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Regulatory Mechanisms of Inhibitory Immune Checkpoint Receptors

Nicolas Curdy[1-3], Olivia Lanvin[1-3], Camille Laurent[1-3], Jean-Jacques Fournié[1-3], Don-Marc Franchini[1-3*]

[1]Cancer Research Center of Toulouse (CRCT), INSERM UMR 1037, CNRS ERL 5294, 31037 Toulouse, France

[2]Université Toulouse III Paul Sabatier, 31330 Toulouse, France

[3]Institut Universitaire du Cancer de Toulouse-Oncopole, 31100 Toulouse, France

*Correspondence: don-marc.franchini@inserm.fr (D.M. Franchini)

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Abstract

T cells responding to persistent tumor or viral antigens progressively lose their functional properties, a feature know as exhaustion. This state is also characterized by cell surface expression of multiple inhibitory immune checkpoint receptors. Cancer immunotherapies targeting immune checkpoints have shown impressive clinical outcomes, but still require substantial improvements due to the limited number of patients who benefit from it. Targeting the mechanisms controlling immune checkpoints expression could represent a step towards this aim. Accumulating data indicate that this strategy limits immune checkpoint expression, with in some instances the simultaneous inhibition of several immune checkpoints. In this review, we discuss various mechanisms through which immune checkpoint receptors are activated or regulated, and discuss ways these mechanisms could be exploited to develop more effective future immunotherapies.

Physiological and pathological function of Inhibitory Immune Checkpoints expression

T cell activation triggers a transcriptional reprogramming allowing T cell effector or memory functions, but also the expression of inhibitory immune checkpoint receptors (IRs) meant for modulating these functions, such as programmed cell death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4). In a physiological context, IRs expression is required to maintain immune homeostasis and self-tolerance, and therefore to avoid autoimmune reaction [1]. In cancer and in chronic viral infection, however, persistent stimulation increases the number and the level of expression of these IRs at the T cell surface, leading to a gradual loss of T cell effector functions, a phenomenon called exhaustion [2,3].

The immune checkpoint blockade (ICB) targeting CTLA-4, PD-1 or its ligand PD-L1 with antibodies gave unprecedented results in patients with previously incurable cancers [4]. Durable benefits, however, are only observed in a subset of patients. To improve this response rate and the number of responding cancer types, combination therapies, by targeting other IRs for instance, are now undertaken.

Tumor cells often overexpress several ligands of various IRs, such as PD-L1, to synergistically use the co-expression of IRs at the T cell surface as an immune escape mechanism. Consistently, tremendous efforts have been made to accumulate insights about PD-L1 regulation in the aim of improving current immunotherapies [5,6]. Along the same line, a better understanding of the mechanisms that control IRs is needed to be able to modulate their expression and counter tumor resistance. Here, we describe the various facets of IRs regulation, and highlight how these regulatory mechanisms are, or could be, exploited to optimize current immunotherapies.

60 **Receptors and Signaling Activating IRs Expression**

61

62 T cell receptor (TCR) stimulation and signaling are the primary drivers of IRs expression, but
63 additional pathways, which, for instance, can be stimulated by factors secreted within the tumor
64 microenvironment (TME), synergize to sustain and increase their expression (**Figure 1**).

65

66 *TCR-Dependent Signaling*

67 Initial studies pointed the TCR and its co-receptor CD28 as the primary trigger for IRs
68 expression. Taylor *et al.* showed that upon T cell activation, the glycogen synthase kinase
69 (GSK) 3α/β is inactivated by phosphorylation induced from the TCR/CD28 signaling involving
70 the PI3K/AKT pathway [7]. GSK-3α/β is a serine-threonine kinase that controls *Tbx21*
71 transcription (encoding for T-bet), which in turn decreases transcription of the PD-1-encoding
72 *PDCD1* gene. Consequently, inhibition of GSK-3α/β induces T-bet expression and PD-1
73 repression, which increases cytotoxic T lymphocytes activity and limits infection from viruses
74 such as herpes virus and lymphocytic choriomeningitis virus (LCMV) in mice models [7].
75 In another study, Balkhi *et al.* showed that TCR/CD28 stimulation phosphorylates and activates
76 the p38MAPK/JNK pathway, which leads to cJun/ATF2 complex activation [8]. A downstream
77 target of this complex is the transcription factor Yin-Yang 1 (YY1), which regulates the
78 expression of PD-1, lymphocyte-activation gene-3 (LAG-3) and T cell immunoglobulin and
79 mucin domain-3 (TIM-3). YY1 binds directly to the PD-1 and LAG-3 promoter regions, but
80 requires GATA3 for TIM-3 promoter binding due to the absence of YY1 consensus binding
81 site. Interestingly, YY1 expression was elevated in exhausted T cells from patients with either
82 melanoma or HIV infection [8].

83

84 *Cytokine Receptors*

85 The common γ-chain cytokines IL-2, IL-7, IL-15 and IL-21, which trigger Janus kinase 1
86 (JAK1), JAK3 and the signal transducer and activator of transcription (STAT) dependent
87 signaling, increase PD-1 [9] and TIM-3 [10] expression in T lymphocytes, but are not
88 necessarily associated with T cell exhaustion. In this regard, it is noteworthy that inhibitory
89 function of TIM-3 is somehow controversial [11].
90 IL-15 secreted by gastric cancer mesenchymal stem cells activates STAT5 signaling in $CD4^+$
91 T cells, which induces further regulatory T cells (Treg) enrichment and PD-1 expression, and
92 eventually promotes tumor progression [12]. Similar results were observed with low-dose of
93 IL-2 in chronic graft-versus-host-disease [13]. In $CD8^+$ tumor-infiltrating lymphocytes (TILs)
94 from breast cancer, IL-15 induced TIM-3 expression, but without enhancing PD-1 expression
95 [14].
96 In triple negative breast cancer, tumor-derived IL-18 induces PD-1 expression on immature,
97 non-cytotoxic NK cells, and is associated with poor prognosis [15]. In patients with follicular
98 lymphoma, IL-12 expressed in the TME favors T cell exhaustion by a STAT4-dependent TIM-
99 3 upregulation [16]. Together, IL-12 and IL-18 act to promote TIM-3 on γδ T cells following
100 malaria infection, and decrease their cytotoxic functions [17].
101 Another member of the IL-12 family cytokine, IL-27, was identified as crucial for TIM-3
102 expression [18]. Mechanistically, through STAT1 and STAT3 signaling, IL-27 induces the
103 expression of the transcription factors NFIL3 and T-bet, which then promote chromatin
104 remodeling at the TIM-3 locus and TIM-3 expression. IL-27 and NFIL3 are required to suppress
105 T helper (Th) 1 cell-mediated colitis, and IL-27 receptor deficiency reduces tumor burden in
106 B16F10 melanoma and Lewis Lung carcinoma-bearing mouse models [18]. In addition to TIM-
107 3, IL-27 controls a wider range of IRs including T cell immunoglobulin and ITIM domain
108 (TIGIT), LAG-3, CTLA-4 and PD-1 and the newly identified IRs PDPN and PROCR [19–22].
109 This control relies on both STAT1 activity [21] and on the transcription factors PRDM1 (also

