

Available online on 15.06.2023 at <http://jddtonline.info>

# Journal of Drug Delivery and Therapeutics

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Review Article

## Dual immune checkpoints inhibition: Cancer treatment and immunological modes of action

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### Article Info:

#### Article History:



Received 21 March 2023

Reviewed 09 May 2023

Accepted 23 May 2023

Published 15 June 2023

#### Cite this article as:

Ramteke AS, Dual immune checkpoints inhibition: Cancer treatment and immunological modes of action, Journal of Drug Delivery and Therapeutics. 2023; 13(6):175-187

DOI: <http://dx.doi.org/10.22270/jddt.v13i6.5880>

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### Abstract

Programmed death receptor 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) are immune checkpoint inhibitors that are effectively esteemed destinations of immunotherapies for the management of melanoma and several cancers. The monotherapy included monoclonal antibodies approved such as Ipilimumab, Pembrolizumab, and Nivolumab planned to restrict with T-cell inhibitory signals to activate the immune responses to cancers. Combined treatment of immune checkpoint blockers can promote results compared to monotherapy in specific patient groups and these clinical advantages can be reported from particular immune mechanisms of action. Although, treatment with checkpoint blockers combinations are present important clinical challenges and raised rates of immune related adverse incidents.

**Keywords:** Cancer, Checkpoint Inhibitors, T-cell, Immunotherapy, Biomarker

### 1. Introduction

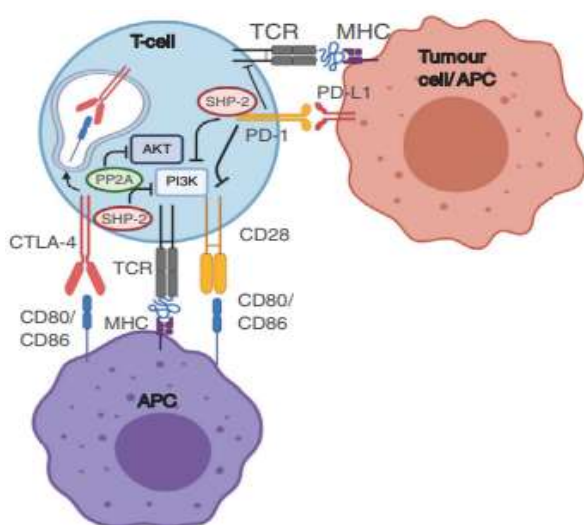
Melanoma is the 5<sup>th</sup> common most cancer in the united nation, with an evaluated 300000 cases recorded globally per year. Melanoma is noticed as the archetypal immunogenic cancer as promoted through clinical investigation of spontaneous cancer regressions and raised melanoma rates in immunosuppressed independent. Melanoma imports a large mutational load, dispersing a range of cancer particular antigens that can pass

the host immune response.<sup>1</sup> Moreover, cancer like melanoma, may invade immunosurveillance by activation of varying immune inhibition involving through immune checkpoint inhibitors and their downstream signals. Checkpoint blockers conduct a role in immune homeostasis supporting negative feedback stimuli to distract autoimmune reactivity. The better describe checkpoint blockers are those of negative regulatory molecules like CTLA-4, PD-1, and its ligands PD-L1 and PD-L2.<sup>2</sup>

Checkpoint inhibitors (CPIs) were created to encourage immune mediated rejection of cancer cells. Monoclonal antibody binding to either PD-1 (Pembrolizumab and Nivolumab) or CTLA-4 (Ipilimumab) outcomes in signaling repeal in response to these inhibitory receptors' ligands in the cancer microenvironment (TME) or withdrawing lymph nodes.<sup>3</sup> Their authorization for the melanoma management has modified prognosis in the last few decades, compared with earlier deficient 5 years survival rates in advanced disease with basic sedative alternative treatment. CPIs were firstly authorized as monotherapies, while more current proof acknowledged that combine immunotherapy may increase the efficiency of these treatments, possibly because of working in an interdependent manner.<sup>4</sup>

## 2. CTLA-4 immune checkpoint pathway

CTLA-4 is an immunoglobulin cell surface receptor which an inhibit activation of T-cell. It is basically displayed on naive T-cells after activation and FoxP3<sup>+</sup> regulatory T-cells (Tregs). T-cell activation is dependent not only TCR binding with an antigen expressed through an APC, but also on the showing of a costimulatory second signal, generally by binding of CD28 presented on the T-cell to CD80/86 initiated on the APC.<sup>5</sup> Absence of this secondary signal can result the T-cell to realize the expressed peptide as a "self-antigen" or to create tolerance against antigen. CTLA-4 is a competing homolog for CD28 that has a higher binding affinity to CD80 (B7-1) and lower to CD86 (B7-2) as compared with CD28 which cause to T-cell blocking co-stimulation (Fig. 1).<sup>6</sup>



