IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ZYMOGENETICS, INC., a Washington Corporation, Plaintiff, v. BRISTOL-MYERS SQUIBB CO., a Delaware Corporation, and DOES 1 through 100, Defendants.	)	Civil Action No: 06-500-SLR-MPT **REDACTED PUBLIC VERSION**

DECLARATION OF DANA LAU IN SUPPORT OF BRISTOL-MYERS SQUIBB COMPANY'S RESPONSE TO ZYMOGENETICS, INC.'S STATEMENT OF UNDISPUTED MATERIAL FACTS AND LEGAL ISSUES UPON WHICH JUDGMENT IS SOUGHT IN SUPPORT FOR SUMMARY JUDGMENT OF INFRINGEMENT OF THE PATENTS-IN-SUIT

I, Dana Lau, hereby declare the following:

1. I am an associate with the law firm of Fitzpatrick, Cella, Harper & Scinto, attorneys for Defendant Bristol-Myers Squibb Company ("Bristol") in this action. I submit this Declaration in support of Bristol's Response to Plaintiff ZymoGenetics, Inc.'s Statement of Undisputed Material Facts And Legal Issues Upon Which Judgment Is Sought In Support For Summary Judgment of Infringement Of Patents-In-Suit.

2. Attached hereto as BMS Exhibit 1 is a true and correct copy of the Orencia® Product Insert.

3. Attached hereto as BMS Exhibit 2 is a true and correct copy of excerpts from the Abatacept Biologics License Application 2005 at BMS001290152-60, BMS001290162, BMS001290170-71, and BMS001290334-357.

4. Attached hereto as BMS Exhibit 3 is a true and correct copy of excerpts of Brunet at al., *A new member of the immunoglobulin superfamily – CTLA-4*, NATURE, 328:267-270 (1987).

5. Attached hereto as BMS Exhibit 4 is a true and correct copy of a Response to Office Action dated March 11, 1993 from European Patent Application, EP89100787.4 at BMS004261722-31.

6. Attached hereto as BMS Exhibit 5 is a true and correct copy of excerpts of Nicholas R.J. Gascoigne, Ph.D. Deposition Transcript, April 29, 2008, at 18-25, 50-53, 118-121, 178-181, 197-200, and 202-213.

7. Attached hereto as BMS Exhibit 6 is a true and correct copy of Walunas et al., *CTLA-4 can function as a negative regulator of T cell activation*, IMMUNITY, 1:405-413 (1994) at BMS001043064-72.

8. Attached hereto as BMS Exhibit 7 is a true and correct copy of Lindsten et al., *Characterization of CTLA-4 structure and expression on human T cells*, JOURNAL OF IMMUNOLOGY, 151:3489-3499 (1993) at BMS001056768-78.

9. Attached hereto as BMS Exhibit 8 is a true and correct copy of June et al., *The B7 and CD28 receptor families*, IMMUNOLOGY TODAY, 15:321-331 (1994) at BMS001043053-63.

10. Attached hereto as BMS Exhibit 9 is a true and correct copy of Magistrelli et al., *A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated*

human T cells, EUROPEAN JOURNAL OF IMMUNOLOGY, 29:3596-3602 (1999) at BMS003334436-42.

11. Attached hereto as BMS Exhibit 10 is a true and correct copy of Oaks et al., *A native form of CTLA-4*, CELLULAR IMMUNOLOGY, 201:144-153 (2000) at BMS001147559-68.

12. Attached hereto as BMS Exhibit 11 is a true and correct copy of Saverino et al., *Soluble CTLA-4 in autoimmune thyroid diseases: Relationship with clinical status and possible role in the immune response dysregulation*, CLINICAL IMMUNOLOGY, 123:190-198 (2007).

13. Attached hereto as BMS Exhibit 12 is a true and correct copy of Linsley et al., *Binding stoichiometry of the cytotoxic T lymphocyte-associated molecule-4 (CTLA-4)*, JOURNAL OF BIOLOGICAL CHEMISTRY, 270: 15417-15424 (1999) at ZG01826-33.