110 known as BLIMP1) and c-MAF [22]. Deletion of both transcription factors results in better
111 tumor growth control in B16F10 melanoma-bearing mice [22].
112 IL-6 also induces c-MAF in a STAT3-dependent signaling, which alters tumor-specific Th1
113 functions and favors tumor progression [23,24]. This effect can be counteracted by combining
114 IL-6 blockade with ICB [25]. In CD8 T cells, IL-6/c-MAF axis is associated with exhaustion
115 [26], and in patients with rheumatoid arthritis, the progression of disease is associated with
116 enhanced IL-6/STAT3 signaling which positively correlates with PD-1 expression [27].
117
118 The immunosuppressive cytokine transforming growth factor β (TGFβ) is generally abundant
119 in the TME. Concurrently to T cell stimulation, TGFβ signaling enhances PD-1 transcription
120 by activating SMAD2/3 transcription factors [28]. However, only SMAD3 appeared essential
121 for PD-1 expression. Furthermore, SMAD3 deficiency decreases tumor growth and is
122 associated with a diminished expression of PD-1 at the $CD8^+$ TILs surface in melanoma and
123 colon cancer mouse models [28]. In addition, in a chronic LCMV infection mouse model,
124 SMAD4, which forms heterodimers with SMAD2/3, was required for optimal $CD4^+$ T cell
125 function and PD-1 expression [29]. Moreover, in a melanoma mouse model, TGFβ induces c-
126 MAF expression [26], a transcription factor involved in multiple IRs expression [22]. TGFβ
127 enhances PD-1 and LAG-3 expression and eventually contributes to CD8 TILs exhaustion [26].
128 The genome organizer SATB1 maintains a repressive chromatin state at the *PDCD1* locus by
129 recruiting the chromatin remodeling/histone deacetylase-containing NuRD repressor complex
130 [30]. Tumor-derived TGFβ signaling reduces the expression of SATB1, thereby increasing
131 histone 3 acetylation (H3ac) at the *PDCD1* locus and subsequent SMAD3-dependent *PDCD1*
132 transcription. Accordingly, $CD8^+$ TILs from ovarian cancer patients present a negative
133 correlation between SATB1 and PD-1 expression, and T cells deficient in SATB1 no longer
134 control tumor growth in ovarian and lung carcinoma mouse models [30].

135

136 Interferon (IFN)-mediated inflammatory response also induces IRs expression. IFNα promotes
137 and sustains *PDCD1* transcription through direct binding of IFN-responsive factor (IRF) 9 to
138 the IFN-stimulated response element located in the promoter region [31]. On the other hand,
139 IFNβ upregulates TIM-3 expression on Th1 cells, increasing the number of PD-1^+TIM-3^+ cells,
140 which results in less severe experimental autoimmune encephalomyelitis in mice [32].
141 Within the TME, IFNγ released by $CD8^+$ T cells induces the production of kynurenine (Kyn)
142 by adjacent tumor cells [33]. Kyn uptake by TILs, performed by the amino acid transporters
143 SLC7A8 and PAT4, then induces and activates the aryl hydrocarbon receptor (AhR), which
144 translocates to the nucleus to upregulate PD-1 expression. Consistently, expression of the
145 Kyn/AhR pathway positively correlates with PD-1 expression on TILs from breast and colon
146 cancer patients. Additionally, blockade of the Kyn/AhR pathways in melanoma-bearing mice
147 results in decreased PD-1 expression on TILs along with controlled tumor growth and better
148 survival of mice [33].
149 Besides immune cells, cytokine-dependent IRs expression has been evidenced on tumors cells.
150 In human melanoma cells, IFNγ-mediated signaling with STAT1 activation recruits CREB-
151 binding protein (CBP)/p300 and induces histone acetylation at the CTLA-4 promoter to
152 promote its expression [34]. In melanoma patients, benefits of anti-CTLA-4 antibody treatment
153 were associated with high expression of CTLA-4. Interestingly, expression of CTLA-4
154 positively correlates with expression of PD-L1, TIM-3 and LAG-3 [34]. In relapsing tumors,
155 sustained IFN signaling increases immunosuppressive mechanisms through the induction of a
156 multigenic program [35]. Among these genes, multiple ligands for IRs are expressed,
157 suggesting a link with IR-based immune escape mechanisms. While ICB alone partially restores
158 the antitumor response, this effect is greatly improved when IFN signaling is compromised.
159 Interestingly, the authors showed that to be effective, ICB must precede IFN signaling

