustly upon TCR activation, a hyper-proliferative response is observed when a heterogenous pool of peripheral T cells is stimulated *ex vivo*. The same study demonstrated that a PTPN6 deficiency causes a skewing toward the T_H2 lineage, associated with sustained IL-4-STAT6 signaling (126). In direct contrast, Martinez et al., also using a T cell specific conditional knockout mouse, showed that PTPN6 depletion lowers the threshold of TCR activation and causes an increase in thymic negative selection and impairs the T cell repertoire (127). Most recently, it has been reported that in an alternate conditional knockout model, in which PTPN6 is deleted in post-selection thymocytes, $CD4^+$ T cells are hyper-responsive to TCR stimulation and are intrinsically more resistant to Treg suppression (128).

An understanding of the link between PTPN6 and IBD still needs to be established. For example, a characterization of colitis induction and progression in a PTPN6 T cell conditional knockout mouse has not been published. It remains unknown therefore, whether PTPN6 plays a role in $CD4^+$ T cell biology in the inflamed gut. Although limited in scope, two published reports suggest a link between PTPN6 in human colitis though. The levels of PTPN6 were quantified in 98 colonic biopsies, and found to be significantly reduced in active UC, quiescent UC and active CD when compared to healthy controls (129). In a separate study, 2 PTPN6 SNPs (rs7310161 and rs759052) were genotyped in 107 IBD patients and 162 healthy controls from Southern Tunisia (130). A weak association with UC was identified which requires confirmation in a larger cohort. Noteworthy, such studies do not demonstrate that it is the deficiency of PTPN6 in $CD4^+$ T cells that is implicated in the disease pathology.

PTPN11

PTPN11 encodes the Src homology 2-containing protein tyrosine phosphatase 2 (SHP-2). Similar to PTPN6, PTPN11 regulates signaling downstream of multiple surface receptors including growth factor receptors, cytokine receptors and integrins. Its broad tissue expression is in line with the embryonic lethality of PTPN11 knockout mouse at mid-gestation in (131–133).

Extensive human genetic data indicates that PTPN11 has an important role in human disease. Specifically, somatic *PTPN11* mutations in patients with multiple cancer types including leukemias, breast cancer and gastric cancer (134) have been genotyped. As well, in a cohort of 114 Japanese patients, a genetic association has been made between a SNP within the PTPN11 locus and UC (135).

Surprisingly, characterization of T cells from multiple PTPN11 dominant negative knock-in mice and PTPN11 knockout mice, has not lead to a conclusive understanding of this PTP's role in T cell biology. Examples of both deficient- and hyper-T cell activation can be found in these studies. This has been in part attributed to the scaffolding properties of PTPN11 that are retained in the catalytically dead knock-in mouse (73, 136–141). As an example of the scaffolding properties of PTPN11, it has been shown to directly interact with STAT1 and retain STAT1 in the cytoplasm thereby impeding its recruitment to the IFNγ receptor. This disruption of IFNγ signaling, was sufficient to inhibit the production of T_H1 cytokines and improve 2, 4, 6-trinitrobenzene sulfonic acid induced colitis (142).

By comparison, a PTPN11 conditional T-cell specific knockout mouse has been reported to exhibit increased susceptibility to DSS induced acute colitis. Phenotypic analysis of the T cell compartments in the PTPN11 T cell deficient mice, did not identify any change in the frequency of peripheral T cells as compared to control mice. However, the severity of DSS-induced colitis was found to be much more pronounced in the deficient mice. This increase in severity manifested as a higher body weight loss, disease activity index and colon shortening. It was also reported that an increase in the infiltration of immune cells was observed. Cytokine profiling identified an increase in pro-inflammatory cytokines including IFN-γ, IL-17A, TNF-α, and IL-6 in the mucosa, which correlated with an increased number of T_H1 and T_H17 cells in the spleen and lamina propria of T cell specific PTPN11 deficient mice (73).The increases risk of development cancer in UC and CD patients in consistent with the role chronic inflammation plays in tumor initiation and progression. It was therefore surprising that despite the increased inflammation observed in mice harboring a PTPN11 deficiency in $CD4^+$ T cells, mice were protected against colitis associated cancer. Specifically, PTPN11 $CD4^+$ T cell deficient mice display enhanced T_H1 immunity and aggravated colitis, but the deficiency inhibited the development of AOM-DSS colitis-associated carcinoma. Mice contained fewer and smaller tumors which expressed reduced levels of proliferation markers. In direct contrast, the progression and metastasis of melanoma was accelerated in the same mouse model. This apparent contradiction suggests that the effects of PTP loss in T cells on tumor progression are highly dependent on the location and stage of the tumor. It must be noted however, that an understanding of how the modulation of PTPs such as SHP-2, impacts the interface between $CD4^+$ T cells and tumors, remains largely unexplored (73).

It is important to note that PTPN11's role in maintaining intestinal homeostasis is not restricted to $CD4^+$ T cells. In fact, an intestinal epithelial specific PTPN11 knockout mouse has been generated and develops severe colitis. Such mice have a severe defect in the intestinal barrier which is associated with impaired tight junction formation, goblet cell differentiation and IEC migration (143, 144).

DUSP2

DUSP2 is a mitogen- and stress-inducible nuclear DUSP that is enriched in lymphoid tissue and differentially expressed in $CD4^+$ T cell subsets. Possibly due to compensation by other DUSP family members, DUSP2 deficient mice are viable and exhibit no gross defects and no alterations in the frequency or absolute number of lymphoid populations in the thymus, spleen or lymph nodes. However, using *in vitro* models of T cell polarization, it was demonstrated that in the absence of DUSP2, T_H17 cell differentiation is promoted while inducible Treg (iTreg) differentiation is suppressed (74).

To question whether such modulation of *in vitro* T_H differentiation relates to effects observed *in vivo,* two mouse models of intestinal inflammation were used. First, in acute and chronic DSS colitis models, *Dusp2*$^{-/-}$ mice exhibited a more severe disease as assessed by weight loss, clinical scoring, survival and histopathology scoring. Higher colonic inflammation was attributed to an increased proportion of colonic Il-17A$^+$ $CD4^+$ T cells in *Dusp2*$^{-/-}$ mice as compared to control mice, whereas T_H1 and Treg populations appeared to be unaltered in DSS treated mice. Second, a T cell transfer colitis model has confirmed the T cell intrinsic effects of a DUSP2 deficiency. To do so, severe combined immunodeficiency (SCID) mice were reconstituted with either WT or *DUSP2*$^{-/-}$ $CD4^+CD45RB^{hi}$ T cells. In this model, an increase in body weight loss, severity of histopathology scoring, and colon thickening were observed following the transfer of *DUSP2*$^{-/-}$ T cells compared to controls. Such clinical signs were corroborated by a rise in the number of colon infiltrating $CD4^+$ T cells expressing a T_H17 gene signature. Moreover, such T_H17 T cells in the inflamed tissue expressed elevated levels of pathogenic T_H17-associated genes but not non-pathogenic genes (74).

Given evidence that Treg differentiation is suppressed in *in vitro* differentiation assays, the ability of *DUSP2*$^{-/-}$ Tregs to suppress colitis in the T cell transfer model was evaluated. Surprisingly, DUSP2 was found to be dispensable, as *DUSP2*$^{-/-}$ Tregs were as efficient as WT Tregs in suppressing inflammation when co-transferred with WT $CD4^+CD45RB^{hi}$ cells into SCID mice (74).

To identify the molecular mechanism by which DUSP2 limits T_H17 differentiation, phosphoproteomic analysis was performed on colon homogenates from DSS treated WT and *DUSP2*$^{-/-}$ mice. Along with follow-up biochemical studies, STAT3 was identified as a direct substrate of DUSP2. DUSP2 was shown to dephosphorylate residues Tyr705 and Ser727. Although comparable levels of basal colonic STAT3 phosphorylation were reported between WT and *DUSP2*$^{-/-}$ mice, the levels were higher in *DUSP2*$^{-/-}$ DSS-challenged mice compared to WT DSS-challenged mice (74). STAT3 is critical in driving disease in T-cell transfer models of colitis (145), the authors propose that the *DUSP2*$^{-/-}$ phenotype is due to elevated STAT3 activity. It should be noted however, that the authors have not formally proven that the phenotype is dependent on STAT3 activity.

DUSP2 has also been implicated in human IBD. The expression of DUPS2 mRNA is reduced in the peripheral blood of UC patients ($n = 24$) and is only increased minimally following PMA stimulation. The low levels of DUPS2 expression were found to be attributed to CpG methylation. Unfortunately, the levels of DUSP2 in colonic biopsies, or colonic T cells, were not quantified. As such, further evidence is required to definitely demonstrate that a reduction in T cell DUSP2 occurs in IBD patients and that this reduction impacts human T cell polarization in the disease state (74).

Based on these findings it has been proposed that under healthy conditions, T cell activation results in a transient increase in DUSP2 which reduces STAT3 activation below levels required to promote T_H17 differentiation. In the absence of DUSP2, elevated levels of STAT3 phosphorylation result in the differentiation of pathogenic T_H17 cells which promote inflammation (74).

DUSP6

Extracellular signal-regulated kinase (ERK) signaling regulates multiple cellular responses to activation including T cell proliferation, cytokine production, survival and adhesion. $CD4^+$ T cell polarization *in vivo* has also been shown to be modulated by ERK signaling, numerous reports suggesting ERK activity impacts T_H1 and T_H2 differentiation and the T_H17:Treg balance. (146–153).

DUSP6 is a cytoplasmic ERK-specific DUSP (154, 155). Surprisingly, genetic ablation of DUSP6 does not cause developmental defects despite an increase in the basal levels of ERK phosphorylation in the heart, spleen, kidney, and brain. Rather, DUSP6 deficient mice are viable with no reported phenotype in the steady state (156). Initial cellular *in vitro* assays suggested a role for DUSP6 in the regulation of $CD4^+$ T cell activation. Specifically, DUSP6 was shown to be upregulated following TLR4 stimulation and then restrained ERK activation and suppressed IFNγ production by TCR stimulation (157).

More recently, a more in depth characterization of DUSP6 in $CD4^+$ T cells has been reported (75). *DUSP6*$^{-/-}$ mice exhibited no change in the frequency or absolute number of peripheral $CD4^+$ or $CD8^+$ T cells. Nevertheless, a higher percentage of memory-effector $CD4^+$ T cells and a lower percentage of naïve $CD4^+$ T cells in *DUSP6*$^{-/-}$ mice, indicates an increase in $CD4^+$ T cell activation. *Ex vivo* TCR-stimulated of *DUSP6*$^{-/-}$ $CD4^+$ T cells display increased ERK activation and proliferation but also an elevated rate of activation induced cell death. Polarization studies demonstrated that DUSP6 depletion promotes T_H1 differentiation and increases IFNγ production, whereas expression levels of IL-2, IL-4, IL-6, or IL-10 are not altered. In contrast T_H17 $CD4^+$ T cells displayed decreased survival and IL-17A secretion, leading to the conclusion that DUSP6 suppresses the T_H1 lineage and promotes T_H17 differentiation (75).

The role of DUSP6 in intestinal inflammation was addressed using an *IL-10*$^{-/-}$ colitis model. *IL-10*$^{-/-}$*DUSP6*$^{-/-}$ double knockout mice were generated and phenotyped. Such mice had accelerated and exacerbated colitis. Histological examination identified elevated epithelial crypt hyperplasia, goblet-cell depletion, and infiltration of mononuclear cells. Moreover, colonic explants were found to secrete higher levels of TNFα and IFNγ, while IL-17A levels

were reduced. Strikingly, ERK inhibition was shown to significantly reduce colitis in *IL-10*$^{-/-}$*DUSP6*$^{-/-}$ mice, both prophylactically and therapeutically. The severity of both crypt hyperplasia and immune cell infiltration was reduced. Despite, these intriguing findings, future studies are needed to demonstrate that the protective role of DUSP6 in colitis is intrinsic to CD4^{+} T cells (75).

PTPs AS THERAPEUTIC TARGETS FOR IBD?

In the late 1990s, multiple research programs sought to identify the PTP(s) that recognize and dephosphorylate the insulin receptor (IR). The regulation of IR activation by a PTP was hypothesized to decrease IR mediated signaling events, and subsequent entry of glucose into insulin receptive tissues. It was believed that a rise in the expression or activity of such a regulatory PTP would result in high levels of blood glucose in the circulation, which would then instigate type II diabetes. This hypothesis was confirmed by two seminal publications which described the PTP1B knockout mouse that presented with an increase in IR phosphorylation. These studies validated PTP1B as an outstanding candidate to be targeted for the treatment of type II diabetes (158, 159) and led to major efforts to identify small inhibitors of PTP1B. Indeed, since their publications these reports were cited over 2500 times, primarily in the context of depicting the isolation of novel PTP1B inhibitors.

In spite of all these reports, there are no small molecule inhibitors against PTP1B (or any PTP) that have been successfully developed beyond initial clinical trials. Multiple reviews have already examined the difficulties associated with developing PTP competitive inhibitors and this subject is beyond the scope of this review (160–163). In brief, the main challenges in developing inhibitors are the high polarity and homology of their catalytic pockets. Hence, chemical screens for competitive small molecule PTP inhibitors, have only isolated inhibitors with poor cell permeability and low specificity. Excitement has been garnered by the recent development of allosteric PTP inhibitors such as the PTP1B carboxyl domain compound (164) and a PTPN11 inhibitor from Novartis (165) which may lead to the development of novel pharmaceuticals.

It is worthy to note that novel therapeutic strategies are also being developed to target PTPs. These include the forced dimerization and inhibition of receptors PTPs (166, 167), intrabodies inhibitors (168), small molecular caged compound inhibitors (169) and even RNA aptamers (170) that modulate the enzymatic activity of PTPs. As well, genetic modulation through CRISPR technologies (171), as well as protein degradation technologies (PDTs) such as PROTACs (PROteolysis TArgeting Chimeras) (172) and SNIPERS (Specific and Non-genetic IAP-dependent Protein Erasers) (173) are now exciting avenues for tackling the difficult PTP gene family.

Significant effort is also being made in the development of cell based therapies. Potential adverse effects that could be associated with systemically inhibiting PTPs are mitigated by using PTP inhibitors in *ex-vivo* cell cultures. This allows for the modulation of PTP activity in a cell-type specific and temporary fashion. For example, we reported a protocol for dendritic cell (DC) vaccination that employs inhibitors of PTPN1-PTPN2 (174). This work demonstrated that in the proper context, PTP inhibitors may have broad application in cancer and infectious diseases. Beyond DCs, other cell types such as macrophages, natural killer cells, and of course T-cells as described above, may also be malleable by PTP inhibition and useful in various cell therapies.

From an IBD perspective, it is clear that inhibiting the PTPs presented above would most likely exacerbate disease by potentiating the effects of pro-inflammatory cytokines and promoting the differentiation of pathogenic T cells. Indeed this contention is supported by the fact that, biologics and small molecules that suppress cytokine receptor signaling have been clinically successful in IBD disease management. One example would be tofacitinib, a JAK inhibitor found to be effective in phase 2 and 3 trials in moderate to severe ulcerative colitis (175).

Our understanding of PTP function in immune cells is expanding beyond cytokine receptor signaling, and our capacity to modulate cellular responses by titrating PTP expression is also evolving at a rapid pace. The cell-dependent context of positive or/and negative modulation bestowed by the nearly 80 PTPs expressed in immune cells, remains an exciting ground for study and clinical improvement. It remains to be seen if this renewed interest in PTP inhibitors would be applicable to inflammatory diseases such as UC and CD.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by an operating grant from the Canadian Institutes of Health Research (CIHR MOP 142497) and by support to MT from the Strauss -Aclon Foundation.

ACKNOWLEDGMENTS

We are thankful for Dr. Noriko Uetani for the art work. MT is a Jeanne and Jean-Louis Lévesque chair in Cancer Research.

REFERENCES

1. Farquhar MG, Palade GE. Junctional complexes in various epithelia. *J Cell Biol.* (1963) 17:375–412. doi: 10.1083/jcb.17.2.375

2. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci USA.* (2008) 105:15064–9. doi: 10.1073/pnas.0803124105

3. Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, et al. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med.* 5:e54. doi: 10.1371/journal.pmed.0050054
4. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol.* (2000) 1:113–8. doi: 10.1038/77783
5. Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, et al. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science* (2011) 334:255–8. doi: 10.1126/science.1209791
6. Slack E, Hapfelmeier S, Stecher B, Velykoredko Y, Stoel M, Lawson M, et al. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science* (2009) 325:617–20. doi: 10.1126/science.1172747
7. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* (2004) 303:1662–5. doi: 10.1126/science.1091334
8. Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. *Science* (2001) 292:1115–8. doi: 10.1126/science.1058709
9. Abraham C, Cho JH. Inflammatory bowel disease. *N Engl J Med.* (2009) 361:2066–78. doi: 10.1056/NEJMra0804647
10. Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* (2007) 369:1641–57. doi: 10.1016/S0140-6736(07)60751-X
11. Bernstein CN, Fried M, Krabshuis JH, Cohen H, Eliakim R, Fedail S, et al. World Gastroenterology Organization Practice Guidelines for the diagnosis and management of IBD in 2010. *Inflamm Bowel Dis.* (2010) 16:112–24. doi: 10.1002/ibd.21048
12. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet.* (2008) 40:955–62. doi: 10.1038/ng.175
13. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui K, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* (2012) 491:119–24. doi: 10.1038/nature11582
14. Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet.* (2015) 47:979–86. doi: 10.1038/ng.3359
15. Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly M, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* (2006) 314:1461–3. doi: 10.1126/science.1135245
16. Franke A, Balschun T, Karlsen TH, Hedderich J, May S, Lu T, et al. Replication of signals from recent studies of Crohn's disease identifies previously unknown disease loci for ulcerative colitis. *Nat Genet.* (2008) 40:713–5. doi: 10.1038/ng.148
17. Sato K, Shiota M, Fukuda S, Iwamoto E, Machida H, Inamine T, et al. Strong evidence of a combination polymorphism of the tyrosine kinase 2 gene and the signal transducer and activator of transcription 3 gene as a DNA-based biomarker for susceptibility to Crohn's disease in the Japanese population. *J Clin Immunol.* (2009) 29:815–25. doi: 10.1007/s10875-009-9320-x
18. Cenit MC, Alcina A, Marquez A, Mendoza JL, Diaz-Rubio MV, de las Heras V, et al. STAT3 locus in inflammatory bowel disease and multiple sclerosis susceptibility. *Genes Immun.* (2010) 11:264–8. doi: 10.1038/gene.2010.10
19. Peter I, Mitchell AA, Ozelius L, Erazo M, Hu J, Doheny D, et al. Evaluation of 22 genetic variants with Crohn's disease risk in the Ashkenazi Jewish population: a case-control study. *BMC Med Genet.* (2011) 12:63. doi: 10.1186/1471-2350-12-63
20. Polgar N, Csongei V, Szabo M, Zambo V, Melegh BI, Sumegi K, et al. Investigation of JAK2, STAT3 and CCR6 polymorphisms and their gene-gene interactions in inflammatory bowel disease. *Int J Immunogenet.* (2012) 39:247–52. doi: 10.1111/j.1744-313X.2012.01084.x
21. Prager M, Buttner J, Haas V, Baumgart DC, Sturm A, Zeitz M, et al. The JAK2 variant rs10758669 in Crohn's disease: altering the intestinal barrier as one mechanism of action. *Int J Colorectal Dis.* (2012) 27:565–73. doi: 10.1007/s00384-011-1345-y
22. Globig AM, Hennecke N, Martin B, Seidl M, Ruf G, Hasselblatt P, et al. Comprehensive intestinal T helper cell profiling reveals specific accumulation of IFN-gamma+IL-17+coproducing CD4+ T cells in active inflammatory bowel disease. *Inflamm Bowel Dis.* (2014) 20:2321–9. doi: 10.1097/MIB.0000000000000210
23. Greenwald B, James SP. Long-term HIV infection with Crohn's disease. *Am J Gastroenterol.* (1995) 90:167–8.
24. Skamnelos A, Tatsioni A, Katsanos KH, Tsianos V, Christodoulou D, Tsianos EV. CD4 count remission hypothesis in patients with inflammatory bowel disease and human immunodeficiency virus infection: a systematic review of the literature. *Ann Gastroenterol.* (2015) 28:337–46.
25. Stronkhorst A, Radema S, Yong SL, Bijl H, ten Berge IJ, Tytgat GN, et al. CD4 antibody treatment in patients with active Crohn's disease: a phase 1 dose finding study. *Gut* (1997) 40:320–7. doi: 10.1136/gut.40.3.320
26. Emmrich J, Seyfarth M, Fleig WE, Emmrich F. Treatment of inflammatory bowel disease with anti-CD4 monoclonal antibody. *Lancet* (1991) 338:570–1. doi: 10.1016/0140-6736(91)91133-F
27. Sandborn WJ, Su C, Panes Tofacitinib as induction and maintenance therapy for ulcerative colitis. J. *N Engl J Med.* (2017) 377:496–7. doi: 10.1056/NEJMc1707500
28. Feagan BG, Sandborn WJ, Gasink C, Jacobstein D, Lang Y, Friedman JR, et al. Ustekinumab as induction and maintenance therapy for Crohn's Disease. *N Engl J Med.* (2016) 375:1946–60. doi: 10.1056/NEJMoa1602773
29. Sandborn WJ, Feagan BG, Marano C, Zhang H, Strauss R, Johanns J, et al. (2014). Subcutaneous golimumab induces clinical response and remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 146:85–95. doi: 10.1053/j.gastro.2013.05.048
30. Colombel JF, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, et al. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* (2007) 132:52–65. doi: 10.1053/j.gastro.2006.11.041
31. Schreiber S, Khaliq-Kareemi M, Lawrance IC, Thomsen OO, Hanauer SB, McColm J, et al. Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med.* (2007) 357:239–50. doi: 10.1056/NEJMoa062897
32. Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* (2002) 359:1541–9. doi: 10.1016/S0140-6736(02)08512-4
33. Duijvestein M, Battat R, Vande Casteele N, D'Haens GR, Sandborn WJ, Khanna R, et al. Novel therapies and treatment strategies for patients with inflammatory bowel disease. *Curr Treat Options Gastroenterol.* (2018) 16:129–46. doi: 10.1007/s11938-018-0175-1
34. Mosmann TR Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone I Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* (1986) 136:2348–57.
35. Del Prete GF, De Carli M, Mastromauro C, Biagiotti R, Macchia D, Falagiani P, et al. Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of Toxocara canis expand *in vitro* human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J Clin Invest.* (1991) 88:346–50. doi: 10.1172/JCI115300
36. Burkett PR, Meyer zu Horste G, Kuchroo VK. Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity. *J Clin Invest.* (2015) 125:2211–9. doi: 10.1172/JCI78085
37. Schmitt E, Klein M, Bopp T. Th9 cells, new players in adaptive immunity. *Trends Immunol.* (2014) 35:61–8. doi: 10.1016/j.it.2013.10.004
38. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
39. Wing JB, Sakaguchi S. Multiple treg suppressive modules and their adaptability. *Front Immunol.* (2012) 3:178. doi: 10.3389/fimmu.2012.00178
40. DuPage M, Bluestone JA. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol.* (2016) 16:149–63. doi: 10.1038/nri.2015.18
41. Hovhannisyan Z, Treatman J, Littman DR, Mayer L. Characterization of interleukin-17-producing regulatory T cells in inflamed intestinal mucosa from patients with inflammatory bowel diseases. *Gastroenterology* (2011) 140:957–65. doi: 10.1053/j.gastro.2010.12.002
42. Ueno A, Jijon H, Chan R, Ford K, Hirota C, Kaplan G, et al. Increased prevalence of circulating novel IL-17 secreting Foxp3 expressing CD4+ T cells and defective suppressive function of circulating Foxp3+ regulatory cells support plasticity between Th17 and regulatory T cells in

inflammatory bowel disease patients. *Inflamm Bowel Dis.* (2013) 19:2522–34. doi: 10.1097/MIB.0b013e3182a85709
43. Iwashima M, Irving BA, van Oers NS, Chan AC, Weissm A. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science* (1994) 263:1136–9. doi: 10.1126/science.7509083
44. Samelson LE, Davidson WF, Morse HC III, Klausner RD. Abnormal tyrosine phosphorylation on T-cell receptor in lymphoproliferative disorders. *Nature* (1986) 324:674–6. doi: 10.1038/324674a0
45. Straus DB, Weiss A. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell* (1992) 70:585–93. doi: 10.1016/0092-8674(92)90428-F
46. van Oers NS, Killeen N, Weiss A. Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. *J Exp Med.* (1996) 183:1053–62. doi: 10.1084/jem.183.3.1053
47. Chan AC, Iwashima M, Turck CW, Weiss A. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* (1992) 71:649–62. doi: 10.1016/0092-8674(92)90598-7
48. van Panhuys N, Klauschen F, Germain RN. T-cell-receptor-dependent signal intensity dominantly controls CD4(+) T cell polarization *in vivo*. *Immunity* (2014) 41:63–74. doi: 10.1016/j.immuni.2014.06.003
49. Apostolou I, von Boehmer H. *In vivo* instruction of suppressor commitment in naive T cells. *J Exp Med.* (2004) 199:1401–8. doi: 10.1084/jem.20040249
50. Curotto de Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ. CD25- T cells generate CD25+Foxp3+ regulatory T cells by peripheral expansion. *J Immunol.* (2004) 173:7259–68. doi: 10.4049/jimmunol.173.12.7259
51. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med.* (2007) 204:1775–85. doi: 10.1084/jem.20070602
52. Fousteri G, Jasinski J, Dave A, Nakayama M, Pagni P, Lambolez F, et al. Following the fate of one insulin-reactive CD4 T cell: conversion into Teffs and Tregs in the periphery controls diabetes in NOD mice. *Diabetes* (2012) 61:1169–79. doi: 10.2337/db11-0671
53. Turner MS, Kane LP, Morel PA. Dominant role of antigen dose in CD4+Foxp3+ regulatory T cell induction and expansion. *J Immunol.* (2009) 183:4895–903. doi: 10.4049/jimmunol.0901459
54. Gottschalk RA, Corse E, Allison JP. TCR ligand density and affinity determine peripheral induction of Foxp3 *in vivo*. *J Exp Med.* (2010) 207:1701–11. doi: 10.1084/jem.20091999
55. Brooks AJ, Dai W, O'Mara ML, Abankwa D, Chhabra Y, Pelekanos RA, et al. Mechanism of activation of protein kinase JAK2 by the growth hormone receptor. *Science* 344:1249783. doi: 10.1126/science.1249783
56. Fu XY, Zhang JJ. Transcription factor p91 interacts with the epidermal growth factor receptor and mediates activation of the c-fos gene promoter. *Cell* (1993) 74:1135–45. doi: 10.1016/0092-8674(93)90734-8
57. Shuai K, Horvath CM, Huang LH, Qureshi SA, Cowburn D, Darnell JE Jr. et al. Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell* (1994) 76:821–8. doi: 10.1016/0092-8674(94)90357-3
58. Horvath CM, Wen Z, Darnell JE Jr. A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. *Genes Dev.* (1995) 9:984–94. doi: 10.1101/gad.9.8.984
59. Shuai K, Stark GR, Kerr IM, Darnell JE Jr. A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. *Science* (1993) 261:1744–6. doi: 10.1126/science.7690989
60. Muller M, Laxton C, Briscoe J, Schindler C, Improta T, Darnell J, et al. Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-alpha and -gamma signal transduction pathways. *EMBO J.* (1993) 12:4221–8. doi: 10.1002/j.1460-2075.1993.tb06106.x
61. Xu X, Sun YL, Hoey T. Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science* (1996) 273:794–7. doi: 10.1126/science.273.5276.794
62. Villarino AV, Kanno Y, O'Shea JJ. Mechanisms and consequences of Jak-STAT signaling in the immune system. *Nat Immunol.* (2017) 18:374–84. doi: 10.1038/ni.3691
63. Tonks NK, Diltz CD, Fischer EH. Purification of the major protein-tyrosine-phosphatases of human placenta. *J Biol Chem.* (1988) 263:6722–30.
64. Tonks NK, Diltz CD, Fischer EH. Characterization of the major protein-tyrosine-phosphatases of human placenta. *J Biol Chem.* (1988) 263:6731–7.
65. Thomas ML, Barclay AN, Gagnon J, Williams AF. Evidence from cDNA clones that the rat leukocyte-common antigen (T200) spans the lipid bilayer and contains a cytoplasmic domain of 80,000 Mr. *Cell* (1985) 41:83–93. doi: 10.1016/0092-8674(85)90063-7
66. Alonso A, Pulido R. The extended human PTPome: a growing tyrosine phosphatase family. *FEBS J.* (2016) 283:2197–201. doi: 10.1111/febs.13748
67. Hale AJ, Ter Steege E, den Hertog J. Recent advances in understanding the role of protein-tyrosine phosphatases in development and disease. *Dev Biol.* (2017) 428:283–92. doi: 10.1016/j.ydbio.2017.03.023
68. Arimura Y, Yagi J. Comprehensive expression profiles of genes for protein tyrosine phosphatases in immune cells. *Sci Signal.* 3:rs1. doi: 10.1126/scisignal.2000966
69. Mustelin T, Vang T, Bottini N. Protein tyrosine phosphatases and the immune response. *Nat Rev Immunol.* (2005) 5:43–57. doi: 10.1038/nri1530
70. Spalinger MR, McCole DF, Rogler G, Scharl M. Protein tyrosine phosphatase non-receptor type 2 and inflammatory bowel disease. *World J Gastroenterol.* (2016) 22:1034–44. doi: 10.3748/wjg.v22.i3.1034
71. Sharp RC, Abdulrahim M, Naser ES, Naser SA. Genetic variations of PTPN2 and PTPN22: role in the pathogenesis of Type 1 Diabetes and Crohn's Disease. *Front Cell Infect Microbiol.* 5:95. doi: 10.3389/fcimb.2015.00095
72. Peloquin JM, Goel G, Kong L, Huang H, Haritunians T, Sartor R, et al. Characterization of candidate genes in inflammatory bowel disease-associated risk loci. *JCI Insight* (2016) 1:e87899. doi: 10.1172/jci.insight.87899
73. Liu W, Guo W, Shen L, Chen Z, Luo Q, Luo X, et al. T lymphocyte SHP2-deficiency triggers anti-tumor immunity to inhibit colitis-associated cancer in mice. *Oncotarget* (2017) 8:7586–97. doi: 10.18632/oncotarget.13812
74. Lu D, Liu L, Ji X, Gao Y, Chen X, Liu Y, et al. The phosphatase DUSP2 controls the activity of the transcription activator STAT3 and regulates TH17 differentiation. *Nat Immunol.* (2015) 16:1263–73. doi: 10.1038/ni.3278
75. Bertin S, Lozano-Ruiz B, Bachiller V, Garcia-Martinez I, Herdman S, Zapater P, et al. Dual-specificity phosphatase 6 regulates CD4+ T-cell functions and restrains spontaneous colitis in IL-10-deficient mice. *Mucosal Immunol.* (2015) 8:505–15. doi: 10.1038/mi.2014.84
76. Brownlie RJ, Miosge LA, Vassilakos D, Svensson LM, Cope A, Zamoyska R, Lack of the phosphatase PTPN22 increases adhesion of murine regulatory T cells to improve their immunosuppressive function. *Sci Signal.* (2012) 5:ra87. doi: 10.1126/scisignal.2003365
77. Spalinger MR, McCole DF, Rogler G, Scharl, M. Role of protein tyrosine phosphatases in regulating the immune system: implications for chronic intestinal inflammation. *Inflamm Bowel Dis.* (2015) 21:645–55. doi: 10.1097/MIB.0000000000000297
78. Cloutier JF, Veillette A. Association of inhibitory tyrosine protein kinase p50csk with protein tyrosine phosphatase PEP in T cells and other hemopoietic cells. *EMBO J.* (1996) 15:4909–18. doi: 10.1002/j.1460-2075.1996.tb00871.x
79. Diaz-Gallo LM, Espino-Paisan L, Fransen K, Gomez-Garcia M, van Sommeren S, Cardena C, et al. Differential association of two PTPN22 coding variants with Crohn's disease and ulcerative colitis. *Inflamm Bowel Dis.* (2011) 17:2287–94. doi: 10.1002/ibd.21630
80. Kyogoku C, Langefeld CD, Ortmann WA, Lee A, Selby S, Carlton VE, et al. Genetic association of the R620W polymorphism of protein tyrosine phosphatase PTPN22 with human SLE. *Am J Hum Genet.* (2004) 75:504–7. doi: 10.1086/423790
81. Michou L, Lasbleiz S, Rat AC, Migliorini P, Balsa A, Westhovens R, et al. Linkage proof for PTPN22, a rheumatoid arthritis susceptibility gene and a human autoimmunity gene. *Proc Natl Acad Sci USA.* (2007) 104:1649–54. doi: 10.1073/pnas.0610250104
82. Begovich AB, Carlton VE, Honigberg LA, Schrodi SJ, Chokkalingam AP, Alexander HC, et al. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am J Hum Genet.* (2004) 75:330–7. doi: 10.1086/422827
83. Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K, Rostamkhani M, et al. A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat Genet.* (2004) 36:337–8. doi: 10.1038/ng1323