14. Attached hereto as BMS Exhibit 13 is a true and correct copy of Davis et al., *Abatacept binds to the Fc receptor CD64 but does not mediate complement-dependent cytotoxicity or antibody-dependent cytoxicity*, JOURNAL OF RHEUMATOLOGY, 34:2204-2210 (2007).

15. Attached hereto as BMS Exhibit 14 is a true and correct copy of Sandor and Lynch, *The biology and pathology of Fc receptors*, JOURNAL OF CLINICAL IMMUNOLOGY, 13:237-246 (1993).

16. Attached hereto as BMS Exhibit 15 is a true and correct copy of excerpts of David A. Ostrov, Ph.D. Deposition Transcript, May 6, 2008, at 258-285.

17. Attached hereto as BMS Exhibit 16 is a true and correct copy of excerpts of Steven Gary Nadler, Ph.D. Deposition Transcript, August 3, 2007, at 37-40, 54-57, 62-65, and 82-89.

18. Attached hereto as BMS Exhibit 17 is a true and correct copy of excerpts of Paul E. Purdue, Deposition Transcript, May 5, 2008 at 237-244.

I declare under penalty of perjury under the laws of the United States of America that the forgoing is true and correct.

Executed this 2nd day of June, 2008, in New York, New York.

Dana Lau

UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CERTIFICATE OF SERVICE

I hereby certify that on June 9, 2008, I electronically filed the foregoing document with the Clerk of the Court using CM/ECF which will send notification of such filing to the following and which has also been served as noted:

BY HAND DELIVERY (on June 6, 2008)

Philip A. Rovner
Potter, Anderson & Corroon
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Wilmington, DE 19899

I hereby certify that on June 6, 2008, the foregoing document was sent to the following non-registered participants in the manner indicted:

BY FEDERAL EXPRESS

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King & Spalding, LLP
1000 Bridge Parkway
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Redwood Shores, CA 94065

Frederick L. Cottrell (#2555)

EXHIBIT 1

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use ORENCIA safely and effectively. See full prescribing information for ORENCIA.

ORENCIA® (abatacept)

Lyophilized Powder for Intravenous Infusion

Initial U.S. Approval: 2005

RECENT MAJOR CHANGES

Indications and Usage, Adult Rheumatoid Arthritis (1.1)	04/2008
Indications and Usage, Juvenile Idiopathic Arthritis (1.2)	04/2008
Indications and Usage, Important Limitations of Use (1.3)	04/2008
Dosage and Administration, Juvenile Idiopathic Arthritis (2.2)	04/2008
Warnings and Precautions, Immunizations (5.4)	04/2008

INDICATIONS AND USAGE

ORENCIA is a selective T cell costimulation modulator indicated for:

Adult Rheumatoid Arthritis (RA) (1.1)

- moderately to severely active RA in adults. ORENCIA may be used as monotherapy or concomitantly with DMARDs other than TNF antagonists (1.1)

Juvenile Idiopathic Arthritis (1.2)

- moderately to severely active polyarticular juvenile idiopathic arthritis in pediatric patients 6 years of age and older. ORENCIA may be used as monotherapy or concomitantly with MTX (1.2)

Important Limitations of Use (1.3)

- Should not be given concomitantly with TNF antagonists (1.3, 5.1)

DOSAGE AND ADMINISTRATION

Adult RA (2.1)

Body Weight of Patient	Dose	Number of Vials
<60 kg	500 mg	2
60 to 100 kg	750 mg	3
>100 kg	1000 mg	4

Juvenile Idiopathic Arthritis (2.2)

- Pediatric patients weighing less than 75 kg receive 10 mg/kg based on the patient's body weight. Pediatric patients weighing 75 kg or more should be administered ORENCIA following the adult dosing regimen, not to exceed a maximum dose of 1000 mg (2.2)

General Dosing Information (2)

- Administer as a 30-minute intravenous infusion (2)
- Following initial dose, give at 2 and 4 weeks, then every 4 weeks (2)
- Prepare ORENCIA (abatacept) using only the silicone-free disposable syringe (2.3)

DOSAGE FORMS AND STRENGTHS

- 250 mg single-use vial (3)