160 inhibition [35]. Consistently, IFN signaling pathway is often mutated in patients with melanoma
161 unresponsive to ICB [36–38]. Along with the fact that PD-1 expression by melanoma cells
162 promotes tumor growth [39], these studies emphasized the importance of understanding the
163 mechanistic regulation of IRs expression in tumor cells to target immunosuppressive
164 mechanisms and thwart tumorigenesis.
165
166 *Non-Cytokine Receptors*
167 Additional receptors and signaling have been described for regulating PD-1 expression. *In vitro*
168 activation of Notch receptor triggers RBPJk and Notch intracellular domain occupancy at the
169 *PDCD1* promoter region to promote *PDCD1* transcription [40]. In early-stage septic shock
170 patients, upregulation of this Notch pathway correlates with PD-1 expression [41].
171 In addition, signaling through glucocorticoid (GC) receptor expressed on NK cells induces PD-
172 1 expression after stimulation by GC produced upon mouse cytomegalovirus infection [42]. As
173 a result, IFNγ overproduction is decreased, preserving from its associated immunopathology
174 but without impairing viral clearance, revealing a fine-tuning by the neuroendocrine system
175 [42].
176 Tumor cells generally secrete immunosuppressive factors into TME. Notably, activation of the
177 cyclic adenosine monophosphate (cAMP) signaling pathway in human and mouse $CD4^+$ T cells
178 increases CTLA-4 expression [43,44]. Moreover, activation of the cAMP signaling pathway by
179 the prostaglandin E2 (PGE2) secreted by tumor cells upregulates TIM-3 expression in Jurkat
180 cell line [45].
181 In addition, tumor cells can express high level of vascular endothelial growth factor A (VEGF-
182 A), an immunosuppressive factor, within the TME. Upon activation, $CD8^+$ T cells upregulate
183 VEGF receptors, which are activated by VEGF-A [46]. The resulting signaling entails the
184 PLCγ-calcineurin-NFAT pathway, which enhances the simultaneous cell surface expression of

185 PD-1, CTLA-4, TIM-3 and LAG-3. Concurrently, the transcription of IRs 2B4, CD160,
186 KLRG1 and BTLA is also triggered. While anti-PD-1 antibody has minimal impact on the
187 tumor in a colorectal mouse model, VEGF-A blockade decreases the proportion of TILs co-
188 expressing IRs and reduces tumor growth, an effect even increased upon association with anti-
189 PD-1 antibody [46]. Similar observations were obtained from small-cell lung cancer mice
190 where anti-PD-L1 resistant tumor-bearing mice developed more exhausted lymphocytes
191 expressing PD-1, TIM-3 and LAG-3 [47]. Addition of anti-VEGF antibody causes IRs
192 downregulation and better survival [47].
193
194 **Epigenetic Regulation of IRs**
195
196 Differentiation of T lymphocytes in response to T cell stimulation by viral infection or tumor
197 cells is accompanied by global changes in chromatin accessibility [48–50]. IRs are among the
198 genes for whose chromatin becomes accessible, correlating with their increased expression
199 levels.
200 These changes stem from DNA methylation and histone modifications that influence the
201 chromatin state (**Figure 2**). Early studies identified *cis*-regulatory elements at the *PDCD1* locus
202 regulated by DNA methylation and histone modifications [51]. Typically, PD-1 expression
203 inversely correlates to the level of DNA methylation, and is associated with an enrichment in
204 the permissive histone marks H3K9ac, H3K27ac or H3K4me1 [51]. In human breast and
205 colorectal cancer, expression of PD-1, CTLA-4, TIM-3, LAG-3 and TIGIT was associated
206 likewise with a decreased in both DNA methylation and repressive histone marks H3K9me3
207 and H3K27me3 [52,53].
208 The plasticity of epigenetic marks allows cells to constantly evolve and adapt to environmental
209 stimuli. Progression to exhaustion status, however, seems to set a more definitive epigenetic

program [48–50,54,55]. Although IRs are associated with accessible chromatin in exhausted T cells, it is unlikely to be a specific feature, as all changes are acquired and do not evolve further from the effector state. An exception is found at the *PDCD1* locus for a region located at -23 kb away from the transcription start site, corresponding to the exhaustion-associated *PDCD1* enhancer that becomes specifically accessible in exhausted T cells [48–50,54,55].

After chronic LCMV infection, the challenged $CD8^+$ T cells keep the *PDCD1* promoter stably unmethylated, along with an impaired clonal expansion long after cells were transferred into uninfected mice, therefore preserving this exhaustion [56]. Nevertheless, the global DNA methylation changes that occur during T cell exhaustion preferentially target both effector and proliferation programs [57]. Instead, IRs genes undergo a stable DNA demethylation preserved from effector to exhausted states. Consequently, the reinvigoration of exhausted T cells by DNA demethylating agents principally occurs through re-induction of these effector and proliferation programs but without affecting IRs expression [57].

Transcriptional Factors Controlling IRs Expression

Chromatin accessibility and epigenetic changes are in close relationship with transcription factors activity (**Figure 2**). The *cis*-regulatory elements at the *PDCD1* gene are recognized by transcription factors such as nuclear factor of activated T cells 2 (NFAT2), activator protein 1 (AP1) subunit c-Fos, forkhead box protein O1 (FoxO1), nuclear factor-κB (NF-κB), B lymphocyte-induced maturation protein 1 (BLIMP-1) and T-bet [51].

NFAT is a key regulator of T cell differentiation, able to balance towards effector or exhaustion programs, according to its association with AP-1. Without AP-1, NFAT1 is enriched at the promoter regions of genes encoding for the IRs PD-1, CTLA-4, TIM-3 and LAG-3. Consequently, NFAT1 binding enhances IRs expression, which is associated with a diminished

