B7-Independent Inhibition of T Cells by CTLA-4

Shunsuke Chikuma, Abul K. Abbas and Jeffrey A. Bluestone

J Immunol 2005; 175:177-181; doi: 10.4049/jimmunol.175.1.177
http://www.jimmunol.org/content/175/1/177

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References  This article cites 26 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/175/1/177.full#ref-list-1

Subscription  Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
B7-Independent Inhibition of T Cells by CTLA-4

Shunsuke Chikuma, Abul K. Abbas, and Jeffrey A. Bluestone

CTLA-4 is an inhibitory molecule that regulates T cell expansion and differentiation. CTLA-4 binding to B7-1/B7-2 is believed to be crucial for its inhibitory signal both by competing for CD28 binding to the same ligands and aggregating CTLA-4 to deliver negative signals. In this study, we demonstrate that B7 binding is not essential for CTLA-4 activity. CTLA-4 knockout T cells are hyperresponsive compared with wild-type T cells in B7-free settings. Expression of a B7-nonbinding CTLA-4 mutant inhibited T cell proliferation, cytokine production, and TCR-mediated ERK activation in otherwise CTLA-4-deficient T cells. Finally, transgenic expression of the ligand-nonbinding CTLA-4 mutant delayed the lethal lymphoproliferation observed in CTLA-4-deficient mice. These results suggest that ligand binding is not essential for the CTLA-4 function and supports an essential role for CTLA-4 signaling during T cell activation. The Journal of Immunology, 2005, 175: 177–181.

Materials and Methods

Reagents

Hamster anti-mouse CD3ε (145-2C11), anti-CD28 (PV-1), anti-B7-1(16-10A1), and anti B7-2 (GL-1) were prepared in our lab. The anti-CD3-foxr anti-CD4-fum bispecific mAb was provided by Dr. J. Tso (Protein Design Labs, Fremont, CA) (18). The rabbit anti-CTLA-4 C-terminal epitope was a gift from Dr. T. Uede (Hokkaido University, Hokkaido, Japan) (19). The Ab pairs used for IL-2 and IFN-γ ELISA (BD Pharmingen) were used according to manufacturer’s recommended protocols. Recombinant mouse B7-1Ig fusion protein (R&D Systems) incubated with protein A-coupled Alexa 633 (Molecular Probes) was used for FACS. Anti-phospho (Thr202/Tyr204)-Erk and anti-Erk mAbs were from Cell Signaling Technology.

Animals

BALB/c mice were purchased from Charles River Laboratories. D0.11 Tcr transgenic (Tg) Rag-2 KO CTLA-4 KO mice as well as BALB/c B7-1/B7-2 double KO (DKO) were kept in specific pathogen-free conditions in the UCSF Animal Barrier Facility.

Retroviral reconstitution of CTLA-4 KO T cells
cDNA constructs of wild-type and mutant CTLA-4 were subcloned into a murine retroviral vector LXIE (a gift from Dr. H. Spits, University Hospital Free University, Amsterdam, The Netherlands) upstream of enhanced GFP (eGFP) (20). The cDNA encoding CTLA-4 and mutant was separated from eGFP by an internal ribosomal entry site. This allowed for simultaneous expression of each protein based on the bicistronic mRNA. The purified CD4+ T cells were activated using irradiated BALB/c mice splenocytes and OVA-derived peptide (aa 323–339) (1 μg/ml). One day later, retroviral DNA-transfected Phoenix cells (from Dr. G. Nolan, Stanford University, Stanford, CA) were added to each well after treatment with 50 μg/ml mitomycin C. At day 3, transduced cells were sorted based on anti-CD4-allophycocyanin staining and eGFP fluorescence using MoFlo cell sorter. The sorted cells were restimulated by irradiated BALB/c splenocytes and OVA peptide. Thymidine incorporation was measured between 16 and 24 h following Ag restimulation. Supernatants were pooled at 16 h for cytokine assay.