CONTRAINDICATIONS

- None (4)

WARNINGS AND PRECAUTIONS

- Concomitant use with a TNF antagonist can increase the risk of infections and serious infections (5.1)
- Hypersensitivity, anaphylaxis, and anaphylactoid reactions (5.2)
- Patients with a history of recurrent infections or underlying conditions predisposing to infections may experience more infections (5.3, 8.5)
- Discontinue if a serious infection develops (5.3)
- Screen for latent TB infection prior to initiating therapy. Patients testing positive should be treated prior to initiating ORENCIA (5.3)
- Live vaccines should not be given concurrently or within 3 months of discontinuation (5.4)
- Patients with juvenile idiopathic arthritis should be brought up to date with all immunizations prior to ORENCIA therapy (5.4)
- Based on its mechanism of action, ORENCIA may blunt the effectiveness of some immunizations (5.4)
- COPD patients may develop more frequent respiratory adverse events (5.5)

ADVERSE REACTIONS

Most common adverse events (≥10%) are headache, upper respiratory tract infection, nasopharyngitis, and nausea (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact Bristol-Myers Squibb at 1-800-721-5072 or FDA at 1-800-FDA-1088 or *www.fda.gov/medwatch*

USE IN SPECIFIC POPULATIONS

- Pregnancy: Registry available. Based on animal data, may cause fetal harm (8.1)

See 17 for PATIENT COUNSELING INFORMATION and FDA-approved patient labeling

Revised: 04/2008

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FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

1.1 Adult Rheumatoid Arthritis (RA)

ORENCIA® (abatacept) is indicated for reducing signs and symptoms, inducing major clinical response, inhibiting the progression of structural damage, and improving physical function in adult patients with moderately to severely active rheumatoid arthritis. ORENCIA may be used as monotherapy or concomitantly with disease-modifying anti-rheumatic drugs (DMARDs) other than tumor necrosis factor (TNF) antagonists

1.2 Juvenile Idiopathic Arthritis

ORENCIA is indicated for reducing signs and symptoms in pediatric patients 6 years of age and older with moderately to severely active polyarticular juvenile idiopathic arthritis. ORENCIA may be used as monotherapy or concomitantly with methotrexate (MTX)

1.3 Important Limitations of Use

ORENCIA should not be administered concomitantly with TNF antagonists. ORENCIA is not recommended for use concomitantly with other biologic rheumatoid arthritis (RA) therapy, such as anakinra.

2 DOSAGE AND ADMINISTRATION

2.1 Adult Rheumatoid Arthritis

For adult patients with RA, ORENCIA should be administered as a 30-minute intravenous infusion utilizing the weight range-based dosing specified in Table 1. Following the initial administration, ORENCIA should be given at 2 and 4 weeks after the first infusion and every 4 weeks thereafter. ORENCIA may be used as monotherapy or concomitantly with DMARDs other than TNF antagonists.

For pediatric juvenile idiopathic arthritis, a dose calculated based on each patient's body weight is used [see *Dosage and Administration (2.2)*].

Table 1: Dose of ORENCIA in Adult RA

Body Weight of Patient	Dose	Number of Vials[a]
<60 kg	500 mg	2
60 to 100 kg	750 mg	3
>100 kg	1000 mg	4

[a] Each vial provides 250 mg of abatacept for administration

2.2 Juvenile Idiopathic Arthritis

The recommended dose of ORENCIA for patients 6 to 17 years of age with juvenile idiopathic arthritis who weigh less than 75 kg is 10 mg/kg calculated based on the patient's body weight at each administration. Pediatric patients weighing 75 kg or more should be administered ORENCIA following the adult dosing regimen, not to exceed a maximum dose of 1000 mg. ORENCIA should be administered as a 30-minute intravenous infusion. Following the initial administration, ORENCIA should be given at 2 and 4 weeks after the first infusion and every 4 weeks thereafter. Any unused portions in the vials must be immediately discarded.

2.3 Preparation and Administration Instructions

Use aseptic technique.