84. Vang T, Congia M, Macis MD, Musumeci L, Orru V, Zavattari P, et al. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat Genet.* (2005) 37:1317–9. doi: 10.1038/ng1673
85. Yu X, Sun JP, He Y, Guo X, Liu S, Zhou B, et al. Structure, inhibitor, and regulatory mechanism of Lyp, a lymphoid-specific tyrosine phosphatase implicated in autoimmune diseases. *Proc Natl Acad Sci USA.* (2007) 104:19767–72. doi: 10.1073/pnas.0706233104
86. Fiorillo E, Orru V, Stanford SM, Liu Y, Salek M, Rapini N, et al. Autoimmune-associated PTPN22 R620W variation reduces phosphorylation of lymphoid phosphatase on an inhibitory tyrosine residue. *J Biol Chem.* (2010) 285:26506–18. doi: 10.1074/jbc.M110.111104
87. Zhang J, Zahir N, Jiang Q, Miliotis H, Heyraud S, Meng X, et al. The autoimmune disease-associated PTPN22 variant promotes calpain-mediated Lyp/Pep degradation associated with lymphocyte and dendritic cell hyperresponsiveness. *Nat Genet.* (2011) 43:902–7. doi: 10.1038/ng.904
88. Hasegawa K, Martin F, Huang G, Tumas D, Diehl L, Chan AC. PEST domain-enriched tyrosine phosphatase (PEP) regulation of effector/memory T cells. *Science* (2004) 303:685–9. doi: 10.1126/science.1092138
89. Zikherman J, Hermiston M, Steiner D, Hasegawa K, Chan A, Weiss A. PTPN22 deficiency cooperates with the CD45 E613R allele to break tolerance on a non-autoimmune background. *J Immunol.* (2009) 182:4093–106. doi: 10.4049/jimmunol.0803317
90. Wu J, Katrekar A, Honigberg LA, Smith A, M, Conn MT, et al. (2006). Identification of substrates of human protein-tyrosine phosphatase PTPN22. *J Biol Chem.* 281:11002–10. doi: 10.1074/jbc.M600498200
91. Gjorloff-Wingren A, Saxena M, Williams S, Hammi D, Mustelin T. Characterization of TCR-induced receptor-proximal signaling events negatively regulated by the protein tyrosine phosphatase PEP. *Eur J Immunol.* (1999) 29:3845–54. doi: 10.1002/(SICI)1521-4141(199912)29:12<3845::AID-IMMU3845>3.0.CO;2-U
92. Hill RJ, Zozulya S, Lu YL, Ward K, Gishizky M, Jallal B. The lymphoid protein tyrosine phosphatase Lyp interacts with the adaptor molecule Grb2 and functions as a negative regulator of T-cell activation. *Exp Hematol.* (2002) 30:237–44. doi: 10.1016/S0301-472X(01)00794-9
93. Ghose R, Shekhtman A, Goger MJ, Ji H, Cowburn D. A novel, specific interaction involving the Csk SH3 domain and its natural ligand. *Nat Struct Biol.* (2001) 8:998–1004. doi: 10.1038/nsb1101-998
94. Wang Y, Shaked I, Stanford SM, Zhou W, Curtsinger JM, Mikulski Z, et al. The autoimmunity-associated gene PTPN22 potentiates toll-like receptor-driven, type 1 interferon-dependent immunity. *Immunity* (2013) 39:111–22. doi: 10.1016/j.immuni.2013.06.013
95. Chang HH, Miaw SC, Tseng W, Sun YW, Liu CC, Tsao HW, et al. PTPN22 modulates macrophage polarization and susceptibility to dextran sulfate sodium-induced colitis. *J Immunol.* (2013) 191:2134–43. doi: 10.4049/jimmunol.1203363
96. You-Ten KE, Muise ES, Itie A, Michaliszyn E, Wagner J, Jothy S, et al. Impaired bone marrow microenvironment and immune function in T cell protein tyrosine phosphatase-deficient mice *J Exp Med.* (1997) 186:683–93. doi: 10.1084/jem.186.5.683
97. Heinonen KM, Nestel FP, Newell EW, Charette G, Seemayer TA, Tremblay ML, et al. T-cell protein tyrosine phosphatase deletion results in progressive systemic inflammatory disease. *Blood* (2004) 103:3457–64. doi: 10.1182/blood-2003-09-3153
98. Wiede F, Shields BJ, Chew SH, Kyparissoudis K, van Vliet C, Galic S, et al. T cell protein tyrosine phosphatase attenuates T cell signaling to maintain tolerance in mice. *J Clin Invest.* (2011) 121:4758–74. doi: 10.1172/JCI59492
99. Spalinger MR, Kasper S, Chassard C, Raselli T, Frey-Wagner I, Gottier C, et al. Scharl (2015). PTPN2 controls differentiation of CD4(+) T cells and limits intestinal inflammation and intestinal dysbiosis. *Mucosal Immunol.* 8:918–29. doi: 10.1038/mi.2014.122
100. Matthews RJ, Bowne DB, Flores E, Thomas ML. Characterization of hematopoietic intracellular protein tyrosine phosphatases: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. *Mol Cell Biol.* (1992) 12:2396–405. doi: 10.1128/MCB.12.5.2396
101. Plutzky J, Neel BG, Rosenberg RD. Isolation of a src homology 2-containing tyrosine phosphatase. *Proc Natl Acad Sci USA.* (1992) 89:1123–7. doi: 10.1073/pnas.89.3.1123
102. Yi TL, Cleveland JL, Ihle JN. Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12-p13. *Mol Cell Biol.* (1992) 12:836–46. doi: 10.1128/MCB.12.2.836
103. Shen SH, Bastien L, Posner BI, Chretien P. A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases. *Nature* (1991) 352:736–9. doi: 10.1038/352736a0
104. Plas DR, Johnson R, Pingel JT, Matthews RJ, Dalton M, Roy G, et al. Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling. *Science* (1996) 272:1173–6. doi: 10.1126/science.272.5265.1173
105. Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, Yi T, et al. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity* (1996) 4:77–85. doi: 10.1016/S1074-7613(00)80300-3
106. Burshtyn DN, Shin J, Stebbins C, Long EO. Adhesion to target cells is disrupted by the killer cell inhibitory receptor. *Curr Biol.* (2000) 10:777–80. doi: 10.1016/S0960-9822(00)00568-6
107. Jiao H, Berrada K, Yang W, Tabrizi M, Platanias LC, Yi T. Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. *Mol Cell Biol.* (1996) 16:6985–92. doi: 10.1128/MCB.16.12.6985
108. Kashiwada M, Giallourakis CC, Pan PY, Rothman PB. Immunoreceptor tyrosine-based inhibitory motif of the IL-4 receptor associates with SH2-containing phosphatases and regulates IL-4-induced proliferation. *J Immunol.* (2001) 167:6382–7. doi: 10.4049/jimmunol.167.11.6382
109. Kim CH, Qu CK, Hangoc G, Cooper S, Anzai N, et al. Abnormal chemokine-induced responses of immature and mature hematopoietic cells from motheaten mice implicate the protein tyrosine phosphatase SHP-1 in chemokine responses. *J Exp Med.* (1999) 190:681–90. doi: 10.1084/jem.190.5.681
110. Klingmuller U, Lorenz U, Cantley LC, Neel BG, Lodish HF. Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* (1995) 80:729–38. doi: 10.1016/0092-8674(95)90351-8
111. Minoo P, Zadeh MM, Rottapel R, Lebrun JJ, Ali S. A novel SHP-1/Grb2-dependent mechanism of negative regulation of cytokine-receptor signaling: contribution of SHP-1 C-terminal tyrosines in cytokine signaling. *Blood* (2004) 103:1398–407. doi: 10.1182/blood-2003-07-2617
112. Nakamura MC, Niemi EC, Fisher MJ, Shultz LD, Seaman WE, Ryan JC. Mouse Ly-49A interrupts early signaling events in natural killer cell cytotoxicity and functionally associates with the SHP-1 tyrosine phosphatase. *J Exp Med.* (1997) 185:673–84. doi: 10.1084/jem.185.4.673
113. Pani G, Kozlowski M, Cambier JC, Mills GB, Siminovitch KA. Identification of the tyrosine phosphatase PTP1C as a B cell antigen receptor-associated protein involved in the regulation of B cell signaling. *J Exp Med.* (1995) 181:2077–84. doi: 10.1084/jem.181.6.2077
114. Roach TI, Slater SE, White LS, Zhang X, Majerus PW, Brown EJ, et al. The protein tyrosine phosphatase SHP-1 regulates integrin-mediated adhesion of macrophages. *Curr Biol.* (1998) 8:1035–8. doi: 10.1016/S0960-9822(07)00426-5
115. Xiao W, Hong H, Kawakami Y, Kato Y, Wu D, Yasudo H, et al. Tumor suppression by phospholipase C-beta3 via SHP-1-mediated dephosphorylation of Stat5. *Cancer Cell* (2009) 16:161–71. doi: 10.1016/j.ccr.2009.05.018
116. Shultz LD, Schweitzer PA, Rajan TV, Yi T, Ihle JN, Matthews RJ, et al. Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. *Cell* (1993) 73:1445–54. doi: 10.1016/0092-8674(93)90369-2
117. Tsui HW, Siminovitch KA, de Souza L, Tsui FW. Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. *Nat Genet.* (1993) 4:124–9. doi: 10.1038/ng0693-124
118. Carter JD, Neel BG, Lorenz U. The tyrosine phosphatase SHP-1 influences thymocyte selection by setting TCR signaling thresholds. *Int Immunol.* (1999) 11:1999–2014. doi: 10.1093/intimm/11.12.1999
119. Zhang J, Somani AK, Yuen D, Yang Y, Love PE, Siminovitch KA. Involvement of the SHP-1 tyrosine phosphatase in regulation of T cell selection. *J Immunol.* (1999) 163:3012–21.
120. Sathish JG, Johnson KG, LeRoy FG, Fuller KJ, Hallett MB, Brennan P, et al. Requirement for CD28 co-stimulation is lower

in SHP-1-deficient T cells. *Eur J Immunol.* (2001) 31:3649–58. doi: 10.1002/1521-4141(200112)31:12<3649::AID-IMMU3649>3.0.CO;2-8
121. Su MW, Yu CL, Burakoff SJ, Jin YJ. Targeting Src homology 2 domain-containing tyrosine phosphatase (SHP-1) into lipid rafts inhibits CD3-induced T cell activation. *J Immunol.* (2001) 166:3975–82. doi: 10.4049/jimmunol.166.6.3975
122. Chen Z, Chen L, Qiao SW, Nagaishi T, Blumberg RS. Carcinoembryonic antigen-related cell adhesion molecule 1 inhibits proximal TCR signaling by targeting ZAP-70. *J Immunol.* (2008) 180:6085–93. doi: 10.4049/jimmunol.180.9.6085
123. Lorenz U, Ravichandran KS, Burakoff SJ, Neel BG. Lack of SHPTP1 results in src-family kinase hyperactivation and thymocyte hyperresponsiveness. *Proc Natl Acad Sci USA*. (1996) 93:9624–9. doi: 10.1073/pnas.93.18.9624
124. Mizuno K, Tagawa Y, Watanabe N, Ogimoto M, Yakura H. SLP-76 is recruited to CD22 and dephosphorylated by SHP-1, thereby regulating B cell receptor-induced c-Jun N-terminal kinase activation. *Eur J Immunol.* (2005) 35:644–54. doi: 10.1002/eji.200425465
125. Stefanova I, Hemmer B, Vergelli M, Martin R, Biddison WE, Germain RN. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat Immunol.* (2003) 4:248–54. doi: 10.1038/ni895
126. Johnson DJ, Pao LI, Dhanji S, Murakami K, Ohashi PS, Neel BG. Shp1 regulates T cell homeostasis by limiting IL-4 signals. *J Exp Med.* (2013) 210:1419–31. doi: 10.1084/jem.20122239
127. Martinez RJ, Morris AB, Neeld DK, Evavold BD. Targeted loss of SHP1 in murine thymocytes dampens TCR signaling late in selection. *Eur J Immunol.* (2016) 46:2103–10. doi: 10.1002/eji.201646475
128. Mercadante ER, Lorenz UM. T cells deficient in the tyrosine phosphatase SHP-1 resist suppression by regulatory T cells. *J Immunol.* (2017) 199:129–37. doi: 10.4049/jimmunol.1602171
129. Christophi GP, Rong R, Holtzapple PG, Massa PT, Landas SK. Immune markers and differential signaling networks in ulcerative colitis and Crohn's disease. *Inflamm Bowel Dis.* (2012) 18:2342–56. doi: 10.1002/ibd.22957
130. Bouzid D, Fourati H, Amouri A, Marques I, Abida O, Haddouk S, et al. Association of ZAP70 and PTPN6, but Not BANK1 or CLEC2D, with inflammatory bowel disease in the Tunisian population. *Genet Test Mol Biomarkers* (2013) 17:321–6. doi: 10.1089/gtmb.2012.0372
131. Feng GS, Hui CC, Pawson T. SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. *Science* (1993) 259:1607–11. doi: 10.1126/science.8096088
132. Freeman RMJr, Plutzky J, Neel BG. Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of Drosophila corkscrew. *Proc Natl Acad Sci USA*. (1992) 89:11239–43. doi: 10.1073/pnas.89.23.11239
133. Saxton TM, Henkemeyer M, Gasca S, Shen R, Rossi DJ, Shalaby F, et al. Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2. *EMBO J.* (1997) 16:2352–64. doi: 10.1093/emboj/16.9.2352
134. Zhang J, Zhang F, Niu R. Functions of Shp2 in cancer. *J Cell Mol Med.* (2015) 19:2075–83. doi: 10.1111/jcmm.12618
135. Narumi Y, Isomoto H, Shiota M, Sato K, Kondo S, Machida H, et al. Polymorphisms of PTPN11 coding SHP-2 as biomarkers for ulcerative colitis susceptibility in the Japanese population. *J Clin Immunol.* (2009) 29:303–10. doi: 10.1007/s10875-008-9272-6
136. Dong B, Gao Y, Zheng X, Gao G, Gu H, Chen X, et al. T cell activation is reduced by the catalytically inactive form of protein tyrosine phosphatase SHP-2. *Int J Clin Exp Med.* (2015) 8:6568–77.
137. Miah SMS, Jayasuriya CT, Salter AI, Reilly EC, Fugere C, Yang W, et al. Ptpn11 Deletion in CD4(+) cells does not affect t cell development and functions but causes cartilage tumors in a T cell-independent manner. *Front Immunol.* 8:1326. doi: 10.3389/fimmu.2017.01326
138. Nguyen TV, Ke Y, Zhang EE, Feng GS. Conditional deletion of Shp2 tyrosine phosphatase in thymocytes suppresses both pre-TCR and TCR signals. *J Immunol.* (2006) 177:5990–6. doi: 10.4049/jimmunol.177.9.5990
139. Salmond RJ, Huyer G, Kotsoni A, Clements L, Alexander DR. The src homology 2 domain-containing tyrosine phosphatase 2 regulates primary T-dependent immune responses and Th cell differentiation. *J Immunol.* (2005) 175:6498–508. doi: 10.4049/jimmunol.175.10.6498
140. Zhang T, Guo W, Yang Y, Liu W, Guo L, Gu Y, et al. Loss of SHP-2 activity in CD4+ T cells promotes melanoma progression and metastasis. *Sci Rep.* 3:2845. doi: 10.1038/srep02845
141. Kwon J, Qu CK, Maeng JS, Falahati R, Lee C, Williams MS. Receptor-stimulated oxidation of SHP-2 promotes T-cell adhesion through SLP-76-ADAP. *EMBO J.* (2005) 24:2331–41. doi: 10.1038/sj.emboj.7600706
142. Wu X, Guo W, Wu L, Gu Y, Gu L, Xu S, et al. Selective sequestration of STAT1 in the cytoplasm via phosphorylated SHP-2 ameliorates murine experimental colitis. *J Immunol.* (2012) 189:3497–507. doi: 10.4049/jimmunol.1201006
143. Coulombe G, Leblanc C, Cagnol S, Maloum F, Lemieux E, Perreault N, et al. Epithelial tyrosine phosphatase SHP-2 protects against intestinal inflammation in mice. *Mol Cell Biol.* (2013) 33:2275–84. doi: 10.1128/MCB.00043-13
144. Yamashita H, Kotani T, Park JH, Murata Y, Okazawa H, et al. Role of the protein tyrosine phosphatase Shp2 in homeostasis of the intestinal epithelium. *PLoS ONE* 9:e92904. doi: 10.1371/journal.pone.0092904
145. Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* (2010) 32:605–15. doi: 10.1016/j.immuni.2010.05.003
146. Chang CF, D'Souza WN, Ch'en IL, Pages G,Pouyssegur J, Hedrick SM. Polar opposites: Erk direction of CD4 T cell subsets. *J Immunol.* (2012) 189:721–31. doi: 10.4049/jimmunol.1103015
147. Luo X, Zhang Q, Liu V, Xia Z, Pothoven KL, Lee C. Cutting edge: TGF-beta-induced expression of Foxp3 in T cells is mediated through inactivation of ERK. *J Immunol.* (2008) 180:2757–61. doi: 10.4049/jimmunol.180.5.2757
148. Altan-Bonnet G, Germain RN. Modeling T cell antigen discrimination based on feedback control of digital ERK responses. *PLoS Biol.* 3:e356. doi: 10.1371/journal.pbio.0030356
149. Liu H, Yao S, Dann SM, Qin H, Elson CO, Cong Y. ERK differentially regulates Th17- and Treg-cell development and contributes to the pathogenesis of colitis. *Eur J Immunol.* (2013) 43:1716–26. doi: 10.1002/eji.201242889
150. Tan AH, Lam KP. Pharmacologic inhibition of MEK-ERK signaling enhances Th17 differentiation. *J Immunol.* (2010) 184:1849–57. doi: 10.4049/jimmunol.0901509
151. Li G, Yu M, Lee WW, Tsang M, Krishnan E, Weyand CM, et al. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. *Nat Med.* (2012) 18:1518–24. doi: 10.1038/nm.2963
152. Agrawal S, Agrawal A, Doughty B, Gerwitz A, Blenis J, Van Dyke T, et al. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos *J Immunol.* (2003) 171:4984–9. doi: 10.4049/jimmunol.171.10.4984
153. Dillon S, Agrawal A, Van Dyke T, Landreth G, McCauley L, Koh A, et al. A Toll-like receptor 2 ligand stimulates Th2 responses *in vivo*, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *J Immunol.* (2004) 172:4733–43. doi: 10.4049/jimmunol.172.8.4733
154. Muda M, Theodosiou A, Rodrigues N, Boschert U, Camps M, Gillieron C, et al. The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. *J Biol Chem.* (1996) 271:27205–8. doi: 10.1074/jbc.271.44.27205
155. Stewart AE, Dowd S, Keyse SM, McDonald NQ. Crystal structure of the MAPK phosphatase Pyst1 catalytic domain and implications for regulated activation. *Nat Struct Biol.* (1999) 6:174–81. doi: 10.1038/5861
156. Maillet M, Purcell NH, Sargent MA, York AJ, Bueno OF, Molkentin JD. DUSP6 (MKP3) null mice show enhanced ERK1/2 phosphorylation at baseline and increased myocyte proliferation in the heart affecting disease susceptibility. *J Biol Chem.* (2008) 283:31246–55. doi: 10.1074/jbc.M806085200
157. Gonzalez-Navajas JM, Fine S, Law J, Datta SK, Nguyen KP, Yu M, et al. TLR4 signaling in effector CD4+ T cells regulates TCR activation and experimental colitis in mice. *J Clin Invest.* (2010) 120:570–81. doi: 10.1172/JCI 40055
158. Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy A, et al. Increased insulin sensitivity and obesity resistance in mice lacking

the protein tyrosine phosphatase-1B gene. *Science* (1999) 283:1544–8. doi: 10.1126/science.283.5407.1544
159. Klaman LD, Boss O, Peroni OD, Kim JK, Martino JL, Zabolotny J, M., et al. (2000). Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. *Mol Cell Biol.* 20:5479–5489. doi: 10.1128/MCB.20.15.5479-5489.2000
160. Lazo JS, McQueeney KE, Burnett JC, Wipf P, Sharlow ER. Small molecule targeting of PTPs in cancer. *Int J Biochem Cell Biol.* (2018) 96:171–81. doi: 10.1016/j.biocel.2017.09.011
161. He R. J., Yu ZH, Zhang RY, Zhang ZY. Protein tyrosine phosphatases as potential therapeutic targets. *Acta Pharmacol Sin.* (2014) 35:1227–46. doi: 10.1038/aps.2014.80
162. Hendriks W, Bourgonje A, Leenders W, Pulido R. Proteinaceous regulators and inhibitors of protein tyrosine phosphatases. *Molecules* (2018) 23. doi: 10.3390/molecules23020395
163. Stanford SM, Bottini N. Targeting tyrosine phosphatases: time to end the stigma. *Trends Pharmacol Sci* (2017) 38:524–40. doi: 10.1016/j.tips.2017.03.004
164. Krishnan N, Koveal D, Miller DH, Xue B, Akshinthala SD, Kragelj J, et al. Targeting the disordered C terminus of PTP1B with an allosteric inhibitor. *Nat Chem Biol.* (2014) 10:558–66. doi: 10.1038/nchembio.1528
165. Chen YN, LaMarche MJ, Chan HM, Fekkes P, Garcia-Fortanet J, Acker M, et al. Allosteric inhibition of SHP2 phosphatase inhibits cancers driven by receptor tyrosine kinases. *Nature* (2016) 535:148–52. doi: 10.1038/nature18621
166. Perron MD, Chowdhury S, Aubry I, Purisima E, Tremblay ML, Saragovi HU. Allosteric noncompetitive small molecule selective inhibitors of CD45 tyrosine phosphatase suppress T-cell receptor signals and inflammation *in vivo*. *Mol Pharmacol.* (2014) 85:553–63. doi: 10.1124/mol.113.089847
167. Wu CL, Hardy S, Aubry I, Landry M, Haggarty A, Saragovi H, et al. Identification of function-regulating antibodies targeting the receptor protein tyrosine phosphatase sigma ectodomain. *PLoS ONE* 12:e0178489. doi: 10.1371/journal.pone.0178489
168. Haque A, Andersen JN, Salmeen A, Barford D, Tonks NK. Conformation-sensing antibodies stabilize the oxidized form of PTP1B and inhibit its phosphatase activity. *Cell* (2011) 147:185–98. doi: 10.1016/j.cell.2011.08.036
169. Tan XF, Uddin Z, Park C, Song YH, Son M, et al. Competitive protein tyrosine phosphatase 1B (PTP1B) inhibitors, prenylated caged xanthones from *Garcinia hanburyi* and their inhibitory mechanism. *Bioorg Med Chem.* (2017) 25:2498–506. doi: 10.1016/j.bmc.2017.03.010
170. Townshend B, Aubry I, Marcellus RC, Gehring K, Tremblay ML. An RNA aptamer that selectively inhibits the enzymatic activity of protein tyrosine phosphatase 1B *in vitro*. *Chembiochem* (2010) 11:1583–93. doi: 10.1002/cbic.201000208
171. Manguso RT, Pope HW, Zimmer MD, Brown FD, Yates KB, Miller B, et al. *In vivo* CRISPR screening identifies Ptpn2 as a cancer immunotherapy target. *Nature* (2017) 547:413–8. doi: 10.1038/nature23270
172. Crew AP, Raina K, Dong H, Qian Y, Wang J, Vigil D, et al. Identification and characterization of von hippel-lindau-recruiting proteolysis targeting chimeras (PROTACs) of TANK-Binding Kinase 1. *J Med Chem.* (2018) 61:583–98. doi: 10.1021/acs.jmedchem.7b00635
173. Ohoka N, Okuhira K, Ito M, Nagai K, Shibata N, Hattori T, et al. *In Vivo* knockdown of pathogenic proteins via specific and nongenetic inhibitor of apoptosis protein (IAP)-dependent protein erasers (SNIPERs) *J Biol Chem.* (2017) 292:4556–70. doi: 10.1074/jbc.M116.768853
174. Penafuerte C, Feldhammer M, Mills JR, Vinette V, Pike KA, Hall A, et al. Downregulation of PTP1B and TC-PTP phosphatases potentiate dendritic cell-based immunotherapy through IL-12/IFNgamma signaling. *Oncoimmunology* (2017) 6:e1321185. doi: 10.1080/2162402X.2017.1321185
175. Olivera P, Danese S, Peyrin-Biroulet L. JAK inhibition in inflammatory bowel disease. *Expert Rev Clin Immunol.* (2017) 13:693–703. doi: 10.1080/1744666X.2017.1291342

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 27 November 2018
doi: 10.3389/fimmu.2018.02737

CTLA-4 and PD-1 Control of T-Cell Motility and Migration: Implications for Tumor Immunotherapy

Monika C. Brunner-Weinzierl[1] *and Christopher E. Rudd*[2,3]*

[1] *Department of Experimental Pediatrics, University Hospital, Health Campus Immunology, Infectiology and Inflammation, Otto-von-Guericke-University, Magdeburg, Germany,* [2] *Research Center-Maisonneuve-Rosemont Hospital (CRHMR), Montreal, QC, Canada,* [3] *Département de Medicine, Université de Montréal, Montreal, QC, Canada*

CTLA-4 is a co-receptor on T-cells that controls peripheral tolerance and the development of autoimmunity. Immune check-point blockade (ICB) uses monoclonal antibodies (MAbs) to block the binding of inhibitory receptors (IRs) to their natural ligands. A humanized antibody to CTLA-4 was first approved clinically followed by the use of antibody blockade against PD-1 and its ligand PD-L1. Effective anti-tumor immunity requires the activation of tumor-specific effector T-cells, the blockade of regulatory cells and the migration of T-cells into the tumor. Here, we review data implicating CTLA-4 and PD-1 in the motility of T-cells with a specific reference to the potential exploitation of these pathways for more effective tumor infiltration and eradication.

Keywords: CTLA4, check-point blockade, cancer, T-cell, motility, migration, PD1, immune surveillance

OPEN ACCESS

Edited by:
Alexandre M. Carmo, i3S, Instituto de Investigação e Inovação em Saúde, Portugal

Reviewed by:
Peter Steinberger, Medizinische Universität Wien, Austria
Ana Mafalda Santos, University of Oxford, United Kingdom

***Correspondence:**
Christopher E. Rudd
christopher.e.rudd@umontreal.ca

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *26 June 2018*
Accepted: *06 November 2018*
Published: *27 November 2018*

Citation:
Brunner-Weinzierl MC and Rudd CE (2018) CTLA-4 and PD-1 Control of T-Cell Motility and Migration: Implications for Tumor Immunotherapy. Front. Immunol. 9:2737. doi: 10.3389/fimmu.2018.02737

INTRODUCTION

T-cells circulate continuously between blood, lymphoid tissues and lymph nodes as a mechanism to encounter and respond to foreign antigen. The movement or motility of T-cells involves integrin and selectin mediated adhesion, increased velocity and arrest, chemotaxis to sites of inflammation, homing back to compartments of initial antigen contact, transmigration to enter tissues and movement inside tissues (**Figure 1**). Antigen-experienced T-cells extravase into non-lymphoid tissue and travel back via lymphatic vessels. In other instances, i.e., in the lymph nodes where foreign antigen is presented to T-cells by dendritic cells (DCs), integrins such as lymphocyte function-associated antigen 1 (LFA-1) are activated by chemokines and antigen-receptor (T-cell receptor; TCR) ligation to bind to their ligands inter-adhesion molecules (ICAMs) to facilitate the "stop signal" for T-cell-dendritic cell (DC) conjugate formation (**Figures 1**, **2A**). The operations of adhesion and chemokine reactivity from blood to tissue involves multi-step transmigration (6).

Integrin-activation supports activation of chemokine receptors that directs migration of T-cells from blood into tissues or back home into lymph nodes and spleen. The movement of T-cells responds to intrinsic and environmental clues. Chemokines play central roles in inducing the movement of mammalian cells to various niches of the immune system (7, 8). Chemokines effect the motility of CD4 and CD8 T-cells, as well as, suppressor regulatory T-cells (Tregs), although not always in a similar fashion (9, 10) (**Figure 1**). T-cells in distinct differentiation states such as naïve, effector, or memory T-cells move differently in the same environment to the same clues. Classically, the presence of sensitive CCR7 mediates homing of T-cells to lymph nodes and spleen, while the presence of CXCR5 in follicular T-cells dictates their movement to germinal centers, whereas CXCR3 and CCR5 directs them to the site of injury and inflammation (11). Antigen-experienced T-cells involve

movement over long distances were infection might occur, while naïve cells tend to explore the local environment over shorter distances in search of presented antigen (12). Co-receptors such as CD28 and CTLA-4 also modulate these pathways for effective migration.