**Figure 1:** Negative regulation of PD-1 and CTLA-4 checkpoint pathways to T-cell activation<sup>6,8,9,22,78</sup>

TCR signaling directly upregulates surface CTLA-4 expression, increasing peak expression at 2-3 days after activation, presenting a negative feedback loop upon T-cell activation. CTLA-4 in intracellular vesicles is possibly carried to the immunologic synapse coming after T-cell activation. At the immunologic synapse, CTLA-4 is balanced through CD80/CD86 binding, performing it to collect and block CD28 binding.<sup>7</sup> CTLA-4 limits CD28 downstream signaling, blocking PI3K and AKT pathways (Fig. 1). CTLA-4 binding to CD80/86 leads an intracellular negative feedback pathway, reached by the tyrosine phosphatase SHP-2 and the serine/threonine phosphatase PP2A, which dephosphorylating signaling kinases further downstream (Fig. 1).<sup>8</sup>

Additionally, CTLA-4 behaves extracellularly to eliminate CD28 ligands CD80/86 from nearby cells through trans-endocytosis in vivo, involving from APCs, more blocking T-cell activation (Fig. 1). Physiologically CTLA-4 is thought to basically exert a modulatory role in T-cell priming in regional secondary lymphoid organs, through blocking T-cell activation and disturbing the effector T-cells production.<sup>9</sup> The unfavorable CTLA-4 function in sustaining self-tolerance is demonstrated through CTLA-4 knockout mice which have been seen to create fatal lympho-proliferative disease at 3 to 4 weeks of age. Also, this important function, CTLA-4 is also view to dampen T-cell activation in the periphery.<sup>10</sup>

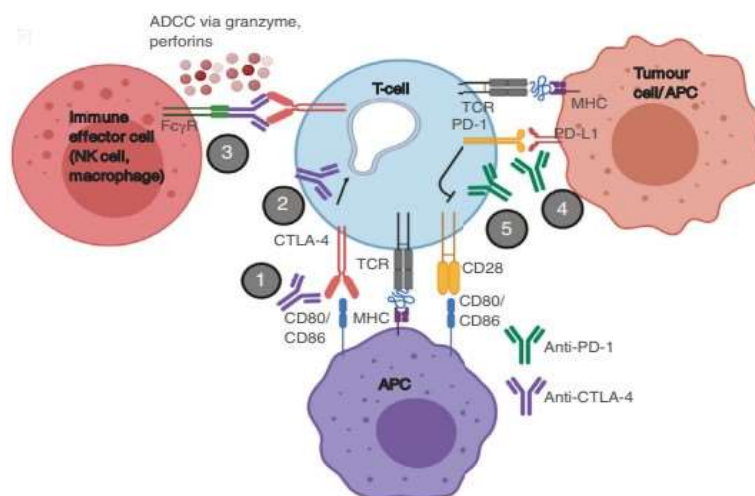
This is assisted through examined basic expression of its ligands CD80/CD86 to different degrees through APC as well as through activated T-cells. However, attenuating T-cell activity by cell-intrinsic functions, CTLA-4 serves cell-extrinsic functions mediated basically by CTLA-4 expressing FoxP3<sup>+</sup> Tregs.<sup>11</sup> Tregs may probably negatively regulate passerby effector T-cells expressing CD80/86 through regulating accessibility of these ligands for CD28 co-stimulation. Particular loss of CTLA-4 expression in Tregs in mice has been correlated with autoimmunity and unnecessary T-cell activation.<sup>12</sup>

## 3. Anti-CTLA-4 activated cancer decline

FDA firstly approved Ipilimumab in 2011, anti-CTLA-4 monoclonal antibody for use in advanced melanoma, which interferes supported positive responses. The main action mechanism of Ipilimumab leads to be by direct blocking of CTLA-4 binding with ligands CD80/CD86 to permitting for CD28 co-stimulation and after T-cell activation (Fig. 2).<sup>13</sup> CD80/86 are basically presented in the secondary lymphoid organs causing to the view that ipilimumab reacts to stop

activation earlier in T-cell development. The mechanisms of anti-CTLA-4 introduced cancer elimination are not fully

clarified but preclinical and clinical investigations observe the major targets as the effector T-cell compartment and Tregs.<sup>14</sup>



**Figure 2:** Mechanisms of action of anti-CTLA-4 and anti-PD-1 antibodies<sup>13,16,78</sup>

Ipilimumab treatment has been connected with evolution of ICOS<sup>+</sup> CD4<sup>+</sup> T-cells in melanoma and further cancers microenvironment. The immunosuppressive effect of cancer cells is in part moderated through engagement of Tregs, while these cancer inhabitants Tregs mostly present CTLA-4.<sup>15</sup> Murine cancer models, *ex-vivo* and neoadjuvant clinical investigations of Ipilimumab have reported a decrease in cancer infiltrating and circulating Tregs following therapy. Ipilimumab introduced Treg reduction is consider to be managed through Fc binding to Fc- $\gamma$  receptors on atypical macrophages in the TME causing to antibody dependent cell mediated cytotoxicity (**Fig. 2**).<sup>16</sup>