Generation of CTLA-4 PYAA mutant Tg mice

The cDNA for mutant PYAA CTLA-4 was subcloned into pUHG-10 vector using XhoI restriction site (21). The 2800-bp fragment containing tetracycline O, the CMV minimal promoter, and the cDNA insert was excised...
using XhoI and AsclI site and microinjected into day 1 embryos isolated from FVB/n mice. Tg founders were crossed to EμSRα-tTA mice on the same background for specific transgene expression on T cells (21). The primers used to screen both transgenes were as follows: tetO PYAA, forward, 5′-gtccagtaggcgtgtacg-3′; tetO PYAA, reverse, 5′-aacaagcaaacgctgaa-3′; tTA, forward, 5′-ctgctaattgagggc-3′; and tTA, reverse, 5′-ctctgcagcgggct-3′. Tg mice were fed a mouse diet containing doxycycline (200 mg/kg food; Bioserv) to turn off the tet-regulated transgene.

Results

CTLA-4 alters the activation threshold in a B7-1/B7-2-free system (B7 DKO)

The importance of B7 ligation for CTLA-4 function was assessed using DO.11 TCR Tg mice bred onto a Rag-deficient background to eliminate the spontaneous activation of T cells observed in CTLA-4-deficient mice. The DO.11 TCR mice survived long term to eliminate the spontaneous activation of T cells observed in CTLA-4-deficient mice. The DO.11 TCR mice responded similarly to OVA peptide-pulsed irradiated H-2d B7 DKO spleen cells in a primary stimulation assay based on proliferation and up-regulation of the activation markers CD25, CD44, and CD69 (data not shown), as well as robust IL-2 production in the presence of anti-CD28 costimulation (Fig. 1A). However, the CTLA-4 KO DO.11 Tg T cells responded better than wild-type Tg T cells to B7 DKO OVA-pulsed APC in a secondary response when stimulated with B7-deficient OVA-pulsed APC. The CTLA-4 KO DO.11 T cells proliferated more (data not shown) and produced significantly more IL-2 compared with WT preprimed cells (Fig. 1B). By comparison, pharmacologic activation of PKC and calcium pathways by PMA and ionomycin activated WT and KO cells similarly consistent with our observations, showing that CTLA-4 functions in the early stages of TCR signaling (22). Anti-B7-1 and anti-B7-2 Abs were added during the entire experimental course to block possible effect of B7-1 or B7-2 expression on the activated T cells. These results suggested that the presence of CTLA-4 on T cells increased the threshold for activation in a B7-independent manner.

A ligand-nonbinding mutant CTLA-4 inhibits T cell activation in vitro

A hexapeptide motif (MYPPPY) present in the CDR3-like domain of CTLA-4 is critical for binding to both B7-1 and B7-2 (23). Therefore, we mutated the proline and tyrosine at positions 5 and 6, respectively, to alanine to create a B7-nonbinding mutant form of CTLA-4. A full-length CTLA-4 MYPPAA mutant form (PYAA mutant), overexpressed in Phoenix cells, did not bind to either recombinant B7-1Ig fusion protein (Fig. 2A) or B7-2Ig (data not shown). This was in sharp contrast to the wild-type CTLA-4 molecule, which bound B7-1Ig at high levels correlating with GFP expression. The lack of B7-1 binding to PYAA mutant was not due to reduced transfection efficiency because internal ribosomal entry site-regulated eGFP expression in the retrovirus construct was comparable between WT and PYAA transfectants (Fig. 2A, middle and right panels). Furthermore, an equal amount of protein was immunoprecipitated from WT and PYAA transfectants by anti-CTLA-4 C-terminal mAb (Fig. 2B).

In vitro-activated DO.11 Rag-2 KO CTLA-4 KO T cells were infected with the CTLA-4 WT and PYAA mutant CTLA-4 and sorted based on eGFP expression. The sorted eGFP+ cells expressed CTLA-4 based on FACS staining using the intracellular domain-specific mAb (Fig. 2C). The sorted eGFP+ cells were re-stimulated using OVA peptide and syngeneic APC. T cells reconstituted with PYAA mutant CTLA-4 inhibited both proliferation and cytokine production compared with empty vector transfectants (Fig. 2, D and E). The inhibition of secondary activation was observed in multiple experiments at all peptide concentrations tested. However, the expression of the B7-binding wild-type molecule was more efficient in blocking T cell activation. Because similar intracellular distribution of the WT and PYAA mutant molecules were observed in the two transfectant cell populations (Fig. 2C), it is likely that the migration of CTLA-4 to the cell surface upon TCR stimulation was similar. Thus, the results suggest that binding of CTLA-4 to B7, although not essential, enhances the regulatory activity of the CTLA-4 molecule by stabilizing CTLA-4 within the IS.

