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*J Immunol* 2000; 164:4433-4442; doi: 10.4049/jimmunol.164.9.4433

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Blockade of T Cell Activation Using a Surface-Linked Single-Chain Antibody to CTLA-4 (CD152)

Matthew D. Griffin,*†‡ David K. Hong,*† Philmore O. Holman,§ Kyung-Mi Lee,*† Matthew J. Whitters,¶ Sean M. O’Herrin,*† Francesca Fallarino,† Mary Collins,¶ David M. Segal,‖ Thomas F. Gajewski, † David M. Kranz,§ and Jeffrey A. Bluestone2*†

CTLA-4 (CD152) engagement can down-regulate T cell activation and promote the induction of immune tolerance. However, the strategy of attenuating T cell activation by engaging CTLA-4 has been limited by sharing of its natural ligands with the costimulatory protein CD28. In the present study, a CTLA-4-specific single-chain Ab (scFv) was developed and expressed on the cell surface to promote selective engagement of this regulatory molecule. Transfectants expressing anti-CTLA-4 scFv at their surface bound soluble CTLA-4 but not soluble CD28. Coexpression of anti-CTLA-4 scFv with anti-CD3ε and anti-CD28 scFv on artificial APCs reduced the proliferation and IL-2 production by resting and preactivated bulk T cells as well as CD4+ and CD8+ T cell subsets. Importantly, expression of anti-CTLA-4 scFv on the same cell surface as the TCR ligand was essential for the inhibitory effects of CTLA-4-specific ligation. CTLA-4-mediated inhibition of tyrosine phosphorylation of components of the proximal TCR signaling apparatus was similarly dependent on coexpression of TCR and CTLA-4 ligands on the same surface. These findings support a predominant role for CTLA-4 function in the modification of the proximal TCR signal. Using T cells from D011.10 and 2C TCR transgenic mice, negative regulatory effects of selective CTLA-4 ligation were also demonstrated during the stimulation of Ag-specific CD4+ and CD8+ T cells by MHC/peptide complexes. Together these studies demonstrate that selective ligation of CTLA-4 using a membrane-bound scFv results in attenuated T cell responses only when coengaged with the TCR during T cell/APC interaction and define an approach to harnessing the immunomodulatory potential of CTLA-4-specific ligation. The Journal of Immunology, 2000, 164: 4433–4442.

During the past decade, there has been a growing appreciation of the importance of negative regulatory pathways in normal and disease-related cellular immune function. Characterization of molecular mechanisms underlying negative immune regulation has revealed the essential role of inhibitory pathways in the maintenance of immune homeostasis, the prevention of autoimmunity, and the orchestration of effective cellular and humoral responses (1–4). Evidence continues to accumulate that these mechanisms are linked to the pathogenesis of immune-mediated disease (1, 5, 6). The intimate relationship that exists between positive and negative regulation during the generation of immune responses is exemplified by the functional profiles of the homologous proteins CD28 and CTLA-4 (CD152). Despite their structural similarities and shared specificity for the ligands B7-1 (CD80) and B7-2 (CD86), it is now clear that the two proteins mediate opposing effects on T cell activation (7–12). The CD28/B7 interaction serves as a positive costimulator in the context of TCR engagement by MHC/Ag complex (13). In contrast, CTLA-4/B7 interactions negatively regulate T cell activation by attenuating cell cycle progression, IL-2 production, and proliferation of T cells following activation (9–12, 14–17). Mice lacking CTLA-4 through targeted gene disruption demonstrate a remarkable dysregulation of T cell homeostasis and die within weeks of birth from unchecked lymphoproliferative disease (18, 19).

The identification of these immune regulatory pathways has generated interest in developing approaches to separately manipulate positive and negative regulation to alter the course of immune-mediated disease. The hypothesis that CTLA-4-mediated negative regulation can critically affect the course of disease processes such as neoplasia, infection, autoimmunity, and allograft rejection has been borne out by studies in which CTLA-4/B7 interactions have been blocked in vivo through administration of CTLA-4-specific mAbs (20–24). In addition, studies of Ag-specific T cell tolerance have shown that CTLA-4 is necessary for unresponsiveness to subsequent Ag exposure (25–28). Although these results imply that enhancement of CTLA-4 function might be used to induce immune hyporesponsiveness or tolerance to disease-related Ags, the pursuit of such a strategy is complicated by the shared specificity of CTLA-4 and CD28 for their natural ligands. The therapeutic potential for manipulating CTLA-4 function will likely be best appreciated by progress in characterizing the intracellular signaling events that mediate its effects, the Ag presentation conditions that preferentially recruit CTLA-4, and the relative influence of CTLA-4 function on different T cell subpopulations.