An effector mechanism is helped through the exploring that germline presence of a most bond polymorphism of the Fc receptor is connected with greater responses to Ipilimumab recommending that Fc-dependent cell eliminate partly presents the anticancer mechanism.<sup>17</sup> An earlier clinical investigation resists this through elaborating that anti-CTLA-4 does not diminish Tregs. Early evaluated this and reported that biopsies timing and sampling bias are more hurdle in the clinical investigations as compared to mouse models.<sup>18</sup>

#### 4. PD-1/PD-L1 immune checkpoint pathway

PD-1 is belonging to immunoglobulin family recognize for immune homeostatic through inducing inhibitory signals on the linking with its ligands, PD-L1 and PD-L2. PD-1 is belief to

be a negative regulator of T-cell function which controlling peripheral tolerance and T-cell responses.<sup>19</sup> PD-1 is displayed more widely than CTLA-4 and may be presented on T-cells, B-cells, NK cells, and different peripheral tissues. The ligand PD-L1 is widely presented through immune cells involving T-cells, B-cells, DCs, and macrophages, and in nonlymphoid tissues involving on cancer cells or in TME.<sup>20</sup>

While the expression of PD-L2 is more confined but it has also been illustrated on a variety of cancers involving melanoma, expression of PD-1 is upregulated on T-cells and B-cells activation. Inflammatory cytokines, like IFN- $\gamma$ , are belief to introduce expression of PD-L1, and to a lower degree expression of PD-L2.<sup>21</sup> Hence, PD-1 mediated modulation of T-cell function is introducible upon IFN- $\gamma$  production generally in the context of cytolytic and effector T-cell events. After PD-1 attracts with its ligands, a negative signal is transmitted by the tyrosine phosphatase SHP2 to relax T-cell activity (**Fig. 1**).<sup>22</sup>

Immunologically, because of its negative costimulatory effects PD-1 is required for tolerance achievement in peripheral tissues as reinforced through the autoimmune abnormalities, involving autoimmune dilated cardiomyopathy and lupus like syndrome. PD-1 expression is connected to an “exhausted” T-cell phenotype in the condition of continued antigen exposure.<sup>23</sup> This happens in the cancer setting or chronic viral infection, whereas continued T-cell stimulation outcomes in a

gradual lower in the effector function of CD8<sup>+</sup> cells. However, PD-1 is a marker of exhaustion and its expression individually does not explain an exhausted T-cell phenotype. PD-1 is a marker for T-cell activation, an exhausted subset of phenotype, which still reserve some effector activity.<sup>24</sup>

PD-1 expression makes extent to achieve than CTLA-4 (6-12 h for PD-1 as compared to 1 h for CTLA-4) (**Table 1**). The CD80/86 expression patterns and PD-L1/PD-L2 are revolve

distinctions between PD-1 and CTLA-4. They show to conclude that CTLA-4 functions recent for introduction of tolerance, whilst PD-1 function is detained to assisted maintenance of tolerance.<sup>25</sup> Intracellularly, CTLA-4 and PD-1 signal through SHP2 and intersect to block downstream PI3K signaling. Similarly, CD28 for which CTLA-4 is a homolog, whereas secondary target for PD-1 mediated dephosphorylation controlling to additional inhibition of co-stimulation.<sup>26</sup>

**Table 1:** Comparison of CTLA-4 and PD-1 signaling and immune pathway<sup>25,78</sup>

	<b>CTLA-4</b>	<b>PD-1</b>
Ligands	CD80 and CD86	PD-L1 and PD-L2
Expression profile of ICIs	Limited expression Naive T-cells, Regulatory T-cells	Broad expression T-cells, B-cells, NK cells, Peripheral tissues
Expression profile of ligands	Limited expression T-cells and APCs	Broad expression T-cells, B-cells, dendritic cells, macrophages, cancer cells
Stimulus	TCR activation upregulates CTLA-4 expression	TCR activation upregulates PD-1 expression BCR activation upregulates PD-1 expression
Timeframe	Expression within 1 h	Expression within 6-12 h
Downstream signaling	Signals via SHP-2 to inhibit PI3K Signals via PP2A to inhibit AKT	Signals via SHP-2 to inhibit ZAP-70 and PI3K
Proposed roles in human physiology	Acting primarily in secondary	Acting primary in peripheral tissues
Modulation of T-cell responses with ICIs	Upregulation of CD4 <sup>+</sup> effector T-cells Depletion of FoxP3 <sup>+</sup> regulatory T-cells	Upregulation of activated CD8 <sup>+</sup> T-cells

## 5. Anti-PD-1/PD-L1 activated cancer decline

Nivolumab and Pembrolizumab, anti-PD-1 antibodies were authorized in advanced melanoma through the FDA in 2014 and European Medicines Agency in 2015. PD-1 is thought to stop cell activity in the effector phase in tissues and cancers,<sup>27</sup> this is in comparison with the role of CTLA-4 which is consider to basically regulate immune functions in the earlier phase of T-cell activation. Preclinical and clinical models recommend that the primary mechanism of action of anti-PD-1 therapy is to decrease the number of phenotypically “exhausted” CD8<sup>+</sup> cytotoxic cells.<sup>28</sup>