235 control of viral infection and tumor growth [58]. Importantly, all members of the NFAT family
236 cooperate to control IRs expression [58,59].
237 Analysis of the transcriptional programs triggered by NFAT1 revealed that genes controlled by
238 NFAT1 overlap with genes involved in exhaustion and anergy [58]. Moreover, these genes
239 match the NFAT-associated chromatin accessibility regions found in exhausted $CD8^+$ T cells
240 from mouse models of chronic infection by LCMV and of B16 melanoma tumor growth
241 [48,49]. Among these genes, IRF4, PRDM1 (BLIMP1) and NR4A-family transcription factors
242 were identified.
243 IRF4 is a transcription factor required for effector $CD8^+$ T cell differentiation and expansion.
244 RNA-sequencing analysis from LCMV-mediated exhausted T cells specifically identified
245 IRF4, along with genes encoding transcription factors such as PRDM1, BATF and NFAT2, and
246 IRs PD-1, LAG-3, TIM-3, CTLA-4, CD160, 2B4 and TIGIT [60]. Reduction of IRF4
247 expression restores T cells functional properties, and downregulates the expression of the
248 aforementioned IRs. Similar observations were obtained with T cells deficient in BATF. In
249 addition, genome-wide DNA binding analysis revealed enrichment of the transcription factors
250 IRF4, BATF and NFAT at genes encoding these IRs, suggesting a cooperative regulation of
251 such genes [60].
252 As aforesaid, PRDM1 was described as a regulator of multiple IRs by cooperating with c-MAF
253 in an IL-27-dependent manner [22]. Moreover, in a study using toxoplasma encephalitis-
254 susceptible model, toxoplasma-specific $CD4^+$ T cells display exhaustion features, associated
255 with elevated expression of PD-1, CTLA-4, LAG-3 and 2B4 [61]. These cells also highly
256 express BLIMP1, which when deleted, improved pathogen control. While BLIMP1-deficient
257 $CD4^+$ T cells display a reduced expression of IRs that is associated with improved functions,
258 they also reduce IRs expression (PD-1, CD160 and LAG-3) and improve functionality of $CD8^+$
259 T cells [61]. Further support for this observation was obtained from patients with acute myeloid

260 leukemia [62]. T lymphocytes from such patients upregulate BLIMP1 expression, which is
261 correlated with the expression of PD-1, TIGIT, TIM-3, 2B4 and CD160 in $CD4^+$ T cells and
262 with PD-1 and TIGIT in $CD8^+$ T cells, and finally associated with reduced T cells functions.
263 Importantly, BLIMP1 directly binds PD-1 and TIGIT promoter and positively regulates their
264 expression [62].
265 NR4A transcription factors family comprises three members, NR4A1, NR4A2 and NR4A3,
266 initially revealed as critical for Treg development [63]. Recently, NR4A factors were identified
267 as essential in mediating T cell exhaustion [64,65]. In a melanoma mouse model, NR4A factors
268 were upregulated in $CD8^+$ T cells with an exhaustion phenotype PD-1^{hi}TIM-3^{hi}, and motifs for
269 NR4A binding were enriched in accessible chromatin of these cells [64]. Additionally, $CD8^+$ T
270 cells transduced with either NR4A factors have higher expression of PD-1, TIM-3, LAG-3,
271 TIGIT and 2B4 IRs. Consequently, all NRA4 cooperatively impair antitumor immunity in
272 melanoma and colorectal mouse models [64]. Similarly, in tolerant $CD4^+$ T cells, transcriptomic
273 and epigenetic analysis revealed homologies with exhausted (virus-induced) and anergic
274 (NFAT-mediated) states [65]. Notably, NR4A1 was substantially expressed in tolerant cells,
275 where NR4A1 deficiency exacerbates $CD4^+$ T cells-mediated colitis. The authors further
276 assessed the role of NR4A1 in exhaustion and found that accordingly, NR4A1 deficiency
277 enhances $CD8^+$ T cells-mediated tumor eradication and virus-specific $CD8^+$ T cells activity.
278 Importantly, the expression of PD-1 and TIM-3 was significantly reduced in both instances,
279 consistent with the fact that NR4A is recruited at the regulatory regions of these genes [65].
280 Altogether, these studies indicate that NR4A induce a transcriptional circuit enforcing
281 exhaustion program throughout IRs expression, among others.
282 Another transcription factor included in the NFAT/IRF4-dependent reprograming is the
283 hypoxia-inducible factor (HIF) 1α [58,60]. HIF pathway is typically induced during hypoxia, a
284 feature of the microenvironment established by tumors or during viral infection. In $CD8^+$ T

285 cells experiencing LCMV infection, upregulation of the HIF pathway was associated with the
286 simultaneous expression of IRs CTLA-4, TIM-3, LAG-3 and 2B4 [66].
287 Interestingly, besides the viral and tumor context, inflammation caused by hypoxia during
288 cerebral ischemia can lead to severe neurological deficits. Using a mouse model of hypoxia-
289 ischemia revealed a HIF1α-dependent TIM-3 expression by glial cells [67]. Consequently,
290 blockade of TIM-3, as observed with HIF1α deficiency, limits expression of the
291 chemoattractants IL-1β and CXCL1 by glial cells, thereby reducing neutrophils infiltration and
292 eventually brain damage [67]. In a similar model, the HIF1α/TIM-3 axis has been shown to be
293 negatively controlled by the cannabinoid type 2 receptors (CB2R), which are mainly expressed
294 on immune cells such as microglia, as CB2R deficiency was associated with TIM-3
295 upregulation and larger brain lesions [68].
296
297
298 **Post-Transcriptional Regulation of IRs Expression**
299
300 *Regulation of IRs Expression by MicroRNAs*
301 MicroRNAs (miR) are short non-coding RNAs that negatively regulate mRNAs at the post-
302 transcriptional level by targeting their 3' untranslated region (UTR) [69]. They are implicated
303 in many aspects of immunity, and play an important role in tumor development and
304 autoimmunity [70], and represent potential targets to improve current ICB therapies [71]
305 (**Figure 3**).
306 MiR-155, described as a central regulator of immune response, directly binds to the 3'UTR of
307 *CTLA-4* and *BTLA* and decreases their protein expression [72,73]. Accordingly, miR-155
308 deficiency reduces $CD8^+$ T cells effector functions and decrease their ability to control viral
309 infection and tumor growth [74,75]. However, these results might not be directly linked to IRs