With these concepts in mind, we generated a cell-surface, CTLA-4-specific ligand with the dual purpose of allowing us to study in isolation the functional and biochemical outcomes of
CTLA-4 engagement as well as that of exploring the immunomodulatory potential of such an agent. The ligand was derived from a well-characterized mAb directed against murine CTLA-4 (9) by modification of a single-chain Ab (scFv)3 (29–31). Along with the anti-CTLA-4 surface-linked scFv, we have also generated similar surface-linked scFvs that interact with murine CD28 and the CD3ε component of the TCR. In the present study, this panel of ligands was used in a flexible artificial APC system to explore the consequences of isolated CTLA-4 engagement during TCR stimulation under Ag-nonspecific and Ag-specific conditions as well as in the presence and absence of additional selective engagement of CD28. Our results lend further support to a mechanistic model of negative regulation in which a physical association between CTLA-4 and the TCR results in modified tyrosine phosphorylation of proximal components of the TCR signaling apparatus. The results also suggest that enhancement of CTLA-4 engagement during primary or subsequent presentation of disease-related Ags represents a feasible approach to attenuating destructive immune responses.

Materials and Methods

Experimental animals and cell lines

Female mice between 6 and 12 wk of age were used for all experiments. Mice were maintained in the University of Chicago animal housing facility in a specific pathogen-free environment. BALB/c mice were purchased from Frederick Cancer Research and Developmental Center (National Cancer Institute, Frederick, MD). DO11.10 and 2C TCR transgenic mice were drawn from colonies maintained at the University of Chicago. The human embryonic kidney cell line 293 was provided by Dr. Craig Thompson (Department of Medicine, University of Pennsylvania). The murine B cell lymphoma cell line A20 was purchased from the American Type Culture Collection (Department of Medicine, University of Pennsylvania). This Ab has no cross-reactivity with murine Ags, this construct was employed as a control protein (40). Surface linkage by the GPI anchor motif alone was employed for mem2C11 scFv, memPV1 scFv, and mem5H7 scFv. For mem4F10 scFv, constructs incorporating both the GPI anchor and the mB7-1-derived motif were generated and tested. While similar functional results were achieved for mem4F10 scFv with both surface-linkage strategies, high levels of surface expression were more consistently achieved using the B7-d-derived motif (data not shown), and it is this protein that was used in the experiments reported.

Complementary DNA sequences for the α- and β-chains of the murine class II MHC protein I-A^d were generated by RT-PCR. Total RNA was extracted from the BALB/c-derived B cell lymphoma cell line A20 using Trizol reagent (Life Technologies) according to the manufacturer’s instructions. Reverse transcription with an oligo(dT) primer was conducted using the Superscript II first-strand cDNA synthesis kit (Life Technologies) by recommended protocol and was followed by PCR using primers derived from published sequence for the coding regions of I-A^a and I-A^b (41–43) and incorporating appropriate restriction endonuclease sites (5’ HindIII, 3’ XbaI) into the mammalian expression vector pCDNA3.1+ (Invitrogen, Carlsbad, CA). A fourth mem-scFv, constructed by the same protocol from the mAb Sh7 (mouse anti-human class I MHC), was provided by Dr. E. S. Woodle (Department of Surgery, University of Cincinnati). As this Ab has no cross-reactivity with murine Ags, this construct was employed as a control protein (40). Surface linkage by the GPI anchor motif alone was employed for mem2C11 scFv, memPV1 scFv, and mem5H7 scFv.

Stable transfection of P1.HTR cells

The cDNA for mem4F10 scFv was ligated into the bicistronic vector LZKSpBMN-linker-internal ribosomal entry site (IRES)-enhanced green fluorescent protein (EGFP) (45), provided by Dr. Hergen Spits (Netherlands Cancer Institute, Amsterdam, The Netherlands). This vector (subsequently referred to as LX1E) is derived from the MFG murine retroviral backbone. The cloning site for insertion of cDNAs is followed by an IRES and the cDNA for EGFP. The resulting transcript allows for the independent translation of the protein of interest and of EGFP. The latter serves as a marker for transfection without functionally altering the product of interest (8). The vector also contains the neomycin gene for the selection of stable transfectants. P1.HTR cells were transfected by calcium phosphate precipitation using the same protocol as described above for 293 cells. Cells were transfected separately with parent vector or with mem4F10 scFv-containing vector and cultured in the presence of 2 μg/ml puromycin. Following outgrowth of puromycin-resistant cells, populations of comparable EGFP expression were purified by flow cytometric sorting. Sorted, puromycin-resistant populations of mem4F10 scFv or vector-transfected cells were maintained in 2 μg/ml puromycin and periodically checked for EGFP expression.