CD28

CD28 plays a central role in providing a second signal needed for T-cell activation (13, 14). Activation signals from the antigen-receptor (TCR) are modified by signals from CD28 and other co-receptors (15–17). CD28 signals via the binding of the lipid kinase phosphatidylinositol- 3-kinase (PI3K) and the adaptor GrB2-SOS complex (18, 19) and $p56^{lck}$ which recruits the protein kinase C (20). It changes the organization of the cytoskeleton (16, 21, 22) and promotes the localization of T-cells to target tissue following antigenic priming (23). With this, it promotes egress from lymphoid tissue and migration to sites of inflammation. Although the downstream pathways that link CD28 to adhesion and migration are not fully understood, loss of CD28 binding to PI3K changes localization to tissues and may favor primed T-cell migration to non-lymphoid tissues (24).

CTLA-4

Check-point blockade of cytotoxic T lymphocyte antigen 4 (CTLA-4, CD152) is a major focus in tumor immunotherapy (25, 26). Ipilimumab, a humanized antibody against the inhibitory co-receptor CTLA-4, was the first checkpoint-block mAb to be approved (27). It is thought to act during neo-antigen presentation in lymph nodes and can affect primary and secondary responses to antigen. The loss of CTLA-4 in mice leads to a dramatic lymphoproliferative disorder where animals die within 3–5 weeks of age. Activated CD4 T-cells show an increased localization and infiltration of non-lymphoid and lymphoid organs where they accumulate in lymph nodes, the heart, liver, and pancreas (28–30). Other *in vivo* models involving antigen-specific T-cell responses combined with CTLA-4 blockade using specific antibodies (31, 32) or reduced CTLA-4 expression (10) support the notion that CTLA-4 can control T-cell infiltration into allo-grafts and tumors.

CTLA-4 dampens T-cell responses via cell intrinsic and extrinsic pathways. Intrinsic events include the inhibition of protein translation, recruitment of phosphatases, activation of ubiquitin ligases, inhibition of cytokine receptor signaling (33–38) and inhibition of lipid microdomain formation on the surface of T-cells (39). CTLA-4 has also been reported to bind to the phosphatases SHP2 and PP2A (34, 40, 41), although the cytoplasmic tail lacks ITIMs for SHP2 binding (42) and PP2A also binds to CD28 (34). Cell extrinsic events include the competition for CD28 in binding to its ligands CD80/86 (43), the removal of CD80/86 (44), the release of suppressive indoleamine (2,3)-dioxygenase (IDO) and the modulation of Treg function (35, 45). Each model has strengths and weaknesses. While competition with CD28 can occur, the induction of autoimmune disease in *Ctla-4*$^{-/-}$ mice depends on a C-terminal intracellular proline CD28 motif in *in vivo* co-stimulation (46). Similarly, while CD80/86 can be trans-endocytosed from the surface of DCs by CTLA-4 (44), the level of CD80/86 removal *in vivo* is low and the ligands can be rapidly re-expressed on presenting cells. Further, whereas the selective deletion of CTLA-4 on FoxP3^{+} Tregs can delay the onset of disease, mice still die within 2–3 months (35, 45). Moreover, the CTLA-4 YVKM motif binding to PI3K activates pro-survival signals (47, 48) and LFA-1 adhesion (49). Beyond this, the TCR/CD3 mediated stop-signal is decoupled in T-cells from CTLA-4 deficient mice (50) and CTLA-4 has regulatory effects on homeostasis which modulates overall levels of peripheral T-cells (35). It is likely that multiple factors account for the auto-proliferative phenotype in the *Ctla-4*$^{-/-}$ mice.

PD-1

PD-1 is a member of the CD28 superfamily which negatively regulates T-cell activation. Blockade of the inhibitory co-receptor PD-1 or its ligand ligand PD-L1 has shown survival rates of 20–30% in treating various types of cancer (27, 51). Negative signals are generated by a cytoplasmic immunoreceptor tyrosine-based switch motif (ITSM) motif that binds to the protein tyrosine phosphatase SHP-2 and which can limit B-cell and T-cell signaling (52, 53). While PD-1-SHP-2 inhibits TCR and/or CD28 signaling (52–54), it is unclear whether PD-1 signals in the same manner in different T-cell subsets. To date, PD-1 has been found to primarily regulate the cytolytic effector function of CD8^{+} cells (55, 56). Anti-PD-1 immunotherapy also depends on the expression of CD28 (57).

CTLA-4 AND PD-1 REGULATION OF T-CELL MOTILITY

The massive infiltration of organs observed in the *Ctla-4*$^{-/-}$ provided the first clue that the co-receptor could alter migration of T-cells. Whether this was due to the hyper-activated state of activated *Ctla-4*$^{-/-}$ CD4 T-cells and/or was related to a direct effect of the co-receptor on mechanisms that affected T-cell motility and/or migration was unclear. An initial clue suggesting that a cell intrinsic pathway might be induced by CTLA-4 was apparent in the observation that T-cells in *Ctla-4*$^{-/-}$mice expressing a tailless form of the gene showed alterations in cell migration (58). Further, the acceleration of allograft rejection by CTLA-4 blockade *in vivo* is associated with more severe mononuclear cell infiltration (59). In addition, depletion of CTLA-4 on T-cell subpopulations *in vivo* showed that while CTLA-4 on Tregs inhibits the aberrant activation of T-cells, the expression of CTLA-4 on conventional T-cells prevents aberrantly activated T-cells from infiltrating and fatally damaging non-lymphoid tissues (60).

CTLA-4 has been shown to engage mechanisms linked to T-cell movement (1–4, 61) (**Figures 1**, **2**). It was first shown to activate LFA-1 adhesion via increased clustering of integrin receptors (49). YVKM motif binding to PI3K mediates this

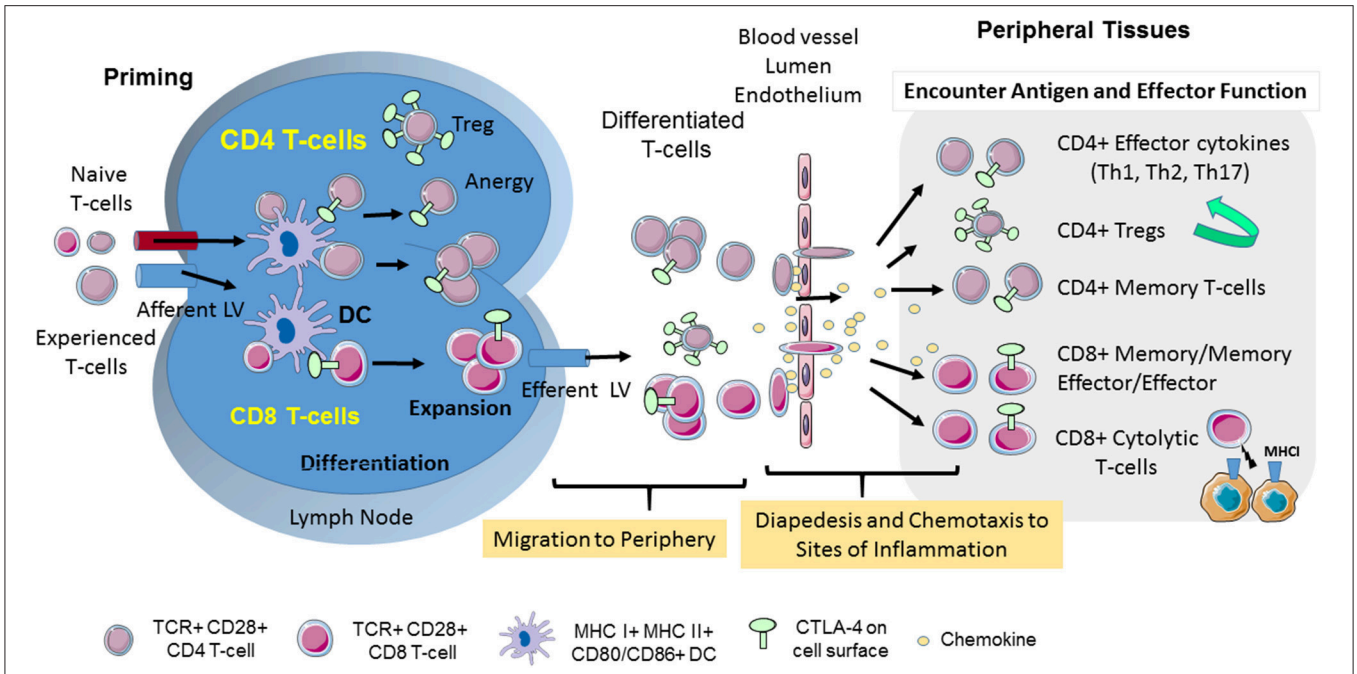


FIGURE 1 | CD28 and CTLA-4-mediated T-cell motility. T-cell response is initiated in secondary lymphoid organs. Naïve and experienced T-cells enter lymph nodes where they encounter antigen presented by DCs. CTLA-4 limits the interaction of CD4+ T-cells with DCs in the reverse-stop signal model involving an increase in T-cell motility, and a raising of the threshold needed to activate T-cells. In the "reverse-stop signal model", CTLA-4 induces T-cell motility and limits T-cell binding to DCs during antigen-presentation (1, 2). Reverse stop-signaling might also promote the egress of T-cells as mediated by responses to Sphingosine-1-phosphate (S1P) and chemokines. T-cells then migrate from the vasculature to infected tissue via a combination of chemokines and CTLA-4. CTLA-4 can alter motility by up-regulating key chemokine receptors CCR5 and CCR7 and the sensitivity toward the chemokines (3, 4). In the presence of antibody blockade, T-cells accumulate in the blood and remain circulating in the body (3). Upon entry into tissues, different T-cell subsets play important roles in determining the immune response to infection. The scheme was drawn using pictures from Servier Medical Art.

adhesion (49). This observation suggested that distinct motifs in co-receptor might mediate different intracellular events. Further, it offered the interesting possibility that CTLA-4 could generate both negative and positive signals. Indeed, a precedent was seen in nerve growth factor (NGF) signaling where the binding of PI3K determined whether positive or negative signals leading to apoptosis or cell death were generated (62). The absence of PI3K binding resulted in proapoptotic signaling via the receptor.

One key function of CTLA-4 is to interfere with the ability of T-cells to form stable conjugates with antigen-presenting cells (APCs) (**Figure 2A**). In the "reverse-stop signal model", CTLA-4 was found to induce T-cell motility and to limit T-cell binding to DCs during antigen-presentation (1, 2). CTLA-4 ligation with specific antibodies activates the motility of T-cells, while CTLA-4 on T-cells interferes with the dwell times of cells with DCs presenting antigenic peptide. Strikingly, antigen-specific *Ctla-4*$^{-/-}$ T-cells continue to move even in the presence of antigen (1). Similarly, the expression of CTLA-4 in transformed cell line, Jurkat promotes its motility (63). In terms of cell biology, CTLA-4 ligation induces a polarized morphology typical of motile T-cells, which in turn depends on the mediator's phosphatidylinositol 3-kinase, Vav-1, Cdc42, and myosin light chain kinase (64). From this, we proposed that the ability of CTLA-4 to limit contact times reduced the efficacy of TCR ligation and signaling which in turn raises the threshold needed to activate T-cells (2). Antigen-attracted T-cells competent for CTLA-4 move specifically to sites of inflammation and easily home to lymph nodes *in vitro* and *in vivo,* whereas CTLA-4 incompetent T-cells migrate to a lesser extent (3, 60).

It is noteworthy that the effects of CTLA-4 on motility may not operate equally in all T-cells. The reverse-stop effects appear limited to conventional T-cells (Tconvs) (9). It does not operate as efficiently in regulatory T-cells (9), or in anergic T-cells (5). Further, in certain antigen-presentation systems, the blockade of CD80/CD86 itself was as effective as CTLA-4 blockade in promoting the dissociation of T-cells from DCs and increased motility (65). While blockade of CD80/86 will also affect the induction of activation signals from CD28, and indirectly act to terminate T-cell-APC binding, it is also possible that the steric blockade of CTLA-4 with CD80/86 might release T-cells in a manner seen with reverse-stop signaling. Lastly, we also observed that T-cells from *Ctla-4*$^{-/-}$ mice are unable to arrest when ligated with anti-CD3 (50). The reason for this is unclear but may involve the heightened activation status of T-cells in an inflamed immune environment. It provides a potential explanation for the massive infiltration of all organs of the *Ctla-4*$^{-/-}$ mice with T-cells. Conversely, the expression of CTLA-4 on conventional T-cells

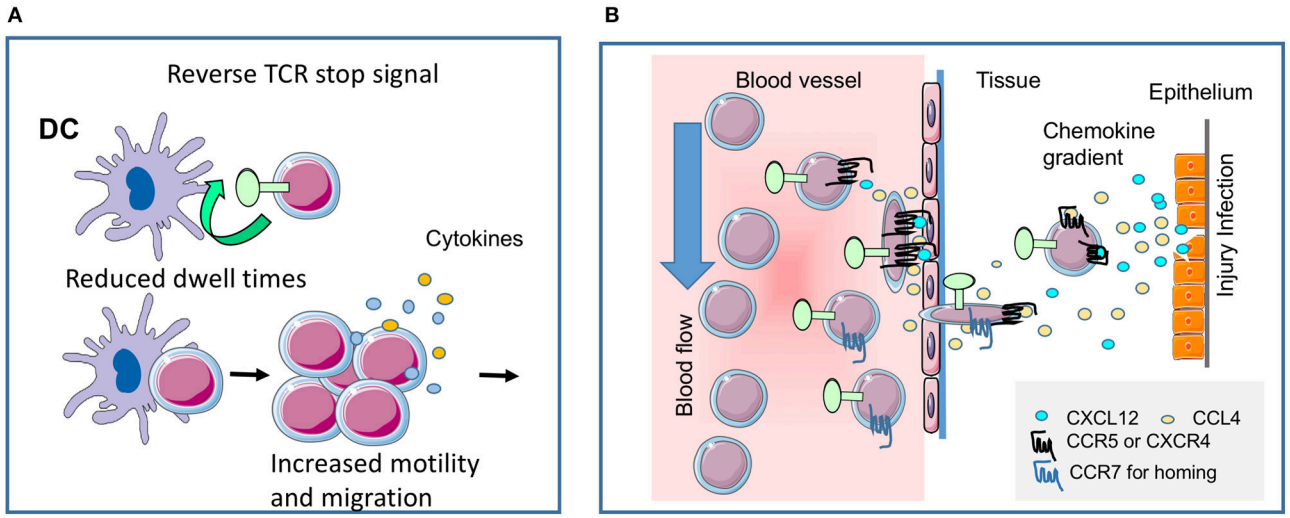


FIGURE 2 | CTLA-4 regulates T-cell motility. **(A)** Reverse-stop signal model of CTLA-4 (and PD-1). CTLA-4 induces T-cell motility and limits T-cell binding to DCs during antigen-presentation (1, 2). Agonistic CTLA-4 ligation could directly activate the motility of T-cells and thereby interfere with the dwell times of cells with DCs presenting antigenic peptide. PD-1 can function in a similar way (5). **(B)** CTLA-4 modulates response to chemokines. Chemokine gradients attract T-cells to the site of injury and inflammation. CTLA-4 can alter motility by up-regulating key chemokine receptors CCR5 and CCR7 and the sensitivity toward the chemokines CCL4 (MIP-1β), CXCL12 (SDF1α) and CCL19, but not CXCL9 (MIG) (3). The scheme was drawn using pictures from Servier Medical Art.

prevents aberrantly activated T-cells from infiltrating and fatally damaging non-lymphoid tissues (60).

In a second pathway of regulation, CTLA-4 can alter motility by up-regulating key chemokine receptors CCR5 and CCR7 and increasing their sensitivity to chemokines CCL4 (MIP-1β), CXCL12 (SDF1α) and CCL19, but not CXCL9 (MIG) (3) (**Figure 1**, middle; **Figure 2B**). We have proposed a model for chemotaxis that integrates CD28 and CTLA-4 signals via the G protein-coupled receptor kinase GRK that its phosphorylation of chemokine receptors for de-sensitization and degradation (4). Whereas, CD28 induces GRK to phosphorylate the CCR5 receptor, CTLA-4 engagement inactivates GRK2, leading to delaying or preventing phosphorylation of CCR5, and thereby halts desensitization. In addition, CTLA-4-enhanced specific migration might be partly the consequence of integrin-supported chemotaxis (66, 67), but is also mediated by TCR-mediated PI3K-Akt phosphorylation which synergizes with CD28-mediated migration (4). Antigen-attracted T-cells competent for CTLA-4 move specifically to sites of inflammation and easily home to lymph nodes *in vitro* and *in vivo* whereas CTLA-4 incompetent T-cells migrate much less (3, 60). Others have shown that T-cells poorly exit an IFN-treated peritoneal cavity, when before antigen recognition by T-cells anti-CTLA-4 antibodies and anti-hamster antibodies were applied (24). T-cells under this treatment did not move and therefore it is unclear whether the antibody-treatment blocked or crosslinked CTLA-4 and to which degree CTLA-4 operated in trans or without CD28 ligation (4).

Anti-CTLA-4 interference with the interaction between T-cells and DCs (1) laid a precedent for the follow-on finding that PD-1 blockade has similar effects in disrupting T-cell bindings to other cells (5, 68). Antibodies to PD-1 also limit contact times of anergic T-cells (5) and CD8 T-cells (68). In the latter study, PD-L1 was found to localize to the central supramolecular activation cluster, to decrease antiviral CD8 T-cell motility, and promote stable immunological synapse formation. Antibodies to PD-1-PD-L1 restored CD8 T-cell motility in the presence of high viral loads (68).

In this model, anti-PD-1 blockade has shared and distinct properties relative to CTLA-4 blockade. PD-L1 ligation of PD-1 appears to enforce adhesion that is released by anti-PD-1 blockade. PD-1 associated SHP-2 does not appear to negatively regulate adhesion. It is likely that CTLA-4 binding to CD80/86 might also promote adhesion and it blockade might release the T-cell from binding to another cell. However, in addition to this event, anti-CTLA-4 also promotes motility (1, 69). CTLA-4 expressing T-cells simply failed to undergo motility arrest *in vivo* in the presence of antigen, without the need for antibody blockade (1, 50). Antibody blockade of receptor binding to ligand and the induction of motility are therefore likely to cooperate in disrupting T-cell binding to other cells. In the presence of blocking antibody, the natural expression of CTLA-4 might limit contact of T-cells, while the additional blockade with anti-CTLA-4 ensures the complete release of the weakly adhesive T-cells. In both instances, anti-CTLA-4 and PD-1 limit T-cell binding to DCs during antigen presentation, thereby reducing the efficacy of TCR signaling and raising the threshold needed for the activation of T-cells. This is further complicated by the observation that T-cells from CTLA-4 deficient mice fail to stop in response to anti-CD3 ligation (50). It is unclear whether this feature is due to chronic stimulation that might over-ride the stop signal over time. Overall, the current data indicates that CTLA-4 and PD-1 alters the interaction of T-cells with other cells, including antigen-presenting cells, and consequently, alters the overall motility and migration of T-cells. The exact nature of the regulatory effect

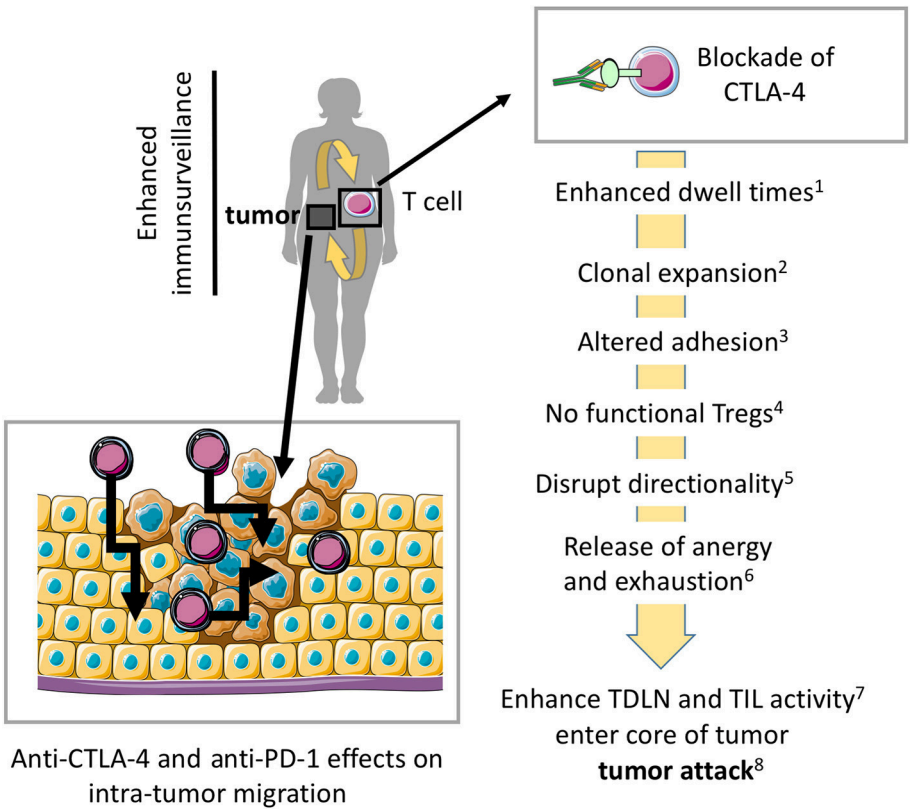


FIGURE 3 | Model where blockade of CTLA-4 and PD-1 enhances migration into tumors and within tumors for more effective tumor rejection. Preventing CTLA-4 engagement, i.e., using anti-CTLA-4-antibodies *in vivo* modulates the entry and migration of T-cells within tumors for more effective tumor elimination. Anti-CTLA-4 and anti-PD-1 effects on antibodies may also modulate T-cell movement within the tumor mass. (1) (5, 43), (2) (28–30, 74), (3) (49), (4) (35, 45), (5) (3, 4, 32), (6) (65, 69), (7) (10, 31, 32), (8) (25, 26, 37, 38). The scheme was drawn using pictures from Servier Medical Art.

may vary depending on the nature of the T-cell, whether CTLA-4 ligation occurs, as well as, the inflammatory conditions in the lymphoid microenvironment.

CTLA-4 AND PD-1 BLOCKADE IN TUMOR MODELS

A prediction from this work has been that CTLA-4 plays a similar role for T-cell entry and movement in tumors. Many tumors express neo-antigens that can be recognized by resident and peripheral T-cells. This aspect might contribute to the synergy seen between anti-CTLA-4 check-point blockade and other modalities of immune intervention (34, 70–73) (**Figure 3**). As mentioned, CTLA-4 limits dwell times with DCs and potential other tissues (1, 2) and the *in vitro* and *in vivo* migration of T-cells is enhanced by CTLA-4 (3, 4). In the presence of antibody blockade, T-cells accumulate in the blood and remain circulating in the body (3). Due to angiogenesis, the enhanced presence of circulating T-cells in the blood may provide an advantage in facilitating tumor access (75). In particular, as vessels at the tumor side are highly branched, irregular, and show a discontinued blood flow (75).

Furthermore, the tumor generates a local immune privileged microenvironment where access by T-cells is limited since integrin-mediated extravasation from blood stream is made difficult as ligands are downregulated at the barrier (75). Some tumors may even grow in immune privileged sides such as the central nervous system. Of note, immune privilege is an active process involving induction of inhibitory mechanisms such as the instructed upregulation of CTLA-4 on T-cells, which can accumulate at the border of the privileged side (76, 77). In addition, T-cells in the tumor microenvironment express CTLA-4 so that blockade releases this localization which enables them to even enter immune privileged microenvironments (32). Therefore, under CTLA-4 blockade using specific antibodies, tumors can be reached by migrating T-cells. However, enhanced motility and migration may also explain immune-related adverse events reported under therapy.

As mentioned, anti-CTLA-4 in tumor models has shown to increase T-cell movement in the tumor (61, 69). In murine breast cancer models, CTLA4 blockade using specific antibodies increased the motility of tumor infiltrating lymphocytes (TILs) in the tumor cavity *in vivo* (69). The expression of NKG2D then offset this effect by enhancing TILs arrest. In some manner,

this combination of anti-CTLA-4 effects on motility combined with stabilization as mediated by NKG2D enhanced tumor eradication. In general, anti-CTLA-4 check-point blockade has been associated with greater tumor entry, although the exact mechanism for this increase in tumor entry has yet to be determined (51). Similarly, in allo-graft models, anti-CTLA-4 blockade increased motility of CD4 effector and Treg cells, it may decrease the motility of CD8 effector T-cells (10). The explanation for these different effects is unclear but may relate to kinetics of CTLA-4 expression on subpopulations and thus, whether it is expressed under anti-CTLA-4 treatment at the cell surface of T-cell helpers and/or CD8 T-cell attackers (47, 74, 78). As CTLA-4 has a much higher affinity to CD80 and CD86 than CD28, the outcome will also be influenced by CD80/86 expression and subsequent ligation in the TDLN and tumor sites (65, 69).

CTLA-4 and PD-1 may have similar effects on T-cell reactivity against tumors; however, the differences in their mode of action may also suggest differences. For example, the more restricted ability of anti-PD-1 to block PD-1 binding to PD-L1 may suggest a more restricted role for T-cells already localized in tumors. Indeed, anti-PD-1 therapy has been reported to have fairly minor effects in promoting an increase in numbers of TILs in tumors such as melanoma. It predominate function on CD8 T-cells may also lead to a restricted effect on this subset. This may operate in conjunction with the effects of anti-PD-1 in restoring functionality to exhausted T-cells (79). By contrast, the combined effects of blockade and direct enhanced motility may be expected to lead to an increase in the migration of T-cells into and within tumors. At the same time, its effect on CD4 and CD8 T-cells might imply a more generalized role on these two major subsets within the T-cell population. Taken together, under CTLA-4 blockade, immune surveillance may be enhanced to sites where T-cells have restricted for tumor entry such as in peri-tumor sites where T-cells can be paralyzed. The synergy of combinational therapy such as CTLA-4 and PD-1 blockade could be due to enhanced motility and a reversal of T-cell exhaustion on different T-cells and in different microenvironments.

CONCLUSION

Although CTLA-4 impinges on many features of T-cell biology, its effect on tissue and tumor infiltration will be the subject of exciting future work. Antibodies to CTLA-4 may act to facilitate tumor entry and alter the movement in tumors, rates of egress. Further studies will elicit and exploit this feature to facilitate tumor entry for more effective tumor eradication.

AUTHOR CONTRIBUTIONS

MB-W and CR contributed equally to the writing of the manuscript.

ACKNOWLEDGMENTS

The work was supported by Deutsch Forschungsgemeinschaft (DFG) SFB854 B14 and DFG Br1860/8.