In mouse melanoma models, cancer growth was terminated in PD-1 KO mice or with anti-PD-1 antibodies. PD-L1 expression is often upregulated in cancers and can be prognostic in melanoma. Although, PD-L1 positivity is not a necessity to achievable anti-PD-1 therapy.<sup>29</sup> Additionally, directly regulating T-cell activation, expression of PD-L1 on macrophages has also been connected with raised T-cells displacement from the TME, recommending the PD-1/PD-L1 axis activity in affecting T-cell migration. T-cells positive for PD-1 are reviewed more possible to be cancer antigen-specific compared with T-cells stopped in the early stage through CTLA-4.<sup>30</sup>

Hence, monoclonal antibodies capable to restrict with the T-cell immunoinhibitory functions of PD-1 would in study be more possibly to activate antigen-specific T-cell responses in cancer patients. In melanoma, anti-PD-1 therapy is concluded to introduce cancer rejection mainly through reactivating CD8<sup>+</sup> T-cells earlier in an “exhausted” state.<sup>31</sup> Continuously patient blood samples on anti-PD-1 therapy illustrate rise in PD-1<sup>+</sup> CD8<sup>+</sup> T-cells. The proliferation of an “exhausted” CD8<sup>+</sup> phenotype in murine melanoma models in response to anti-PD-1 therapy and this negatively associated with cancer growth.<sup>32</sup>

An anti-PD-1 treatment response is dependent on a T-cell response in the TME, with coming PD-L1 expression on cancer cells as a result of IFN- $\gamma$  production upon T-cell activation. This is a quickly developing area and various further inhibitory checkpoint molecules are being explored,<sup>33</sup> involving lymphocyte activation gene 3 (LAG3), T-cell immunoglobulin 3 (TIM3), T-cell immunoglobulin, ITIM domain TIGIT, and programmed death 1 homologue (PD-1H). A numerous clinical investigations are presently examining targeting these checkpoint molecules either alone or in combination with ongoing therapies.<sup>34</sup>

## 6. Clinical trial: Monotherapy vs. combined therapy

The antibodies Ipilimumab (anti-CTLA-4) with Nivolumab and Pembrolizumab (anti-PD-1) are authorized, which use as monotherapy for the advanced metastatic melanoma treatment, and also presently as adjuvant therapy in resected disease.<sup>35</sup> Combined therapy with Ipilimumab and Nivolumab is authorized for metastatic melanoma cancer. Nivolumab and Pembrolizumab have reported better long-term efficiency and safety rules vs the anti-CTLA-4 antibody Ipilimumab in face-to-face phase III cases in advanced melanoma.<sup>36</sup>

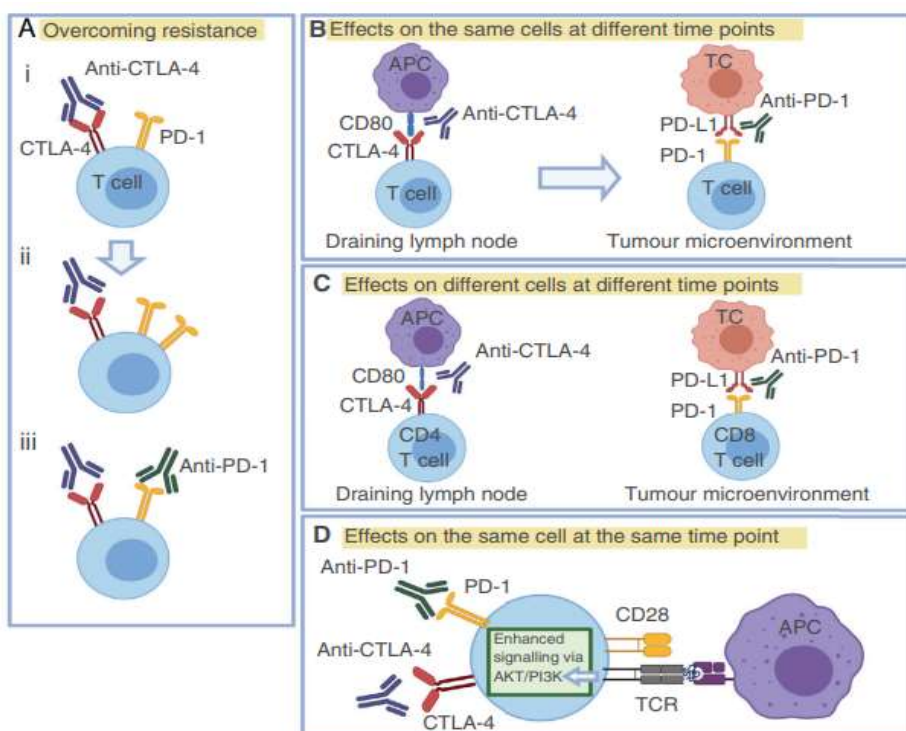
The major proof for the clinical use of combined checkpoints immunotherapy arrives from the landmark Check-Mate 67 clinical trial (NCT01844505). In 945 melanoma patients with stage III/IV, this investigation direct reported combined therapy with Nivolumab and Ipilimumab in contrast with