Flow cytometric analysis

Transiently transfected 293 cells or stably transfected P1.HTR cells were suspended in FACS buffer (PBS, 0.1% BSA, 0.01% NaN3). Aliquots of 100 μl were incubated with and without fluorescein-coupled soluble murine fusion proteins (mCTLA-4-Ig or mCD28-Ig) for 30 min at 4°C.
washed, resuspended in 250 μl of FACS buffer, and analyzed on a FACscan flow cytometer (Becton Dickinson) with the CellQuest software package. Dual expression of EGFP and mem4F10 scFv was conducted by incubation of transfectants with purified mCTLA-4-Ig (1 μg/10^5 cells for 30 min at 4°C) followed by incubations with biotinylated goat-anti-mouse IgG and PE-coupled streptavidin. FACS was conducted on a Vantage cell sorter (Becton Dickinson).

Purification and preactivation of murine lymph node T cells and 2C TCR transgenic splenic T cells

Purified T cells were prepared from the inguinal, axillary, and mesenteric lymph nodes of BALB/c mice by nylon wool column separation and complement-mediated lysis following incubation with the following hybridoma supernatants: anti-heat stable Ag (hybridoma J11d; Ref. 46) and anti-class II MHC (hybridoma MKD6; Ref. 47). For purification of CD4^+ T cell or CD8^+ T cell populations supernatants containing mAbs against CD4 (hybridoma RL172.4; Ref. 48) or against CD8 (hybridoma 3.155; Ref. 49) were also added. Ab binding cells were depleted by addition of an equal volume of rabbit complement (Pel-Freez Clinical Systems, Brown Deer, WI) diluted 1:5 in sterile PBS with incubation at 37°C for 45 min. Viable cells were then isolated by density-gradient centrifugation using Ficoll-Hypaque. Purity of the desired cell populations was between 95 and 99%. For studies in which preactivated T cells were employed, freshly purified T cells were added to six-well tissue culture plates that had been coated with goat anti-hamster IgG (10 μg/ml in PBS; ICN Pharmaceuticals, Costa Mesa, CA) followed by 145-2C11 and PV-1 mAb (2 μg/ml each in PBS). Between 60 and 72 h later, the cells were removed, washed in complete medium, and cultured at 37°C for a further 8 h before use in coculture studies. Freshly dissected spleens from 2C TCR transgenic mice were gently disrupted, filtered, depleted of RBC by a 5-min incubation in ACK lysis buffer (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4), and then mixed with irradiated (2500 rad), RBC-depleted BALB/c splenocytes at 1:1 ratio in 24-well tissue culture plates. After 5 days of culture, activated 2C T cells were separated by density-gradient centrifugation with Ficoll-Hypaque, washed, resuspended in fresh medium, and cultured at 37°C for a further 8 h before use in coculture studies.

In vitro proliferation and cytokine assays

Transfected 293 cells and P1.HTR transfectants were treated with 50 μg/ml mitomycin C (Sigma-Aldrich) for 1 h at 37°C followed by extensive washing then added to wells of 96-well tissue culture plates. The numbers of cells used per well for individual experiments are indicated in the relevant figure legends. In experiments where mixtures of 293 transfectants were used, cell suspensions were premixed at 1:1 ratio just before their addition to the wells. Resting or preactivated murine lymph node T cells were then added to wells containing transfectants and incubated at