REFERENCES

1. Schneider H, Downey J, Smith A, Zinselmeyer BH, Rush C, Brewer JM, et al. Reversal of the TCR stop signal by CTLA-4. *Science* (2006) 313:1972–5. doi: 10.1126/science.1131078
2. Rudd CE. The reverse stop-signal model for CTLA4 function. *Nat Rev Immunol.* (2008) 8:153–60. doi: 10.1038/nri2253
3. Knieke K, Hoff H, Maszyna F, Kolar P, Schrage A, Hamann A, et al. CD152 (CTLA-4) determines CD4 T-cell migration *in vitro* and *in vivo*. *PLoS ONE* (2009) 4:e5702. doi: 10.1371/journal.pone.0005702
4. Knieke K, Lingel H, Chamaon K, Brunner-Weinzierl MC. Migration of Th1 lymphocytes is regulated by CD152 (CTLA-4)-mediated signaling via PI3 kinase-dependent Akt activation. *PLoS ONE* (2012) 7:e31391. doi: 10.1371/journal.pone.0031391
5. Fife BT, Pauken KE, Eagar TN, Obu T, Wu J, Tang Q, et al. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. *Nat Immunol.* (2009) 10:1185–92. doi: 10.1038/ni.1790
6. Andrian UH, von Chambers JD, McEvoy LM, Bargatze RF, Arfors KE, Butcher EC. Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins *in vivo*. *Proc Nat Acad Sci USA.* (1991) 88:7538–42. doi: 10.1073/pnas.88.17.7538
7. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science* (1996) 272:60–6. doi: 10.1126/science.272.5258.60
8. Dustin ML, Bivona TG, Philips MR. Membranes as messengers in T-cell adhesion signaling. *Nat Immunol.* (2004) 5:363–72. doi: 10.1038/ni1057
9. Lu Y, Schneider H, Rudd CE. Murine regulatory T-cells differ from conventional T-cells in resisting the CTLA-4 reversal of TCR stop-signal. *Blood* (2012) 120:4560–70. doi: 10.1182/blood-2012-04-421420
10. Miska J, Abdulreda MH, Devarajan P, Lui JB, Suzuki J, Pileggi A, et al. Real-time immune cell interactions in target tissue during autoimmune-induced damage and graft tolerance. *J Exp Med.* (2014) 211:441–56. doi: 10.1084/jem.20130785
11. Crotty S. Follicular helper CD4 T-cells (TFH). *Ann Rev Immunol.* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
12. Krummel MF, Bartumeus F, Gérard A. T-cell migration, search strategies and mechanisms. *Nat Rev Immunol.* (2016) 16:193–201. doi: 10.1038/nri.2015.16
13. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nature Rev Immunol.* (2001) 1:220–8. doi: 10.1038/35105024
14. Rudd CE, Schneider H. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat Rev Immunol.* (2003) 3:544–56. doi: 10.1038/nri1131
15. Rudd CE. Upstream-downstream: CD28 cosignaling pathways and T-cell function. *Immunity* (1996) 4:527–34. doi: 10.1016/S1074-7613(00)80479-3
16. Zell T, Hunt SW, Mobley JL, Finkelstein LD, Shimizu Y. CD28-mediated up-regulation of beta 1-integrin adhesion involves phosphatidylinositol 3-kinase. *J Immunol.* (1996) 156:883–6.
17. Kaga S, Ragg S, Rogers KA, Ochi A. Stimulation of CD28 with B7-2 promotes focal adhesion-like cell contacts where Rho family small G proteins accumulate in T-cells. *J Immunol.* (1998) 160:24–7.
18. Prasad KV, Cai YC, Raab M, Duckworth B, Cantley L, Shoelson SE, et al. T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. *Proc Nat Acad Sci USA.* (1994) 91:2834–8. doi: 10.1073/pnas.91.7.2834
19. Schneider H, Cai YC, Prasad KV, Shoelson SE, Rudd CE. T-cell antigen CD28 binds to the GRB-2/SOS complex, regulators of p21ras. *Eur J Immunol.* (1995) 25:1044–50. doi: 10.1002/eji.1830250428
20. Kong K-F, Yokosuka T, Canonigo-Balancio AJ, Isakov N, Saito T, Altman A. A motif in the V3 domain of the kinase PKC-θ determines its localization in the

immunological synapse and functions in T-cells via association with CD28. *Nat Immunol*. (2011) 12:1105–12. doi: 10.1038/ni.2120
21. Raab M, Pfister S, Rudd CE. CD28 signaling via VAV/SLP-76 adaptors: regulation of cytokine transcription independent of TCR ligation. *Immunity* (2001) 15:921–33. doi: 10.1016/S1074-7613(01)00248-5
22. Wang H, Lim D, Rudd CE. Immunopathologies linked to integrin signalling. *Semi Immunopathol.* (2010) 32:173–82. doi: 10.1007/s00281-010-0202-3
23. Chang TT, Jabs C, Sobel RA, Kuchroo VK, Sharpe AH. Studies in B7-deficient mice reveal a critical role for B7 costimulation in both induction and effector phases of experimental autoimmune encephalomyelitis. *J Exp Med.* (1999) 190:733–40. doi: 10.1084/jem.190.5.733
24. Mirenda V, Jarmin SJ, David R, Dyson J, Scott D, Gu Y, et al. Physiologic and aberrant regulation of memory T-cell trafficking by the costimulatory molecule CD28. *Blood* (2007) 109:2968–77. doi: 10.1182/blood-2006-10-050724
25. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* (1996) 271:1734–6. doi: 10.1126/science.271.5256.1734
26. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. (2010) 363:711–23. doi: 10.1056/NEJMoa1003466
27. Page DB, Postow MA, Callahan MK, Allison JP, Wolchok JD. Immune modulation in cancer with antibodies. *Annu Rev Med*. (2014) 65:185–202. doi: 10.1146/annurev-med-092012-112807
28. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* (1995) 3:541–7. doi: 10.1016/1074-7613(95)90125-6
29. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* (1995) 270:985–8. doi: 10.1126/science.270.5238.985
30. Chambers CA, Krummel MF, Boitel B, Hurwitz A, Sullivan TJ, Fournier S, et al. The role of CTLA-4 in the regulation and initiation of T-cell responses. *Immunol Rev*. (1996) 153:27–46. doi: 10.1111/j.1600-065X.1996.tb00919.x
31. Schwaiger T, van den Brandt C, Fitzner B, Zaatreh S, Kraatz F, Dummer A, et al. Autoimmune pancreatitis in MRL/Mp mice is a T-cell-mediated disease responsive to cyclosporine A and rapamycin treatment. *Gut* (2014) 63:494–05. doi: 10.1136/gutjnl-2012-303635
32. Yshii LM, Gebauer CM, Pignolet B, Mauré E, Quériault C, Pierau M, et al. CTLA4 blockade elicits paraneoplastic neurological disease in a mouse model. *Brain* (2014) 139:2923–34. doi: 10.1093/brain/aww225
33. Marengère LE, Waterhouse P, Duncan GS, Mittrücker HW, Feng GS, Mak TW. Regulation of T-cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. *Science* (1996) 272:1170–3.
34. Chuang E, Fisher TS, Morgan RW, Robbins MD, Duerr JM, Vander Heiden MG, et al. The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity* (2000) 13:313–22. doi: 10.1016/S1074-7613(00)00031-5
35. Kolar P, Knieke K, Hegel JKE, Quandt D, Burmester G-R, Hoff H, et al. CTLA-4 (CD152) controls homeostasis and suppressive capacity of regulatory T-cells in mice. *Arthrit Rheumat.* (2009) 60:123–32. doi: 10.1002/art.24181
36. Hoff H, Kolar P, Ambach A, Radbruch A, Brunner-Weinzierl MC. CTLA-4 (CD152) inhibits T-cell function by activating the ubiquitin ligase Itch. *Mol Immunol.* (2010) 47:1875–81. doi: 10.1016/j.molimm.2010.03.017
37. Arra A, Lingel H, Kuropka B, Pick J, Schnoeder T, Fischer T, et al. The differentiation and plasticity of Tc17 cells are regulated by CTLA-4-mediated effects on STATs. *Oncoimmunology* (2017) 6:e1273300. doi: 10.1080/2162402X.2016.1273300
38. Lingel H, Wissing J, Arra A, Schanze D, Lienenklaus S, Klawonn F, et al. CTLA-4-mediated posttranslational modifications direct cytotoxic T-lymphocyte differentiation. *Cell Death Diff.* (2017) 24:1739–49. doi: 10.1038/cdd.2017.102
39. Martin M, Schneider H, Azouz A, Rudd CE. Cytotoxic T lymphocyte antigen 4 and CD28 modulate cell surface raft expression in their regulation of T-cell function. *J Exp Med.* (2001) 194:1675–81. doi: 10.1084/jem.194.11.1675
40. Lee KM, Chuang E, Griffin M, Khattri R, Hong DK, Zhang W, et al. Molecular basis of T-cell inactivation by CTLA-4. *Science* (1998) 282:2263–6. doi: 10.1126/science.282.5397.2263
41. Baroja ML, Vijayakrishnan L, Bettelli E, Darlington PJ, Chau TA, Ling V, et al. Inhibition of CTLA-4 function by the regulatory subunit of serine/threonine phosphatase 2A. *J Immunol.* (2002) 168:5070–8. doi: 10.4049/jimmunol.168.10.5070
42. Schneider H, Rudd CE. Tyrosine phosphatase SHP-2 binding to CTLA-4: absence of direct YVKM/YFIP motif recognition. *Biochem Biophy Res Commun.* (2000) 269:279–83. doi: 10.1006/bbrc.2000.2234
43. Schildberg FA, Klein SR, Freeman GJ, Sharpe AH. Coinhibitory pathways in the B7-CD28 ligand-receptor family. *Immunity* (2016) 44:955–72. doi: 10.1016/j.immuni.2016.05.002
44. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* (2011) 332:600–3. doi: 10.1126/science.1202947
45. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T-cell function. *Science* (2008) 322:271–5. doi: 10.1126/science.1160062
46. Tai X, van Laethem F, Sharpe AH, Singer A. Induction of autoimmune disease in CTLA-4-/- mice depends on a specific CD28 motif that is required for *in vivo* costimulation. *Proc Nat Acad Sci USA*. (2007) 104:13756–61. doi: 10.1073/pnas.0706509104
47. Pandiyan P, Gärtner D, Soezeri O, Radbruch A, Schulze-Osthoff K, Brunner-Weinzierl MC. CD152 (CTLA-4) determines the unequal resistance of Th1 and Th2 cells against activation-induced cell death by a mechanism requiring PI3 kinase function. *J Exp Med*. (2004) 199:831–42. doi: 10.1084/jem.20031058
48. Schneider H, Valk E, Leung R, Rudd CE. CTLA-4 Activation of phosphatidylinositol 3-Kinase (PI 3-K) and Protein Kinase B (PKB/AKT) sustains T-cell anergy withouT-cell death. *PLoS ONE* (2008) 3:e3842. doi: 10.1371/journal.pone.0003842
49. Schneider H, Valk E, da Rocha Dias S, Wei B, Rudd CE. CTLA-4 up-regulation of lymphocyte function-associated antigen 1 adhesion and clustering as an alternate basis for coreceptor function. *Proc Nat Acad Sci USA*. (2005) 102:12861–6. doi: 10.1073/pnas.0505802102
50. Downey J, Smith A, Schneider H, Hogg N, Rudd CE. TCR/CD3 mediated stop-signal is decoupled in T-cells from Ctla4 deficient mice. *Immunol Lett*. (2008) 115:70–2. doi: 10.1016/j.imlet.2007.09.004
51. Sharma P, Wagner K, Wolchok JD, Allison JP. Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. *Nat Rev Cancer* (2011) 11:805–12. doi: 10.1038/nrc3153
52. Okazaki T, Maeda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Nat Acad Sci USA*. (2001) 98:13866–71. doi: 10.1073/pnas.231486598
53. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T-cell stimulation, but only receptor ligation prevents T-cell activation. *J Immunol*. (2002) 173:945–54. doi: 10.4049/jimmunol.173.2.945
54. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T-cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* (2017) 355:1428–33. doi: 10.1126/science.aaf1292
55. Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat Immunol.* (2013) 14:1212–8. doi: 10.1038/ni.2762
56. Baumeister SH, Freeman GJ, Dranoff G, Sharpe AH. Coinhibitory pathways in immunotherapy for cancer. *Ann Rev Immunol.* (2016) 34:539–73. doi: 10.1146/annurev-immunol-032414-112049
57. Kamphorst AO, Wieland A, Nasti T, Yang S, Zhang R, Barber DL, et al. Rescue of exhausted CD8 T-cells by PD-1-targeted therapies is CD28-dependent. *Science* (2017) 355:1423–7. doi: 10.1126/science.aaf0683
58. Masteller EL, Chuang E, Mullen AC, Reiner SL, Thompson CB. Structural analysis of CTLA-4 function *in vivo*. *J Immunol.* (2000) 164:5319–27. doi: 10.4049/jimmunol.164.10.5319
59. Lin H, Rathmell JC, Gray GS, Thompson CB, Leiden JM, Alegre ML. Cytotoxic T lymphocyte antigen 4 (CTLA4) blockade accelerates the acute rejection of cardiac allografts in CD28-deficient mice: CTLA4 can function independently of CD28. *J Exp Med.* (1998) 188:199–204. doi: 10.1084/jem.188.1.199

60. Jain N, Nguyen H, Chambers C, Kang J. Dual function of CTLA-4 in regulatory T-cells and conventional T-cells to prevent multiorgan autoimmunity. *Proc Nat Acad Sci USA*. (2010) 107:1524–8. doi: 10.1073/pnas.0910341107
61. Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T-cells in organ transplantation. *Sci Transl. Med.* (2010) 2:17ra10. doi: 10.1126/scitranslmed.3000116
62. Minshall C, Arkins S, Freund GG, Kelley KW. Requirement for phosphatidylinositol 3'-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. *J Immunol.* (1996) 156:939–47.
63. Schneider H, Smith X, Liu H, Bismuth G, Rudd CE. CTLA-4 disrupts ZAP70 microcluster formation with reduced T-cell/APC dwell times and calcium mobilization. *Eur J Immunol.* (2008) 38:40–7. doi: 10.1002/eji.200737423
64. Wei B, da Rocha Dias S, Wang H, Rudd CE. CTL-associated antigen-4 ligation induces rapid T-cell polarization that depends on phosphatidylinositol 3-kinase, Vav-1, Cdc42, and myosin light chain kinase. *J Immunol.* (2007) 179:400–8. doi: 10.4049/jimmunol.179.1.400
65. Pentcheva-Hoang T, Simpson TR, Montalvo-Ortiz W, Allison JP. Cytotoxic T lymphocyte antigen-4 blockade enhances antitumor immunity by stimulating melanoma-specific T-cell motility. *Cancer Immunol Res.* (2014) 2:970–80. doi: 10.1158/2326-6066.CIR-14-0104
66. Schneider H, Valk E, Dias SDR, Wei B, Rudd CE. CTLA-4 regulation of T-cell function via RAP-1-mediated adhesion. *Adv Exp Med Biol.* (2006) 584:115–26. doi: 10.1007/0-387-34132-3_9
67. Marelli-Berg FM, Fu H, Vianello F, Tokoyoda K, Hamann A. Memory T-cell trafficking: new directions for busy commuters. *Immunology* (2010) 130:158–65. doi: 10.1111/j.1365-2567.2010.03278.x
68. Zinselmeyer BH, Heydari S, Sacristán C, Nayak D, Cammer M, Herz J, et al. PD-1 promotes immune exhaustion by inducing antiviral T-cell motility paralysis. *J Exp Med.* (2013) 210:757–74. doi: 10.1084/jem.20121416
69. Ruocco MG, Pilones KA, Kawashima N, Cammer M, Huang J, Babb JS, et al. Suppressing T-cell motility induced by anti-CTLA-4 monotherapy improves antitumor effects. *J Clin Invest.* (2012) 122:3718–30. doi: 10.1172/JCI61931
70. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T-cell exhaustion and restore anti-tumor immunity. *J Exp Med.* (2010) 207:2187–94. doi: 10.1084/jem.20100643
71. Huang R-Y, Eppolito C, Lele S, Shrikant P, Matsuzaki J, Odunsi K. LAG3 and PD1 co-inhibitory molecules collaborate to limit CD8+ T-cell signaling and dampen antitumor immunity in a murine ovarian cancer model. *Oncotarget* (2015) 6:27359–77. doi: 10.18632/oncotarget.4751
72. Nguyen LT, Ohashi PS. Clinical blockade of PD1 and LAG3 — potential mechanisms of action. *Nat Rev Immunol.* (2015) 15:45–56. doi: 10.1038/nri3790
73. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory receptors with specialized functions in immune regulation. *Immunity* (2016) 44:989–1004. doi: 10.1016/j.immuni.2016.05.001
74. Maszyna F, Hoff H, Kunkel D, Radbruch A, Brunner-Weinzierl MC. Diversity of clonal T-cell proliferation is mediated by differential expression of CD152 (CTLA-4) on the cell surface of activated individual T lymphocytes. *J Immunol.* (2003) 171:3459–66. doi: 10.4049/jimmunol.171.7.3459
75. Sanctis F, de Ugel S, Facciponte J, Facciabene A. The dark side of tumor-associated endothelial cells. *Semi Immunol.* (2018) 35:35–47. doi: 10.1016/j.smim.2018.02.002
76. Gimsa U, ØRen A, Pandiyan P, Teichmann D, Bechmann I, Nitsch R, et al. Astrocytes protect the CNS: antigen-specific T helper cell responses are inhibited by astrocyte-induced upregulation of CTLA-4 (CD152). *J Mol Med.* (2004) 82:364–72. doi: 10.1007/s00109-004-0531-6
77. Gimsa U, Mitchison NA, Brunner-Weinzierl MC. Immune privilege as an intrinsic CNS property: astrocytes protect the CNS against T-cell-mediated neuroinflammation. *Med Inflamm.* (2013) 2013:1–11. doi: 10.1155/2013/320519
78. Pandiyan P, Hegel JKE, Krueger M, Quandt D, Brunner-Weinzierl MC. High IFN- production of individual CD8 T lymphocytes is controlled by CD152 (CTLA-4). *J Immunol.* (2007) 178:2132–40. doi: 10.4049/jimmunol.178.4.2132
79. Wherry EJ. T cell exhaustion. *Nat Immunol.* (2011) 12:492–9. doi: 10.1038/ni.2035

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 27 November 2018
doi: 10.3389/fimmu.2018.02755

The Roles of Coinhibitory Receptors in Pathogenesis of Human Retroviral Infections

Keiko Yasuma-Mitobe[1] *and Masao Matsuoka*[2,3]*

[1] Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan, [2] Department of Hematology, Rheumatology and Infectious Disease, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan, [3] Laboratory of Virus Control, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan

Costimulatory and coinhibitory receptors play a key role in regulating immune responses to infection and cancer. Coinhibitory receptors include programmed cell death 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and T cell immunoglobulin and ITIM domain (TIGIT), which suppress immune responses. Coinhibitory receptors are highly expressed on exhausted virus-specific T cells, indicating that viruses evade host immune responses through enhanced expression of these molecules. Human retroviruses, human immunodeficiency virus (HIV) and human T-cell leukemia virus type 1 (HTLV-1), infect T cells, macrophages and dendritic cells. Therefore, one needs to consider the effects of coinhibitory receptors on both uninfected effector T cells and infected target cells. Coinhibitory receptors are implicated not only in the suppression of immune responses to viruses by inhibition of effector T cells, but also in the persistence of infected cells *in vivo*. Here we review recent studies on coinhibitory receptors and their roles in retroviral infections such as HIV and HTLV-1.

Keywords: co-inhibitory receptor, HTLV-1, HIV-1, PD-1, TIGIT

OPEN ACCESS

Edited by:
Alexandre M. Carmo, i3S, Instituto de Investigação e Inovação em Saúde, Portugal

Reviewed by:
Ulf Dittmer, Universität Duisburg-Essen, Germany
Cosima T. Baldari, Università degli Studi di Siena, Italy

***Correspondence:**
Masao Matsuoka
mamatsu@kumamoto-u.ac.jp

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *26 June 2018*
Accepted: *08 November 2018*
Published: *27 November 2018*

Citation:
Yasuma-Mitobe K and Matsuoka M (2018) The Roles of Coinhibitory Receptors in Pathogenesis of Human Retroviral Infections. Front. Immunol. 9:2755. doi: 10.3389/fimmu.2018.02755

INTRODUCTION

Various viruses cause acute and chronic infections in humans. Since the host immune system functions to eliminate exogenous virus and infected cells, viruses that cause chronic infections must evade host immune surveillance by various strategies. Some viruses that cause persistent infections are also associated with viral oncogenesis; these include hepatitis C virus (HCV) (1), hepatitis B virus (HBV) (2, 3), human T-cell leukemia virus type 1 (HTLV-1) (4, 5), and Epstein-Barr virus (EBV) (6). Human immunodeficiency virus (HIV) also contributes to the development of cancers (7).

Costimulatory and coinhibitory receptor molecules play a key role in regulating immune responses to infections and cancers (8). When bound by their ligands, coinhibitory receptors suppress excess immune responses. In several cancers, tumor-infiltrating T cells express coinhibitory molecules that enable the tumors to escape the host immune response. Recent studies show that antibodies that block coinhibitory receptors, called immune checkpoint blocking antibodies, enhance immune responses to various cancers and exhibit remarkable clinical efficacy in cancer treatment (9).

Both virus-specific T cells and tumor-infiltrating T cells express coinhibitory molecules, and immune checkpoint pathways play a role in maintaining an exhausted T cell phenotype characterized by impaired cytokine production and cytotoxicity (10). The attenuated responses cannot eliminate viruses. In this review, we focus on coinhibitory receptors in retroviral infections.

COINHIBITORY RECEPTORS

An increasing number of coinhibitory molecules and pathways have now been identified (**Table 1**). There are two major families of T cell cosignaling molecules: the immunoglobulin superfamily (IgSF), which includes the B7-CD28 subfamily, and the tumor necrosis factor superfamilies of ligands (TNFSF) and receptors (TNFRSF). The B7-CD28 subfamily includes the classic coinhibitory receptor cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (35). B7 family molecules, such as CD80 and CD86, enhance TCR-mediated responses through binding to the co-stimulatory receptor CD28. On the other hand, CTLA-4 competes with CD28 for binding to CD80 and CD86 (36), thus playing a critical role in regulating T-cell activation and expansion (37–39). Thus, coinhibitory CD28 subfamily molecules negatively regulate T cell responses (8). Another representative molecule of this subfamily is programmed cell death 1 (PD-1: also known as Pdcd1). The IgSF also includes T cell immunoglobulin and mucin domain 3 (Tim-3), T cell immunoglobulin and ITIM domain (TIGIT), Lymphocyte activation gene-3 (Lag-3), and 2B4 (CD244, a member of the signaling lymphocyte activation molecule (SLAM) family of CD2-related receptors) (32, 40–42).

The other major group of cosignaling molecules, members of the TNFSF and TNFRSF, elicit costimulatory and coinhibitory signals between various cells. Most TNFRSF members bind to their specific TNFSF ligands and elicit costimulatory signals; however, herpesvirus entry mediator (HVEM) binds to several different ligands, including both TNFSF members and IgSF members. These ligands provide both stimulatory and inhibitory signals from HVEM. Binding of HVEM to the IgSF member B and T lymphocyte attenuator (BTLA) triggers inhibitory signals (43). HVEM also binds to the IgSF member CD160 and elicits a coinhibitory signal (44). CD160 is involved in both NK cell activation and T cell exhaustion (43). Another TNFRSF member, Death receptor 6, has also been reported to be a regulatory receptor (45). Coinhibitory receptors that are expressed during retrovirus infection are shown in **Table 1**.

T CELL EXHAUSTION AND VIRAL INFECTION

When T cells are chronically activated by viral infections, T cells tend to express coinhibitory receptors, and acquire exhausted phenotypes. Although most murine retroviruses establish chronic infection only when they infect neonatal mice, Friend virus (FV) causes acute and chronic infection even when it infects adult immunocompetent mice, suggesting that FV can evade the host immune responses and cause persistent infection (46). Regarding this, FV is similar to HIV and HTLV. As well as lymphocytic choriomeningitis virus (LCMV) infection (47, 48), FV-specific effector CD8 T cells express multiple coinhibitory receptors, such as PD-1, Tim-3, Lag-3, and CTLA-4 during chronic FV infection. Those cells were shown to be dysfunctional and associated with exhaustion (49, 50). In murine retrovirus model of FV chronic infection, blocking of CTLA-4 showed augmented T cell response and decreased the viral load (51). In addition to enhanced expression of coinhibitory receptors, regulatory T (Treg) cells also increase, which is associated with inhibition of effector T cells during FV infection (49, 52, 53). Thus, combined treatment of depletion of Treg cells and blockade of coinhibitory receptors recover CD8 T cell responses to FV (52).

Although FV infection is one model of retrovirus infection, it should be pointed out that FV is a retrovirus which infects mainly erythroid precursor cells and causes erythroleukemia. The target cell type is different from that of human retroviruses, such as HIV and HTLV-1, which target immune cells including T cells. Next, we review the coinhibitory receptors and their roles in pathogenesis of human retrovirus infections, HIV and HTLV-1.

RETROVIRUS INFECTION AND COINHIBITORY RECEPTORS

Coinhibitory receptors are also implicated in persistent infection with human retroviruses, HIV, and HTLV-1. However, one difference between these viruses and most others is that the target cells of these human retroviruses are the immune cells themselves, including T cells, macrophages and dendritic cells—cells that also express coinhibitory receptors. Moreover, inhibitory ligands are also expressed on retrovirus infected cells (54), which can cause dysfunction of effector cells through interaction with coinhibitory receptors. Therefore, we need to consider the effects of coinhibitory receptors on two types of cells: uninfected effector cells to the virus, and cells that are infected with the virus.

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

To established chronic infection, human retroviruses have to evade the host immune response. One mechanism is the escape mutations of epitopes that are recognized by cytotoxic T lymphocytes (CTL). Since viral reverse transcriptase is an error-prone DNA polymerase, vigorous viral replication generates vast number of mutations in the provirus. If the target epitope of CTL is mutated, this mutation enables the virus to escape from CTL responses. Furthermore, HIV impairs the immune function of effector cells to HIV infected cells through co-inhibitory receptors, which also helps virus to escape from immune responses (55). We are going to discuss about several co-inhibitory receptors.

PD-1

PD-1 expression is upregulated on HIV-specific CD8 and CD4 T cells in humans (**Figure 1**, upper left). The expression of PD-1 on these cells is positively correlated with viral load and disease progression (13), suggesting that PD-1 expression allows viral replication *in vivo*. HIV infection also upregulates PD-L1 expression on infected cells, which impairs T-cell function through interaction with increased PD-1 on effector T cells (54). Blockade of the PD-1/PD-L1 pathway using antibodies against PD-L1 *ex vivo* restores the function of HIV-specific CD4 and CD8 T-cells from anti-retroviral therapy naïve patients (13). Further

TABLE 1 | Inhibitory Ig superfamily and TNF superfamily receptors and their stimulatory molecules expressing during retrovirus infection*.

Supperfamily	Receptor subfamily	Molecules	Expression in infection**	Receptor expressing cells during infection	Signaling	Known ligands	References
Ig SF	CD28	CTLA-4	HIV	CD4+	Inhibitory	CD80, CD86	(11, 12)
		PD-1	HIV, SIV, HTLV-1	CD4+, CD8+	Inhibitory	PD-L1, PD-L2	(12–18)
		BTLA	HTLV-1 (decreased)	CD4+ (ATL cells)	Inhibitory	HVEM, UL144	(19)
	CD226	TIGIT	HIV, SIV, HTLV-1	CD4+, CD8+	Inhibitory	CD155, CD112, CD113	(18, 20)
	TIM	Tim-3	HIV, HTLV-1 (decreased)	CD4+, CD8+	Inhibitory	Galectin9, PS	(12, 21–26)
	CD2/SLAM	2B4(CD244)	HIV, HTLV-1	CD8+	Stimulatory/inhibitory	CD48	(27–29)
	LAIR	LAIR1	HTLV-1 (decreased)	CD4+ (ATL cells)	Inhibitory	Collagens	(19)
	Orphans	Lag-3(CD223)	HIV	CD4+	Stimulatory/inhibitory	MHC2/ unknown	(30)
		CD160	HIV	CD8+	Stimulatory/inhibitory	HVEM	(27, 28)
TNFRSF	Type-L	HVEM	HIV	Monocytes, DCs	Stimulatory	LIGHT	(31)
					Inhibitory	BTLA, CD160	

*This table is based on modified (32–34).

**Decreased expression is mentioned specifically, otherwise expression is elevated during indicated infection.

studies investigated the effect of blocking the PD-1 pathway using an *in vivo* mouse model. The effect of PD-L1 blocking antibodies was analyzed in humanized mice chronically infected with HIV-1. The blockade of the PD-1 pathway decreased HIV-1 viral loads and suppressed disease progression, especially in animals with high levels of PD-1 expression on CD8 T cells (14, 15). A recent study showed that antibodies targeting BTLA and Tim-3 in combination with PD-1 antibody also enhanced HIV-specific CD8 T cells proliferation *in vitro* (56). These studies suggest that the blocking of these coinhibitory receptors is an effective strategy to restore the anti-virus T cell responses and suppress viral load in HIV-infected individuals. In particular, this strategy combined with "shock-and-kill" therapy and/or ART might be beneficial for control of HIV.

The SIV infected rhesus macaque is the *in vivo* model of HIV-1 infection. An *in vivo* experiment using rhesus macaques also showed that PD-1 blockade enhances SIV-specific CD8 T cell responses, reduced viremia, and prolonged survival of SIV-infected macaques (57, 58), especially in combination with antiretroviral therapy (ART) (31).

CTLA-4

CTLA-4, another inhibitory receptor, is also upregulated in HIV-specific CD4 T cells, most of which co-express it with PD-1 (11) (**Figure 1**, upper left). CTLA-4 expression also positively correlates with disease progression. Blocking of CTLA-4 enhances HIV-specific CD4 T cell proliferation in response to HIV protein (11).

Tim-3

The exhaustion of HIV-specific CD8 T cells is also mediated by Tim-3 (**Figure 1**). The frequency of Tim-3 expressing dysfunctional T cells was elevated in HIV-1 infected individuals. In particular, Tim-3 expression was upregulated in HIV-specific CD8 T cells. Tim-3 expression was positively correlated with viral load and inversely correlated with CD4 T cell count (21). Tim-3 triggers cell death after interaction with its ligand, Galectin-9 (Gal-9) (22–24). Treg cells constitutively express Gal-9 and suppress proliferation of HIV-specific CD8 T cells with high level of Tim-3 expression (59). Furthermore, Tim-3 expressing HIV-specific CD8 T cells are defective in regard of degranulation (25). It has also been reported that PD-1, CTLA-4, and Tim-3 are co-expressed on HIV-specific CD4 T cells from untreated infected patients, and the co-expression of these three inhibitory receptors was strongly correlated with viral load (12).

TIGIT

TIGIT is often coexpressed with PD-1 at higher levels on HIV-specific CD8 T cells in HIV-infected patients, and this expression correlates with exhaustion of T cells and disease progression (**Figure 1**). TIGIT is highly expressed on intermediately differentiated memory CD8 T cells that are not fully mature effectors, which expand in HIV infection (20, 60). It has been reported that TIGIT+ cells produce less IL-2, TNF-α and IFN-γ and degranulate less (20). In addition, TIGIT expression on CD4 T cells is also associated with HIV viral load. As was the case for the other inhibitory receptors described above, blocking TIGIT and/or PD-L1 restores CD8 T cell responses *in vitro* (20).

Other Inhibitory Receptors in HIV Infection

Other inhibitory molecules are also implicated in HIV infection. HIV-specific CD8 T cells expressing PD-1 also express CD160 and 2B4 (27, 28) (**Figure 1**). Co-expression of these inhibitory receptors correlates with virus load and T cell responses. ART

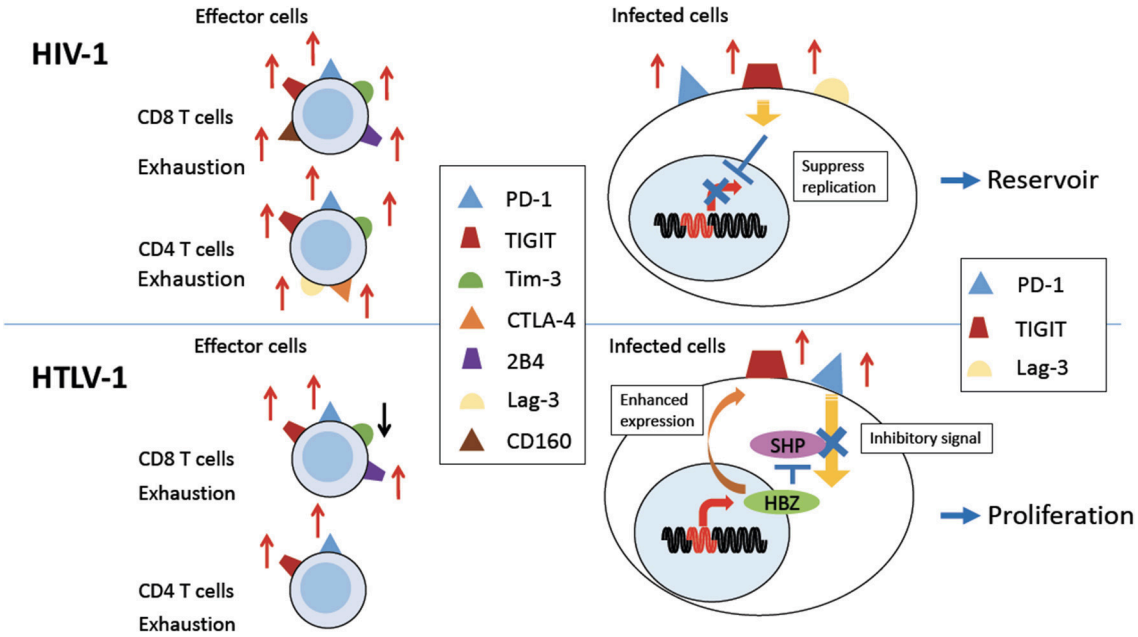


FIGURE 1 | Expression of coinhibitory receptors in HIV-1 and HTLV-1 infection. Persistent HIV-1 **(Upper Left)** and HTLV-1 **(Bottom Left)** infection induces expression of various coinhibitory receptors on uninfected effector CD8 T cells, and some uninfected CD4 T cells, causing exhaustion of T cells (left). PD-1 and TIGIT and/or Lag-3 are also expressed on HIV-1 or HTLV-1 infected CD4 T cells (right). In HIV-1 infection, coinhibitory receptor expression is implicated in establishment of a viral reservoir **(Upper Right)**. In HTLV-1 infection, expression of coinhibitory receptors is enhanced by the viral protein HBZ. Inhibitory signals from coinhibitory receptors are impaired by HBZ. Thus, infected cells are able to proliferate despite of increased expression of coinhibitory receptors **(Bottom Right)**.

reduces the expression of these inhibitory molecules on the surfaces of HIV-specific CD8 T cells. Furthermore, blocking of the PD-1/PD-L1 and 2B4/CD48 pathways enhances the proliferation of virus-specific CD8 T cells (27). Higher expression of Lag-3 on CD4 T cells was also reported in rapid progressors of HIV infection (30) (**Figure 1**, upper left).