ORENCIA is provided as a lyophilized powder in preservative-free, single-use vials. Each ORENCIA vial provides 250 mg of abatacept for administration. The ORENCIA powder in each vial must be reconstituted with 10 mL of Sterile Water for Injection, USP, using **ONLY the SILICONE-FREE DISPOSABLE SYRINGE PROVIDED WITH EACH VIAL** and an 18- to 21-gauge needle. After reconstitution, the concentration of abatacept in the vial will be 25 mg/mL. If the ORENCIA powder is accidentally reconstituted using a siliconized syringe, the solution may develop a few translucent particles. Discard any solutions prepared using siliconized syringes.

If the **SILICONE-FREE DISPOSABLE SYRINGE** is dropped or becomes contaminated, use a new **SILICONE-FREE DISPOSABLE SYRINGE** from inventory. For information on obtaining additional **SILICONE-FREE DISPOSABLE SYRINGES**, contact Bristol-Myers Squibb 1-800-ORENCIA.

During reconstitution, to minimize foam formation in solutions of ORENCIA, the vial should be rotated with gentle swirling until the contents are completely dissolved. Avoid prolonged or vigorous agitation. DO NOT SHAKE. Upon complete dissolution of the lyophilized powder, the vial should be vented with a needle to dissipate any foam that may be present. The solution should be clear and colorless to pale yellow. Do not use if opaque particles, discoloration, or other foreign particles are present.

1) To reconstitute the ORENCIA powder, remove the flip-top from the vial and wipe the top with an alcohol swab. Insert the syringe needle into the vial through the center of the rubber stopper and direct the stream of Sterile Water for Injection, USP, to the glass wall of the vial. Do not use the vial if the vacuum is not present. Rotate the vial with gentle swirling until the contents are completely dissolved.

2) Upon complete dissolution of the lyophilized powder, the vial should be vented with a needle to dissipate any foam that may be present. After reconstitution, each milliliter will contain 25 mg (250 mg/10 mL).

3) The reconstituted ORENCIA (abatacept) solution must be further diluted to 100 mL as follows. From a 100 mL infusion bag or bottle, withdraw a volume of 0.9% Sodium Chloride Injection, USP, equal to the volume of the reconstituted ORENCIA solution required for the patient's dose. Slowly add the reconstituted ORENCIA solution into the infusion bag or bottle using the same SILICONE-FREE DISPOSABLE SYRINGE PROVIDED WITH EACH VIAL. Gently mix. DO NOT SHAKE THE BAG OR BOTTLE. The final concentration of abatacept in the bag or bottle will depend upon the amount of drug added, but will be no more than 10 mg/mL. Any unused portions in the vials must be immediately discarded.

4) Prior to administration, the ORENCIA solution should be inspected visually for particulate matter and discoloration. Discard the solution if any particulate matter or discoloration is observed.

5) The entire, fully diluted ORENCIA solution should be administered over a period of 30 minutes and must be administered with an infusion set and a **STERILE, NON-PYROGENIC, LOW-PROTEIN-BINDING FILTER** (pore size of 0.2 µm to 1.2 µm).

6) The infusion of the fully diluted ORENCIA solution must be completed within 24 hours of reconstitution of the ORENCIA vials. The fully diluted ORENCIA solution may be stored at room temperature or refrigerated at 2°C to 8°C (36°F to 46°F) before use.

7) ORENCIA should not be infused concomitantly in the same intravenous line with other agents. No physical or biochemical compatibility studies have been conducted to evaluate the coadministration of ORENCIA with other agents.

3 DOSAGE FORMS AND STRENGTHS

250 mg single-use vial

4 CONTRAINDICATIONS

None.

5 WARNINGS AND PRECAUTIONS

5.1 Concomitant Use with TNF Antagonists

In controlled clinical trials in patients with adult RA, patients receiving concomitant ORENCIA and TNF antagonist therapy experienced more infections (63%) and serious infections (4.4%) compared to patients treated with only TNF antagonists (43% and 0.8%, respectively) [see *Adverse Reactions (6.1)*]. These trials failed to demonstrate an important enhancement of efficacy with concomitant administration of ORENCIA with TNF antagonist; therefore, concurrent therapy with ORENCIA and a TNF antagonist is not recommended. While transitioning from TNF antagonist therapy to ORENCIA therapy, patients should be monitored for signs of infection.