310 expression, as miR-155 is expressed in exhausted T cells from melanoma-bearing mice [76],
311 and is associated with T cell exhaustion in a chronic viral infection mouse model and correlates
312 positively with several IRs expression (PD-1, CD160, 2B4) [77].
313 In melanoma-bearing mice, $CD4^+$ T cells expressing PD-1 downregulate the expression of miR-
314 28 as compared to the PD-1 negative cells [76]. MiR-28 binds to the 3'UTR of *PDCD1*, *TIM-*
315 *3* and *BTLA*, whose expression consistently increase upon miR-28 silencing.
316 MiR-138 binds to the 3'UTR of *PDCD1* and *CTLA-4* mRNAs, and inhibits their protein
317 expression [78]. Moreover, the administration of miR-138 in mice with glioma induces a
318 reduction of tumor burden, and a decrease in PD-1 and CTLA-4 expression on T cells in the
319 TME. Using the same model, Yang *et al.* showed that miR-15a/16 deficiency correlates with a
320 lower expression of PD-1, TIM-3 and LAG-3 [79].
321 Several other miRs have been described to independently modulate IRs expression. MiR-4717
322 [80] and miR-374b [81] bind the *PDCD1* 3'UTR and inhibit PD-1 expression, while EBV-miR-
323 BART cluster correlates with PD-1 expression [82]. CTLA-4 is a potential target for miR-487a-
324 3p, which can bind the *CTLA-4* 3'UTR [83], and for miR-181a [84]. MiR-584-5p binds to the
325 3'UTR of *KLRG1* and decreases its expression [85], while miR-330-5p and miR-498 expression
326 correlate with TIM-3 downregulation [86,87]. MiR-23a, a downstream target of the long-
327 noncoding RNA MEG3, modulates TIGIT expression by binding to the 3'UTR of its mRNA
328 [88]. In $CD4^+$ T cells derived from patients with autoimmune anaplastic anemia, MEG3 is
329 downregulated, allowing the overexpression of miR-23a and inhibition of TIGIT expression
330 [88].
331
332 *Control of IRs mRNA Translation*
333 Recently, we identified a post-transcriptional mechanism that simultaneously modulate the
334 expression of several IRs [89] (**Figure 3**). Following T lymphocytes stimulation, the newly

335 induced mRNAs of IRs associate with stress granule-specific factors, such as G3BP1. These
336 ribonucleoprotein complexes are then transported by the kinesin 1, a molecular motor
337 composed of KIF5B and KLC1, along the microtubules to form stress granules [89]. Without
338 this molecular motor or without the microtubules, the maturation of the stress granules remains
339 locked in a state that does not allow their disassembly. Hence, this prevents the transport and
340 delivery of these mRNAs to the polysomes for their translation. Most of the IRs (PD-1, CTLA-
341 4, LAG-3, TIM-3, BTLA and TIGIT) are regulated by these stress granules and the microtubule
342 network, whereas stimulatory receptors (OX40, GITR, 4 -1BB) are not [89]. An essential
343 feature to follow stress granules regulation seems to lie within the higher length of the 3'UTR
344 mRNAs. In IRs, their mean length is similar to that of neuronal mRNAs displaying microtubule
345 transport for synaptic translation. Consequently, microtubule-targeting agents decrease IRs
346 expression on TILs of patients with Hodgkin's lymphoma, and display a significantly increased
347 risk for immune-related adverse events, as observed with ICB antibodies [89].
348
349 **Control of IRs at the Protein Level**
350
351 Protein stability, transport and turnover are crucial to maintain the proper homeostasis within
352 the cells and the surrounding environment. These different processes are undertaken by
353 conjugating partners or through specific post-translational modifications (PTM).
354 Huang *et al.* showed that co-expression of CEACAM1, a TIM-3 ligand also expressed by
355 lymphocytes, is required for TIM-3 stabilization and cell surface expression [90]. Indeed,
356 CEACAM1 deficiency causes TIM-3 hypoglycosylation and intracellular retention. In mice,
357 CEACAM1 deficiency enhances mucosa-associated inflammation owing to TIM-3 lower
358 expression at the $CD4^+$ T cell surface. On the other hand, in a colorectal cancer mouse model,

359 CEACAM1-deficient mice exhibited lower tumor burden and decreased TIM-3 expression by
360 the $CD8^+$ TILs [90].
361 CTLA-4 cell surface retention also depends on N-glycan branching [91], a hallmark altered in
362 the multiple sclerosis autoimmune disease [92]. Moreover, Treg suppressive functions depend
363 on glycosylation, whose level correlates with cell surface marker PD-1 and CTLA-4 [93].
364 CRISPR-Cas9-based screening identified core fucosylation, catalyzed by FUT8, as required for
365 PD-1 cell surface expression [94]. Impeding this modification causes intracellular retention of
366 PD-1 and enhances T cell activity. Consequently, B16 melanoma-bearing mice treated with
367 fucosylation inhibitors present a decreased tumor growth associated with increased rates of
368 TILs [94]. Moreover, the dynamics of PD-1 expression at the T cell surface is regulated by
369 ubiquitination-induced degradation. This reaction is mediated by the F-box protein FBXO38,
370 an E3 ubiquitin ligase member of the SKP1-CUL1-F-box protein family [95]. In B16F10
371 melanoma and MC38 colorectal carcinoma mouse models deficient in FBXO38, anti-tumor
372 immunity is compromised due to the higher expression of PD-1 at the surface of TILs. Anti-
373 PD-1 immunotherapy counteracts the FBXO38 deficiency, while IL-2 treatment could enhance
374 FBXO38 gene expression [95].
375
376 **Concluding Remarks and future Perspectives**
377
378 IRs targeting-based immunotherapies have shown unprecedented clinical benefits and durable
379 responses. Yet, despite these progresses, biomarkers identification, efficacy, and drug
380 resistance still pose significant therapeutic challenges. Because immune escape is partly due to
381 simultaneous expression of IRs, increasing the knowledge underlying their expression
382 represents a major concern (**see Outstanding Questions**).