HVEM, which is a member of the TNFRSF and the ligand of CD160, is upregulated on monocytes and dendritic cells (DCs) in HIV chronically infected patients. Blocking the interaction between CD160 and HVEM also enhanced the proliferation of HIV-specific CD8 T cells (61).

Implication of Coinhibitory Receptors for Persistence of HIV

So far we have discussed the effects of coinhibitory receptors on uninfected effector T cells to HIV (**Figure 1**, upper left). Next we need to consider the effects of these coinhibitory receptors on HIV infected cells themselves. Coinhibitory receptors suppress the proliferation and activation of infected cells (62) (**Figure 1**, upper right). First, PD-1 has been shown to associate with persistence of HIV in patients under ART treatment (63). The expression of the co-inhibitory receptors PD-1, TIGIT, and LAG-3, correlates with HIV-infected cells persisting in individuals treated with ART (64) (**Figure 1**, upper right). Thus, these studies suggest that co-inhibitory receptors play a important role in the formation of an HIV reservoir. It has been clarified that more than 95% of HIV-1 provirus in patients who receive ART are defective (65, 66). Such defective provirus does not kill infected cells since it cannot produce infectious virion. However, infected cells with defective provirus can produce viral proteins, which cause inflammation. It is likely that continuous inflammation upregulates PD-1, resulting in immune exhaustion and escape of infectious HIV-1 from host immune responses.

HUMAN T-CELL LEUKEMIA VIRUS TYPE 1 (HTLV-1)

HTLV-1 causes adult T-cell leukemia (ATL) and inflammatory diseases including HTLV-1-asscociated myelopathy/tropical spastic paraparesis (HAM/TSP) (4, 67). The *HTLV-1 bZIP factor* (*HBZ*) gene plays a critical role in oncogenesis and inflammation. HBZ is constantly expressed in ATL cells and HTLV-1-infected cells in carriers, and furthermore, transgenic expression of HBZ induces T-cell lymphomas and systemic inflammatory diseases resembling those found in HTLV-1-infected individuals (68, 69). One prominent feature of HTLV-1 is that this virus transmits primarily through cell-to-cell contact. To facilitate its transmission, HTLV-1 increases the number of infected cells *in vivo*. Therefore, this virus has evolved strategies to promote the proliferation of infected cells and evade host immune surveillance. Although HTLV-1 can infect a variety of cells, it primarily infects $CD4^+CD45RO^+CCR4^+$ T cells *in vivo*. It is thought that

HTLV-1 increases this special subtype of $CD4^+$ T cells *in vivo*. HBZ is thought to be critical for this special phenotype since HBZ converts infected cells to this special phenotype T cells (70).

Coinhibitory Receptors in HTLV-1 Infection

Since HTLV-1 mainly infects CD4 T cells *in vivo*, we again need to consider the roles of coinhibitory receptors on two different kinds of cells: infected CD4 T cells and uninfected effector T cells (**Figure 1**). It has been reported that during chronic HTLV-1 infection, PD-1 expression is increased on HTLV-1-specific CD8 T cells (**Figure 1**) (16). At the same time, ATL cells and HTLV-1 infected CD4 T cells of HAM/TSP patients express high levels of PD-1 (17, 18). These infected cells also express high levels of TIGIT (**Figure 1**) (18). Interestingly, the expression of PD-1 and TIGIT is enhanced in HBZ gene-transduced T cells (18), whereas the expressions of other coinhibitory receptors, BTLA and LAIR-1, is suppressed (19). This selective enhanced expression of particular coinhibitory receptors appears to be unique to HTLV-1. Co-blocking of PD-1 and TIGIT *ex vivo* partially restores anti-Tax T-cell responses in some HAM/TSP patients.

Flow cytometry showed that PD-L1 expression is also upregulated in 21.7% of ATL cases (16). One possible mechanism of the upregulation of PD-L1 has been identified: 27% of ATL cases possess structural variations that commonly disrupt the 3' untranslated region of the *PD-L1* gene, resulting in increased *PD-L1* transcripts (71). This upregulated PD-L1 expression enables ATL cells to evade the host CTL responses by causing exhaustion of effector T cells.

Immune Impairment by Coinhibitory Receptors on HTLV-1 Infected Cells

HBZ-induced coinhibitory receptors on HTLV-1 infected cells likely impair anti-virus T cell responses (18). As a mechanism, high expression levels of TIGIT promote production of IL-10 from CD155 positive DCs by reverse signaling (i.e., signaling from coinhibitory receptor ligands, such as PD-L1 and/or PD-L2 for PD-1, and CD155 for TIGIT on DCs). IL-10 not only suppresses the host immune response, but also promotes proliferation of HTLV-1 infected cells and ATL cells (72). The reverse signaling reduces maturation of DCs and changes them to a suppressive phenotype (73, 74). Furthermore, TIGIT competes for the binding of CD155 with CD226, a stimulatory receptor on T cells (75). Thus, HTLV-1 induces expression of coinhibitory receptors on effector T cells not only by chronic infection resulting in exhaustion of effector T cells but also by direct effect of HBZ on HTLV-1 infected T cells (18).

HBZ-Mediated Escape From the Growth Suppressive Effect of Coinhibitory Receptors

Coinhibitory receptors normally inhibit the proliferation of expressing T cells through binding of their ligands (76). However, the enigma is that ATL cells and HTLV-1 infected T cells do proliferate *in vivo*. Coinhibitory receptors like PD-1 and TIGIT normally inhibit cell proliferation through the ITIM or ITSM domains of their cytoplasmic regions, which interact with the phosphatases SHP1 and SHP2. However, HBZ expressing cells are resistant to the growth-inhibitory effects of TIGIT. THEMIS forms complexes with Grb2 and SHP and recruits them to the ITIM or ITSM domains of the coinhibitory receptors (77). HBZ interacts with THEMIS and impairs the growth-suppressive signal through SHP (**Figure 1**, bottom right). Thus, HBZ induces the expression of coinhibitory receptors while it blocks their suppressive effects on the proliferation of expressing cells (19).

IL-10

Mice with impaired IL-10 signaling were reported to develop autoimmune colitis, suggesting a critical role of IL-10 in regulating inflammation (78). IL-10 has various effects on many hematopoietic cells: IL-10 causes dendritic cells to downregulate stimulatory IL-12 production and expression of costimulatory molecules (78, 79). In addition, IL-10 decreases T-cell cytokine production (78). Thus, IL-10 is critical for suppressing immune responses. High levels of TIGIT induce IL-10 production, leading to a suppressed host immune response (74).

Anti-PD-1 Antibody for Treatment of ATL

Recently, it has been reported that anti-PD-1 antibody induced rapid progression of ATL after its administration (80). It has been reported that PD-1 functions as a tumor suppressor in T-cell lymphomas (81). This is the case in some ATL cases. This finding suggests that HBZ partially hinders the suppressive effects of PD-1. Thus, the significance of coinhibitory receptors for ATL cells needs further study.

CD244 and Tim-3

Another inhibitory receptor, 2B4 (CD244), is expressed at elevated levels on CD8 T cells in HTLV-1 infected patients and especially on HTLV-1 specific CD8 T cells. Blockade of the interaction between 2B4 and its ligand CD48 by antibody to CD48 *in vitro* enhances HTLV-1 specific CD8 T cell effector function measured by increased CD107a degranulation and perforin expression (29). On the other hand, Tim-3 expression is reduced on CD4 and CD8 T cells of HTLV-1 infected patients (26). This selective expression may be modulated by virus genes, such as HBZ, in order to have an advantage of virus survival.

PERSPECTIVES AND CONCLUSION

Since exhausted T cells are also implicated in chronic viral infections as described in this review, immune checkpoint therapy could be a novel treatment for diseases associated with persistent viral infections as well as anti-tumor therapy (82). Notably for human retroviral infections, coinhibitory receptors on both effector cells and infected target cells play different roles in the pathogenesis. Coinhibitory receptors on target cells infected with HIV or HTLV-1 likely promote their

survival by protecting target cells from immune responses or inhibiting viral production. Further studies are necessary to clarify the roles of coinhibitory receptors in chronic viral infections.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

We thank Dr. Linda Kingsbury for proofreading. This work was supported by JSPS KAKENHI (JP16H05336 to MM), the Project for Cancer Research and Therapeutic Evolution (P-CREATE) (17cm0106306h0002 to MM), the Research Program on Emerging and Re-emerging Infectious Diseases (17fk0108227h0002 to MM). This study was also supported in part by the JSPS Core-to-Core Program A, Advanced Research Networks.

REFERENCES

1. Urbani S, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, et al. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol.* (2006) 80:11398–403. doi: 10.1128/JVI.01177-06
2. Alter MJ. Epidemiology of viral hepatitis and HIV co-infection. *J Hepatol.* (2006) 44(Suppl. 1):S6–9. doi: 10.1016/j.jhep.2005.11.004
3. Stanaway JD, Flaxman AD, Naghavi M, Fitzmaurice C, Vos T, Abubakar I, et al. The global burden of viral hepatitis from 1990 to 2013: findings from the global burden of disease study 2013. *Lancet* (2016) 388:1081–8. doi: 10.1016/S0140-6736(16)30579-7
4. Matsuoka M. Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer* (2007) 7:270–80. doi: 10.1038/nrc2111
5. Ishitsuka K, Tamura K. Human T-cell leukaemia virus type I and adult T-cell leukaemia-lymphoma. *Lancet Oncol.* (2014) 15:e517–26. doi: 10.1016/S1470-2045(14)70202-5
6. Cohen JI. Epstein-Barr virus infection. *N Engl J Med.* (2000) 343:481–92. doi: 10.1056/NEJM200008173430707
7. Yarchoan R,Tosato G, Little RF. Therapy insight: AIDS-related malignancies–The influence of antiviral therapy on pathogenesis and management. *Nat Clin Pract Oncol.* (2005) 2:406–15. doi: 10.1038/ncponc0253
8. Chen L. Co-inhibitory molecules of the B7–CD28 family in the control of T-cell immunity. *Nat Rev Immunol.* (2004) 4:336–47. doi: 10.1038/nri1349
9. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science* (2015) 348:56–61. doi: 10.1126/science.aaa8172
10. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol.* (2003) 77:4911–27. doi: 10.1128/JVI.77.8.4911-4927.2003
11. Kaufmann DE, Kavanagh DG, Pereyra F, Zaunders JJ, Mackey EW, Miura T, 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) 8:1246–54. doi: 10.1038/ni1515
12. Kassu A, Marcus RA, D'Souza MB, Kelly-McKnight EA, Golden-Mason L, Akkina R, et al. Regulation of virus-specific CD4+ T cell function by multiple costimulatory receptors during chronic HIV infection. *J Immunol.* (2010) 185:3007–18. doi: 10.4049/jimmunol.1000156
13. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* (2006) 443:350–4. doi: 10.1038/nature05115
14. Palmer BE, Neff CP, Lecureux J, Ehler A, Dsouza M, Remling-Mulder L, et al. *In vivo* blockade of the PD-1 receptor suppresses HIV-1 viral loads and improves CD4+ T cell levels in humanized mice. *J Immunol.* (2013) 190:211–9. doi: 10.4049/jimmunol.1201108
15. Seung E, Dudek TE, Allen TM, Freeman GJ, Luster AD, Tager AM. PD-1 blockade in chronically HIV-1-infected humanized mice suppresses viral loads. *PLoS ONE* (2013) 8:e77780. doi: 10.1371/journal.pone.0077780
16. Kozako T, Yoshimitsu M, Fujiwara H, Masamoto I, Horai S, White Y, et al. PD-1/PD-L1 expression in human T-cell leukemia virus type 1 carriers and adult T-cell leukemia/lymphoma patients. *Leukemia* (2009) 23:375–82. doi: 10.1038/leu.2008.272
17. Shimauchi T, Kabashima K, Nakashima D, Sugita K, Yamada Y, Hino R, et al. Augmented expression of programmed death-1 in both neoplastic and non-neoplastic CD4+ T-cells in adult T-cell leukemia/lymphoma. *Int J Cancer* (2007) 121:2585–90. doi: 10.1002/ijc.23042
18. Yasuma K, Yasunaga J, Takemoto K, Sugata K, Mitobe Y, Takenouchi N, et al. HTLV-1 bZIP Factor impairs anti-viral immunity by inducing Co-inhibitory molecule, T cell immunoglobulin and ITIM domain (TIGIT). *PLoS Pathog.* (2016) 12:e1005372. doi: 10.1371/journal.ppat.1005372
19. Kinosada H, Yasunaga JI, Shimura K, Miyazato P, Onishi C, Iyoda T, et al. HTLV-1 bZIP factor enhances T-cell proliferation by impeding the suppressive signaling of co-inhibitory receptors. *PLoS Pathog.* (2017) 13:e1006120. doi: 10.1371/journal.ppat.1006120
20. Chew GM, Fujita T, Webb GM, Burwitz BJ, Wu HL, Reed JS, et al. TIGIT marks exhausted T cells, correlates with disease progression, and serves as a target for immune restoration in HIV and SIV infection. *PLoS Pathog.* (2016) 12:e1005349. doi: 10.1371/journal.ppat.1005349
21. Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, 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) 205:2763–79. doi: 10.1084/jem.20081398
22. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol.* (2005) 6:1245–52. doi: 10.1038/ni1271
23. Rangachari M, Zhu C, Sakuishi K, Xiao S, Karman J, Chen A, et al. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3-mediated cell death and exhaustion. *Nat Med.* (2012) 18:1394–400. doi: 10.1038/nm.2871
24. Poonia B, Pauza CD. Levels of CD56+TIM-3- effector CD8 T cells distinguish HIV natural virus suppressors from patients receiving antiretroviral therapy. *PLoS ONE* (2014) 9:e88884. doi: 10.1371/journal.pone.0088884
25. Sakhdari A, Mujib S, Vali B, Yue FY, MacParland S, Clayton K, et al. Tim-3 negatively regulates cytotoxicity in exhausted CD8+ T cells in HIV infection. *PLoS ONE* (2012) 7:e40146. doi: 10.1371/journal.pone.0040146
26. Abdelbary NH, Abdullah HM, Matsuzaki T, Hayashi D, Tanaka Y, Takashima H, et al. Reduced Tim-3 Expression on Human T-lymphotropic Virus Type I (HTLV-I) Tax-specific Cytotoxic T Lymphocytes in HTLV-I Infection. *J Infect Dis.* (2011) 203:948–59. doi: 10.1093/infdis/jiq153
27. Yamamoto T, Price DA, Casazza JP, Ferrari G, Nason M, Chattopadhyay PK, et al. Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. *Blood* (2011) 117:4805–15. doi: 10.1182/blood-2010-11-317297
28. Buggert M, Tauriainen J, Yamamoto T, Frederiksen J, Ivarsson MA, Michaëlsson J, et al. T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection. *PLoS Pathog.* (2014) 10:e1004251. doi: 10.1371/journal.ppat.1004251
29. Ezinne CC, Yoshimitsu M, White Y, Arima N. HTLV-1 specific CD8+ T cell function augmented by blockade of 2B4/CD48 interaction in HTLV-1 infection. *PLoS ONE* (2014) 9:e87631. doi: 10.1371/journal.pone.0087631
30. Rotger M, Dalmau J, Rauch A, McLaren P, Bosinger SE, Martinez R, et al. Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque. *J Clin Invest.* (2011) 121:2391–400. doi: 10.1172/JCI45235

31. McGary CS, Silvestri G, Paiardini M. Animal models for viral infection and cell exhaustion. *Curr Opin HIV AIDS* (2014) 9:492–9. doi: 10.1097/COH.0000000000000093
32. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol.* (2013) 13:227–42. doi: 10.1038/nri3405
33. Odorizzi PM, Wherry EJ. Inhibitory receptors on lymphocytes: insights from infections. *J Immunol*. (2012) 188:2957–65. doi: 10.4049/jimmunol.1100038
34. Attanasio J, Wherry EJ. Costimulatory and coinhibitory receptor pathways in infectious disease. *Immunity* (2016) 44:1052–68. doi: 10.1016/j.immuni.2016.04.022
35. Schildberg FA, Klein SR, Freeman GJ, Sharpe AH. Coinhibitory pathways in the B7-CD28 ligand-receptor family. *Immunity* (2016) 44:955–72. doi: 10.1016/j.immuni.2016.05.002
36. Carreno BM, Collins M. Theb7 family ofligands anditsreceptors: new pathways for costimulation and inhibition of immune responses. *Ann Rev Immunol.* (2002) 20:29–53. doi: 10.1146/annurev.immunol.20.091101.091806
37. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* (1995) 270:985–8. doi: 10.1126/science.270.5238.985
38. Bluestone JA. New perspectives of CD28-B7-mediated T cell costimulation. *Immunity* (1995) 2:555–9. doi: 10.1016/1074-7613(95)90000-4
39. Jenkins MK. The ups and downs of T cell costimulation. *Immunity* (1994) 1:443–6. doi: 10.1016/1074-7613(94)90086-8
40. Waggoner SN, Kumar V. Evolving role of 2B4/CD244 in T and NK cell responses during virus infection. *Front Immunol*. (2012) 3:377. doi: 10.3389/fimmu.2012.00377
41. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity* (2016) 44:989–1004. doi: 10.1016/j.immuni.2016.05.001
42. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell* (2014) 26:923–37. doi: 10.1016/j.ccell.2014.10.018
43. Ward-Kavanagh LK, Lin WW, Šedý JR, Ware CF. The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses. *Immunity* (2016) 44:1005–19. doi: 10.1016/j.immuni.2016.04.019
44. Cai G, Freeman GJ. The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation. *Immunol Rev*. (2009) 229:244–58. doi: 10.1111/j.1600-065X.2009.00783.x
45. Liu J, Na S, Glasebrook A, Fox N, Solenberg PJ, Zhang Q, et al. Enhanced CD4+ T cell proliferation and Th2 cytokine production in DR6-deficient mice. *Immunity* (2001) 15:23–34. doi: 10.1016/S1074-7613(01)00162-5
46. Hasenkrug KJ, Chesebro B. Immunity to retroviral infection: the friend virus model. *Proc Natl Acad Sci USA*. (1997) 94:7811–6. doi: 10.1073/pnas.94.15.7811
47. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, et al. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med*. (1998) 188:2205–13. doi: 10.1084/jem.188.12.2205
48. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol*. (2009) 10:29–37. doi: 10.1038/ni.1679
49. Zelinskyy G, Robertson SJ, Schimmer S, Messer RJ, Hasenkrug KJ, Dittmer U. CD8+ T-cell dysfunction due to cytolytic granule deficiency in persistent Friend retrovirus infection. *J Virol*. (2005) 79:10619–26. doi: 10.1128/JVI.79.16.10619-10626.2005
50. Takamura S, Tsuji-Kawahara S, Yagita H, Akiba H, Sakamoto M, Chikaishi T, et al. Premature terminal exhaustion of Friend virus-specific effector CD8+ T cells by rapid induction of multiple inhibitory receptors. *J Immunol*. (2010) 184:4696–707. doi: 10.4049/jimmunol.0903478
51. Teigler JE, Zelinskyy G, Eller MA, Bolton DL, Marovich M, Gordon AD, et al. Differential inhibitory receptor expression on T cells delineates functional capacities in chronic viral infection. *J Virol*. (2017) 91:e01263–17. doi: 10.1128/JVI.01263-17
52. Dietze KK, Zelinskyy G, Liu J, Kretzmer F, Schimmer S, Dittmer U. Combining regulatory T cell depletion and inhibitory receptor blockade improves reactivation of exhausted virus-specific CD8+ T cells and efficiently reduces chronic retroviral loads. *PLoS Pathog*. (2013) 9:e1003798. doi: 10.1371/journal.ppat.1003798
53. Iwashiro M, Messer RJ, Peterson KE, Stromnes IM, Sugie T, Hasenkrug KJ. Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection. *Proc Natl Acad Sci USA*. (2001) 98:9226–30. doi: 10.1073/pnas.151174198
54. Akhmetzyanova I, Drabczyk M, Neff CP, Gibbert K, Dietze KK, Werner T, et al. PD-L1 expression on retrovirus-infected cells mediates immune escape from CD8+ T cell killing. *PLoS Pathog*. (2015) 11:e1005224. doi: 10.1371/journal.ppat.1005224
55. Letvin NL, Walker BD. Immunopathogenesis and immunotherapy in AIDS virus infections. *Nat Med*. (2003) 9:861–6. doi: 10.1038/nm0703-861
56. Grabmeier-Pfistershammer K, Stecher C, Zettl M, Rosskopf S, Rieger A, Zlabinger GJ, et al. Antibodies targeting BTLA or TIM-3 enhance HIV-1 specific T cell responses in combination with PD-1 blockade. *Clin Immunol*. (2017) 183:167–73. doi: 10.1016/j.clim.2017.09.002
57. Velu V, Titanji K, Zhu B, Husain S, Pladevega A, Lai L, et al. Enhancing SIV-specific immunity *in vivo* by PD-1 blockade. *Nature* (2009) 458:206–10. doi: 10.1038/nature07662
58. Dyavar Shetty R, Velu V, Titanji K, Bosinger SE, Freeman GJ, Silvestri G, et al. PD-1 blockade during chronic SIV infection reduces hyperimmune activation and microbial translocation in rhesus macaques. *J Clin Invest*. (2012) 122:1712–6. doi: 10.1172/JCI60612
59. Elahi S, Dinges WL, Lejarcegui N, Laing KJ, Collier AC, Koelle DM, et al. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med*. (2011) 17:989–95. doi: 10.1038/nm.2422
60. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med*. (2002) 8:379–85. doi: 10.1038/nm0402-379
61. Peretz Y, He Z, Shi Y, Yassine-Diab B, Goulet JP, Bordi R, et al. CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction. *PLoS Pathog*. (2012) 8:e1002840. doi: 10.1371/journal.ppat.1002840
62. Wykes MN, Lewin SR. Immune checkpoint blockade in infectious diseases. *Nat Rev Immunol*. (2018) 18:91–104. doi: 10.1038/nri.2017.112
63. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, et al. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. *Nat Med*. (2009) 15:893–900. doi: 10.1038/nm.1972
64. Fromentin R, Bakeman W, Lawani MB, Khoury G, Hartogensis W, DaFonseca S, et al. CD4+ T cells expressing PD-1, TIGIT and LAG-3 contribute to HIV persistence during ART. *PLoS Pathog*. (2016) 12:e1005761. doi: 10.1371/journal.ppat.1005761
65. Imamichi H, Dewar RL, Adelsberger JW, Rehm CA, O'Doherty U, Paxinos EE, et al. Defective HIV-1 proviruses produce novel protein-coding RNA species in HIV-infected patients on combination antiretroviral therapy. *Proc Natl Acad Sci USA*. (2016) 113:8783–8. doi: 10.1073/pnas.1609057113
66. Hughes SH, Coffin JM. What integration sites tell us about HIV persistence. *Cell Host Microbe* (2016) 19:588–98. doi: 10.1016/j.chom.2016.04.010
67. Mesri EA, Feitelson MA, Munger K. Human viral oncogenesis: a cancer hallmarks analysis. *Cell Host Microbe* (2014) 15:266–82. doi: 10.1016/j.chom.2014.02.011
68. Matsuoka M, Green PL. The HBZ gene, a key player in HTLV-1 pathogenesis. *Retrovirology* (2009) 6:71. doi: 10.1186/1742-4690-6-71
69. Satou Y, Yasunaga J, Zhao T, Yoshida M, Miyazato P, Takai K, et al. HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation *in vivo*. *PLoS Pathog*. (2011) 7:e1001274. doi: 10.1371/journal.ppat.1001274
70. Tanaka A, Matsuoka M. HTLV-1 alters T cells for viral persistence and transmission. *Front Microbiol*. (2018) 9:461. doi: 10.3389/fmicb.2018.00461
71. Kataoka K, Shiraishi Y, Takeda Y, Sakata S, Matsumoto M, Nagano S, et al. Aberrant PD-L1 expression through 3'-UTR disruption in multiple cancers. *Nature* (2016) 534:402–6. doi: 10.1038/nature18294
72. Sawada L, Nagano Y, Hasegawa A, Kanai H, Nogami K, Ito S, et al. IL-10-mediated signals act as a switch for lymphoproliferation in human T-cell leukemia virus type-1 infection by activating the STAT3 and IRF4 pathways. *PLoS Pathog*. (2017) 13:e1006597. doi: 10.1371/journal.ppat.1006597
73. Kuipers H, Muskens F, Willart M, Hijdra D, van Assema FB, Coyle AJ, et al. Contribution of the PD-1 ligands/PD-1 signaling pathway to dendritic

cell-mediated CD4+ T cell activation. *Eur J Immunol*. (2006) 36:2472–82. doi: 10.1002/eji.200635978

74. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol*. (2009) 10:48–57. doi: 10.1038/ni.1674
75. Stengel KF, Harden-Bowles K, Yu X, Rouge L, Yin J, Comps-Agrar L, et al. Structure of TIGIT immunoreceptor bound to poliovirus receptor reveals a cell-cell adhesion and signaling mechanism that requires cis-trans receptor clustering. *Proc Natl Acad Sci USA*. (2012) 109:5399–404. doi: 10.1073/pnas.1120606109
76. Arasanz H, Gato-Cañas M, Zuazo M, Ibañez-Vea M, Breckpot K, Kochan G, et al. PD1 signal transduction pathways in T cells. *Oncotarget* (2017) 8:51936–45. doi: 10.18632/oncotarget.17232
77. Paster W, Bruger AM, Katsch K, Grégoire C, Roncagalli R, Fu G, et al. A THEMIS:SHP1 complex promotes T-cell survival. *EMBO J*. (2015) 34:393–409. doi: 10.15252/embj.201387725
78. Coombes JL, Robinson NJ, Maloy KJ, Uhlig HH, Powrie F. Regulatory T cells and intestinal homeostasis. *Immunol Rev*. (2005) 204:184–94. doi: 10.1111/j.0105-2896.2005.00250.x
79. Liu J, Cao S, Kim S, Chung EY, Homma Y, Guan X, et al. Interleukin-12: an update on its immunological activities, signaling and regulation of gene expression. *Curr Immunol Rev*. (2005) 1:119–37. doi: 10.2174/1573395054065115
80. Ratner L, Waldmann TA, Janakiram M, Brammer JE. Rapid progression of adult T-cell leukemia-lymphoma after PD-1 inhibitor therapy. *N Engl J Med*. (2018) 378:1947–8. doi: 10.1056/NEJMc1803181
81. Wartewig T, Kurgyis Z, Keppler S, Pechloff K, Hameister E, Öllinger R, et al. PD-1 is a haploinsufficient suppressor of T cell lymphomagenesis. *Nature* (2017) 552:121–5. doi: 10.1038/nature24649
82. Cox MA, Nechanitzky R, Mak TW. Check point inhibitors as therapies for infectious diseases. *Curr Opin Immunol*. (2017) 48:61–7. doi: 10.1016/j.coi.2017.07.016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

PERSPECTIVE
published: 07 December 2018
doi: 10.3389/fimmu.2018.02900

CD5, an Undercover Regulator of TCR Signaling

Guillaume Voisinne[1], *Anne Gonzalez de Peredo*[2] *and Romain Roncagalli*[1]*

[1] Centre d'Immunologie de Marseille-Luminy, Aix Marseille Université, INSERM, CNRS, Marseille, France, [2] Institut de Pharmacologie et de Biologie Structurale, Département Biologie Structural Biophysique, Protéomique Génopole Toulouse Midi Pyrénées CNRS UMR 5089, Toulouse, France

T cells are critical components of adaptive immunity. As such, their activation is regulated by the T cell receptor (TCR) that constantly scan peptides associated with major histocompatibility complexes (MHC). TCR engagement initiates a series of molecular events leading to cytokine secretion, proliferation, and differentiation of T cells. As a second coincident event, activation of co-stimulatory molecules, such as CD28, synergize with the TCR in order to prolong and/or amplify intracellular signals. With the recent advances in immunotherapies targeting T cells, co-inhibitory receptors are of growing interest for immunologists due to their potential modulatory properties on T cell functions. However, special attention should be dedicated to avoid unwanted clinical outcomes (1). In particular, Manichean categorization of receptors based on incomplete functional knowledge can lead to an over-simplistic view of complex cellular regulations. Thus, analysis of the functions that characterize these receptors in diverse physiological contexts remains essential for their rational use in therapeutic protocols. Here we focus on CD5, a transmembrane receptor that regulates T cell functions and development but remains poorly characterized at the molecular level. We will review its roles in physiological conditions and suggest potential molecular effectors that could account for CD5-dependent regulation of TCR signaling.

Keywords: CD5, TCR-T cell receptor, signaling/signaling pathways, coreceptor, inhibition

OPEN ACCESS

Edited by:
Alexandre M. Carmo, i3S, Instituto de Investigação e Inovação em Saúde, Portugal

Reviewed by:
Nicole L. La Gruta, Monash University, Australia
Georges Bismuth, INSERM U1016 Institut Cochin, France

***Correspondence:**
Romain Roncagalli
roncagalli@ciml.univ-mrs.fr

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *15 August 2018*
Accepted: *26 November 2018*
Published: *07 December 2018*

Citation:
Voisinne G, Gonzalez de Peredo A and Roncagalli R (2018) CD5, an Undercover Regulator of TCR Signaling. Front. Immunol. 9:2900. doi: 10.3389/fimmu.2018.02900

REGULATION OF T CELL DEVELOPMENT AND FUNCTION BY CD5

Seminal studies have identified CD5 as an activation marker of T cells (2, 3). Thus, expression of CD5 increases according to the magnitude of the signal delivered by the TCR. Consequently, CD5 expression reflects the heterogeneity of the signal strength associated with each individual TCR within a polyclonal T cell population. This observation has been also documented with various TCR transgenic mice, for which CD5 expression levels correlated with the affinity of the TCR with its known agonist peptide (4, 5). The study of CD5 deficient mice allowed to position CD5 not only as an activation marker but also as an active player of the TCR signaling pathway (6). Indeed, absence of CD5 enhanced signaling and activation of double positive (DP) thymocytes induced by TCR stimulation. Moreover, CD5 deficient DP thymocytes from TCR transgenic mice have a shifted windows of selection toward a lower threshold, resulting in an enhanced positive or negative selection with TCRs of low or high avidities, respectively (7, 8). These results established CD5 as a negative regulator of the TCR signaling pathway in immature thymocytes.