Nivolumab or Ipilimumab monotherapy.<sup>37</sup> The median overall survival (OS) was more than 60 months, at a minimal follow-up time of 60 months (median not progressed). For the Nivolumab with Ipilimumab group, in comparison with 19.9 months for the Ipilimumab and 36.9 months for the Nivolumab group. The 5 years of OS rates were recorded as 52, 44, and 26 %, respectively.<sup>38</sup>

While OS shows bettered with combined therapy, the trial was not pushed to see a statistical variation between the 2 Nivolumab including groups (HR) for Nivolumab with Ipilimumab vs Nivolumab alone is not considerable (HR 0.83 95 % CI, 0.67 to 1.03).<sup>39</sup> The frequency of adverse incidents was raised in combined therapy in this case, and attracts the challenges in estimating patient response. Combined immunotherapy of Ipilimumab and Pembrolizumab is not presently authorized in clinical trial. An open label, phase-I b trial has illustrated possibility of this combined immunotherapy in advanced melanoma.<sup>40</sup>

## 7. Possible modes and merits of combine immunotherapy

PD-1 and CTLA-4 are inhibitory receptors, they function to block T-cell activation through definite needed mechanisms, likely functioning at separate positions and time in T-cell evolution (**Fig. 3A-C**). Combined anti-CTLA-4 and anti-PD-1 therapy can provide boosted clinical results in comparison to monotherapy. Although, we don't know if combined therapies manage in a complementary fashion and various investigations have demonstrated whether these receptors can even function in a symbiotic way (**Fig. 3A-D**).<sup>41</sup> Apparent cellular mechanisms underlying the CTLA-4/PD-1 checkpoints, the expression characteristics of CTLA-4/PD-1 and their ligands signals that they may react for visible times in T-cell evolution, at the secondary lymphoid organs (CTLA-4) vs TME (PD-1).<sup>42</sup>



**Figure 3:** Possible effects of combined checkpoint immunotherapy on T-cells<sup>41,43,50,78</sup>

This is assisted through the different interval over which CTLA-4 and PD-1 are displayed (6-12 h for PD-1 in comparison 1 h for CTLA-4) upon T-cell activation. Although, we remember that there is a convergence degree with regards to the cell intrinsic mechanisms of these pathways. Both pathways connect on the PI3K/AKT pathway (**Fig. 3D**) and PD-1 blocks CD28 for which CTLA-4 is a competitive homolog, causing to

coincide and improvement of T-cell activity.<sup>43</sup> The underlying mechanisms of combined therapy have been investigated in animal models and in clinical trial. While there is unclear agreement as to the immunological features and their significances shown in the setting of combined therapy, further investigations may create on live imagination on arising immune responses (**Table 2**).<sup>44</sup>

**Table 2:** Comparison of immune variations with monotherapy vs combined therapy<sup>44,78</sup>

Treatment	Tumor model	Reference by
Anti-CTLA-4 monotherapy	Preclinical murine tumor model Clinical PBMCs	Curran et al, Wei et al Wei et al, Das et al
Anti-PD-1 monotherapy	Preclinical murine tumor model Clinical PBMCs	Curran et al, Wei et al Wei et al, Huang et al, Das et al
Combined therapy	Preclinical murine tumor model Clinical PBMCs	Curran et al, Wei et al Wei et al, Das et al

### 8. Preclinical outcomes

Various murine models illustrate proof of symbiotic effects of combined therapy, generally highlighting on the function of T-cells in the TME. An *in-vivo* mouse model has reported

combined therapy to be related with raised numbers of IL-2 producing and proliferating CD8<sup>+</sup> T-cells.<sup>45</sup> Fascinating, the outcomes of this study recommend that this is a basic response and not through T-cell engagement from the periphery. These

variations are related to cancer regression. Cancer derived T-cells has been phenotype through mass cytometry with monotherapy (anti-CTLA-4 or anti-PD-1) and combined therapy.<sup>46</sup>

They displayed that combined therapy to be the better treatment, conducted through a definite T-cell phenotype. Combined CPI noticeably raised the efficiency of activated effector CD8<sup>+</sup> cells, while continuously lowering the efficacy of exhausted effector CD8<sup>+</sup> cells. Although, anti-PD-1 monotherapy had the reverse effect, which showed to progress of exhausted CD8<sup>+</sup> T-cells, with no effect on activated CD8<sup>+</sup> T-cells.<sup>47</sup> These records demonstrated that combination immunotherapy can disturb or reverse exhaustion of CD8<sup>+</sup> cells. It was recommended that there is no variation in the proliferation of phenotypically exhausted CD8<sup>+</sup> T-cells between treatment traits.<sup>48</sup>