Generation of ligand-nonbinding CTLA-4 Tg mice

To study the ligand-independent function of CTLA-4 in a physiological setting, we generated Tg mice that expressed the PYAA mutant transgene in T lineage cells under the control of a tetracycline-responsive minimal promoter (tet-o-PYAA) (Fig. 3A). Selected founder mice were bred with a second Tg line that expressed the tTA under the control the Ig H chain enhancer and the SRα promoter (EμSR-tTA) (21) (Fig. 3A). Assessment of CTLA-4 expression on freshly isolated lymph node cells using the C-terminal epitope-specific mAb in Western blot analyses demonstrated significant amounts of transgene protein in Tg+ but not Tg− littermate T cells (Fig. 3B). The Tg protein was specifically expressed...
on thymocytes (data not shown) and peripheral lymph node T cells (Fig. 3C), but not B cells (data not shown). Furthermore, the expression of the transgene was highly regulated by the tetracycline promoter because feeding mice with doxycycline-containing diet for 1 wk turned off transgene expression (Fig. 3C, right panels).
The biological significance of these observations has been illustrated in recent genetic analysis of the mouse *ctla4* gene, which identified an alternatively spliced form of the molecule that encoded a protein completely lacking extracellular domain (lCTLA-4) (16, 17). In fact, a single nucleotide mutation that inhibits the alternative splicing has been mapped as the critical polymorphism in the NOD mouse *ctla4* gene, which identified an alternatively spliced form of the molecule that encoded a protein completely lacking extracellular domain (lCTLA-4) (16, 17).
might help to explain the autoimmune propensity of this inbred strain of mice (16). Importantly, the naturally occurring liCTLA-4 is expressed in naive T cells rather than activated T cells and the truncated molecule effectively inhibited activation in vitro (17).

Thus, we propose that the liCTLA-4 form has distinct temporal expression and biological functions compared with full-length CTLA-4. The late expression of the full-length form of CTLA-4 is consistent with a critical role in attenuating ongoing Ag-driven immune responses and maintaining tolerance. In this regard, we and others have demonstrated that blockade of full-length CTLA-4 engagement with B7 can reverse tolerance and that direct engagement of CTLA-4 using mAbs directed at the extracellular domain can attenuate responses (4, 5, 24). In fact, treatment of autoreactive mice with an anti-CTLA-4 mAb directed at the full-length B7 ligand binding form exacerbates immunity only after the disease has been initiated—not when administered at birth (5). Similarly, anti-CTLA-4 therapy in the tumor setting enhances immunity by up-regulating Ag-specific immunity (26). In contrast, the function of li form has not been understood. It is tempting to speculate that this isoform controls survival and/or homeostasis of naive T cell subsets based on the observation that the liCTLA-4 form is expressed in naive T cells and the CTLA-4 KO mouse (which eliminates both forms) manifests a lymphoproliferative disease that occurs in a potentially Ag-independent manner. The expression of liCTLA-4 might set a higher threshold of TCR signaling that keeps autoproliferation as well as homeostatic proliferation in the young “lymphopenic” mice in check.

Both ligand-dependent and -independent function of CTLA-4 should be further examined in the future study for understanding the clinical importance of CTLA-4 manipulation in autoimmunity.

Acknowledgments
We thank Jason Dietrich for Tg injection; Paul Wegfahrt, Yousuf Bhaijee, and Mike Lee for expert assistance with the mice; and Shuwei Jiang for cell script, Dr. T. Uede for anti-CTLA-4 Ab, Dr. H. Spits for LIIE vector, and Mike Lee for expert assistance with the mice; and Shuwei Jiang for cell

Disclosures
The authors have no financial conflict of interest.

References