383 Many researches revealed one layer of the regulatory mechanisms for one specific IR, and in
384 some instances, mechanisms controlling several IRs simultaneously have been identified.
385 Because the expression of IRs is not restricted to T lymphocytes but is found in basically all
386 immune cell types, it will be important to verify and validate the regulatory mechanisms in
387 these cells. Moreover, pathological context influences IRs expression on immune cells, for
388 instance through secreting factors. This cell- and context-dependent regulation emphasizes the
389 need to further improve the characterization of IRs regulation. This fundamental knowledge has
390 the potential to facilitate the development of alternative strategies able to improve therapeutic
391 outcomes unrestrictedly in cancer (**Box 1**) as well as in viral infection, autoimmune diseases
392 including neurological disorders.
393 Besides IRs, targeting of PD-L1 has met clear-cut clinical success. As for IRs, deciphering the
394 mechanisms regulating PD-L1 expression is indispensable to counteract immune escape
395 mechanisms, and tremendous efforts have been made to achieve this objective [5,6].
396 Interestingly, looking at the mechanisms regulating on one hand the IRs and on the other hand
397 PD-L1 reveals striking overlaps (**Box 2**). Future research will evaluate the targeting of such
398 regulatory pathways for enhancing antitumor immunity, and will inspire the design of new
399 therapeutic strategies in the upcoming years.
400

401 **Box 1. Potential of Targeting IRs Regulatory Mechanism for Immunotherapies.**

402

403 The different regulatory levels controlling IRs expression offer a multitude of theoretical targets
404 to modulate IR mRNA or protein levels and to enhance antitumor immunity (Figure I).
405 Conceivably, epigenetic drugs such as 5-azacytidine currently used for treating myelodysplastic
406 syndromes and acute myeloid leukemia might owe their efficacy to both impairing DNA
407 methylation of the cancer cells while reinvigorating cytolytic lymphocytes. Thus, associations
408 of epigenetic therapy and immunotherapy can target the epigenetic control of immune cells [96]
409 as well as the IR expression profiles. Ongoing phase II trial with azacitidine, entinostat, and
410 nivolumab *versus* nivolumab alone in patients with recurrent metastatic non-small cell lung
411 cancer (https://clinicaltrials.gov/) will determine the efficacy of such combinations, and
412 validate their ability to simultaneously hit multiple IR targets.
413 The therapeutic targeting of transcription factors in cancer was clinically validated with drugs
414 targeting nuclear hormone receptors [97]. Despite their potential, no inhibitor of HIF1/2 has
415 met both safety and clinical efficacy so far [98]. On the other hand, cannabinoid agonists of
416 CB2R might reinvigorate cytolytic lymphocytes, and could be tested in combination with ICB
417 of cancers such as neuroblastoma, hepatocellular carcinoma or pancreatic ductal
418 adenocarcinoma.
419 In addition, drugs selectively inhibiting either stress granules assembly or mRNA transport,
420 such as integrated stress response inhibitor (ISRIB) or microtubule inhibitors, respectively, can
421 impair both the expression and downstream function of IRs. In lymphocytes stimulated within
422 tumors, these agents inhibit simultaneously the translation of several IRs. These drugs now
423 warrant clinical investigation, notably in combination with nivolumab in cancer patients
424 relapsed after first-line ICB.

425 PTM have a pivotal role in immune function but also in cancer formation and progression. In
426 particular, as observed with the regulation of PD-1 expression by FBXO38 [95], F-box-
427 containing E3 ligases might represent future targets to selectively modulate IRs expression [99],
428 and inhibitors of cullin neddylation pevonedistat could potentiate immunotherapy [100].
429 Moreover, targeting glycosylation could bring benefits in autoimmunity or tumor control [101].
430 Indeed, in melanoma mouse model, fucosylation inhibition can not only act on tumor
431 aggressiveness [102] but also reduce PD-1 expression on T lymphocytes and enhance their
432 effector functions, which is even strengthened with anti-PD-1 immunotherapy [94]. Benefits of
433 such an inhibitor, the 2-fluorofucose, combined with immunotherapy will be determined by
434 ongoing clinical trial (NCT02952989).
435 Altogether, identification of key regulatory elements able to modulate IRs expression will allow
436 the design of new therapeutic approaches or molecules eventually effective for human cancer
437 treatment.
438

439 **Box 2. Similarities between PD-L1 and IRs Expression Regulation.**

440

441 Among all immune checkpoints, the PD-1/PD-L1axis is the best characterized. The regulation
442 underlying PD-L1 expression has been the focus of remarkable research, and expanding the
443 knowledge concerning PD-L1 regulation has shown great promises for improving current
444 immunotherapies or by suggesting alternative strategies [5,6]. These have revealed rather
445 complex multifactorial regulatory mechanisms that occur from triggering receptors to PTM.
446 Interestingly, some of these mechanisms stand out by the fact that they are also described to
447 regulate IRs. Therefore, future therapeutics targeting these shared pathways could be designed
448 to inhibit both receptors and ligands, which could further alleviate immune escape mechanisms.
449 For instance, IL-27 regulates the expression of multiple IRs on T cells [21,22]. Similarly, PD-
450 L1 expression on T cells was shown to require IL-27/STAT1 signaling [21,103], being involved
451 in suppressing Th17 differentiation [103]. In addition, IL-27 exerts immune regulatory
452 properties by inducing the expression of PD-L1 on dendritic cells and human epithelial ovarian
453 cancer cells [5].
454 Furthermore, several miR involved in IRs expression have also been shown to interact with the
455 3'UTR of *PD-L1* mRNA and suppress its translation [5,71]. Reduction of PD-L1 was observed
456 on colorectal cancer cell lines by miR-138, on primary human dermal lymphatic endothelial
457 cells by miR-155, and on malignant pleural mesothelioma cells by miR-15/16 [5,71].
458 Whole genome and RNA sequencing analyses of tumor cells overexpressing PD-L1 revealed
459 chromosomal aberration at the PD-L1 locus consisting in 3'UTR disruption [104]. In T cells, it
460 was suggested that stress granules-dependent IRs expression relies on the 3'UTR of IRs
461 mRNAs [89]. These data suggest that the 3'UTR of mRNAs restrain PD-L1 and IRs expression.
462 It is therefore tempting to speculate that stabilization of these interactions could reduce both
463 PD-L1 and IRs expression. Along the same line, inhibition of IL-27 expression or signaling, as

464 well as use of miR represent challenging approaches to consider for harnessing the potential of

465 immunotherapies.