The reported lower in efficiency of exhausted T-cells in response to combined therapy is not because of an updated cells proliferation. The investigation revealed that, CD4<sup>+</sup> effector T-cell population was positively regulated through combined therapy. T-cells of a Th<sub>1</sub> CD4<sup>+</sup> effector phenotype inflated in efficiency coming after anti-CTLA-4 monotherapy instead of anti-PD-1 therapy.<sup>49</sup> Contrarily, combined therapy leads a several rise in CD4<sup>+</sup> effector T-cell efficacy compared with anti-CTLA-4 monotherapy. This addition raised of the Th<sub>1</sub> CD4<sup>+</sup> effector cell compartment in combined therapy condition recommends a possible linkage of the two pathways. An anti-CTLA-4 monotherapy can upregulate PD-1 expression restricting several developments of CD4<sup>+</sup> cells (**Fig. 3A**).<sup>50</sup>

Regulation of the CD4<sup>+</sup> T-cells through anti-PD-1 monotherapy would be dependent on early assigning of anti-CTLA-4. Combined therapy also caused to lowered Treg efficiency, after the suppression progressed through every monotherapy. Combined inhibition also collaboratively raised T-cell effector functions in a B16 murine model of melanoma.<sup>51</sup> Particular checkpoint blockade using anti-PD-1 or anti-CTLA-4 was assisted through raised effector CD8<sup>+</sup> T-cell infiltration in the TME. Hence, this expansion was restricted because of a satisfactory rise of CTLA-4 and PD-1 expression.<sup>52</sup>

Contrarily, continuous blockade of both immune checkpoints permitted effector T-cells to simultaneous proliferate in the TME and caused to favorable upregulation of effector T-cells compared with inflammation supporting Tregs.<sup>53</sup> The same investigation reported raised synthesis of pro-inflammatory

cytokines IFN- $\gamma$  and TNF- $\alpha$  from effector CD8<sup>+</sup> T-cells in cancers in the circumstances of combined therapy. Their outcomes recommend that combined therapy can be double as beneficial as monotherapy in progressing rejection of B16 melanoma and the cancers connect this with expansion of the effector T-cell compartment.<sup>54</sup>

## 9. Discrimination from treating with checkpoint inhibitor

The results of anti-CTLA-4 with anti-PD-1 combined treatments have been investigated in peripheral blood samples from melanoma patients, particularly diagnosing T-cells in samples from patients treated with monotherapy Nivolumab, Ipilimumab, and Pembrolizumab, or combination of both Ipilimumab and Nivolumab. Dual immunotherapy allowed an evolution of circulating PD-1<sup>+</sup> and PD-1<sup>-</sup> CD8<sup>+</sup> T-cells presenting the proliferation and cell cycling marker Ki-67.<sup>55</sup> Compared with samples from ones that were explored with anti-PD-1 or anti-CTLA-4 monotherapies, where Ki-67 was find out at most in the activated PD-1<sup>+</sup> CD8<sup>+</sup> T cell subsets. Since, CD8<sup>+</sup> T-cell efficiency was raised in the outfit who received combined therapy in comparison to a trait that received anti-PD-1 monoclonal antibody alone.<sup>56</sup>

Ultimately modified meta-cluster 1 CD8<sup>+</sup> T-cell ranges in the blood were beneficially higher after combination immunotherapy as resisted to individual anti-CTLA or anti-PD-1 therapies. Combined anti-CTLA-4 with anti-PD-1 therapy leads increase to distinctive transcriptional events comparison to monotherapy. Blood samples from 45 patients evaluated experience monotherapy with anti-PD-1 or anti-CTLA-4 vs combined CPI.<sup>57</sup> They early employed a genome wide strategy to investigate gene expression characteristics in peripheral T-cells and monocytes before and 3 weeks after either monotherapy (Ipilimumab or Nivolumab) or combine (Ipilimumab and Nivolumab). They displayed that variations in peripheral T-cells were higher marked comparison to variations in monocytes.<sup>58</sup>

Combined inhibition caused to non-overlapping variations in gene expression compared with monotherapy. For instance, only combined therapy upregulated gene expression of IL-8 and HLA-DR and also anti-CTLA-4 and combined therapy both induced Ki-67 (proliferation marker). Mass cytometry of peripheral blood T-cells recognized that Ki-67<sup>+</sup> T-cells in the assisting of anti-CTLA-4 and combined therapy have a developmental cell memory phenotype.<sup>59</sup> Preclinical

investigations illustrating CTLA-4 mediated proliferation depletion and raised memory following CTLA-4 blockade in mice. Evaluation of variously displayed coding transcripts reported that the major pathway presented in the setting of anti-CTLA-4 therapy and combined therapy was cell cycle or proliferation.<sup>60</sup>