FIGURE 1. Strategy for construction of surface-linked scFvs and flow cytometric analysis of 293 cells transfected with anti-CTLA-4 and anti-CD28 scFv constructs. A, Sequences encoding the variable regions of the L and H chains of a mAb (V_L and V_H) are amplified from RNA by RT-PCR using primers derived from the variable (5′) and constant or joining (3′) regions and joined by sequence for a flexible peptide linker. This basic scFv cDNA is further modified by a second round of PCR using primers with extended tails to add a 5′ leader peptide and a 3′ spacer/surface anchor motif. The modified mem-scFv is then transferred to a mammalian expression vector and, when transfected into eukaryotic cell lines, results in surface expression of a protein in which the V_L and V_H domains reconstitute the Ag-binding domain of the parent mAb. B, Four surface-linked scFvs, generated by the strategy outlined in A, were used for the creation of artificial APC populations. The symbols shown are employed in subsequent figures to represent the expression characteristics of the stimulating cells used in individual experiments. C, Human embryonic kidney (293) cells, transiently transfected with cDNA encoding anti-CTLA-4 (mem4F10), anti-CD28 (memPV1) scFv, in the amounts shown or with both constructs together were incubated with (solid lines) or without (dashed lines) FITC-coupled soluble murine fusion proteins mCTLA-4-Ig and mCD28-Ig as indicated and analyzed by flow cytometry for surface staining. Transfection with mem4F10 scFv (upper panels) results in surface staining by mCTLA-4-Ig-FITC but not mCD28-FITC, while transfection with memPV1 scFv (middle panels) produces the opposite staining pattern. Doubly transfected cells are capable of binding both labeled fusion proteins (lower panels).
37°C. For coculture experiments using 293 cells, flat-bottom plates were used, while for experiments involving coculture with P1.HTR cells, round-bottom plates were used. At defined time points, plates were pulsed with 1 μCi of tritiated thymidine and incubated for a further 8–16 h. Plates were freeze-thawed and then harvested onto fiberglass filters and analyzed using a Filtermate 196 cell harvester and Topcount Scintillation Counter (Packard Instrument, Meriden, CT). Three or six identical wells were analyzed for each condition, and results (in cpm) are expressed as mean ± SD. In all cases, background thymidine incorporation for mitomycin C-treated transfectants was determined and was subtracted from final counts for experimental conditions. For analysis of cytokine concentrations, culture supernatants were withdrawn from wells at defined time points, and levels of the cytokines IL-2, IL-4, and IFN-γ were measured by commercial ELISA kits according to the manufacturer’s recommendations (IL-2 and IL-4, Endogen, Woburn, MA; IFN-γ, PharMingen, San Diego, CA).

Detection of T cell protein tyrosine phosphorylation

Aliquots of 5 × 10⁶ preactivated lymph node T cells were mixed on ice in 1.5-ml tubes with aliquots of 2.5 × 10⁶ transiently transfected 293 cells suspended in complete culture medium. The transfectants used in individual experiments are indicated in the relevant figure legends. Cell mixtures were then pelleted by brief centrifugation, transferred to a heating block prewarmed to 37°C, and incubated for 2 and 5 min followed by lysis in 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, pH 8.0, 1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μM aprotinin, 1 mM PMSF. Lysates were precleared once with protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) and once with protein A-Sepharose beads coated with an irrelevant hamster mAb (UC3-10A6; Ref. 50). Immunoprecipitation was performed overnight at 4°C with the anti-phosphotyrosine mAb FB2 coated onto protein A-Sepharose beads. Immunoprecipitates were separated on a reducing 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and immunoblotted with mAb 4G10 to phosphotyrosine (Upstate Biotechnology, Lake Placid, NY). Bound proteins were detected by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce, Rockford, IL).

**Results**

A surface-linked scFv with binding specificity for CTLA-4 can attenuate the proliferative response of resting T cells

A panel of three surface-linked scFvs (mem-scFvs) directed against the murine T cell proteins CD3ε, CD28, and CTLA-4 as well as a control-scFv with no murine ligand was generated (see Fig. 1, A and B). In preliminary experiments using mem2C11 scFv and memPV1 scFv constructs separately and together, varying amounts of plasmid construct per transfection were tested to generate populations of 293 cells capable of inducing significant T cell proliferation. We found that combined transient transfection of low amounts of mem2C11 scFv (0.5–1.0 μg per 2 × 10⁶ cells) and memPV1 scFv (1.0–2.0 μg per 2 × 10⁶ cells) resulted in a potent immunostimulatory effect (data not shown). Untransfected cells and cells transfected with memPV1 scFv alone induced no T cell proliferation, while mem2C11 scFv alone induced low-level proliferation. Increased amounts of the transfected cDNA were associated with qualitatively similar effects (data not shown).

Surface expression and ligand binding of mem4F10 scFv vs memPV1 scFv were determined by flow cytometric analysis (Fig. 1C). Cells expressing mem4F10 scFv bound soluble murine CTLA-4 (mCTLA-4-Ig) but not soluble murine CD28 (mCD28-Ig). Those expressing memPV1 scFv bound mCD28-Ig but not mCTLA-4-Ig. Cotransfection of both constructs resulted in binding of both soluble fusion proteins at levels comparable to those of singly transfected cells. The functional properties of mem4F10 scFv were first characterized in experiments in which 293 cells, transfected with combinations of the surface-linked scFvs, were coincubated with resting murine T cells (Fig. 2). Transfectants...
expressing mem2C11 scFv and memPV1 scFv were used as artificial APCs for purified resting T cells. Coexpression of the control scFv was associated with a strong proliferative response. In contrast, engagement of CTLA-4 by coexpression of mem4F10 scFv resulted in substantial attenuation of T cell proliferation and IL-2 secretion (Fig. 2A). A similar result was observed in a repeated experiment (Fig. 2B). For these and subsequent experiments, expression of mem4F10 scFv was maximized. Lower levels of expression of the anti-CTLA-4 ligand were associated with qualitatively similar but less potent inhibitory effects (data not shown). As the proliferative response to combined CD3ε and CD28 engagement was found to be similar in the presence or absence of control scFv, in subsequent experiments cells transfected with equivalent amounts of empty vector or mem4F10 scFv were used. Together

