

1 **Commentary Title:** CTLA-4 – checkpoints beyond the membrane

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12 **Commentary on article:** Kennedy et al (2023) Soluble CTLA-4 attenuates T-cell activation and
13 modulates anti-tumour immunity.

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16 **Commentary**

17 In 2018 the Nobel Prize in Physiology or Medicine was awarded to Professors James Allison and
18 Tasuku Honjo for their pioneering research on CTLA-4 and PD-1. The discovery that checkpoint
19 receptors CTLA-4 and PD-1 regulate anti-tumour T-cell immunity has revolutionised the field of
20 cancer therapy since ipilimumab, a monoclonal antibody (mAb) specific for CTLA-4, was first shown
21 to prolong survival in patients with difficult-to-treat metastatic melanoma over 10 years ago. While
22 the assumption has been that CTLA-4-targeting mAb target the membrane-bound form of CTLA-4, an
23 understudied soluble form of CTLA-4 (sCTLA-4) has also been described. In this issue of Molecular
24 Therapy, Kennedy et al comprehensively demonstrate that sCTLA-4 alone confers a growth
25 advantage to tumour cells and importantly, a monoclonal antibody specific for sCTLA-4, and not
26 membrane (m)CTLA-4, proved an effective therapeutic agent in vivo [1]. The authors provide further
27 insights into the cellular mechanisms by which sCTLA-4, and anti-sCTLA-4 alter the tumour
28 microenvironment (TME). These findings challenge long-held assumptions about the mechanism(s)
29 of action of anti-CTLA-4 mAb and provide a basis for considering the clinical potential of sCTLA-4
30 targeting mAb.

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32 CTLA-4 is a dimeric receptor upregulated to moderate levels on effector T cells and constitutively
33 highly expressed on regulatory T cells (Tregs). Professional antigen presenting cells, via their
34 expression of CD80 and CD86 (also known as a B7-1 and B7-2 respectively), are able to bind with
35 relatively high affinity to CTLA-4 and with lower affinity to the classical T-cell co-stimulatory receptor
36 CD28. CTLA-4 is therefore considered a competitive inhibitor of T-cell activation, acting in opposition
37 to CD28. Mab-mediated blockade of CTLA-4 may therefore increase the bioavailability of CD80/86
38 for co-stimulation through CD28 by sequestering CTLA-4 as well as reducing CTLA-4-mediated
39 transendocytosis of CD80/86 [2]. Some anti-CTLA-4 mAb have a complementary mode of action by
40 depletion of CTLA-4^{hi} Tregs via an Fc-dependent mechanism, although the importance of Treg
41 depletion after ipilimumab treatment in humans remains controversial [3, 4]. A recent study has also

42 shown an anti-CTLA-4 mAb, to drive a Fc-dependent increase in inflammatory myeloid populations in
43 the TME [5]. The relative dominance of these mechanisms downstream of anti-CTLA-4 mAb is
44 influenced by several factors including the isotype of the relevant antibody with some (e.g. human
45 IgG1, ipilimumab) predicted to be superior at depletion, and others (e.g. human IgG2) more adept at
46 promoting effector function [4]. For optimal efficacy, animal models indicate that both promotion of
47 effector cells and depletion of Treg cells are required [6].

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49 A commonality of these studies is lack of consideration of the soluble form of CTLA-4, despite sCTLA-
50 4-encoding transcripts detected in human lung adenocarcinoma and melanoma, albeit at lower
51 frequencies than full length CTLA-4 transcripts (Kennedy et al, this issue), and prior evidence that
52 circulating sCTLA-4 correlates with cancer progression [7]. sCTLA-4 lacks the transmembrane and
53 intracellular domains present in mCTLA-4 and instead incorporates a novel C-terminal sequence of
54 22 residues, providing a unique target for the generation of sCTLA-4-specific mAb [8]. Importantly
55 the B7-binding domain is identical in both isoforms implying that sCTLA-4 has similar ability to
56 compete with CD28 as mCTLA-4. Kennedy et al generated cell lines expressing sCTLA-4 and showed
57 that sCTLA-4 adopted a dimeric structure, akin to mCTLA-4, further suggestive of similar B7-binding
58 stoichiometry by the two isoforms. Importantly, sCTLA-4 expression conferred a significant
59 growth/survival advantage apparent only in cells grown in the presence of PBMCs or in immune-
60 competent mice, and thereby providing clear evidence of an immune-modulatory influence of sCTLA-
61 4. Furthermore, sCTLA-4 expressing cells (but not empty vector controls) inhibited T-cell proliferation
62 and were resistant to cell death when cultured in the presence of NK-cell depleted PBMCs. Taken
63 together, these data strongly implicate the T-cell compartment as a significant target for sCTLA-4-
64 mediated modulation.

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66 To gain further insight into the mechanism(s) through which sCTLA-4 influences the TME, the authors
67 used mass cytometry to compare the TME in mice seeded with sCTLA-4-transduced or control MCA-
68 205 fibrosarcoma. Perhaps surprisingly, the most striking difference was not in a T-cell population
69 but instead was a substantial sCTLA-4-associated increase in an 'undetermined' cell type (expressing
70 amongst other markers CD44, Foxp3, F4/80 and CTLA-4) with a corresponding decrease in
71 macrophage and Ly6C^{hi} monocyte populations. The nature of this undetermined cell population is
72 intriguing and requires further characterisation; while tumour-associated F4/80+Foxp3+
73 macrophages have been described [9] and are associated with high efferocytic activity in stroke [10],
74 the significance of such cells in a TME is not clear. Targeted analysis of the relatively small (<5%)
75 population of CD8+ tumour infiltrating cells revealed a relative decrease in CD69+ cells in the sCTLA-
76 4+ TME but no consistent changes in Treg/effector cell ratios. Subsequent experiments made use of
77 a sCTLA-4-specific mAb (JMW-3B3) [8], to show that sCTLA-4 targeting alone is sufficient to slow the
78 growth of colorectal native MC38 tumour in vivo. The anti-sCTLA-4 mAb used in this study was of
79 mIgG1 isotype, predicted to have blocking/effector-promoting rather than depleting activity. This
80 seems a logical choice given that sCTLA-4 is unlikely to be Treg associated and therefore it is difficult
81 to envision how a cell-depleting anti-sCTLA-4 mAb might be of benefit (Figure 1). Indeed, Kennedy et
82 al, did not observe changes in Treg frequency in the TME after anti-sCTLA-4 treatment. Cells
83 enriched in the TME after anti-sCTLA-4 treatment were broadly the reciprocal of those enriched in
84 sCTLA-4-secreting tumours, with decreased frequency of an 'undetermined' myeloid-like population
85 (expressing F4/80, Foxp3 and CTLA-4) and enrichment of 'effector' like (granzyme B^{hi}, perforin^{hi})
86 CD8+ T cells. The extent to which these populations (CD8+ T cells, or 'undetermined') contribute
87 directly to the impairment of tumour growth remains to be seen. Of interest, others have shown
88 that a mIgG1 anti-CTLA-4 mAb (presumably targeting both soluble and membrane CTLA-4) given to
89 MC38-bearing mice fails to confer protection, and no significant differences were observed in the
90 TME with or without anti-CTLA-4 treatment [5]. However, tumour protection and significant
91 modifications to the myeloid compartment were observed after administration of a mIgG2a isotype

92 anti-CTLA-4 in the same model [5]. Future studies comparing the TME after anti-CTLA-4 and anti-
93 sCTLA-4 mAb treatment will be informative to identify common mechanisms of action. Important
94 insights may also be gained from study of the tumour draining lymph nodes (TDLN) after anti-sCTLA-
95 4 treatment given the high frequencies of CD80/86-expressing cells and CTLA-4^{hi} Tregs at this site [5].

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97 This important study by Kennedy et al, highlights a previously unappreciated role for sCTLA-4 in
98 suppressing tumour immunity and may herald a new subclass of soluble-specific check-point
99 inhibitors for clinical exploitation. Given the significant immune related adverse events suffered by
100 patients treated with existing anti-CTLA-4 mAb [2], and absence of detectable peripheral immune
101 activation in mice given anti-sCTLA-4 (Kennedy et al, this issue), the possibility that anti-sCTLA-4 mAb
102 might allow decoupling of anti-tumour immunity from significant side effects offers additional
103 grounds for optimism.

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105 **Declaration of Interest**

106 The author declares no competing interests.

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146 **Figure 1. Mechanisms through which anti-CTLA-4 mAb may influence T-cell activation**

147 Conventional T (Tcon) and T regulatory cells (Treg) express CTLA-4. While much CTLA-4 is
148 intracellular, for simplicity, mCTLA-4 is shown here only on the cell surface. (A) In homeostasis,
149 bioavailability of CD80 and CD86 is limited by the presence of mCTLA-4 which mediates CD80/86
150 internalisation through transendocytosis (not shown). A reasonable assumption is that sCTLA-4 also
151 precludes binding of CD80/86 to CD28 by steric hindrance. (B) Anti-CTLA-4 mAbs increase availability
152 of CD80/86 to provide co-stimulatory signals through CD28 on Tcon, and/or remove the suppressive
153 influence of Tregs by direct cytotoxicity, antibody dependent cellular cytotoxicity or by blockade of
154 CTLA-4 on Tregs. Anti-CTLA-4 mAb would also be predicted to bind sCTLA-4. (C) Anti-sCTLA-4 mAbs
155 would not be predicted to exert direct influence on Tregs but may increase bioavailability of CD80/86
156 for Tcon co-stimulation. A further intriguing possibility is that anti-sCTLA-4 mAb (and indeed anti-
157 CTLA-4 mAb) may cross-link CD80/86-bound sCTLA-4 to promote reverse signalling through CD80/86
158 thereby increasing APC activation, although this remains speculation.

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