The upregulation of these pathways was noticed in the assisting of combined therapy. Contrarily, anti-CTLA-4 or combined immunotherapy, genes altered through anti-PD-1 therapy didn't form a proliferation signature and rather illustrated enrichment for genes included in cytolytic events and resist of effector T-cell and NK cell function. This suggests that every monotherapy and combine therapies cause to various results in evolving human T-cells *in-vivo*.<sup>61</sup> Different variations in hindering T-cells, Ipilimumab, Nivolumab, and combined therapy can result diverse variations in systemic cytokine levels. Particularly, soluble IL-2R is upregulated fallow combine therapy. Moreover, IL-1 $\alpha$  levels raised after anti-PD-1 and combined therapy with anti-PD-1 and anti-CTLA-4.<sup>62</sup>

CXCL10 levels may effort to immune cell triggering events in the TME, were promoted followed anti-CTLA-4, anti-PD-1, and combine treatment. These outcomes demonstrate the different variations of peripheral blood cytokine ranges shown with individual type of checkpoint immunotherapy.<sup>63</sup> While oppose to CPI monotherapy is not whole explained, it is possible that the boosted efficiency reported with combined can be by reducing several major resistance mechanisms. As an example, a satisfactory rise T-cell related checkpoints has been illustrated with CPI monotherapy. PD-L1 expression on peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T-cells can forecast resistance to anti-CTLA-4 therapy, recommending a require for combine therapy.<sup>64</sup>

PD-L1 expression in cancers has been investigated as a feasible marker for patient selection for CPI. While PD-L1 is seen to act as an inoperative biomarker, PD-L1 enhances only marginally the forecast compared to random assignment.<sup>65</sup> Although, in the similar investigation, an underpowered subgroup evaluation disclosed that the progression free survival (PFS) of the PD-L1 positive population trialed with Nivolumab monotherapy is the similar as the PFS for that of combined therapy with Ipilimumab plus Nivolumab (14 months). This recommended that there can be advantage in linking PD-L1 positivity as a biomarker for patient groups and may notify a subgroup of patients that can't get added merit from combined CPI.<sup>66</sup>

MHC class I molecules levels presented in melanoma can provide various reactivities to anti-CTLA-4 and anti-PD-1 inhibition, whereas with the downregulation of MHC I being a primary resistance mechanism to anti-CTLA-4 inhibition, but not to anti-PD-1 inhibition, related with accelerating disease and absence of clinical response. This corporation was not reported seen to affect responses in combined therapy,<sup>67</sup> several supporting the idea that various action mechanisms in these agents act equivalent to create superior anticancer immune responses. Notifying patients with decrease MHC I levels on observation can act as a selector for patients who can be capable to advantage from combined therapy long-lasting response to diminishing the disease progression risk related with monotherapy.<sup>68</sup>

While CPIs are planned to upregulate T-cell effector functions, there is raising alert on the support of B-cell responses to patient results and the progress of immune related adverse events. CPI can enhance the B-cell phenotype in the periphery and the melanoma TME, hence, relation with clinical response is different. Some investigations have illustrated a different B-cell phenotype in the setting of combined therapy compared to monotherapy. It was recorded that in 23 patients following a combine therapy cycle with Nivolumab and Ipilimumab, there was an overall decrease in the entire numbers of circulating CD19<sup>+</sup> B-cells but raised plasma blast and CD21 less B-cell subset estimates.<sup>69</sup>

This result was not found in 16 patients who gained monotherapy with either Nivolumab or Ipilimumab. PD-1 expression was increase on the CD21 low B-cells compare to other B-cells, which recommending that these cells can be particularly regulated through anti-PD-1 therapy. This observation did not illustrate cooperative between variations in B-cell response in combined therapy and clinical response. Furthermore, the conclusion of these outcomes can be insufficient because of the minimal sample size and on treatment the peripheral blood samples were only drawn at before time point after CPI initiation and can't be indicative of the full or long-term response.<sup>70</sup>

Latest details have come to light notifying a group of patients who not only absence to respond to immunotherapy but also promote rise of disease progression in the setting of checkpoint blockade. This is introduced to as "hyper-progression" and has been outlined in a variety of cancers involving select trials of melanoma. It is not promptly mentioned, hyper-progression is introduced to through few

investigations as a >50 % rise in cancer volume at first estimation following treatment in comparison to baseline. The huge majority of trials of hyper-progression experienced CPI monotherapy individually. Furthermore, there are evaluated patient's cases creating hyper- progressive disease after combined CPI with anti-PD-1 and anti-CTLA-4.<sup>71</sup>

The basic mechanisms are still not well defined, but are possible to be diverse based on cancer type, regulation of TME through earlier therapy and the patient's immune system. A mechanism considered is that earlier medications with chemotherapy can select disrupt cancer cell clones which are eventually or afterward produced to proliferate after CPI. Regulation of immune subsets is suitable to play a role. For instance, rise of intra-cancerous regulatory T-cells in patient's treatment with gastric cancer who experienced hyper-progressive disease. Several mechanistic perceptions are required earlier it may be civilized whether combined CPI is protective or harmful.<sup>72</sup>