466

Disclaimer Statement

N.C. and C.L. declare no conflict of interest. O.L., J.J.F and D.M.F. co-authored two INSERM patents on immune checkpoint control pathways.

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721

722 **Figure Legends**

723

724 **Figure 1. Overview of the Receptors and the Underlying Signaling Pathways Leading to**
725 **IRs Expression.**

726 In T cells, IRs expression is induced by several receptors following their stimulation, and
727 involves different signaling pathways. Kinases are illustrated in blue and transcription factors
728 in yellow. In green are represented PLCγ (phospholipase C γ), DAG (diacylglycerol) and CaN
729 (calcineurin) that do not belong to the kinases or transcription factors. The endoplasmic
730 reticulum (ER) is represented in purple. The IRs regulated by a signaling pathway are indicated
731 in green boxes at the bottom of each corresponding pathway.

732

733 **Figure 2. Epigenetic and Transcriptional Regulation of IRs.**

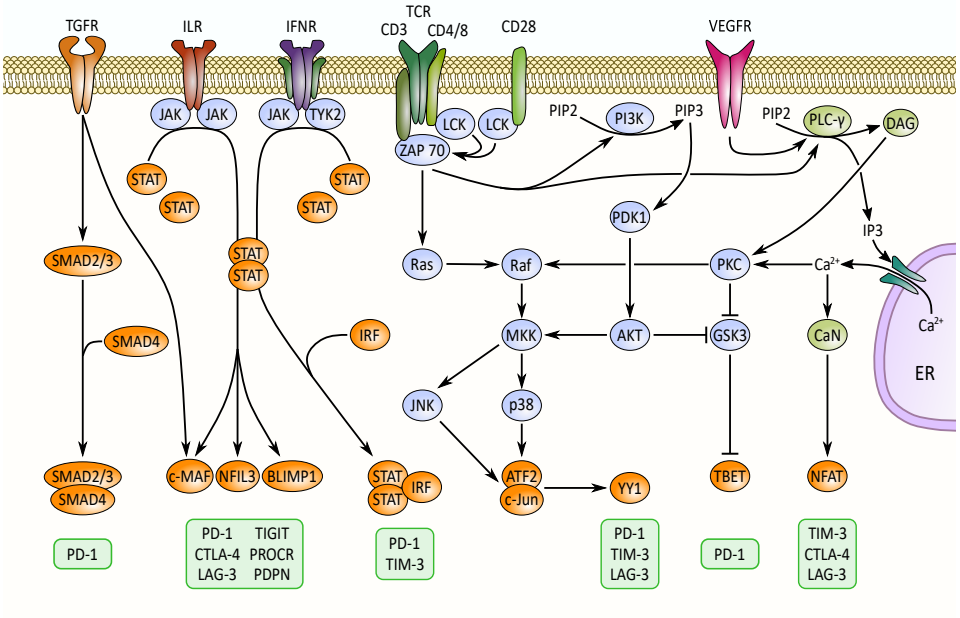
734 Upper panel: accessible chromatin that possesses unmethylated cytosines and permissive
735 histone marks such as H3K4me1, H3K9ac and H3K27ac. Transcription factors can then bind
736 to the regulatory regions (i.e. promoter) to allow transcription. Main transcription factors
737 involved in the control of multiple IRs are represented. Lower panel: within closed chromatin,
738 constituted of methylated cytosines and repressive histone marks such as H3K9me3 and
739 H3K27me3, transcription factors binding to the regulatory regions is precluded, thereby
740 preventing transcription of IRs.