In contrast to its role in the thymus, the functions of CD5 in the periphery remain unclear. On the one hand CD5 deficient peripheral T cells showed better proliferative responses following TCR stimulation than their wild-type counterparts (8), suggesting that CD5 also acts as a negative regulator of TCR signaling in mature T cells. On the other hand, analysis of polyclonal and TCR transgenic T cells showed that effector functions of mature T cells positively correlate with CD5 expression (4, 5, 9). As a result, it has been proposed that the abundance of CD5 can predict TCR avidities with self and foreign peptides (4). Also, other reports suggested that $CD5^{hi}$ cells acquired intrinsic properties during thymic selection against self-peptides that could be maintained in periphery leading to improved reactivity against foreign antigens (5, 9).

These complex results illustrate the difficulty of assessing the impact of altered thymic selection on T cell reactivity in the periphery. Indeed, comparing results obtained in the periphery and in the thymus raises several issues. The first issue is that phenotypic differences observed in peripheral T cells could result from perturbed thymocyte education. Hence, in the case of CD5 deficient mice, the increased proliferation observed in periphery could be due to an alteration of the TCR repertoire selected during thymic development. Also, changes in selection pressure could modify the abundance of other regulators of the TCR signaling pathway. For example, it has been shown that the abundance of CD6 (a transmembrane receptor structurally related to CD5) was higher in peripheral T cells deficient for CD5 (10).The second issue is related to the difficulty of tracking the same cell during the processes of thymic selection and egression *in vivo*. Thus, although CD5 expression correlates with the magnitude of the TCR signal during DP selection and of tonic TCR signals in periphery, it does not necessarily indicate that a $CD5^{hi}$ cell in the thymus remains $CD5^{hi}$ in the periphery. Indeed, it is possible that selecting self-peptides are absent in the periphery or do not induce a similar TCR reactivity as they did during thymocyte selection. The above issues make it difficult to distinguish between direct CD5 signaling effects in peripheral T cells from indirect consequences of perturbed thymic selection. Conditional deletion of CD5 in peripheral T cells would greatly help elucidate the role of CD5 in periphery independently of its effect on thymic selection.

STRUCTURAL BASIS FOR THE NEGATIVE REGULATION EXERTED BY CD5

From a structural point of view, CD5 is a type-I transmembrane glycoprotein with an extracellular region composed of three scavenger receptor cysteine-rich (SRCR) domains. Several CD5 ligands have been reported such as CD72, the IgV(H) framework region and several polypeptides (gp40-80, gp150) whose identity remains undetermined (3, 11, 12). CD5 can also establish low stoichiometric homophilic interactions in *cis* or in *trans* (13). Whether these molecules bind to CD5 and modulate its activity in physiological settings remains a matter of debate. Even so, it has been reported that cross-linking of antibodies targeting the extracellular domain of CD5 induces signaling in the Jurkat cell line (14). In the absence of binding of CD5 with potential ligands, TCR stimulation triggers CD5 phosphorylation on tyrosine residues (15) and its translocation into the immunological synapse (16), thereby indicating a direct regulation of CD5 by TCR signals. Both types of stimulation suggest that CD5-mediated signaling inhibition could be potentiated by spatial confinement in areas where phosphatases are excluded and kinases enriched.

On its cytoplasmic tail, CD5 contains four tyrosine residues at position 402, 453, 464, and 486 in human (historically Y378, Y429, Y441, and Y463 if the signal peptide sequence is not included) exposed to potential phosphorylation regulations. Although the tyrosine Y402 was initially associated with the CD5 inhibitory signal through its association with the SH2 domain containing-tyrosine phosphatase 1 (SHP-1) (17), cumulative data from mass spectrometry analysis failed to detect phosphorylation at this position even though the corresponding peptide bearing this tyrosine residue is frequently observed (source: phosphosite.org and peptideatlas.org). In contrast, the three distal tyrosine residues (Y453, Y464, and Y486) have been frequently observed in their phosphorylated form. Moreover, studies using either phosphopeptides coding for CD5 tyrosine motifs or B cells transfected with a chimeric molecule composed of the extracellular and the transmembrane domains of FcgRIIB with the cytoplasmic domain of CD5 did not detect SHP-1 interaction (18, 19). Consistently, analysis of truncated mutants of CD5 demonstrated that the cytoplasmic tail of CD5 comprising these three distal tyrosines residues could account for global CD5 phosphorylation following pervanadate stimulation and was required for CD5 signaling activity (7). These three distal tyrosine residues are subjected to Src kinases regulation and have been proposed as docking sites for several effectors such as the RasGAP or the phosphatidylinositol 3-kinase (PI3K) (18, 20). In addition to the tyrosine dependent interactions, it has been shown that the two carboxy-terminal serine residues of CD5 allow constitutive binding with the casein kinase 2 (CK2) (21). Transgenic mouse models for which the CD5 serine motif has been deleted display abnormal T cell development and perturbed differentiation of mature T cells (22, 23). Moreover, T cells from these mice exhibit reduced survival capacity and hypoproliferate in response to TCR stimulation. These studies illustrate that CD5 signal transduction relies on both tyrosine and serine motifs.

More recently, our group demonstrated that CD5 could associate with CBL, CBLB, and GRB2 in mature $CD4^+$ T cells upon TCR stimulation (24, 25). To do so, we developed mouse models suitable for proteomics analysis in primary T lymphocytes. These mice are genetically engineered to express proteins bearing an OST tag at their N terminal, thereby serving as "baits" allowing affinity purification (AP) of protein complexes. AP samples are subjected to tandem mass spectrometry (MS-MS) and specific binding partners are identified by comparing protein intensities in samples from cells bearing the endogenous or the OST-tagged proteins. Using this approach, the set

of specific binding partners for a protein of interest, its "interactome," can be quantified in a comprehensive manner. We discuss in the following how the molecular mechanisms of CD5 signaling might be revisited in light of these recent results.

COOPERATIVITY BETWEEN CD5 AND THE UBIQUITIN LIGASES CBL AND CBLB IN MATURE T CELLS

CBL molecules (CBL and CBLB) are E3-ubiquitin ligases involved in the negative regulation of the TCR signaling pathway via different complementary mechanisms (26). CBL has been shown to control ubiquitination and degradation of the CD3 chains and activities of the proximal tyrosine kinases LCK and ZAP70 (27–29) whereas CBLB negatively regulates the CD28 co-stimulatory pathway by dampening the PI3K activity (30–32).

CBL and CBLB both target specific substrates for ubiquitination. Globally, CBLB proximal molecular environment contains more ubiquitinated species than CBL, suggesting a predominant role of CBLB over CBL for this post-translational regulation in mature T cell (24). This observation correlated with the severe phenotype of the *Cbl-b*$^{-/-}$ T cells exhibiting an increased capacity to proliferate and secrete cytokines when activated (30, 31).

Because CBL and CBLB interact together (24, 33) (**Figure 1**), it is possible that the scaffolding property of each ubiquitin ligase allows *trans*-ubiquitination of contiguous proteins not subjected to *cis*-ubiquitination. For example, although PI3K subunits are specifically associated with CBL in peripheral T cells, their ubiquitination is mainly regulated by CBLB (24, 32).

In peripheral T cells, both CBL and CBLB associated with CD5 upon TCR stimulation (24). This suggests that CD5 could play a scaffolding role, facilitating the CBL-CBLB relocalization to the plasma membrane in proximity of the tyrosine kinases required for their activities. This cooperativity between CBL, CBLB, and CD5 could also be important for enhancing ubiquitination within supra molecular complexes assembled upon TCR stimulation. In line with this model, mature T cell from CD5 deficient mouse showed reduced CBL-dependent ubiquitination in activated T cells (24). More specifically, ubiquitination of PI3K subunits following TCR stimulation was reduced in the absence of CD5 suggesting that CD5 could facilitate *trans*-ubiquitination.

In the CBLB deficient T cells, association of CD5 with CBL was preserved. Interestingly, the absence of CBLB enhanced the interaction between CD5 and CBL and increased global protein ubiquitination within the complex formed around CBL (24). These results suggest that CBL molecules compete for binding to shared docking sites on CD5 and in the ubiquitination of shared substrates. Also, they indicate that despite a molecular reorganization in the absence of CBLB, CBL is unable to fully compensate for CBLB deficiency in mature T cells. Hence, in peripheral T cells, CD5 could negatively control TCR signaling by coordinating ubiquitination through its interaction with CBL and CBLB.

INTERACTIONS BETWEEN CD5 AND CBL IN THYMOCYTES

The situation described above is modified in thymocytes where abundances of CBL molecules differ from that in mature T cells. While CBL and CBLB have similar abundances in peripheral T cells, protein expression of CBLB is much lower than CBL in thymocytes (24). In agreement with observations reported in CBLB deficient mature T cells, analysis of the CBL interactome in thymocytes revealed that the association between CD5 and CBL is maintained despite the low abundance of CBLB (**Figure 1**). These results suggest the existence of a functional relationship between CBL and CD5 in thymocytes. The comparison of the phenotypes between the CBL and CD5 deficient mice partially supports this hypothesis. Indeed, both CBL and CD5 deficient DP thymocytes show enhanced intracellular signaling which, onto a low avidity TCR transgenic background, lead to increased positive selection (35, 36). However, in contrast to *CD5*$^{-/-}$ mice, DP thymocytes of *CBL*$^{-/-}$ mice have elevated TCR levels due to reduced TCR degradation and increased TCR recycling (37, 38). Thus, increased TCR reactivity in *CBL*$^{-/-}$ DP thymocytes could essentially reflects the increased abundance of the TCR at the plasma membrane. This phenotype might mask another function of CBL. Indeed, considering that the interaction between CBL and CD5 depends on TCR stimulation, the increased TCR responses observed in CD5 deficient mouse could reflect a specific role of CBL strictly dependent on TCR engagement. Hence, two mechanisms of TCR signaling regulation involving CBL could coexist (**Figure 2A**). One where CBL, independently of CD5, regulates the constitutive TCR pool at the surface of DP thymocytes and another one, triggered by the TCR stimulation, relocating a fraction of CBL molecules to the synapse via CD5 and promoting its inhibitory activity (ubiquitination) in this particular cellular localization. In this molecular context, specific effectors of the proximal TCR signaling pathway could be negatively controlled by CBL.

Hence, both in thymocytes and in mature T cells, CBL molecules are possible molecular mediators of CD5 inhibition of TCR signaling (**Figure 2B**).

CONTRIBUTIONS OF UBASH3A/B MOLECULES TO CD5 INHIBITION

Other molecules than CD5 associated with both CBL and CBLB in TCR stimulated mature T cells (24). Among them, the Ubiquitin-associated and SH3 domain-containing protein A and B (UBASH3A, UBASH3B also known as STS-2 and STS-1) have been associated with negative regulation of the TCR signaling pathway (39) and might therefore participate in CD5 inhibition.

Association of CBL with UBASH3A was detected in unstimulated thymocytes and mature T cells and remained unchanged upon TCR stimulation (**Figure 1**). In contrast, UBASH3B was associated with CBL only upon TCR stimulation and this recruitment correlated with that of CBLB. This suggests preferential associations of CBL with UBASH3A and CBLB with UBASH3B. In support of this statement, the expression pattern of

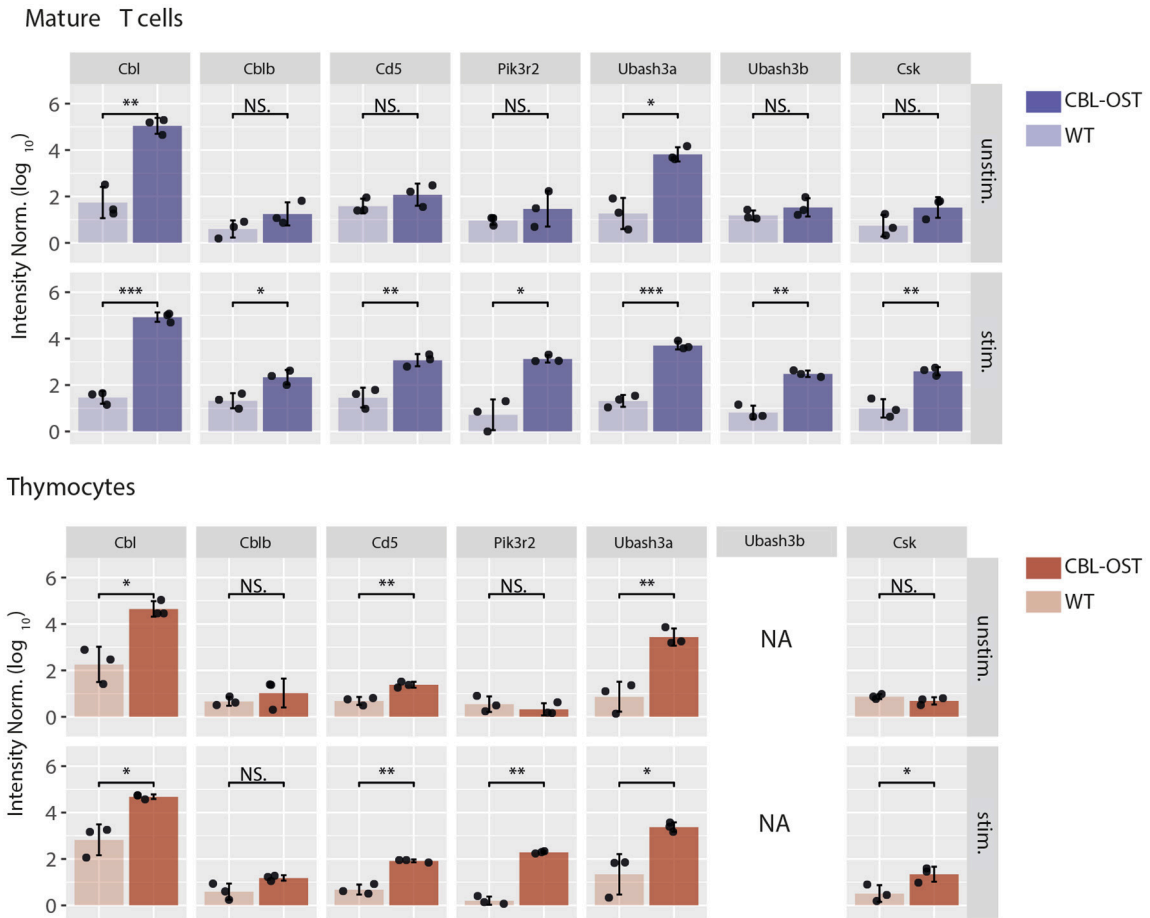


FIGURE 1 | Association of selected proteins with CBL in thymocytes and mature T cells. Mature CD4$^+$ T cells and thymocytes from wild-type (WT) and gene-targeted mice expressing One-STrEP-tag at the carboxyl-terminus of endogenous CBL (CBL-OST) were left unstimulated (unstim.) or stimulated for 30 s (stim.) with anti-CD3 plus anti-CD4 antibodies and subsequently lysed. Protein lysates were subjected to OST affinity purification coupled to mass spectrometry analysis (AP-MS) (24, 34). For each sample, protein intensities were log transformed and normalized by the sample median intensity. Intensities were then averaged across technical replicates and missing values imputed by values simulating noise around the detection limit. After missing values imputation, log-transformed intensities from WT and CBL-OST cells were compared using a two-sided Welch *t*-test (symbols used according to the *t*-test *P*-value: N.S., $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). Intensities were divided by the minimum intensity across all intensities represented to ensure that all log-transformed values were positive. Data used for mature CD4$^+$ T cells are from Voisinne et al. (24) (NA, non-applicable).

UBASH3A and UBASH3B proteins in alpha/beta T cells is similar to that of CBL and CBLB, respectively (39) (www.immgen.org). Hence, UBASH3A is highly expressed in DP thymocytes whereas expression of UBASH3B starts in single positive (SP) thymocytes.

Both UBASH3 molecules exert phosphatase activities and bind ubiquitinated proteins though their UBA domains (40, 41). When both molecules are inactivated, mature T cells showed an enhanced capacity to proliferate and secrete cytokines, a phenotype reminiscent of those observed with CD5 and CBLB deficient mice (39). In addition, in dually UBASH3 deficient mouse, TCR stimulation triggers increased tyrosine phosphorylation and ubiquitination of signaling effectors (39). The simultaneous increase of these post-translational modifications could be due to the fact that the activation-deactivation sequence of specific effectors is stopped at a stage where they have been phosphorylated by tyrosine kinases, ubiquitinated by CBL molecules but subsequently improperly dephosphorylated or targeted for degradation as they should when UBASH molecules bind to ubiquitin. As confirmed by recent studies, one of the first targets of this regulation is ZAP-70 (42, 43). In this context, it is possible that CD5 allows molecular cooperativity between CBL and UBASH3 molecules to terminate TCR induced signaling by dampening the activity of ZAP-70 kinase and by contributing to its degradation (**Figure 2B**).

CD5-MEDIATED REGULATION OF CSK

Another potential mediator of the CD5 inhibition that was also detected with both CBL and CBLB after TCR engagement is the tyrosine kinase CSK. The recruitment of CD5 and CSK to both CBL molecules was correlated indicating a possible physical

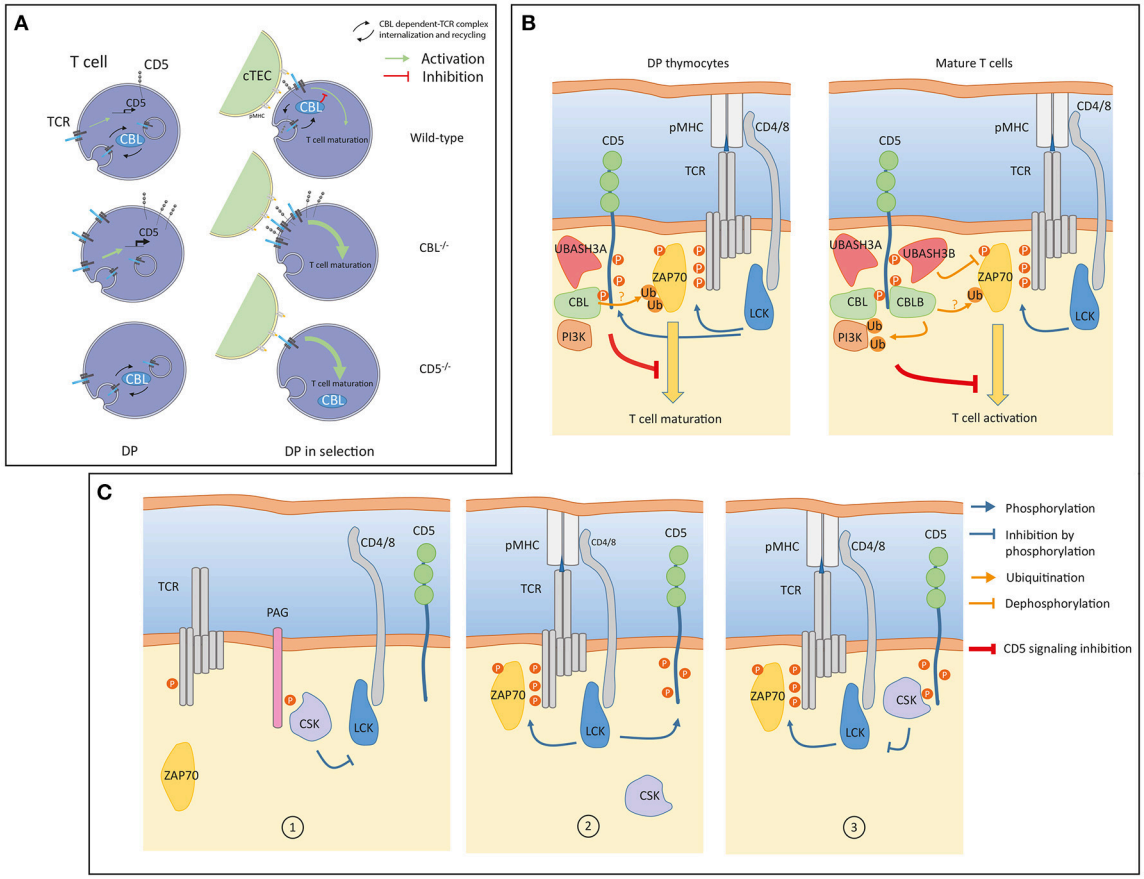


FIGURE 2 | (A) A model of signaling in double positive (DP) thymocytes from wild-type, *CBL*$^{-/-}$ and *CD5*$^{-/-}$ mice. Prior selection (left), constitutive TCR internalization, recycling and degradation are regulated by CBL. CD5 is not involved in these processes but CD5 protein level is transcriptionally controlled by weak constitutive TCR signaling (green arrow). In the absence of CBL, surface TCR concentration increases which enhances transcription of CD5. Surface TCR concentration is unaffected in CD5 deficient cells and remains controlled by CBL. During selection (right), CBL associates with CD5 within the immunological synapse (IS) to negatively control TCR signaling. In the absence of CBL, TCRs accumulate at the cell surface leading to increased TCR signaling. The inhibition of TCR signaling by CD5 is impaired in the absence of CBL. In CD5 deficient cells, recruitment of CBL to the immunological synapse (IS) is impaired which leads to enhanced TCR signaling. (cTEC: Cortical thymic epithelial cell) **(B)** A model of CD5 signaling in thymocytes and mature T cells. Upon TCR engagement, LCK phosphorylates the CD3 chains and CD5 (blue arrows). Phosphorylation of CD5 allows interactions of inhibitory molecules such as CBL and UBASH3 proteins triggering post-translation modifications (ubiquitination, dephosphorylation) of positive effectors (ZAP70, PI3K) involved in the proximal TCR signaling pathway. The global negative signal mediated by CD5 is symbolized by the inhibitory red line. In thymocytes, CBLB and UBASH3B expressions are undetectable, CD5 associated only with CBL and UBASH3A. The PI3K interacts with CBL in thymocytes and in peripheral T cells. PI3K regulation by ubiquitination is essentially mediated by CBLB. **(C)** An alternative model of CD5 signaling involving CSK. In quiescent T cells CSK interacts with PAG to negatively control LCK (1). Upon TCR engagement LCK phosphorylates CD5 (2). CSK molecules associated with phosphorylated CD5 localized into the IS. CD5-associated CSK phosphorylates the inhibitory tyrosine residue of LCK thereby reducing the magnitude of TCR signaling (3). Panel **(A)** was modified from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

association between them. This association was confirmed by co-immunoprecipation of CSK with CD5 upon TCR stimulation (24). CSK has been shown to control the activity of Src kinases by phosphorylating their C-terminal tyrosine residue (44). In turn, CSK activity depends on its association with the transmembrane adaptor PAG (45, 46). In this context, CD5 ligation was shown to induce the phosphorylation of the Src kinase Fyn at its C-terminal inhibitory residue and attenuate its activity (14). To explain this observation, it has been proposed that CD5 could interfere with the disassembly of the CSK-PAG complexes during T cell activation. However, in contrast to CSK and CD5, PAG was not identified as a binding partner of either CBL or CBLB in TCR stimulated mature T cells. This suggest that different pools of CSK are present in T cells, within different protein complexes. Moreover, a recent study has demonstrated that PAG-regulated TCR signaling is essentially active in effector T cells (47). Thus, it is conceivable that the facilitation of CSK recruitment to the synapse could operate through alternative transmembrane adaptors, and possibly directly with CD5, depending of the activation state of T cells. An attractive model could be that CD5 binds to CSK through its SH2 domain. In this setting, the interaction between CD5 and CSK, induced by TCR stimulation,

could participate in a negative feedback loop by reducing the activity of Src kinase recruited to the synapse (**Figure 2C**).

CD5 AND IMMUNOTHERAPY

Accumulated knowledge on immunomodulatory properties of CD5 positions this receptor as a putative checkpoint inhibitor, potentially useful in the context of immunotherapies. In this context, one way to harness the inhibitory functions of CD5 would be the development of anti-CD5 monoclonal antibodies (mAb) having diverse functional properties. Thus, mAb with the ability to sequester the receptor away from the T cell synapse could be useful to reduce CD5 inhibitory signaling and increase T cell responses against tumors. Alternatively, anti-CD5 mAbs enhancing the inhibitory role of the receptor could help improve autoimmune diseases by reducing effector functions of autoreactive T cells.

Prior the emergence of antibody-based cancer treatments, results of clinical trials using anti-CD5 mAb have established moderated benefit in patients with chronic lymphocyte leukemia or cutaneous T-cell lymphomas (48, 49). With the recent advances in immunotherapies, experimental protocols have evolved and critical factors have been identified to improve treatment efficacies. For example, manipulation of antibody structure to avoid rapid clearance and immune response against the therapeutic mAb is one of the issue that could be investigated with CD5. Also, evaluation of biological effects provided by combination with other antibodies in a broader spectrum of malignancies could reveal CD5 as a potent target to control cancer.

However, immunotherapy strategies targeting CD5 should be the object of cautious attention. Indeed, as CD5 is expressed in all T cell subsets and on B-1a B cells, *in vivo* administration of CD5 specific antibodies will result in the sum of individual cell type responses. For example, it has been shown that generation of induced Treg (iTreg) cells is altered among CD5 low or CD5 deficient T cell populations (50). It is therefore likely that inhibition of CD5 would simultaneously reduce iTreg cell number and activate effector functions on conventional T cells, thereby increasing T cell reactivity against self and potentiating auto-immune disorders. In addition and as proposed by studies using a mouse expressing a serine-truncated CD5 form, signaling of the receptor can also affect T cell differentiation toward specific Th subsets (51). Therefore, all these parameters must be taken into consideration in order to avoid the onset of undesirable reactions resulting from complex global effects.

CONCLUDING REMARKS

Overall, it appears that distinct molecular mechanisms remain possible to explain the negative regulation of TCR signaling exerted by CD5 in thymocytes, naïve and effector T cells. As illustrated by the different signaling models presented here, CD5 could act as a scaffold coordinating the action of CBL, UBASH3 and CSK molecules within the immunological synapse. In conclusion, CD5 and the identified effectors involved in the same signaling pathway offer great potential for the development of new drugs. However, complexity of the molecular relationships and difficulties to predict perturbations of the system must be taken into account prior to the design of new therapeutic strategies.

AUTHOR CONTRIBUTIONS

GV, AGP, and RR generated, analyzed the data and wrote the manuscript.

ACKNOWLEDGMENTS

We thank Bernard Malissen and Renaud Lesourne for discussion. This work was supported by CNRS, INSERM, Agence Nationale de la Recherche (LymphoScan project to RR), the Investissement d'Avenir program ProFI (Proteomics French Infrastructure project; ANR-10-INBS-08) and PHENOMIN (French National Infrastructure for Mouse Phenogenomics), Région Midi-Pyrénées.

REFERENCES

1. Schraven B, Kalinke U. CD28 superagonists: what makes the difference in humans? *Immunity* (2008) 28:591–5. doi: 10.1016/j.immuni.2008.04.003
2. Azzam HS, Grinberg A, Lui K, Shen H, Shores EW, Love PE. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J Exp Med.* (1998) 188:2301–11. doi: 10.1084/jem.188.12.2301
3. Biancone L, Bowen MA, Lim A, Aruffo A, Andres G, Stamenkovic I. Identification of a novel inducible cell-surface ligand of CD5 on activated lymphocytes. *J Exp Med.* (1996) 184:811–9. doi: 10.1084/jem.184.3.811
4. Mandl JN, Monteiro JP, Vrisekoop N, Germain RN. T cell-positive selection uses self-ligand binding strength to optimize repertoire recognition of foreign antigens. *Immunity* (2013) 38:263–74. doi: 10.1016/j.immuni.2012.09.011
5. Persaud SP, Parker CR, Lo WL, Weber KS, Allen PM. Intrinsic CD4$^+$ T cell sensitivity and response to a pathogen are set and sustained by avidity for thymic and peripheral complexes of self peptide and MHC. *Nat Immunol.* (2014) 15:266–74. doi: 10.1038/ni.2822
6. Tarakhovsky A, Kanner SB, Hombach J, Ledbetter JA, Muller W, Killeen N, et al. A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Science* (1995) 269:535–7. doi: 10.1126/science.7542801
7. Azzam HS, DeJarnette JB, Huang K, Emmons R, Park CS, Sommers CL, et al. Fine tuning of TCR signaling by CD5. *J Immunol.* (2001) 166:5464–72. doi: 10.4049/jimmunol.166.9.5464
8. Pena-Rossi C, Zuckerman LA, Strong J, Kwan J, Ferris W, Chan S, et al. Negative regulation of CD4 lineage development and responses by CD5. *J Immunol.* (1999) 163:6494–501.
9. Fulton RB, Hamilton SE, Xing Y, Best JA, Goldrath AW, Hogquist KA, et al. The TCR's sensitivity to self peptide-MHC dictates the ability of naive CD8 T cells to respond to foreign antigens. *Nat Immunol.* (2014) 16:107–17. doi: 10.1038/ni.3043
10. Orta-Mascaro M, Consuegra-Fernandez M, Carreras E, Roncagalli R, Carreras-Sureda A, Alvarez P, et al. CD6 modulates thymocyte selection and peripheral T cell homeostasis. *J Exp Med.* (2016) 213:1387–97. doi: 10.1084/jem.20151785