Preclinical and clinical investigations recommend that combined anti-CTLA-4 and anti-PD-1 immunotherapy can cause to a different immune outline assisting improved cancer rejection. This involves upregulation of effector T-cell CD8<sup>+</sup> and CD4<sup>+</sup> populations and also downregulation of repressive T-cell populations, known as CD8<sup>+</sup> exhausted T-cells and Tregs. After this, combined CPI can cause to different cytokine and transcriptional characteristics. The mechanisms of improved efficiency underlying this are not much understood but possible link to defeat resistance mechanisms of compensatory rise in checkpoint proteins against CPI monotherapy.<sup>73</sup>

## 10. Problems of combining checkpoint blockade antibodies

Immune checkpoint blockade may lower basic immune self-tolerance and cause to the onset of immune related adverse events (irAEs) mimicking autoimmunity. irAEs distinction with the classical immunosuppression of cytotoxic chemotherapy. Combine CPI enhances efficiency in melanoma and toxicity, whilst has minimal the use of combined therapy for targeted patients. It is unknown if the mechanisms of irAE in the setting of combined therapy are differ from those in monotherapy. irAEs suddenly cause to disruption or disturbance, of the CPI therapy. *Ex-vivo* peripheral blood investigates concentrated on autoimmunity have illustrated that T-cell exhaustion relates with a lower the autoimmune disease activity state.<sup>74</sup>

CPI therapy is connected with a variation in the phenotype of T-cells from an exhausted phenotype to an active effector phenotype. Earlier investigations display improvements in circulating T-cell repository in Ipilimumab induced patients leads the onset of irAEs. Combine Nivolumab and Ipilimumab but not monotherapy has been related with a differ in whole B-cell counts in the peripheral blood but a correlative rise in CD21<sup>low</sup> and plasma blast B-cell subsets. variations in B-cells after combined CPI related with efficiency and irAEs strength. Patients demonstrating changed B-cell responses with combined treatments had raised chance of multiorgan immunotoxicity. These improvements in B-cells were experienced throughout 3 weeks earlier to the onset of toxicity.<sup>75</sup>

Exploration of inflammatory markers can give significant mechanism to examine patients to notify those at risk of evolving irAEs and provide insight in to the mechanisms of irAE in combined therapy. For instance, a group induced 65 cytokines into 98 melanoma patients treated with combine anti-CTLA-4 and anti-PD-1 therapy or agent anti-PD-1 alone. A composition of 11 cytokines at pretreatment sampling and premature on treatment were related with acute irAEs in the combine regiment. These cytokines have proinflammatory events, involving enhancing immune cell recruitment, proliferation, survival, differentiation and effector functions, and several cytokines (IL-1 $\alpha$ , IL-1b, IL-2, IFN- $\alpha$ 2, and IL-12p70) have been involved in autoimmune diseases.<sup>76</sup>

Continuity of irAE to restrict the use of combine CPI and cause to high rates of treatment disruption. The notification of biomarkers to forecast treatment result and irAE is highly beneficial. Anticipative biomarkers are beneficial in the condition of different patient results in combine anti-PD-1 and anti-CTLA-4 therapy, but would be very feasible in classifying patients in monotherapy and combined immunotherapy. In spite of, enhanced the biological knowledge of melanoma, our capacity to realize specific molecular profiles, and the considerable progress of results related with targeted and immune therapies, there is an agreement absence on biomarker directed treatment strategies, after the estimation of baseline BRAF mutational level.<sup>77</sup>

Direct analysis of the PD-1/PD-L1 expression on cancers would develop to be a logical option as a possible biomarker for treatment response for anti PD-L/PD-L1 therapies. Furthermore, in the setting of melanoma environment, PD-L1 absorptions didn't create to be good in possibly treatment

response to Nivolumab, or combined therapy with Nivolumab and Ipilimumab. The changeability in the proof related to PD-L1 expression to feasible immune response can falsie in the absence of standardization over investigations in describing an important PD-L1 threshold. Likewise, PD-L1 is not a fixed biomarker, and its expression shows dynamic, possibly reflecting the compound signaling interactions in cancer and immune cells during treatment. Hence, expression can differ with timing of the biopsy.<sup>78</sup>

## Conclusion

The medical perspective for advanced melanoma and other cancers has been transformed through immune checkpoint blockers (ICBs) with boosted or improved median and long-term survival in comparison to normal cytotoxic chemotherapy. Combined anti-PD-1 and anti-CTLA-4 immunotherapy gives capable for better efficiency and this can be allocated to every agent functioning in a corresponding or possibly even symbiotic way. It is clear that combined ICBs may cause to a different immunological outline, improving T-cell and B-cell populations with various cytokine and transcriptional reactions. several mechanistic investigations are required to enable us to possibly patient responses and toxicity.

## Acknowledgements

The author thankful to Visvesvaraya National Institute of Technology, Nagpur to motivated for research work. The author also thankful to Faculty of Science, Janata Junior College, Nagbhid to encouraged for research work.

## Author contribution

The author has alone responsible for research work and writing this article.

## Funding source

None

## Competing interest

The authors declare no competing interest.

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