FIGURE 3. Anti-CTLA-4 scFv reduced proliferation and cytokine production during secondary stimulation of preactivated murine T cells. A panel of five mitomycin C-treated 293 transfectants was used to provide secondary stimulation to murine T cells that had been preactivated with plate-bound 2C11 and PV-1 mAbs for 60 h and rested for a further 12 h. The transfections used were: 1, empty vector (5.0 μg); 2, mem2C11 scFv (0.5 μg) plus empty vector (5.0 μg); 3, mem2C11 scFv (0.5 μg) plus mem4F10 scFv (5.0 μg); 4, mem2C11 scFv (0.5 μg) plus PV1 scFv (0.5 μg) plus empty vector (5.0 μg); 5, mem2C11 scFv (0.5 μg) plus memPV1 scFv (0.5 μg) plus mem4F10 scFv (5.0 μg). T cells were added at 2.5 × 10^4 cells per well and 293 cells at 1.5 × 10^4 per well. Proliferation was measured by tritiated thymidine incorporation between 72 and 84 h of culture and cytokine levels in culture supernatants after 48 h. Upper panels, Stimulation with mem2C11 scFv alone was sufficient to induce significant T cell proliferation and production of IL-2 and IFN-γ, while cells transfected with empty vector alone induced no response. No IL-4 was detected under these stimulation conditions. Coexpression of mem4F10 scFv with an equal amount of mem2C11 scFv was associated with significantly reduced proliferation and cytokine production. Lower panels, Combined expression of mem2C11 scFv and memPV1 scFv resulted in ~10-fold enhancement of proliferative response and production of IL-2 and IFN-γ compared with mem2C11 scFv alone as well as detectable production of IL-4. All these responses were significantly reduced by coexpression of mem4F10 scFv on the stimulating cells. Results are expressed as mean ± SD of six identical wells for each condition. The results shown represent one of eight coculture experiments that were conducted using preactivated T cells. The range of reduction among individual experiments was 33–84% for proliferation (eight experiments) and 30–100% for IL-2 production (four experiments). Reductions in IL-4 and IFN-γ production by preactivated T cells was observed in three individual experiments.

FIGURE 4. Both CD4⁺ and CD8⁺ T cells can be negatively regulated by selective CTLA-4 engagement during secondary activation. Purified CD4⁺ and CD8⁺ T cells were preactivated with plate-bound 2C11 and PV-1 mAbs for 60 h and rested for a further 12 h. A panel of three mitomycin C-treated 293 transfectants, plated at 1.5 × 10⁴ per well, was then used to provide secondary stimulation. Preactivated T cells were added at 2.5 × 10⁶ per well. The transfectants used were: 1, empty vector (5.0 μg); 2, mem2C11 scFv (0.5 μg) plus empty vector (5.0 μg); 3, mem2C11 scFv (0.5 μg) plus mem4F10 scFv (5.0 μg). Vector-transfected 293 cells did not induce proliferation in either CD4⁺ or CD8⁺ populations, while expression of mem2C11 scFv alone was associated with a proliferative response in both subsets and with the secretion of detectable levels of IL-2 and IL-4 (CD4⁺ only) and IFN-γ (CD8⁺ only). Coexpression of mem2C11 scFv and mem4F10 scFv resulted in marked reduction of proliferation and cytokine production for both T cell subsets. Peak proliferation shown occurred between 64 and 76 h of coculture for CD4⁺ T cells and between 30 and 42 h for CD8⁺ T cells. All cytokine levels were measured following 48 h of coculture. Results are expressed as mean ± SD of six identical wells for each condition.
these results confirmed that the various mem-scFvs are expressed on the cell surface and mediate functional effects during cell-cell interactions wherein anti-CD3ε and anti-CD28 scFvs function as immune activators while selective engagement of CTLA-4 exerts a negative regulatory effect on primary T cell activation.