11. Van de Velde H, von Hoegen I, Luo W, Parnes JR, Thielemans K. The B-cell surface protein CD72/Lyb-2 is the ligand for CD5. *Nature* (1991) 351:662–5. doi: 10.1038/351662a0
12. Calvo J, Places L, Padilla O, Vila JM, Vives J, Bowen MA, et al. Interaction of recombinant and natural soluble CD5 forms with an alternative cell surface ligand. *Eur J Immunol.* (1999) 29:2119–29. doi: 10.1002/(SICI)1521-4141(199907)29:07<2119::AID-IMMU2119>3.0.CO;2-F
13. Brown MH, Lacey E. A ligand for CD5 is CD5. *J Immunol* (2010) 185:6068–74. doi: 10.4049/jimmunol.0903823
14. Bamberger M, Santos AM, Goncalves CM, Oliveira MI, James JR, Moreira A, et al. A new pathway of CD5 glycoprotein-mediated T cell inhibition dependent on inhibitory phosphorylation of Fyn kinase. *J Biol Chem.* (2011) 286:30324–36. doi: 10.1074/jbc.M111.230102
15. Davies AA, Ley SC, Crumpton MJ. CD5 is phosphorylated on tyrosine after stimulation of the T-cell antigen receptor complex. *Proc Natl Acad Sci USA.* (1992) 89:6368–72. doi: 10.1073/pnas.89.14.6368
16. Brossard C, Semichon M, Trautmann A, Bismuth G. CD5 inhibits signaling at the immunological synapse without impairing its formation. *J Immunol.* (2003) 170:4623–9. doi: 10.4049/jimmunol.170.9.4623
17. Perez-Villar JJ, Whitney GS, Bowen MA, Hewgill DH, Aruffo AA, Kanner SB. CD5 negatively regulates the T-cell antigen receptor signal transduction pathway, involvement of SH2-containing phosphotyrosine phosphatase SHP-1. *Mol Cell Biol.* (1999) 19:2903–12. doi: 10.1128/MCB.19.4.2903
18. Dennehy KM, Broszeit R, Ferris WF, Beyers AD. Thymocyte activation induces the association of the proto-oncoprotein c-cbl and ras GTPase-activating protein with CD5. *Eur J Immunol.* (1998) 28:1617–25. doi: 10.1002/(SICI)1521-4141(199805)28:05<1617::AID-IMMU1617>3.0.CO;2-7
19. Gary-Gouy H, Bruhns P, Schmitt C, Dalloul A, Daeron M, Bismuth G. The pseudo-immunoreceptor tyrosine-based activation motif of CD5 mediates its inhibitory action on B-cell receptor signaling. *J Biol Chem.* (2000) 275:548–56. doi: 10.1074/jbc.275.1.548
20. Dennehy KM, Broszeit R, Garnett D, Durrheim GA, Spruyt LL, Beyers AD. Thymocyte activation induces the association of phosphatidylinositol 3-kinase and pp120 with CD5. *Eur J Immunol.* (1997) 27:679–86. doi: 10.1002/eji.1830270316
21. Raman C, Kuo A, Deshane J, Litchfield DW, Kimberly RP. Regulation of casein kinase 2 by direct interaction with cell surface receptor CD5. *J Biol Chem.* (1998) 273:19183–9. doi: 10.1074/jbc.273.30.19183
22. Axtell RC, Xu L, Barnum SR, Raman C. CD5-CK2 binding/activation-deficient mice are resistant to experimental autoimmune encephalomyelitis, protection is associated with diminished populations of IL-17-expressing T cells in the central nervous system. *J Immunol.* (2006) 177:8542–9. doi: 10.4049/jimmunol.177.12.8542
23. Sestero CM, McGuire DJ, De Sarno P, Brantley EC, Soldevila G, Axtell RC, et al. CD5-dependent CK2 activation pathway regulates threshold for T cell anergy. *J Immunol.* (2012) 189:2918–30. doi: 10.4049/jimmunol.1200065
24. Voisinne G, Garcia-Blesa A, Chaoui K, Fiore F, Bergot E, Girard L, et al. Co-recruitment analysis of the CBL and CBLB signalosomes in primary T cells identifies CD5 as a key regulator of TCR-induced ubiquitylation. *Mol Syst Biol.* (2016) 12:876. doi: 10.15252/msb.20166837
25. Caron E, Roncagalli R, Hase T, Wolski WE, Choi M, Menoita MG, et al. Precise temporal profiling of signaling complexes in primary cells using SWATH mass spectrometry. *Cell Rep.* (2017) 18:3219–26. doi: 10.1016/j.celrep.2017.03.019
26. Huang F, Gu H. Negative regulation of lymphocyte development and function by the Cbl family of proteins. *Immunol Rev.* (2008) 224:229–38. doi: 10.1111/j.1600-065X.2008.00655.x
27. Wang HY, Altman Y, Fang D, Elly C, Dai Y, Shao Y, et al. Cbl promotes ubiquitination of the T cell receptor zeta through an adaptor function of Zap-70. *J Biol Chem.* (2001) 276:26004–11. doi: 10.1074/jbc.M010738200
28. Thien CB, Bowtell DD, Langdon WY. Perturbed regulation of ZAP-70 and sustained tyrosine phosphorylation of LAT and SLP-76 in c-Cbl-deficient thymocytes. *J Immunol.* (1999) 162:7133–9.
29. Thien CB, Langdon WY. c-Cbl and Cbl-b ubiquitin ligases, substrate diversity and the negative regulation of signalling responses. *Biochem J.* (2005) 391:153–66. doi: 10.1042/BJ20050892
30. Chiang YJ, Kole HK, Brown K, Naramura M, Fukuhara S, Hu RJ, et al. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* (2000) 403:216–20. doi: 10.1038/35003235
31. Bachmaier K, Krawczyk C, Kozieradzki I, Kong YY, Sasaki TA, Oliveira-dos-Santos, A, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* (2000) 403:211–16. doi: 10.1038/35003228
32. Fang D, Liu YC. Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. *Nat Immunol.* (2001) 2:870–5. doi: 10.1038/ni0901-870
33. Rorsman C, Tsioumpekou M, Heldin CH, Lennartsson J. The ubiquitin ligases c-Cbl and Cbl-b negatively regulate platelet-derived growth factor (PDGF) BB-induced chemotaxis by affecting PDGF receptor beta (PDGFRbeta) internalization and signaling. *J Biol Chem.* (2016) 291:11608–18. doi: 10.1074/jbc.M115.705814
34. Roncagalli R, Hauri S, Fiore F, Liang Y, Chen Z, Sansoni A, et al. Quantitative proteomics analysis of signalosome dynamics in primary T cells identifies the surface receptor CD6 as a Lat adaptor-independent TCR signaling hub. *Nat Immunol.* (2014) 15:384–92. doi: 10.1038/ni.2843
35. Murphy MA, Schnall RG, Venter DJ, Barnett L, Bertoncello I, Thien CB, et al. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. *Mol Cell Biol.* (1998) 18:4872–82. doi: 10.1128/MCB.18.8.4872
36. Naramura M, Kole HK, Hu RJ, Gu H. Altered thymic positive selection and intracellular signals in Cbl-deficient mice. *Proc Natl Acad Sci USA.* (1998) 95:15547–52. doi: 10.1073/pnas.95.26.15547
37. Naramura M, Jang IK, Kole H, Huang F, Haines D, Gu H. c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. *Nat Immunol.* (2002) 3:1192–9. doi: 10.1038/ni855
38. Myers MD, Sosinowski T, Dragone LL, White C, Band H, Gu H, et al. Src-like adaptor protein regulates TCR expression on thymocytes by linking the ubiquitin ligase c-Cbl to the TCR complex. *Nat Immunol.* (2006) 7:57–66. doi: 10.1038/ni1291
39. Carpino N, Turner S, Mekala D, Takahashi Y, Zang H, Geiger TL, et al. Regulation of ZAP-70 activation and TCR signaling by two related proteins, Sts-1 and Sts-2. *Immunity* (2004) 20:37–46. doi: 10.1016/S1074-7613(03)00351-0
40. Carpino N, Chen Y, Nassar N, Oh HW. The Sts proteins target tyrosine phosphorylated, ubiquitinated proteins within TCR signaling pathways. *Mol Immunol.* (2009) 46:3224–31. doi: 10.1016/j.molimm.2009.08.015
41. Hoeller D, Crosetto N, Blagoev B, Raiborg C, Tikkanen R, Wagner S, et al. Regulation of ubiquitin-binding proteins by monoubiquitination. *Nat Cell Biol.* (2006) 8:163–9. doi: 10.1038/ncb1354
42. Yang M, Chen T, Li X, Yu Z, Tang S, Wang C, et al. K33-linked polyubiquitination of Zap70 by Nrdp1 controls $CD8^+$ T cell activation. *Nat Immunol.* (2015) 16:1253–62. doi: 10.1038/ni.3258
43. Hu H, Wang H, Xiao Y, Jin J, Chang JH, Zou Q, et al. Otud7b facilitates T cell activation and inflammatory responses by regulating Zap70 ubiquitination. *J Exp Med.* (2016) 213:399–414. doi: 10.1084/jem.20151426
44. Okada M, Nada S, Yamanashi Y, Yamamoto T, Nakagawa H. CSK, a protein-tyrosine kinase involved in regulation of src family kinases. *J Biol Chem.* (1991) 266:24249–52.
45. Brdicka T, Pavlistova D, Leo A, Bruyns E, Korinek V, Angelisova P, et al. Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. *J Exp Med.* (2000) 191:1591–604. doi: 10.1084/jem.191.9.1591
46. Davidson D, Bakinowski M, Thomas ML, Horejsi V, Veillette A. Phosphorylation-dependent regulation of T-cell activation by PAG/Cbp, a lipid raft-associated transmembrane adaptor. *Mol Cell Biol.* (2003) 23:2017–28. doi: 10.1128/MCB.23.6.2017-2028.2003
47. Davidson D, Zhong MC, Pandolfi PP, Bolland S, Xavier RJ, Seed B, et al. The Csk-associated adaptor PAG inhibits effector T cell activation in cooperation with phosphatase PTPN22 and dok adaptors. *Cell Rep.* (2016) 17:2776–88. doi: 10.1016/j.celrep.2016.11.035
48. Dillman RO, Shawler DL, Dillman JB, Royston I. Therapy of chronic lymphocytic leukemia and cutaneous T-cell lymphoma with T101 monoclonal antibody. *J Clin Oncol.* (1984) 2:881–91. doi: 10.1200/JCO.1984.2.8.881

49. Foss FM, Raubitscheck A, Mulshine JL, Fleisher TA, Reynolds JC, Paik CH, et al. Phase I study of the pharmacokinetics of a radioimmunoconjugate, 90Y-T101, in patients with CD5-expressing leukemia and lymphoma. *Clin Cancer Res*. (1998) 4:2691–700.
50. Henderson JG, Opejin A, Jones A, Gross C, Hawiger D. CD5 instructs extrathymic regulatory T cell development in response to self and tolerizing antigens. *Immunity* (2015) 42:471–83. doi: 10.1016/j.immuni.2015.02.010
51. McGuire DJ, Rowse AL, Li H, Peng BJ, Sestero CM, Cashman KS, et al. CD5 enhances Th17-cell differentiation by regulating IFN-gamma response and RORgammat localization. *Eur J Immunol*. (2014) 44:1137–42. doi: 10.1002/eji.201343998

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 18 December 2018
doi: 10.3389/fimmu.2018.02994

CD6, a Rheostat-Type Signalosome That Tunes T Cell Activation

Carine M. Gonçalves[1,2], *Sónia N. Henriques*[1,2,3], *Rita F. Santos*[1,2,3] *and Alexandre M. Carmo*[1,2]*

[1] Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal, [2] Instituto de Biologia Molecular e Celular, Porto, Portugal, [3] Instituto de Ciências Biomédicas Abel Salazar and Faculdade de Ciências, Universidade do Porto, Porto, Portugal

Following T cell receptor triggering, T cell activation is initiated and amplified by the assembly at the TCR/CD3 macrocomplex of a multitude of stimulatory enzymes that activate several signaling cascades. The potency of signaling is, however, modulated by various inhibitory components already at the onset of activation, long before co-inhibitory immune checkpoints are expressed to help terminating the response. CD5 and CD6 are surface glycoproteins of T cells that have determinant roles in thymocyte development, T cell activation and immune responses. They belong to the superfamily of scavenger receptor cysteine-rich (SRCR) glycoproteins but whereas the inhibitory role of CD5 has been established for long, there is still controversy on whether CD6 may have similar or antagonistic functions on T cell signaling. Analysis of the structure and molecular associations of CD5 and CD6 indicates that these molecules assemble at the cytoplasmic tail a considerable number of signaling effectors that can putatively transduce diverse types of intracellular signals. Biochemical studies have concluded that both receptors can antagonize the flow of TCR-mediated signaling; however, the impact that CD5 and CD6 have on T cell development and T cell-mediated immune responses may be different. Here we analyze the signaling function of CD6, the common and also the different properties it exhibits comparing with CD5, and interpret the functional effects displayed by CD6 in recent animal models.

Keywords: inhibitory receptors, CD5, CD6, signalosome, T lymphocytes

OPEN ACCESS

Edited by:
Bernard Malissen, INSERM U1104 Centre d'Immunologie de Marseille-Luminy, France

Reviewed by:
Renaud Lesourne, INSERM U1043 Centre de Physiopathologie de Toulouse Purpan, France
Jacques A. Nunes, INSERM U1068 Centre de Recherche en Cancérologie de Marseille, France

***Correspondence:**
Alexandre M. Carmo
acarmo@ibmc.up.pt

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *31 July 2018*
Accepted: *04 December 2018*
Published: *18 December 2018*

Citation:
Gonçalves CM, Henriques SN, Santos RF and Carmo AM (2018) CD6, a Rheostat-Type Signalosome That Tunes T Cell Activation. Front. Immunol. 9:2994. doi: 10.3389/fimmu.2018.02994

INTRODUCTION

Antigen-specific T cell activation is triggered by the T cell receptor (TCR) recognition of a cognate peptide presented by antigen presenting cells (APC), but it is overall controlled by a plethora of other cell surface receptors that either increase or repress the strength of the signals, the combination of which determines the outcome of T cell-mediated responses. Most of the receptors do not contain intrinsic enzymatic activities so their function relies on the establishment of interactions with signaling effectors, and also on an appropriate localization where they can exert their role, determined extracellularly by the binding to specific ligands expressed on the APC and intracellularly through connecting with the cytoskeleton.

Inhibitory co-receptors, such as the immune checkpoints Programmed cell death protein 1 (PD-1), Cytotoxic T-lymphocyte-associated protein 4 (CTLA4), T cell immunoreceptor with Ig and ITIM domains (TIGIT), Lymphocyte-activation gene 3 (LAG-3), and B- and T-lymphocyte attenuator (BTLA) are crucial to halt the progression or to terminate cell activation once they become expressed, given that they are strongly induced upon activation (1–5). They exert their

inhibitory effect intracellularly through very limited and defined interactions utilizing ITIM, ITSF, or other sequences of their cytoplasmic tails that are bound by inhibitory enzymes, typically serine/threonine or tyrosine phosphatases (6–10). Moreover, some of these inhibitory co-receptors also interfere with co-stimulatory receptors through extracellular competition for the same ligands (11–14).

A different class of inhibitory receptors that can be active immediately following the triggering of the TCR and thus modulate T cell signaling at the onset of activation is exemplified by CD5: constitutively expressed on nearly all T cell subsets, including naïve and non-activated cells (15, 16), and loosely interacting with the TCR/CD3 complex (17), CD5 is able to modulate responses concomitantly with TCR triggering (18, 19). The expression of CD5 can increase significantly upon thymocyte and mature T cell activation such that the potency of the inhibition is proportionally adjusted in accordance to the affinity of TCR recognition of peptide/MHC and TCR-dependent signaling intensity (20, 21). This indicates that the variable levels of CD5 expression are important to counteract the strength of TCR signaling (22).

CD6 shares with CD5 many genetic, structural, and functional characteristics, among them the capacity to interact with the TCR/CD3 complex, to be tyrosine-phosphorylated and activated upon TCR triggering, and slightly increases its surface expression upon T cell activation (23–26). However, some differences between the kinetics of expression of CD5 and CD6 during thymocyte ontogeny and selection and T cell subset polarization as well as apparent different requirements for ligand binding suggest that CD5 and CD6 may have non-coincident roles during thymocyte development, T cell activation and immune responses. Nevertheless, in cellular systems it was also shown that the strength of activation correlates inversely with the expression of CD6 (27). Therefore, similarly to CD5 and distinctive from the immune checkpoints that can shut down activation at later stages, CD6 may be a rheostat-type regulator of activation, fine-tuning the response depending on the strength of the antigenic challenge.

CD6 IS A HUB FOR THE ASSEMBLY OF STIMULATORY AND INHIBITORY EFFECTORS

At the time of the cloning of their genes and initial functional characterization of the proteins, CD5 and CD6 were regarded as co-stimulatory receptors that amplified TCR-dependent activation (28–31). In the case of CD5, the function of the molecule was soon after revised following the development of mice with a disrupted *Cd5* gene (18). The lack of similar definitive models addressing the role of CD6 *in vivo* until very recently delayed significantly the progress on CD6 research, and caused that the knowledge on the function of CD6 is still lagging considerably behind.

There are many common aspects in the biochemical behavior of CD5 and CD6 and in fact they can interact with each other in non-activated T cells (32, 33). Upon antigen recognition and T cell-APC conjugation, both receptors localize at the center of the immunological synapse (33). In contact with the TCR/CD3 signaling machinery, CD5 and CD6 are very rapidly phosphorylated on tyrosine residues (19, 24), presumably by the SRC-family kinase LCK, with the concomitant docking of intracellular mediators that contain SH2 domains, semi-autonomous conserved structural domains that bind to phosphorylated tyrosine residues. The net contribution of either CD5 and CD6 appears to be inhibitory, given that cells that lack any of the receptors are significantly more responsive to antigenic or mitogenic stimulation (22, 34). However, the number and diversity of effectors that associate with CD5 and/or CD6, depending or not on tyrosine phosphorylation, would not give an obvious idea of the repressive potential of the receptors, given that many interacting partners are effectively protein tyrosine kinases that are normally associated with signaling progression. These include LCK, FYN, ZAP70, and additionally in the case of CD6, the TEC-family kinase ITK (32, 35–37).

Perhaps this aggregation of kinases at the cytoplasmic tail of CD5 and CD6 explains the behavior observed in their initial characterization when either receptor, when triggered together with the TCR/CD3 complex with monoclonal antibodies, amplified the activation signals originated at the TCR complex. Notwithstanding this possibly artifactual contribution to activation determined by the *in vitro* experimental design, it is also possible that the kinases may actually contribute to positive signaling via CD5 and CD6 in very defined contexts, thus explaining the dual function that has been many times attributed to CD6 and occasionally to CD5.

CD5 contains four tyrosine residues on its cytoplasmic domain, that when phosphorylated constitute putative sites for the docking of SH2 domain-containing cytoplasmic molecules. Tyrosine 402 is close or even buried within the plasma membrane and therefore it is disputable whether it can actually be phosphorylated. Nonetheless, the remaining tyrosine residues of CD5, when phosphorylated, have been for a long time shown to bind to the tyrosine kinase LCK (35), the tyrosine phosphatase SHP1 (38, 39), the ubiquitin ligases CBL and CBLB (40, 41), the GTPase activating protein for RAS (RASGAP) (40) and the lipid kinase PI3K (42), while the associations of CD5 with the protein kinases FYN and ZAP70 have not been shown to be direct (**Figure 1A**).

CD6 possesses possibly the longest cytoplasmic tail of the known receptors of leukocytes, containing amongst other signaling motifs nine tyrosine residues. However, fewer than expected interactions of CD6 with SH2 domain-containing effectors have been reported to date, possibly because research on CD6 function has been performed less systematically than that on CD5 (**Figure 1B**). Moreover, most of the disclosed interactors of CD6 seem to be more related to activation pathways rather than to repression mechanisms, including the tyrosine kinases LCK, FYN, ZAP70, and ITK that were shown to be associated with CD6 but not confirmed to be dependent on phosphotyrosine-SH2 domain binding. Additionally, the adaptors SLP76 (27), TSAD (43), GADS, and GRB2 (44), that have established roles in T cell activation have been shown to bind to the two most carboxyl-terminal

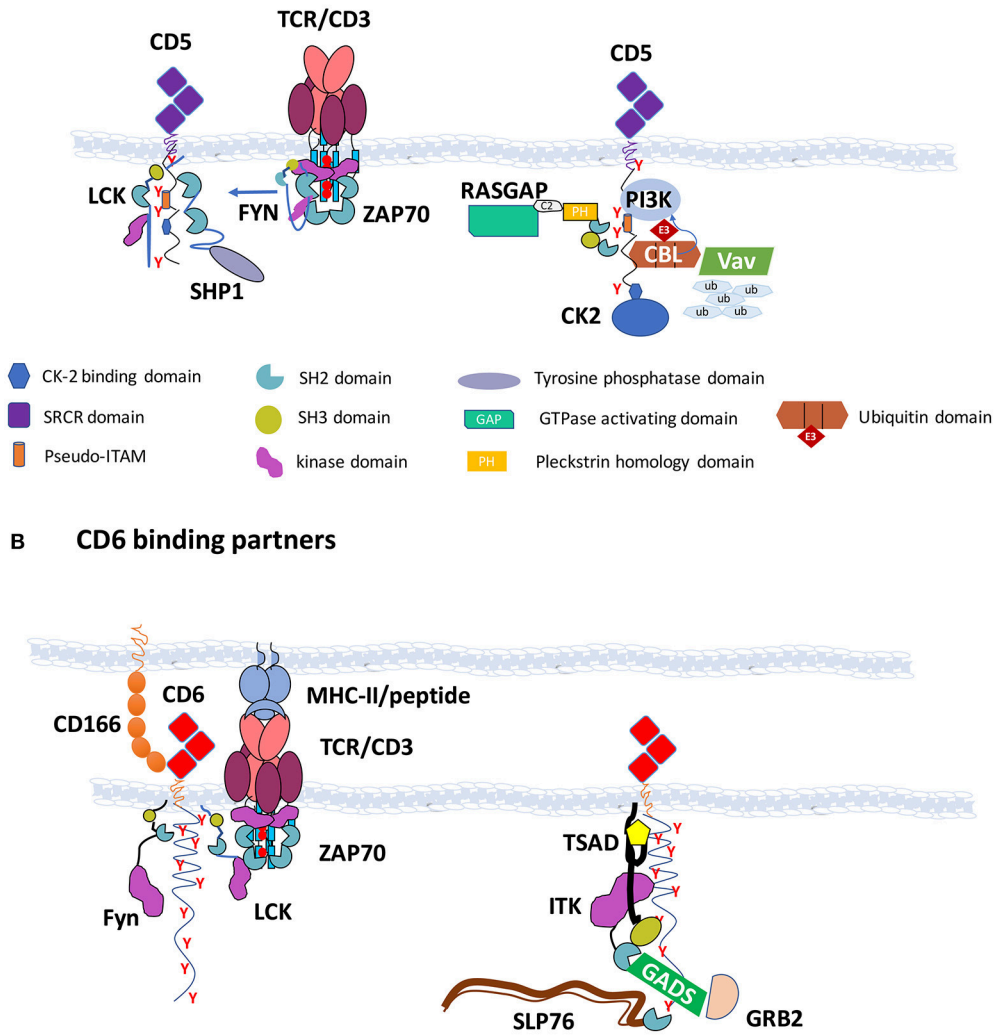


FIGURE 1 | CD5 and CD6 are hubs for the assembly of effector enzymes and adaptors—**(A)** CD5 binding partners: CD5 contains in its cytoplasmic tail four tyrosine residues, of which three (Y453, Y465, and Y487) are believed to be phosphorylated upon TCR triggering and can bind the SH2 domains of LCK, RASGAP, CBL, CBLB, SHP1, and PI3K. Recruitment of CBL to the C-terminal region of CD5 is important for the ubiquitylation and degradation of several substrates following TCR engagement, including VAV. CK2 is also able to bind to the cytoplasmic tail of CD5 through other mechanisms. The interaction with FYN is also not dependent on tyrosine phosphorylation. CSK associates with the CD5 signalosome possibly through the cooperation with PAG, CBL, or CBLB. CD5 is represented in duplicate to accommodate all binding partners; **(B)** CD6 binding partners: CD6 contains in its cytoplasmic tail nine tyrosine residues that when phosphorylated can dock the SH2 domains of SLP76, TSAD, GADS, GRB2, and SHP1. The interactions with LCK, FYN, ZAP70, and ITK were not shown to be dependent on SH2 domain binding to phosphotyrosine residues, but ITK may be recruited through its association with TSAD. CD6 binds through the C-terminal sequence to the PDZ domains of syntenin. The CD6 signalosome is depicted in the right. Structures are not drawn to scale.

phosphotyrosines of CD6. CD6 also binds to the scaffolding protein syntenin-1, but the interaction is likely mediated by the tandemly arranged PDZ domains of syntenin-1 (45).

Interestingly, some of the papers that reported the phosphoprotein-dependent molecular interactions of CD6 described the coincident finding that the same molecules could also dock onto the phosphotyrosine sites of Linker for activation of T cells (LAT), a membrane-bound adaptor of the main axis of the TCR-mediated pathway. In an unbiased *in vivo* proteomics screening, Roncagalli et al. described the LAT-independent association of SLP76 to CD6 (46), while Hem et al. showed that TSAD bound to both LAT and CD6 (43). Given that additionally GRB2 and GADS are well-established binders of LAT (47), it emerges that CD6 displays some characteristics of membrane-bound adaptors, such as LAT and Phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG) in that it contains multiple tyrosine residues that once phosphorylated can couple to a diverse set of signaling effectors, possibly feeding onto various, convergent or divergent, signaling pathways (48).

Therefore, and much like LAT and PAG, CD6 seems to constitute a signalosome that assembles many different enzymes and adaptors that can impact on signal propagation in different pathways and potentially with divergent outcomes. *Lat* knockout mice, however, show a very different behavior than those deficient of CD6 (described later in this paper), displaying a block in the development of thymocytes at the double negative stage and a complete absence of mature T cells (49). This established LAT as a crucial adaptor for T cell signaling leading to lymphocyte differentiation and also of T cell activation. On the other hand, mice with a disrupted *Pag* gene, much similarly to *Cd6*-deficient but also to *Cd5*-deficient animals, have no overall differences in total numbers of T cells than wild-type mice; and effector T cells, although not naïve, are more prone to activation upon TCR targeting (50). PAG is essential for the phosphotyrosine-dependent docking of the protein tyrosine kinase CSK, a major inhibitory enzyme of T cell activation that phosphorylates the inhibitory carboxyl-terminal tyrosine residues of LCK and FYN, inactivating these kinases (51, 52). Interestingly, CD5 seems to be a relevant alternative docking receptor for CSK, and in fact PAG and CD5 may cooperate in the inhibition of FYN (36, 41). Overall, it appears that while LAT is a hub for the assembling of positive effectors, CD5 as well as PAG can be viewed as inhibitory signalosomes. Whether CD6 is an activating, inhibitory, or multi-purpose scaffolding transmembrane receptor is still under debate.

Given that CD6, besides being structurally very similar to CD5, shares with it many features, such as profile of expression and functional roles in the biochemical repression of T cell activation, it would be expected or at least plausible that, similarly to CD5, it could couple to analogous signaling inhibitory partners. A recent report has in fact described the interaction of CD6 with the protein phosphatase SHP1 (26), constituting this the first solid biochemical evidence that CD6 can couple to inhibitory signaling.

CAN LIGAND BINDING AND SIGNALING INHIBITION BE UNCOUPLED EVENTS?

Unlike PAG or LAT, CD6 contains structured ectodomains that are suitable to establish interactions with extracellular ligands. CD6 binds to CD166, widely expressed in many cell types and tissues (53, 54). and recently CD318 was identified as an alternative ligand in cells derived from human thymus, skin, synovium, and cartilage (55, 56). However, there is no absolute requirement for CD6 to bind to ligands to be able to exert its inhibitory function (34). This is a characteristic common to CD5 (57). Therefore, these two receptors can be general attenuators of TCR-mediated signaling independent of any mechanical effects of ligand binding or of any particular membrane localization. So, if there can be a functional uncoupling between these two features of the molecules, what may be the role of the ectodomains and the consequences of binding to ligands?

During thymocyte development, the expression of CD6 increases steadily from double negative (DN) to double positive (DP) and to single $CD4^+$ or $CD8^+$ thymocytes, decreasing then slightly in the negatively selected single $CD4^+$ or $CD8^+$ thymocytes just before thymic emigration, and to mature T cells (58). The increasing expression of CD6 favors the interaction with CD166, highly expressed on thymic epithelium, possibly providing anti-apoptotic signals and also increasing the adhesion of thymocytes to thymic epithelial cells. However, this signal tuning-related variation of expression is a general characteristic and not a differentiation feature, i.e., thymocytes are exposed to APCs that all express the same putative CD6 ligands and thus there should be no distinctive outcome in selection or subset polarization between different thymocytes based solely on whether or not CD6 binds to its ligand.

The scenario can be remarkably different regarding the activation of mature T cells, though. The interaction between CD6 and CD166 is one of the strongest between cell surface adhesion molecules, with a dissociation constant (K_D) of 0.4–1.0 μM measured by surface plasmon resonance (59), and contributing to binding between T cells and APC with forces equivalent to those of integrins, as quantified by atomic force microscopy (60). Integrating this information with the inhibitory contribution toward signaling, it emerges that CD6 can possibly have a dual role, the first of which is to promote cellular adhesion, facilitating the TCR scanning of specific peptides. Upon antigen recognition and the formation of immunological synapses, CD6 can then adjust the strength of T cell activation through the attenuation of the signaling cascades. These features are also generic considering T cell activation as a whole, given that most APCs do express the ligand CD166. But the fact is that in different contexts with distinct APCs, possibly expressing varied levels of CD166 but also armed with different sets of costimulatory or co-inhibitory ligands for the many cell surface receptors of T cells, CD6 may impact differently on the signaling pathways and can eventually influence on the polarization of T cell subsets and responses, namely in the development of Th1 and Th17 sub-populations (61).

As for CD5, no APC-expressed ligand has been demonstrated so far; however, it was recently shown that it can serve as an alternative receptor for IL-6, leading to the activation of the transcription factor STAT3 (62). Although this observation was made in B1a cells, it nevertheless opens the perspective of IL-6 being able to promote Th17 responses when binding to T cell-expressed CD5.

THE MODULATORY ROLE OF CD6 DURING THYMOCYTE DEVELOPMENT

Two recent reports on independent *Cd6* knockout models have finally confirmed that at the cellular level, the net contribution of CD6 to signaling is generally inhibitory (24, 25). Isolated $CD4^+$ and $CD8^+$ T cells from the mutant mice displayed significantly augmented activation upon anti-CD3 triggering than cells isolated from wild-type mice (24, 25), highlighting the inhibitory role of CD6 in T cell activation. It appears, however, that the strength of inhibition is milder than that of CD5.

The generation of *Cd6* knockout mouse models has been helpful in understanding the role of the protein during thymocyte selection. Although the frequency and total numbers of most

cell populations in the periphery are identical comparing wild-type and CD6$^{-/-}$ mice, in the thymus there seems to be a partial impairment in the transition from double-positive to single positive thymocytes (63). Namely, there is an increase in the percentage of DP thymocytes undergoing selection, which is accompanied by a decrease of CD4^{+} or CD8^{+} SP thymocytes that complete selection. Besides, the percentage of CD4^{+} SP thymocytes is reduced in these animals. The percentage of CD8^{+} SP cells is unaltered but a deeper look into that subpopulation revealed that, in *Cd6* knockout mice, the percentage of CD8^{+} immature cells was increased while that of CD8^{+} mature cells was decreased (63). In summary, CD6 is able to modulate signaling during T cell development as the lack of the molecule changes the threshold for negative selection in the thymus resulting in a reduced number of T cells that fully mature (**Figure 2**).

Similar studies had been conducted to assess the role of CD5 expression in development and how it affects the generation of either CD8^{+} or CD4^{+} T cell populations (18, 64). Despite the fact that the overall effect of CD5 expression in development was initially disguised by the large repertoire of expressed TCRs (65), the use of TCR-transgenic mice allowed to understand that CD5 acts as a negative regulator during T cell maturation (18, 64). In fact, a subsequent study encompassing different TCR-transgenic animal models clarified that the effect of CD5 is related to its levels of expression (22). In T cells with high-affinity TCRs (and consequent high expression of CD5), lack of CD5 markedly decreases positive selection, while increasing negative selection. In low-affinity TCR-expressing T cells, however, the loss of CD5 expression did not result in such significant changes (22).