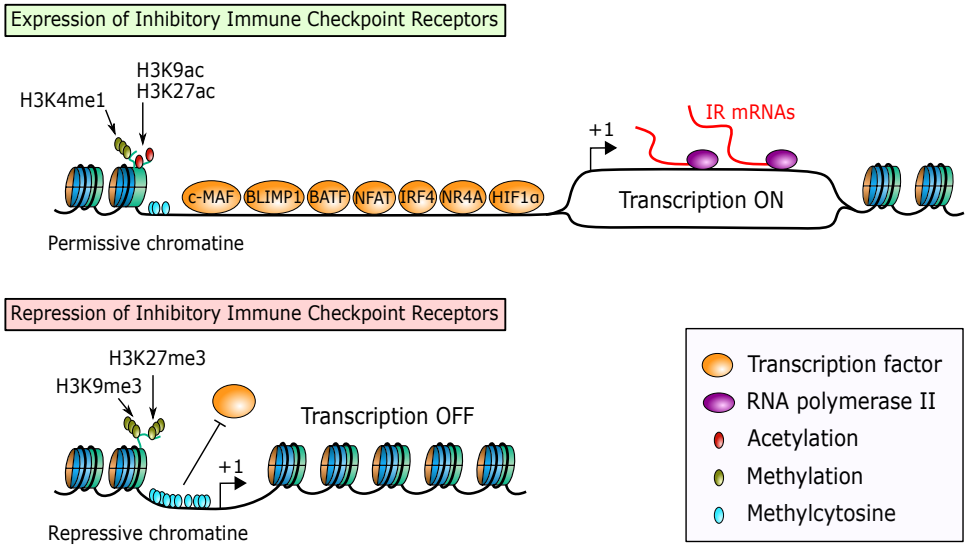
741

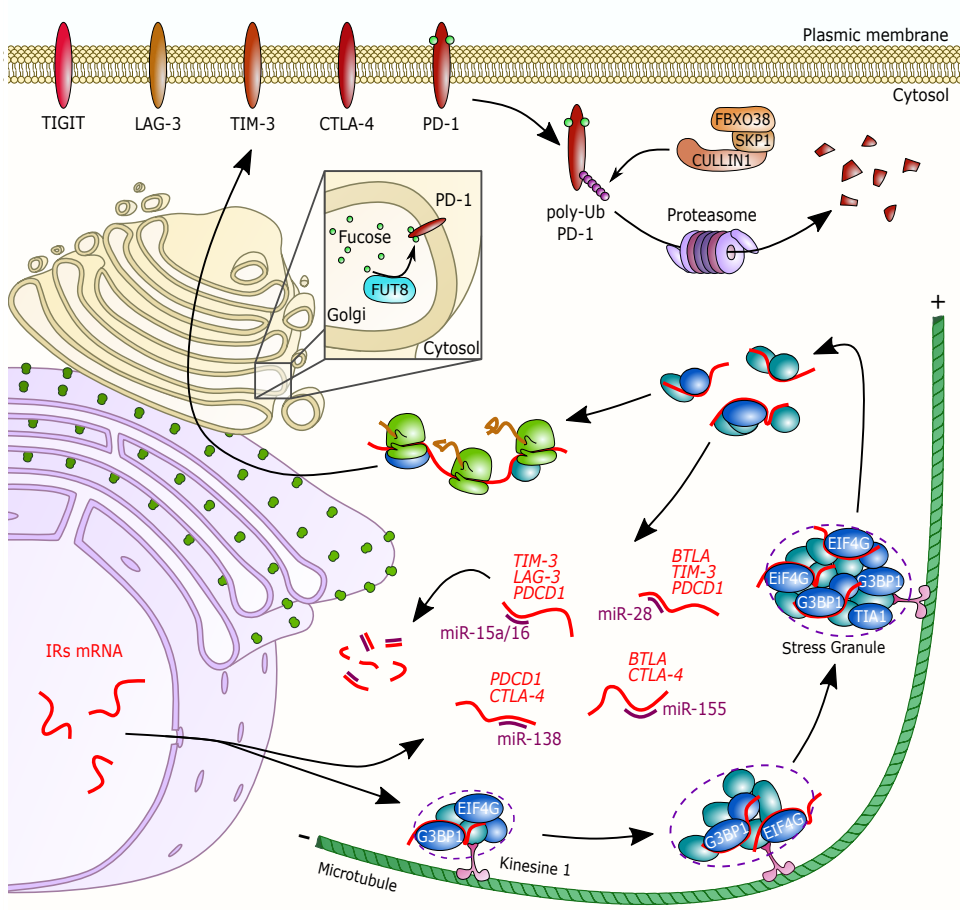
742 **Figure 3. Post-Transcriptional and Post-Translational Regulations of IRs.**

743 The mRNAs-encoding IRs that exit from the nucleus undergo post-translational regulatory
744 controls. These regulations operate through miR and stress granules targeting, and control
745 translation of multiple IRs. For miR regulation, mRNAs are depicted as "naked" for
746 simplification. Once translated, a further control step occurs through post-translational

747 modifications such as fucosylation and ubiquitination (Ub), which finalize the regulation of
748 PD-1 cell surface expression.
749
750 **Figure I. Targeting regulatory mechanisms of IRs expression along with ICB to potentiate**
751 **antitumor immunity.**
752 The biological processes leading to IR expression and eventually to T cell functions inhibition
753 are indicated in the blue boxes. Various small molecule inhibitors (written in red) can modulate
754 IR expression at the chromatin, RNA or protein levels to limit IRs cell surface expression. In
755 contrast, ICB (in green) target the function of IRs expressed at the cell surface. Combination of
756 both types of targeting can synergize to enhance antitumor immunity. Targeting agents are not
757 exhaustively listed, and new therapeutic agents could be developed at all targeting levels. The
758 graphic at the right-hand side represents an immune reaction of T cells before (blue) and after
759 (red) treatment with targeting agents, against a tumor cell. Expression of IR at the T cell surface
760 (blue cell) and interaction with its corresponding IR-ligand, expressed at the tumor cell surface,
761 lead to T cell functions inhibition and dampen antitumor immunity. Treatment with small
762 molecule inhibitors and ICB reduces IR expression and function, respectively, on T cell (in red)
763 to eventually improve antitumor immunity. DNMTi, DNA methyl transferase inhibitor;
764 HDACi, histone deacetylase inhibitor; MTD, microtubule-targeting drugs.
765


TGFR
ILR
IFNR
TCR
CD3
CD4/8
CD28
VEGFR
JAK
JAK
JAK
TYK2
LCK
LCK
ZAP 70
PIP2
PI3K
PIP3
PIP2
PLC-γ
DAG
STAT
STAT
STAT
STAT
STAT
STAT
PDK1
IP3
SMAD2/3
Ras
Raf
PKC
Ca2+
Ca2+
ER
SMAD4
IRF
MKK
AKT
GSK3
CaN
JNK
p38
SMAD2/3
SMAD4
c-MAF
NFIL3
BLIMP1
STAT
STAT
IRF
ATF2
c-Jun
YY1
TBET
NFAT
PD-1
PD-1
CTLA-4
LAG-3
TIGIT
PROCR
PDPN
PD-1
TIM-3
PD-1
TIM-3
LAG-3
PD-1
TIM-3
CTLA-4
LAG-3


Expression of Inhibitory Immune Checkpoint Receptors
H3K4me1
H3K9ac
H3K27ac
c-MAF
BLIMP1
BATF
NFAT
IRF4
NR4A
HIF1α
+1
IR mRNAs
Transcription ON
Permissive chromatine
Repression of Inhibitory Immune Checkpoint Receptors
H3K27me3
H3K9me3
Transcription OFF
+1
Repressive chromatine
Transcription factor
RNA polymerase II
Acetylation
Methylation
Methylcytosine


Plasmic membrane
Cytosol
TIGIT
LAG-3
TIM-3
CTLA-4
PD-1
FBXO38
SKP1
CULLIN1
poly-Ub
PD-1
Proteasome
PD-1
Fucose
FUT8
Golgi
Cytosol
+
TIM-3
LAG-3
PDCD1
miR-15a/16
BTLA
TIM-3
PDCD1
miR-28
EIF4G
EIF4G
G3BP1
G3BP1
TIA1
Stress Granule
IRs mRNA
PDCD1
CTLA-4
miR-138
BTLA
CTLA-4
miR-155
EIF4G
G3BP1
Kinesine 1
G3BP1
EIF4G
-
Microtubule