**Engagement of CTLA-4 by surface-linked anti-CTLA-4 scFv during secondary stimulation of T cells and T cell subsets alters proliferation and cytokine secretion**

The concept of a protracted role for CTLA-4 in shaping the magnitude, nature, and duration of an ongoing immune response is compatible with its persistent expression in activated helper and cytotoxic T cells (51–55), in memory T cells (56), and in T cell-derived clones of a variety of phenotypes (55, 57). With this in mind, experiments were performed using preactivated T cells and T cell subsets (see Materials and Methods). The activated T cells can be induced to rapidly proliferate and secrete cytokines upon restimulation by a TCR signal alone or combined with a CD28 signal as seen in Fig. 3. Artificial APCs expressing low levels of mem2C11 scFv alone (upper graphs) or a combination of mem2C11 scFv and memPV1 scFv (lower graphs) were cotransfected with either empty vector or mem4F10 scFv. Peak proliferative responses and levels of IL-2, IFN-γ, and IL-4 of the activated T cells were shown. While untransfected 293 cells did not stimulate preactivated T cells, the presence of a TCR ligand was sufficient to induce proliferation and production of IL-2 and IFN-γ by the preactivated cells. No detectable IL-4 was produced under these conditions. Coexpression of mem4F10 scFv resulted in significant reductions in both T cell proliferation and cytokine production. The addition of low-level expression of memPV1 scFv resulted in a ~10-fold increase in the measured responses as well as inducing measurable secretion of IL-4. In the presence of combined TCR and CD28 signals, the magnitude of the anti-CTLA-4 effect on proliferation and secretion of IL-2 and IFN-γ was similar to that observed in the absence of additional CD28 engagement. Attenuation of IL-4 secretion was also observed. Expression of mem4F10 scFv inhibited proliferation and IL-2 production by 80 and 67%, respectively, with mem2C11 scFv alone and by 66 and 69% with combined mem2C11 scFv and PV1 scFv. Fig. 4 illustrates the results of a similar experiment performed using preactivated purified CD4+ T cells and CD8+ T cells. CTLA-4 coengagement was associated with significant reductions in peak responses of both subsets of T cells. Negative regulation of T cell proliferation and secretion of IL-2 and IL-4 were observed in the CD4+ subset, while in the case of CD8+ T cell proliferation and secretion of IFN-γ were negatively regulated by CTLA-4 ligation. Thus, selective engagement of CTLA-4 upon restimulation of activated T cells resulted in a substantial inhibition of their proliferative capacity and cytokine production. Moreover, this effect could be observed both in the presence and absence of CD28 ligation.

**Coordinate ligation of CTLA-4 and TCR on the same cell surface is essential for signal attenuation**

We have previously reported that coengagement of TCR and CTLA-4 results in attenuation of tyrosine phosphorylation of components of the proximal TCR signaling apparatus, thus providing a mechanistic basis for negative regulation of T cell activation by CTLA-4 (58). To further ask whether physical proximity to the TCR is essential for negative regulation to occur through CTLA-4, we compared the effect of mem4F10 scFv expression on the same cell surface (“in cis”) as mem2C11 scFv with that of expression on a separate population of cells (“in trans”). Preactivated T cells were employed and secondary stimulation was provided through the TCR alone. Fig. 5A shows that, while providing TCR and CTLA-4 engagement on the same cell surface resulted in a significant attenuation of proliferation and IL-2 production, when the two ligands were provided in trans no attenuating effects were observed. In fact, under these circumstances a modest enhancement of the T cell responses was seen. Complementary results were observed in biochemical studies in which preactivated T cells were incubated in suspension with similar mixtures of transfectants for defined time periods (Fig. 5B). As shown, coexpression of mem4F10 scFv on the same cell surface as mem2C11 scFv resulted in reduced levels of tyrosine phosphorylation of proteins of approximate molecular masses of 36 and 23 kDa in preactivated T cells. In contrast, the expression of the CTLA-4 ligand on a separate, admixed population of cells did not attenuate tyrosine phosphorylation of these proteins. We have previously identified these phosphoproteins by immunoprecipitation with specific Abs (58) as.
linker for activated T cells (LAT) (59) and hyperphosphorylated TCRζ, both integral components of the proximal TCR signaling apparatus. These results are consistent with the hypothesis that a physical association between CTLA-4 and the TCR underlies the primary mechanism of negative regulation and imply that the interaction of CTLA-4 with a cell-surface ligand may result in widely differing outcomes depending on the point of ligation relative to the TCR.

Surface-linked anti-CTLA-4 scFv attenuates proliferation of CD4+ T cells when coexpressed with MHC/peptide complex

Although multiple studies have been published demonstrating the ability of CTLA-4 ligation to attenuate Ag nonspecific T cell stimulation, limited data is available to support similar effects on bona fide Ag-specific responses. Therefore, we used the mem-scFv system to examine the effects of the membrane-bound anti-CTLA-4 scFv on primary and secondary activation in a well-characterized model of Ag-driven CD4+ T cell activation. T cells derived from mice transgenic for the DO11.10 TCR (with specificity for the OVA-derived peptide OVA323–339 presented by the murine class II MHC I-A\(^d\); Ref. 60) were coincubated with 293 cells transfected with cDNAs encoding the Ag protein I-A\(^d\) complexed with Ag peptide (as represented by an additional symbol). For each mixture, one transfectant expressed I-A\(^d\) (10 \(\mu\)g each of the \(\alpha-\) and \(\beta\)-chain cDNAs) along with empty vector (5 \(\mu\)g) or mem4F10 scFv (5 \(\mu\)g) and the other expressed memPV1 scFv (2 \(\mu\)g) also with either empty vector or mem4F10 scFv (5 \(\mu\)g). Resting T cells were added at 10\(^5\) per well, preactivated T cells at 2.5 \(\times\) 10\(^5\) per well, and 293 mixtures at 4 \(\times\) 10\(^3\) per well. Proliferation was measured by thymidine incorporation between 72 and 84 h of culture for resting T cells and between 60 and 72 h for preactivated T cells. In both experiments, incubation of DO11.10 T cells with cells separately expressing MHC/peptide and memPV1 scFv (1) resulted in a proliferative response, which was significantly reduced by coexpression of mem4F10 scFv on the same cell surface as the MHC/peptide complex (2). Coexpression of mem4F10 scFv on the same cell surface as memPV1 scFv (3) resulted in a mild increase in proliferation. Results are expressed as mean \(\pm\) SD of six identical wells for each condition.

Selective CTLA-4 engagement inhibits Ag-driven proliferation and cytokine production by preactivated CD8+ T cells using stable mem4F10 scFv transfectants

For studies performed using a transient transfection system, it was not possible to ensure that the levels of the anti-CD3 scFv were completely matched. Therefore, a second Ag-specific system was employed using a stable population of P1.HTR transfectants generated with dual expression of EGFP and mem4F10 scFv or with EGFP alone using a bicistronic vector (Fig. 7A). Flow cytometric analysis of these stable transfectants showed no difference in the levels of Ld expression between the mem4F10 scFv and control transfectants (data not shown). In a series of studies, the ability of the Ld-expressing murine mastocytoma P1.HTR to stimulate preactivated CD8\(^+\) 2C TCR transgenic T cells in the absence of additional CD28 engagement was analyzed (32). Fig. 7, A and C demonstrate that expression of mem4F10 scFv by P815 cells was associated with significant reductions in proliferation and production of IL-2 and IFN-\(\gamma\) by 2C T cells. Similar results were observed in four repeat experiments. Thus, the stable expression of mem4F10 scFv negatively regulated the activation of Ag-specific CD8\(^+\) T cells responses.

Discussion

Single-chain Abs have been shown to have a variety of applications as investigational tools and immunomodulatory agents including modification to generate surface-expressed ligands (29–35, 38, 61, 62). Productive outcomes mediated by surface-expressed scFvs directed against human CD28 as well as other
markers have been reported and demonstrate that this strategy can be used successfully for cell surface-specific immunomodulation (62). We have used this approach in a novel way to generate a panel of ligands that has permitted us to separately engage CD28 and CTLA-4 in the context of interactions between T cells and cells presenting a TCR stimulus. This approach has allowed us to gain new insights into the range and mechanisms of CTLA-4-mediated immune regulation and to begin to predict how this function might be harnessed in vivo to attenuate unwanted immune responses such as allograft rejection and autoimmunity.

We found that transfection with small amounts of the anti-CD3ε and anti-CD28 scFv constructs together provided a significant mitogenic stimulus. In contrast, the combination of surface-linked anti-CTLA-4 scFv with anti-CD3ε and anti-CD28 during primary and secondary activation of T cells demonstrated significant reductions in proliferation and IL-2, IL-4, and IFN-γ production as compared with conditions where no CTLA-4 ligand was provided. The level of expression required to observe functional effects for the individual surface-linked constructs reflects both signaling characteristics of the targeted T cell proteins as well as properties of the engineered ligands themselves such as binding affinity and stability of the refolded Ag-binding domains. The functional effects mediated by each of the surface-linked scFvs represent outcomes that had been predicted on the basis of the functional properties of the parent mAb (9, 36, 37). Although some variability in level of expression of each construct between transfectant populations is likely to occur in any given experiment, we have observed qualitatively similar results in multiple experiments. More importantly, we were able to demonstrate similar inhibition of proliferation and levels of IL-2 and IFN-γ. Results represent mean ± SD of six identical wells for each condition.