CD6 is also able to impact on the efficiency of Tregs, a particular subset of T cells responsible for suppressing immune responses by inducing antigen tolerance. The ability of Tregs to downregulate both effector and helper T cells in response to self-antigens renders them important players in preventing autoimmune diseases. Tregs isolated from the spleen of *Cd6* knockout mice were shown to be less efficient in suppressing the proliferation of conventional T cells than those extracted from wild-type mice (63). Taking into consideration the many features common to both CD6 and CD5, this observation was somewhat surprising, as the opposite phenotype had been seen for *Cd5* knockout mice (66). Specifically, it was observed that Tregs from *Cd5* knockout mice were more efficient in their suppressive activity than cells from wild-type mice, consistent with CD5 being a negative regulator (66). The explanation for this discrepancy may be found in the global view of the system: CD4^{+} T cells from the spleen of *Cd5* knockout mice were described to express slightly more CD6; on the other hand, splenic CD4^{+} T cells from *Cd6* knockout mice tend to express less CD5 (63). Therefore, *Cd5* knockout mice ultimately display Tregs that are more able to repress immune responses whereas *Cd6* knockout mice, expressing less CD5 but also less CD6, are characterized by less efficient Tregs. This translates into less suppression of the immune system. In summary, when CD6 expression is increased directly or indirectly, the overall result seems to be a system more equipped to tone down immune responses.

LIGAND BINDING IN THE PROMOTION OF T CELL MIGRATION VS. SUBSET POLARIZATION

Mice with disrupted *Cd6* genes display differing responses in the two main inflammatory disease settings investigated so far. In the collagen-induced arthritis (CIA) model, *Cd6* knockout mice were characterized by earlier disease onset and increased clinical score as well as worsened hallmarks for the disease, namely IL-6 and TNF expression in the joints (63), whereas in a model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), the absence of CD6 confers resistance to the demyelinating disorder (61). Although the mice strains were different and not the conventional usually used in the two different disease models, the disparate responses could again

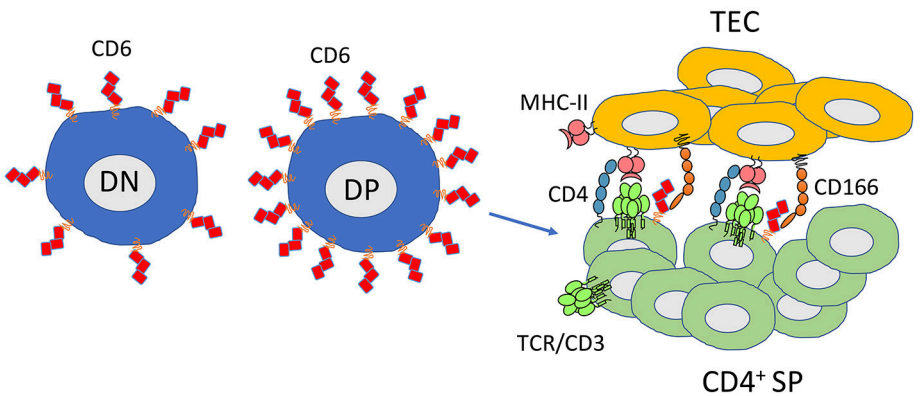


FIGURE 2 | CD6 favors thymocyte differentiation and maturation and is required for selection of thymocytes with high-avidity TCRs. CD6 is expressed at all stages of thymocyte development. Its expression is increased at the double positive stage in cells that are assigned to maturation (CD69^{high}). CD6 has an important role limiting the threshold for negative selection. The CD6-CD166 interaction promotes higher affinity TCR-MHC-II/peptide interactions contributing to CD4^{+} selection. CD6$^{-/-}$ mice have an increased frequency of DP cells undergoing selection. Conversely, CD4^{+} SP and CD8^{+} SP have lower numbers or are less mature, respectively. DN, double negative thymocytes; DP, double positive thymocytes; CD4^{+} SP, single positive CD4 thymocytes; TEC, thymic epithelial cells.

point to dual alternative roles of CD6 in T cell activation leading to divergent immune responses. However, when isolated and responding *in vitro* to antigenic or antibody-induced activation, CD6-negative cells were more responsive in both cases (61, 63). This observation again suggests that CD6 may have other roles than just its participation in signal transduction mechanisms.

Whereas, in the CIA model the augmented reactivity of $CD6^{-/-}$ T cells is consistent with a cellular signaling inhibitory function for CD6, or an inhibitory CD6-mediated T cell response, in EAE the absence of CD6 was suggested, rather, to impair T cell migration through brain microvascular endothelial cells resulting in a lower infiltrate in the spinal cord of, among others, the autoreactive T cells. Although the experiments performed did not address leukocyte transmigration *in vivo* but actually used brain microvascular endothelial cells isolated from wild-type and knockout mice (61), these observations are concordant with a recent report where the re-engineered additional expression in CD6 molecules of the domain (d3) that interacts with CD166 significantly promotes T cell migration into the brain in a brain cancer model, where cancer endothelium upregulates CD166 expression (67). On the whole, a plausible explanation for the divergent impact of the lack of CD6 in the two models is that in CIA, $CD6^{neg}$ cells are more autoreactive and inflict tissue damage whereas in EAE the lower T cell infiltration into the brain is the dominant effect.

The hypothesis that the lower EAE scores in the absence of CD6 are due to less transmigration is, however, in apparent contradiction with the interpretation that is given for the CD6-dependent corresponding human pathology: *CD6* is a susceptibility gene for multiple sclerosis (68), and individuals carrying a disease-related polymorphism at the locus rs17828933 within the first intron have increased levels of CD6Δd3 (69), a naturally occurring CD6 isoform that lacks the CD166-binding domain (70). Thus, in both mouse and human studies, the defective T cells are in disadvantage to cross the blood-brain barrier; but whereas in the mouse model the lack of cell infiltration is protective, in the human setting, paradoxically, the conditions are set to induce or aggravate the illness.

One main difference is that in the mouse the whole molecule is missing whereas in humans the full signaling potential is present. Furthermore, cells carrying the CD6Δd3 isoform are more reactive upon mAb challenge than cells expressing wild-type CD6. How can all these features be reconciled to explain the progression and severity of the disease? Unless we consider that the mouse and human studies are too different to be comparable, there is at present no simple answer to that question if only the signaling aspects of CD6 are taken into account. Therefore, a possible function of CD6 that should be considered is in fact its impact on the polarization of different T cell subsets in different disease conditions.

THERAPEUTIC CD6 MABS

Bughani et al. have developed an anti-mouse CD6 mAb, mCD6D1, that recognizes the membrane distal domain of CD6 (d1), to ameliorate the incidence of EAE in C57BL/6 mice (26), while Li et al. have used a mouse anti-human CD6-d1 mAb, UMCD6, to reverse EAE progression in DBA/1 humanized mice (61). Itolizumab is a mouse mAb that also binds to CD6-d1 (71) and that has been found to be very efficient in treating autoimmune pathologies (72). Clinical trials for Itolizumab have been conducted to treat rheumatoid arthritis and psoriasis, yielding very encouraging results (73, 74). This antibody has already received approval from the Drugs Controller General of India as treatment for chronic plaque psoriasis in 2013, with treated patients presenting less proliferative T cells and decreased levels of pro-inflammatory cytokines in the serum (75). Despite the positive outcomes that are emerging from the use of this antibody, very little is known about its mechanism of action and how exactly it impacts on T cell signaling (72).

UMCD6 and Itolizumab recognize overlapping epitopes and can partly inhibit the binding of soluble CD166 to T cell-expressed CD6, although substantially less than anti-CD6-d3 (76). The structure of the CD6 extracellular region has only recently been solved (77), and it was speculated that antibody binding to CD6-d1 could perhaps hinder the ability of CD6 to interact with CD166 (26). However, in different experimental settings the effect of blocking of the direct interaction between T cell expressed-CD6 and APC-expressed CD166 by anti-CD6d1 mAbs, or even the reduction of T cell-APC conjugates, has produced contradictory results (70, 71, 78). Although it is possible that the presence of massive amounts of antibodies decorating the surface of T cells may actually reduce the number of T cell-APC conjugates, another plausible explanation is that the action of the reagents may have a direct effect on CD6-mediated signaling, or alternatively on CD6-mediated T cell polarization. The first case has been extensively documented *in vitro* and delivers very disparate results depending on the mAb clone used and conditions of cell culture and activation, ranging from significant activation to marked inhibition (79). As for the second, there is still insufficient documentation of *in vivo* studies to allow for any conclusions to be withdrawn for the time being; nevertheless, Itolizumab has proven efficacy to treat human diseases that are characterized by having Th17 polarization and concordantly, in human PBMC cultured in Th17 polarizing conditions, addition of Itolizumab seems to decrease the differentiation of $CD4^{+}$ T cells into the Th17 sub-set and decrease the production of IL-17 (26).

CONCLUDING REMARKS

Although perhaps counterintuitively at a first glance, signal inhibition relies heavily on intracellular tyrosine phosphorylation. Moreover, the phosphorylation of activation and inhibitory motifs can be catalyzed by exactly the same kinases and also be coincidental in time. Therefore, it cannot be unexpected that T cell-expressed inhibitory receptors functionally and physically associate with kinases that are known to be crucial to T cell activation. CD6 associates with LCK, FYN, ZAP70, and ITK, protein tyrosine kinases that are regarded as components of signaling progression, and yet, this complex molecule has been shown to bestow T cells with a strong inhibitory potential both *in vitro* as well as in *ex vivo* systems (34, 61, 63).

However, a straightforward categorization of CD6 as an inhibitor, or alternatively, as a co-stimulatory receptor, does not suffice to explain the apparent diversity of functions that the molecule displays in many different situations. CD6 seems to exhibit many features analogous to membrane-bound intracellular adaptors, such as LAT and PAG, as it has multiple tyrosine residues on its cytoplasmic domain that once phosphorylated can couple to a range of different enzymes and adaptors, possibly feeding into multiple parallel signaling pathways. Perhaps this can explain the antagonistic roles attributed to the molecule in different experimental setups. On the other hand, the fact that CD6 establishes robust extracellular interactions with APC-expressed ligands strongly favors the adhesion between T cells and APCs and unequivocally promotes T cell activation. Whether binding to CD166 can, alternatively in different situations, promote T cell activation or repression, or impact on T cell subset polarization requires further investigation. CD6 is being recognized as an important target for therapy against several autoimmune diseases and the use of therapeutic CD6 mAbs is steadily increasing. A major challenge facing ahead is to understand how these reagents can regulate CD6 function to be able to devise the most appropriate treatment for human disease.

AUTHOR CONTRIBUTIONS

CMG, SNH, RFS, and AMC wrote the manuscript. CMG and AMC designed the figures.

ACKNOWLEDGMENTS

This work was financed by FEDER-Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020-Operacional Programme for Competitiveness and Internationalization (POCI), Portugal 2020, and by Portuguese funds through FCT-Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior in the framework of the project POCI-01-0145-FEDER-032296 (PTDC/MED-IMU/32296/2017). Additional funds, including funding for CMG, were from the project Norte-01-0145-FEDER-000012-Structured program on bioengineered therapies for infectious diseases and tissue regeneration, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (FEDER). SNH and RFS are recipients of Ph.D. studentships from FCT, references SFRH/BD/133312/2017 and SFRH/BD/110691/2015, respectively.

REFERENCES

1. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol.* (1996) 8:765–72. doi: 10.1093/intimm/8.5.765
2. Metzler B, Burkhart C, Wraith DC. Phenotypic analysis of CTLA-4 and CD28 expression during transient peptide-induced T cell activation *in vivo*. *Int Immunol.* (1999) 11:667–75. doi: 10.1093/intimm/11.5.667
3. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol.* (2009) 10:48–57. doi: 10.1038/ni.1674
4. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med.* (1990) 171:1393–405. doi: 10.1084/jem.171.5.1393
5. Han P, Goularte OD, Rufner K, Wilkinson B, Kaye J. An inhibitory Ig superfamily protein expressed by lymphocytes and APCs is also an early marker of thymocyte positive selection. *J Immunol.* (2004) 172:5931–9. doi: 10.4049/jimmunol.172.10.5931
6. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med.* (2012) 209:1201–17. doi: 10.1084/jem.20112741
7. Chuang E, Fisher TS, Morgan RW, Robbins MD, Duerr JM, Vander Heiden MG, et al. The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity* (2000) 13:313–22. doi: 10.1016/S1074-7613(00)00031-5
8. Liu S, Zhang H, Li M, Hu D, Li C, Ge B, et al. Recruitment of Grb2 and SHIP1 by the ITT-like motif of TIGIT suppresses granule polarization and cytotoxicity of NK cells. *Cell Death Differ.* (2013) 20:456–64. doi: 10.1038/cdd.2012.141
9. Workman CJ, Dugger KJ, Vignali DA. Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. *J Immunol.* (2002) 169:5392–5. doi: 10.4049/jimmunol.169.10.5392
10. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol.* (2003) 4:670–9. doi: 10.1038/ni944
11. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a second receptor for the B cell activation antigen B7. *J Exp Med.* (1991) 174:561–9. doi: 10.1084/jem.174.3.561
12. Azuma M, Ito D, Yagita H, Okumura K, Phillips JH, Lanier LL, et al. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* (1993) 366:76–9. doi: 10.1038/366076a0
13. Lozano E, Dominguez-Villar M, Kuchroo V, Hafler DA. The TIGIT/CD226 axis regulates human T cell function. *J Immunol.* (2012) 188:3869–75. doi: 10.4049/jimmunol.1103627
14. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol.* (2008) 180:5916–26. doi: 10.4049/jimmunol.180.9.5916
15. Huang HJ, Jones NH, Strominger JL, Herzenberg LA. Molecular cloning of Ly-1, a membrane glycoprotein of mouse T lymphocytes and a subset of B cells: molecular homology to its human counterpart Leu-1/T1 (CD5). *Proc Natl Acad Sci USA.* (1987) 84:204–8. doi: 10.1073/pnas.84.1.204
16. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *J Exp Med.* (1983) 157:202–18. doi: 10.1084/jem.157.1.202
17. Beyers AD, Spruyt LL, Williams AF. Molecular associations between the T-lymphocyte antigen receptor complex and the surface antigens CD2, CD4, or CD8 and CD5. *Proc Natl Acad Sci USA.* (1992) 89:2945–9. doi: 10.1073/pnas.89.7.2945
18. Tarakhovsky A, Kanner SB, Hombach J, Ledbetter JA, Müller W, Killeen N, et al. A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Science* (1995) 269:535–7.
19. Burgess KE, Yamamoto M, Prasad KV, Rudd CE. CD5 acts as a tyrosine kinase substrate within a receptor complex comprising T-cell receptor zeta chain/CD3 and protein-tyrosine kinases p56lck and p59fyn. *Proc Natl Acad Sci USA.* (1992) 89:9311–5. doi: 10.1073/pnas.89.19.9311

20. Groves T, Parsons M, Miyamoto NG, Guidos CJ. TCR engagement of CD4+CD8+ thymocytes *in vitro* induces early aspects of positive selection, but not apoptosis. *J Immunol.* (1997) 158:65–75.
21. Azzam HS, Grinberg A, Lui K, Shen H, Shores EW, Love PE. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J Exp Med.* (1998) 188:2301–11. doi: 10.1084/jem.188.12.2301
22. Azzam HS, DeJarnette JB, Huang K, Emmons R, Park CS, Sommers CL, et al. Fine tuning of TCR signaling by CD5. *J Immunol.* (2001) 166:5464–72. doi: 10.4049/jimmunol.166.9.5464
23. Gimferrer I, Calvo M, Mittelbrunn M, Farnós M, Sarrias MR, Enrich C, et al. Relevance of CD6-mediated interactions in T cell activation and proliferation. *J Immunol.* (2004) 173:2262–70. doi: 10.4049/jimmunol.173.4.2262
24. Wee S, Schieven GL, Kirihara JM, Tsu TT, Ledbetter JA, Aruffo A. Tyrosine phosphorylation of CD6 by stimulation of CD3: augmentation by the CD4 and CD2 coreceptors. *J Exp Med.* (1993) 177:219–23. doi: 10.1084/jem.177.1.219
25. da Gloria VG, de Araujo MM, Santos AM, Leal R, de Almeida SF, Carmo AM, et al. T Cell activation regulates CD6 alternative splicing by transcription dynamics and SRSF1. *J Immunol.* (2014) 193:391–9. doi: 10.4049/jimmunol.1400038
26. Bughani U, Saha A, Kuriakose A, Nair R, Sadashivarao RB, Venkataraman R, et al. T cell activation and differentiation is modulated by a CD6 domain 1 antibody Itolizumab. *PLoS ONE* (2017) 12:e0180088. doi: 10.1371/journal.pone.0180088
27. Hassan NJ, Simmonds SJ, Clarkson NG, Hanrahan S, Puklavec MJ, Bomb M, et al. CD6 regulates T-cell responses through activation-dependent recruitment of the positive regulator SLP-76. *Mol Cell Biol.* (2006) 26:6727–38. doi: 10.1128/MCB.00688-06
28. Ceuppens JL, Baroja ML. Monoclonal antibodies to the CD5 antigen can provide the necessary second signal for activation of isolated resting T cells by solid-phase-bound OKT3. *J Immunol.* (1986) 137:1816–21.
29. June CH, Rabinovitch PS, Ledbetter JA. CD5 antibodies increase intracellular ionized calcium concentration in T cells. *J Immunol.* (1987) 138:2782–92.
30. Gangemi RM, Swack JA, Gaviria DM, Romain PL. Anti-T12, an anti-CD6 monoclonal antibody, can activate human T lymphocytes. *J Immunol.* (1989) 143:2439–47.
31. Osorio LM, Garcia CA, Jondal M, Chow SC. The anti-CD6 mAb, IOR-T1, defined a new epitope on the human CD6 molecule that induces greater responsiveness in T cell receptor/CD3-mediated T cell proliferation. *Cell Immunol.* (1994) 154:123–33. doi: 10.1006/cimm.1994.1062
32. Castro M, Nunes R, Oliveira M, Tavares P, Simoes C, Parnes J, et al. OX52 is the rat homologue of CD6: evidence for an effector function in the regulation of CD5 phosphorylation. *J Leuk Biol.* (2003) 73:183–90. doi: 10.1189/jlb.0902437
33. Gimferrer I, Farnós M, Calvo M, Mittelbrunn M, Enrich C, Sánchez-Madrid F, et al. The accessory molecules CD5 and CD6 associate on the membrane of lymphoid T cells. *J Biol Chem.* (2003) 278:8564–71. doi: 10.1074/jbc.M209591200
34. Oliveira MI, Gonçalves CM, Pinto M, Fabre S, Santos AM, Lee SF, et al. CD6 attenuates early and late signaling events, setting thresholds for T-cell activation. *Eur J Immunol.* (2012) 42:195–205. doi: 10.1002/eji.201040528
35. Raab M, Yamamoto M, Rudd CE. The T-cell antigen CD5 acts as a receptor and substrate for the protein-tyrosine kinase p56lck. *Mol Cell Biol.* (1994) 14:2862–70. doi: 10.1128/MCB.14.5.2862
36. Bamberger M, Santos AM, Gonçalves CM, Oliveira MI, James JR, Moreira A, et al. A new pathway of CD5 glycoprotein-mediated T cell inhibition dependent on inhibitory phosphorylation of fyn kinase. *J Biol Chem.* (2011) 286:30324–36. doi: 10.1074/jbc.M111.230102
37. Gary-Gouy H, Lang V, Sarun S, Boumsell L, Bismuth G. *In vivo* association of CD5 with tyrosine-phosphorylated ZAP-70 and p21 phospho-zeta molecules in human CD3+ thymocytes. *J Immunol.* (1997) 159:3739–47.
38. Perez-Villar JJ, Whitney GS, Bowen MA, Hewgill DH, Aruffo AA, Kanner SB. CD5 negatively regulates the T-cell antigen receptor signal transduction pathway: involvement of SH2-containing phosphotyrosine phosphatase SHP-1. *Mol Cell Biol.* (1999) 19:2903–12. doi: 10.1128/MCB.19.4.2903
39. Carmo A, Castro M, Arosa F. CD2 and CD3 associate independently with CD5 and differentially regulate signaling through CD5 in Jurkat T cells. *J Immunol.* (1999) 163:4238–45.
40. Dennehy KM, Broszeit R, Ferris WF, Beyers AD. Thymocyte activation induces the association of the proto-oncoprotein c-cbl and ras GTPase-activating protein with CD5. *Eur J Immunol.* (1998) 28:1617–25. doi: 10.1002/(SICI)1521-4141(199805)28:05<1617::AID-IMMU1617>3.0.CO;2-7
41. Voisinne G, García-Blesa A, Chaoui K, Fiore F, Bergot E, Girard L, et al. Co-recruitment analysis of the CBL and CBLB signalosomes in primary T cells identifies CD5 as a key regulator of TCR-induced ubiquitylation. *Mol Syst Biol.* (2016) 12:876. doi: 10.15252/msb.20166837
42. Dennehy KM, Broszeit R, Garnett D, Durrheim GA, Spruyt LL, Beyers AD. Thymocyte activation induces the association of phosphatidylinositol 3-kinase and pp120 with CD5. *Eur J Immunol.* (1997) 27:679–86. doi: 10.1002/eji.1830270316
43. Hem CD, Ekornhol M, Granum S, Sundvold-Gjerstad V, Spurkland A. CD6 and linker of activated T cells are potential interaction partners for T cell-specific adaptor protein. *Scand J Immunol.* (2017) 85:104–12. doi: 10.1111/sji.12513
44. Breuning J, Brown MH. T cell costimulation by CD6 is dependent on bivalent binding of a GADS/SLP-76 complex. *Mol Cell Biol.* (2017) 37:e00071–00017. doi: 10.1128/MCB.00071-17
45. Gimferrer I, Ibáñez A, Farnós M, Sarrias MR, Fenutría R, Roselló S, et al. The lymphocyte receptor CD6 interacts with syntenin-1, a scaffolding protein containing PDZ domains. *J Immunol.* (2005) 175:1406–14. doi: 10.4049/jimmunol.175.3.1406
46. Roncagalli R, Hauri S, Fiore F, Liang Y, Chen Z, Sansoni A, et al. Quantitative proteomics analysis of signalosome dynamics in primary T cells identifies the surface receptor CD6 as a Lat adaptor-independent TCR signaling hub. *Nat Immunol.* (2014) 15:384–92. doi: 10.1038/ni.2843
47. Zhang W, Trible RP, Zhu M, Liu SK, McGlade CJ, Samelson LE. Association of Grb2, Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell angigen receptor-mediated signaling. *J Biol Chem.* (2000) 275:23355–61. doi: 10.1074/jbc.M000404200
48. Santos RF, Oliveira L, Carmo AM. Tuning T cell activation: the function of CD6 at the immunological synapse and in T cell responses. *Curr Drug Targets* (2016) 17:630–9. doi: 10.2174/1389450116666150531152439
49. Zhang W, Sommers CL, Burshtyn DN, Stebbins CC, DeJarnette JB, Trible RP, et al. Essential role of LAT in T cell development. *Immunity* (1999) 10:323–32. doi: 10.1016/S1074-7613(00)80032-1
50. Dobenecker MW, Schmedt C, Okada M, Tarakhovsky A. The ubiquitously expressed Csk adaptor protein Cbp is dispensable for embryogenesis and T-cell development and function. *Mol Cell Biol.* (2005) 25:10533–42. doi: 10.1128/MCB.25.23.10533-10542.2005
51. Brdicka T, Pavlistová D, Leo A, Bruyns E, Korínek V, Angelisová P, et al. Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. *J Exp Med.* (2000) 191:1591–604. doi: 10.1084/jem.191.9.1591
52. Kawabuchi M, Satomi Y, Takao T, Shimonishi Y, Nada S, Nagai K, et al. Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. *Nature* (2000) 404:999–1003. doi: 10.1038/35010121
53. Bowen MA, Patel DD, Li X, Modrell B, Malacko AR, Wang WC, et al. Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand. *J Exp Med.* (1995) 181:2213–20. doi: 10.1084/jem.181.6.2213
54. Patel DD, Wee SF, Whichard LP, Bowen MA, Pesando JM, Aruffo A, et al. Identification and characterization of a 100-kD ligand for CD6 on human thymic epithelial cells. *J Exp Med.* (1995) 181:1563–8. doi: 10.1084/jem.181.4.1563
55. Saifullah MK, Fox DA, Sarkar S, Abidi SM, Endres J, Piktel J, et al. Expression and characterization of a novel CD6 ligand in cells derived from joint and epithelial tissues. *J Immunol.* (2004) 173:6125–33. doi: 10.4049/jimmunol.173.10.6125
56. Enyindah-Asonye G, Li Y, Ruth JH, Spassov DS, Hebron KE, Zijlstra A, et al. CD318 is a ligand for CD6. *Proc Natl Acad Sci USA.* (2017) 114:E6912–21. doi: 10.1073/pnas.1704008114
57. Bhandoola A, Bosselut R, Yu Q, Cowan ML, Feigenbaum L, Love PE, et al. CD5-mediated inhibition of TCR signaling during

intrathymic selection and development does not require the CD5 extracellular domain. *Eur J Immunol.* (2002) 32:1811–7. doi: 10.1002/1521-4141(200206)32:6<1811::AID-IMMU1811>3.0.CO;2-G
58. Singer NG, Fox DA, Haqqi TM, Beretta L, Endres JS, Prohaska S, et al. CD6: expression during development, apoptosis and selection of human and mouse thymocytes. *Int Immunol.* (2002) 14:585–97. doi: 10.1093/intimm/dxf025
59. Hassan NJ, Barclay AN, Brown MH. Optimal T cell activation requires the engagement of CD6 and CD166. *Eur J Immunol.* (2004) 34:930–40. doi: 10.1002/eji.200424856
60. Te Riet J, Zimmerman AW, Cambi A, Joosten B, Speller S, Torensma R, et al. Distinct kinetic and mechanical properties govern ALCAM-mediated interactions as shown by single-molecule force spectroscopy. *J Cell Sci.* (2007) 120:3965–76. doi: 10.1242/jcs.004010
61. Li Y, Singer NG, Whitbred J, Bowen MA, Fox DA, Lin F. CD6 as a potential target for treating multiple sclerosis. *Proc Natl Acad Sci USA.* (2017) 114:2687–92. doi: 10.1073/pnas.1615253114
62. Zhang C, Xin H, Zhang W, Yazaki PJ, Zhang Z, Le K, et al. CD5 Binds to interleukin-6 and induces a feed-forward loop with the transcription factor STAT3 in B cells to promote cancer. *Immunity* (2016) 44:913–23. doi: 10.1016/j.immuni.2016.04.003
63. Orta-Mascaró M, Consuegra-Fernández M, Carreras E, Roncagalli R, Carreras-Sureda A, Alvarez P, et al. CD6 modulates thymocyte selection and peripheral T cell homeostasis. *J Exp Med.* (2016) 213:1387–97. doi: 10.1084/jem.20151785
64. Peña-Rossi C, Zuckerman LA, Strong J, Kwan J, Ferris W, Chan S, et al. Negative regulation of CD4 lineage development and responses by CD5. *J Immunol.* (1999) 163:6494–501.
65. Tarakhovsky A, Müller W, Rajewsky K. Lymphocyte populations and immune responses in CD5-deficient mice. *Eur J Immunol.* (1994) 24:1678–84.
66. Dasu T, Qualls JE, Tuna H, Raman C, Cohen DA, Bondada S. CD5 plays an inhibitory role in the suppressive function of murine CD4(+) CD25(+) T(reg) cells. *Immunol Lett.* (2008) 119:103–13. doi: 10.1016/j.imlet.2008.05.008
67. Samaha H, Pignata A, Fousek K, Ren J, Lam FW, Stossi F, et al. A homing system targets therapeutic T cells to brain cancer. *Nature* (2018) 561:331–7. doi: 10.1038/s41586-018-0499-y
68. De Jager PL, Jia X, Wang J, de Bakker PI, Ottoboni L, Aggarwal NT, et al. Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. *Nat Genet.* (2009) 41:776–82. doi: 10.1038/ng.401
69. Kofler DM, Severson CA, Mousissian N, De Jager PL, Hafler DA. The CD6 multiple sclerosis susceptibility allele is associated with alterations in CD4+ T cell proliferation. *J Immunol.* (2011) 187:3286–91. doi: 10.4049/jimmunol.1100626
70. Castro M, Oliveira M, Nunes R, Fabre S, Barbosa R, Peixoto A, et al. Extracellular Isoforms of CD6 generated by alternative splicing regulate targeting of CD6 to the immunological synapse. *J Immunol.* (2007) 178:4351–61. doi: 10.4049/jimmunol.178.7.4351
71. Alonso R, Huerta V, de Leon J, Piedra P, Puchades Y, Guirola O, et al. Towards the definition of a chimpanzee and human conserved CD6 domain 1 epitope recognized by T1 monoclonal antibody. *Hybridoma* (2008) 27:291–301. doi: 10.1089/hyb.2008.0007
72. Hernández P, Moreno E, Aira LE, Rodríguez PC. Therapeutic targeting of CD6 in autoimmune diseases: a review of cuban clinical studies with the antibodies IOR-T1 and itolizumab. *Curr Drug Targets* (2016) 17:666–77. doi: 10.2174/1389450117666160201114308
73. Rodriguez PC, Torres-Moya R, Reyes G, Molinero C, Prada D, Lopez AM, et al. A clinical exploratory study with itolizumab, an anti-CD6 monoclonal antibody, in patients with rheumatoid arthritis. *Results Immunol.* (2012) 2:204–11. doi: 10.1016/j.rinim.2012.11.001
74. Krupashankar DS, Dogra S, Kura M, Saraswat A, Budamakuntla L, Sumathy TK, et al. Efficacy and safety of itolizumab, a novel anti-CD6 monoclonal antibody, in patients with moderate to severe chronic plaque psoriasis: results of a double-blind, randomized, placebo-controlled, phase-III study. *J Am Acad Dermatol.* (2014) 71:484–92. doi: 10.1016/j.jaad.2014.01.897
75. Aira LE, López-Requena A, Fuentes D, Sánchez L, Pérez T, Urquiza A, et al. Immunological and histological evaluation of clinical samples from psoriasis patients treated with anti-CD6 itolizumab. *MAbs* (2014) 6:783–93. doi: 10.4161/mabs.28376
76. Garner LI, Hartland A, Breuning J, Brown MH. CD6 monoclonal antibodies differ in epitope, kinetics and mechanism of action. *Immunology* (2018) 155:273–82. doi: 10.1111/imm.12952
77. Chappell PE, Garner LI, Yan J, Metcalfe C, Hatherley D, Johnson S, et al. Structures of CD6 and its ligand CD166 give insight into their interaction. *Structure* (2015) 23:1426–36. doi: 10.1016/j.str.2015.05.019
78. Singer NG, Mitra R, Lialios F, Richardson BC, Marks RM, Pesando JM, et al. CD6 dependent interactions of T cells and keratinocytes: functional evidence for a second CD6 ligand on gamma-interferon activated keratinocytes. *Immunol Lett.* (1997) 58:9–14. doi: 10.1016/S0165-2478(97)02707-7
79. Pinto M, Carmo AM. CD6 as a therapeutic target in autoimmune diseases: successes and challenges. *Biodrugs* (2013) 27:191–202. doi: 10.1007/s40259-013-0027-4

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership