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Cutting Edge: CTLA-4 on Effector T Cells Inhibits In Trans

Emily Corse and James P. Allison

CTLA-4 is thought to inhibit effector T cells both intrinsically, by competing with CD28 for B7 ligands, and extrinsically, through the action of regulatory T cells (Tregs). We studied in vivo responses of normal and CTLA-4–deficient Ag-specific murine effector CD4+ T cells. We directly demonstrate that effector T cell–restricted CTLA-4 inhibits T cell responses in a cell-extrinsic manner. Cotransfer experiments show that CTLA-4 on normal effector CD4+ T cells completely abrogates the dramatically increased expansion normally experienced by their CTLA-4–deficient counterparts. Neither the wild-type nor the CTLA-4–deficient T cells express the Treg transcription factor Foxp3 when transferred alone or together. Thus, cell-extrinsic inhibition of T cell responses by CTLA-4 is not limited to Tregs but is also a function of effector T cells.

The T cell transmembrane receptor CTLA-4 is a critical negative regulator of T cell responses (1). Blocking its function increases antitumor T cell responses (2), and anti–CTLA-4 Ab has been approved by the U.S. Food and Drug Administration for treatment of late-stage melanoma (3). Despite prolific investigation, the mechanisms by which CTLA-4 inhibits T cell responses are actively debated (4–6). Initial in vitro experiments showed that ligation of CTLA-4 by cross-linking specific Ab inhibited T cell proliferation and IL-2 production, whereas blocking function with Ab Fab fragments augmented proliferation (7, 8). Nonmutually exclusive cell-autonomous mechanisms by which CTLA-4 is thought to downregulate T cell responses include competition with the CD28 costimulatory receptor for their shared ligands, B7-1 and B7-2; transcriptional inhibition of cell cycle regulators; and inhibition of signal-transduction pathways downstream of TCR (9, 10). Fatal lymphoproliferation and autoimmunity in mice deficient for CTLA-4 exemplify its downstream of TCR (9, 10). Fatal lymphoproliferation and autoimmunity in mice deficient for CTLA-4 exemplify its importance in the physiological negative regulation of T cells (11–13).

In contrast to rampant proliferative disease in CTLA-4–deficient animals, the healthy phenotype of chimeras of wild-type (WT) and CTLA-4–deficient bone marrow first suggested the existence of a cell-extrinsic mode of CTLA-4 inhibition (14). Experiments involving suppression of colitogenic CD45RB<sup>high</sup> CD4+ T cells by their CD45RB<sup>low</sup> counterparts attributed cell-extrinsic CTLA-4 function to Foxp3<sup>+</sup> regulatory T cells (Tregs), which constitutively express significantly more surface CTLA-4 than do activated effector T cells (15, 16). Indeed, Treg-specific CTLA-4 deficiency results in decreased suppressive function and causes disease, albeit milder than seen in mice fully deficient in CTLA-4 (17). Blockade of CTLA-4 on both effector T cells and Tregs contributes to antitumor immunity (18), and a vague consensus has emerged in which CTLA-4 on effector T cells is purported to act cell intrinsically, whereas cell-extrinsic CTLA-4 function is commonly attributed to Tregs (4, 19).

Recent work suggests that CTLA-4 may cell extrinsically suppress immune responses by sequestration or removal of B7-1 and B7-2 molecules from APC membranes (17, 20, 21). Theoretically, this type of inhibitory function is not limited to Tregs but could apply to any cell expressing surface CTLA-4 (i.e., activated effector T cells). Although these studies (17, 20, 21) showed that interaction with effector T cells could modulate B7 levels on APCs, effects were more dramatic with Tregs, consistent with their higher surface CTLA-4 expression. Thus, the relevance of cell-extrinsic suppression by CTLA-4–expressing effector T cells for physiological immune responses remains unclear, given the lack of functional data (reviewed in Ref. 4).

In this study, we provide direct evidence that CTLA-4 expressed on Ag-specific, Foxp3<sup>−</sup> effector T cells functionally inhibits T cell responses cell extrinsically. In the setting of a normal immune system, we examined responses of normal and CTLA-4–deficient Ag-specific CD4+ T cells. The absence of CTLA-4 allowed greatly increased expansion and accumulation of deficient T cells. Cotransfer of WT T cells completely suppressed the hyperexpansion seen when CTLA-4–deficient T cells are present alone, indicating that CTLA-4 expressed on effector T cells functions non-cell autonomously to inhibit proliferation. These results directly demonstrate that cell-extrinsic suppression of T cell responses by CTLA-4 is not limited to Tregs but is also an important functional property of effector T cells.

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The microarray data presented in this article have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE37563.
Materials and Methods

Mice, adoptive transfers, and immunizations

T cells from 5C.C7 RAG2^{−/−} CD45.1 and 5C.C7 RAG2^{−/−} CD45.1 CTLA-4^{−/−} mice were transferred and activated by immunization with moth cytochrome c (MCC) 88–103 peptide and LPS, as previously described (22). Experiments were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

Flow cytometry and Abs

Flow cytometry was performed with a BD LSRII, and data were analyzed with FlowJo software (TreeStar). Abs were from BD PharMingen, eBioscience, or BioLegend.

Cell sorting, microarray samples, and data analysis

Activated T cells were sorted on a FACSAria (BD) from lymph nodes (LNs) based on the expression of CD4 and CD45.1. RNA isolation, labeling, and hybridization to the mouse MOE430 2.0 array (Affymetrix), as well as analysis of raw expression data, were done by the Memorial Sloan-Kettering Cancer Center Genomics Core Lab. Heat maps of differential gene expression, relative to WT 5C.C7 T cells, were generated using median ratio values from Partek software and normalizing log-scaled fold change values by the difference between the largest and smallest values (the range). Microarray data were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE37563. Expression data were analyzed and displayed using MetaCore software version 6.8 (GeneGo).

Results and Discussion

CTLA-4 deficiency allows greatly increased expansion of effector T cells

To study CTLA-4 function on effector CD4^{+} T cells in a physiological model of immunity to foreign Ag, we transferred 10^{4} CD45.1^{+} RAG2-deficient 5C.C7 T cells with a normal or knockout allele of CTLA-4 into normal syngeneic B10.A recipient mice. 5C.C7 T cells are specific for a peptide from MCC 88–103 presented in the context of H2-I-Ek (23), and their activation by peptide/LPS immunization results in acquisition of full effector function, including expansion, cytokine production, contraction, and maintenance (22). In contrast to CTLA-4–deficient T cells from RAG-sufficient mice, the 5C.C7 CTLA-4^{−/−} T cells are naive prior to immunization, as shown by analysis of CD25, CD62L, and CD44, consistent with lack of Ag exposure (Fig. 1A). Expansion of WT or CTLA-4–deficient 5C.C7 T cells in response to immunization was analyzed by flow cytometry in LNs (Fig. 1B, 1C) and blood (Fig. 1D). The greatly increased amount of CTLA-4–deficient 5C.C7 T cells compared with WT 5C.C7 T cells 6 d after immunization demonstrates that CTLA-4 significantly inhibits the peak accumulation of effector T cells (Fig. 1B). Compiled data from analysis of 5C.C7 T cell frequency in LN at 3, 4, and 7 d after immunization is shown in Fig. 1C; they indicate that a difference in the accumulation of WT and CTLA-deficient 5C.C7 T cells is not apparent until day 4. An expanded version of the day-3
The presence of CTLA-4 reduces, by ways in the absence of CTLA-4 (Supplemental Table IA, IB), significant upregulation of cell cycle-related genes and pathway activation of active negative regulation of T cell signaling by CTLA-4 (4). T cells present in LNs 7 d after immunization showed that CTLA-4 on effector T cells, we analyzed the responses of cotransferred differentially marked 5C.C7 WT and 5C.C7 CTLA-4 KO T cells in response to immunization with MCC peptide (Fig. 2A). Quantification of the frequency of 5C.C7 T cells present in LNs 7 d after immunization showed that cotransfer of WT T cells prevented hyperaccumulation of CTLA-4 KO T cells, restoring them to WT T cell frequencies at the peak of expansion (Fig. 2B, open symbols).

**CTLA-4 inhibits expression of many positive regulators of the cell cycle**

We used microarray analysis to compare transcripts in WT and CTLA-4 knockout (KO) 5C.C7 T cells 4 d after immunization (Fig. 1F). This is the first instance at which differences are observed in the extent of accumulation of WT and CTLA-4 KO 5C.C7 T cells (Fig. 1C, Supplemental Fig. 1). The complete microarray dataset can be found at http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE37563. Most changes correspond to genes whose expression is upregulated in CTLA-4 KO 5C.C7 T cells compared with WT 5C.C7 T cells (Fig. 1F, red genes). In fact, only 10 independent genes, including the Cda4 gene itself, have increased expression (>2-fold) in WT T cells compared with CTLA-4 KO T cells (Fig. 1F, blue genes). There is no obvious signature of active negative regulation of T cell signaling by CTLA-4 in the WT T cells. MetaCore software analysis showed highly significant upregulation of cell cycle-related genes and pathways in the absence of CTLA-4 (Supplemental Table IA, IB). The presence of CTLA-4 reduces, by >2-fold, the transcription of 27 genes related to cell cycle progression, including those encoding cyclin A and B, CDK1, Aurora A and B kinases, and CDC6 (Fig. 1F, Supplemental Table IA). This is consistent with previous data showing inhibition of cyclin D, CDK4, and CDK6 protein expression by Ab ligation of CTLA-4 on polyclonal CD4+ T cells (25). The data in Fig. 1F and Supplemental Table I illustrate the wide footprint of CTLA-4 in terms of decreasing the expression of positive regulators of the cell cycle during an in vivo immune response.

**CTLA-4 expressed on effector T cells inhibits T cell responses cell extrinsically**

CTLA-4 on Tregs is thought to inhibit effector T cell responses by downregulation or sequestration of B7-1 and B7-2 molecules on APCs (17, 20, 21). Although this mode of action is theoretically applicable to CTLA-4 expressed on activated effector T cells, its functional significance has not been demonstrated (4). To investigate potential non-cell autonomous inhibition by CTLA-4 on effector T cells, we analyzed the responses of cotransferred differentially marked 5C.C7 WT and 5C.C7 CTLA-4 KO T cells in response to immunization with MCC peptide (Fig. 2A). Quantification of the frequency of 5C.C7 T cells present in LNs 7 d after immunization showed that...
Suppression of the increased accumulation of CTLA-4–deficient 5C.C7 T cells by cotransfer of WT 5C.C7 T cells is also evident in the peripheral blood (Fig. 2C–E). Thus, enhanced egress from LNs does not account for the decreased frequency of CTLA-4 KO 5C.C7 T cells in the presence of WT 5C.C7 T cells. Longitudinal analysis of T cell responses in blood shows that the presence of CTLA-4 on WT T cells decreases the expansion of CTLA-4 KO T cells to WT levels at all time points (Fig. 2C). In fact, in mice harboring both types of T cells, the CTLA-4–deficient 5C.C7 T cells exhibit expansion, contraction, and maintenance exactly equivalent to those of WT 5C.C7 T cells (Fig. 2C). Considering potential competition effects from twice the number of input 5C.C7 T cells in the cotransferred mice, we transferred twice the number of CTLA-4 KO 5C.C7 T cells in some experiments as a control (Fig. 2D, open squares). This does not reduce the level of expansion to normal levels; thus, suppression of hyperresponsive CTLA-4–deficient 5C.C7 T cells by cotransfer of WT 5C.C7 T cells can only be due to the presence of WT CTLA-4. The degree of suppression is influenced by the ratio of WT/CTLA-4 KO 5C.C7 T cells, as the titration in Fig. 2E shows. Only a ratio of 1:1 or 1:2 (red and green open symbols, respectively) restores the response of 5C.C7 CTLA-4 KO T cells to WT levels, although lower frequencies of WT cells have a suppressive effect.

Because cell-extrinsic suppression of T cell responses by CTLA-4 has only been attributed previously to Foxp3+ Tregs (4), we looked for Foxp3 expression in the WT and CTLA-4 KO 5C.C7 T cells when transferred individually and together. The CD45.1+ 5C.C7 T cells were completely Foxp3− in all cases, as shown by the lack of CD45.1+ Foxp3+ cells (Fig. 2F). This is consistent with our previous work showing that immunization with MCC peptide and LPS does not result in Foxp3 expression by 5C.C7 T cells (22).

CTLA-4 was proposed to “reverse signal” through 7B molecules, via the induction of IDO, a tryptophan catabolizer that inhibits T cells through tryptophan depletion (reviewed in Ref. 6). Treatment of mice with 1-methyl-tryptophan pellets to inhibit IDO had no effect on cell-extrinsic inhibition of T cell responses by CTLA-4 on effector T cells (data not shown). Although production of inhibitory factors by APCs cannot be ruled out, a reasonable explanation for our results is that the CTLA-4 on WT T cells prevents access to B7 ligands by CD28 on the CTLA-4–deficient T cells. This could occur through competition, downregulation, or sequestration of the B7 molecules (reviewed in Ref. 6). Thus, inhibition of T cell responses by CTLA-4 on effector T cells and Tregs could both be explained by limiting levels of B7 ligands that occur following CTLA-4 binding.

The significance of our results lies in the direct demonstration of functional cell-extrinsic suppression of effector T cell responses by CTLA-4 expressed on the effector T cell population. Indeed, there is no need to invoke cell-intrinsic inhibition to explain hyperexpansion of CTLA-4–deficient T cells in our experiments (Fig. 1), although its existence cannot be excluded. It is tempting to speculate that cell-extrinsic CTLA-4 function could explain the relatively late time point at which differences in normal and CTLA-4–deficient effector T cells are first observed (day 4; Fig. 1C, Suppential Fig. 1); functional suppression of T cell responses could depend on accumulation of a quorum of CTLA-4–expressing effector T cells.

Our data suggest that effector T cells contribute to non-cell autonomous inhibition of immune responses by CTLA-4 commonly attributed to Tregs (14, 19, 26–28). Our results, and results described in a companion paper (29), provide functional evidence for a model in which CTLA-4 expressed on activated effector T cells and Tregs inhibits T cell responses in similar ways (4, 6). In general, Tregs may be more potent negative regulators simply because of the substantially greater amounts of surface CTLA-4 that they constitutively express, but our data show that the cell-extrinsic inhibitory power of CTLA-4–expressing effector T cells should not be underestimated.

Finally, these data highlight the possibility that CTLA-4 on an activated effector T cell of a particular specificity may cross-regulate responding T cells of another specificity, thus exerting broad influence over the quantity and quality of polyclonal T cell responses. We previously showed that blockade of CTLA-4 on effector T cells is necessary for optimal antitumor T cell responses (18). Our results, and the results of Wang et al. (29), mean that blockade of CTLA-4 on activated effector T cells could enhance responses of naive or less activated T cells, thus allowing their contribution to overall antitumor reactivity.

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Disclosures
The authors have no financial conflicts of interest.

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