FIGURE 7. Anti-CTLA-4 scFv inhibits Ag-driven proliferation and cytokine production by preactivated CD8+ T cells. A, Left, 293 cells were transiently transfected with a bicistronic vector expressing either EGFP alone (LXIE) or mem4F10 scFv followed by EGFP (mem4F10 scFv LXIE) and analyzed by two-color flow cytometry for expression of EGFP and surface binding of mCTLA-Ig. Transfection with LXIE resulted in significant expression of EGFP but no binding of mCTLA-4-Ig, whereas transfection with mem4F10 scFv LXIE resulted in comparable EGFP expression with additional expression of a surface ligand for mCTLA-Ig. Right, P1.HTR (P815) cells transfected with LXIE or with mem4F10 scFv LXIE were selected in puromycin and sorted for comparable expression of EGFP (solid lines, sorted P1.TR transfectants; broken lines, untransfected P815). B and C, Thymidine incorporation and production of IL-2 and IFN-γ by preactivated 2C TCR transgenic T cells during coculture with mitomycin C-treated control (Vector-P815) or mem4F10 scFv-expressing P1.HTR. T cells and P815 cells were cocultured in 96-well round-bottom plates at a 1:1 ratio (1.25 × 10^3 of each per well). Thymidine incorporation was measured between 24 and 60 h, cytokines were measured following 24 h of culture. Expression of mem4F10 scFv resulted in significantly reduced proliferation and levels of IL-2 and IFN-γ. Results represent mean ± SD of six identical wells for each condition.
Wu et al. (63) and Zheng et al. (64), we did not observe augmentation of T cell proliferation as a result of selective CTLA-4 engagement except under circumstances in which the TCR and CTLA-4 were ligated on separate cell surfaces (discussed in further detail below).

One of the strengths of the current system was the ability to replace the nonspecific TCR mitogen with bona fide class I and class II MHC/peptide complexes. Under these conditions, selective CTLA-4 engagement suppressed both primary and secondary T cell activation of CD4+ DO11.10 T cells in a well-characterized Ag-driven system representing the first demonstration of CTLA-4-mediated attenuation of normal Ag-specific T cells responses. Of note, we could also clearly observe negative regulation of Ag-specific, preactivated CD8+ T cells following CD4+ TCR/CTLA-4 engagement using the 2C T cells. Thus, although recent studies have shown that CTLA-4 may not exert significant effects during primary CD8+ T cell activation (52, 65), our results and those of others (53, 54) suggest that CTLA-4-mediated negative regulation of CD8+ T cells may occur both indirectly, through inhibition of CD4+ helper functions, and directly, through inhibition of expansion and cytokine production during secondary Ag encounters.

We have recently demonstrated that coligation of TCR and CTLA-4 results in reduced tyrosine phosphorylation of early components of the TCR signaling apparatus including p23 (hyperphosphorylated) TCRζ and p36 LAT (58). In this study, we have expanded this observation by showing, both in proliferation and IL-2 assays as well as in biochemical studies, that the negative regulatory effects of CTLA-4 engagement are abolished by expression of CD28 ligation. Furthermore, by separating the TCR and CD28 ligands during Ag-driven system representing the first demonstration of CTLA-4 engagement using the 2C T cells. Thus, although recent studies have shown that CTLA-4 may not exert significant effects during primary CD8+ T cell activation (52, 65), our results and those of others (53, 54) suggest that CTLA-4-mediated negative regulation of CD8+ T cells may occur both indirectly, through inhibition of CD4+ helper functions, and directly, through inhibition of expansion and cytokine production during secondary Ag encounters.

In conclusion, we have shown that positive and negative regulation of the TCR signal in different subpopulations of T cells, the possibility of additional mechanisms of action for CTLA-4 remains an important unanswered question.

In conclusion, we have shown that positive and negative regulation of T cell activation can be separately manipulated through the generation of surface-linked ligands with specific affinity for CD28 and CTLA-4. Using this approach, it is possible to selectively recruit the inhibitory influence of CTLA-4 to T cell activation events delivered either by Ag-nonspecific signals or by antigenic peptide presented on MHC. Our extended observations of CTLA-4-mediated attenuation of proximal TCR signaling events and of the requirement for intimate coligation of TCR and CTLA-4 contribute to a growing understanding of how this essential protein functions. The outcomes of specifically engaging CTLA-4 during a variety of T cell/APC interactions suggest that a strategy of enhanced CTLA-4 recruitment by expression of such a ligand could be used to favor immune hypo-responsiveness or tolerance to selected Ags in the context of tissue transplantation or tissue-specific autoimmunity.

Acknowledgments

We acknowledge the contributions of Julie Auger (University of Chicago Flow Cytometry Facility) for expert assistance and consultation and Ulf Körthauer, Keshav Rajagopal, Ingrid Rulifson, Benoit Salomon, Anne Sterling, Qizhi Tang, and Theresa Walunas for many helpful discussions and suggestions.

References

fat fatal multigorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 3:541.