5.2 Hypersensitivity

Of 2688 patients with adult RA treated with ORENCIA in clinical trials, there were two cases of anaphylaxis or anaphylactoid reactions. Other events potentially associated with drug hypersensitivity, such as hypotension, urticaria, and dyspnea, each occurred in less than 0.9% of ORENCIA-treated patients. Of the 190 patients with juvenile idiopathic arthritis treated with ORENCIA in clinical trials, there was one case of a hypersensitivity reaction (0.5%). Appropriate medical support measures for the treatment of hypersensitivity reactions should be available for immediate use in the event of a reaction [see *Adverse Reactions (6.1, 6.2)*].

5.3 Infections

Physicians should exercise caution when considering the use of ORENCIA in patients with a history of recurrent infections, underlying conditions which may predispose them to infections, or chronic, latent, or localized infections. Patients who develop a new infection while undergoing treatment with ORENCIA should be monitored closely. Administration of ORENCIA should be discontinued if a patient develops a serious infection [see *Adverse Reactions (6.1)*]. A higher rate of serious infections has been observed in adult RA patients treated with concurrent TNF antagonists and ORENCIA [see *Warnings and Precautions (5.1)*].

Prior to initiating immunomodulatory therapies, including ORENCIA, patients should be screened for latent tuberculosis infection with a tuberculin skin test. ORENCIA has not been studied in patients with a positive tuberculosis screen, and the safety of ORENCIA in individuals with latent tuberculosis infection is unknown. Patients testing positive in tuberculosis screening should be treated by standard medical practice prior to therapy with ORENCIA.

Anti-rheumatic therapies have been associated with hepatitis B reactivation. Therefore, screening for viral hepatitis should be performed in accordance with published guidelines before starting therapy with ORENCIA. In clinical studies with ORENCIA, patients who screened positive for hepatitis were excluded from study.

5.4 Immunizations

Live vaccines should not be given concurrently with ORENCIA or within 3 months of its discontinuation. No data are available on the secondary transmission of infection from persons receiving live vaccines to patients receiving ORENCIA. The efficacy of vaccination in patients receiving ORENCIA is not known. Based on its mechanism of action, ORENCIA may blunt the effectiveness of some immunizations.

It is recommended that patients with juvenile idiopathic arthritis be brought up to date with all immunizations in agreement with current immunization guidelines prior to initiating ORENCIA therapy.

5.5 Use in Patients with Chronic Obstructive Pulmonary Disease (COPD)

Adult COPD patients treated with ORENCIA (abatacept) developed adverse events more frequently than those treated with placebo, including COPD exacerbations, cough, rhonchi, and dyspnea. Use of ORENCIA in patients with RA and COPD should be undertaken with caution and such patients should be monitored for worsening of their respiratory status [see *Adverse Reactions (6.1)*]

5.6 Immunosuppression

The possibility exists for drugs inhibiting T cell activation, including ORENCIA, to affect host defenses against infections and malignancies since T cells mediate cellular immune responses. The impact of treatment with ORENCIA on the development and course of malignancies is not fully understood [see *Adverse Reactions (6.1)*]. In clinical trials in patients with adult RA, a higher rate of infections was seen in ORENCIA-treated patients compared to placebo [see *Adverse Reactions (6.1)*]

6 ADVERSE REACTIONS

6.1 Clinical Studies Experience in Adult RA

Because clinical trials are conducted under widely varying and controlled conditions, adverse reaction rates observed in clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not predict the rates observed in a broader patient population in clinical practice.

The data described herein reflect exposure to ORENCIA in patients with active RA in placebo-controlled studies (1955 patients with ORENCIA, 989 with placebo). The studies had either a double-blind, placebo-controlled period of 6 months (258 patients with ORENCIA, 133 with placebo) or 1 year (1697 patients with ORENCIA, 856 with placebo). A subset of these patients received concomitant biologic DMARD therapy, such as a TNF blocking agent (204 patients with ORENCIA, 134 with placebo).

The majority of patients in RA clinical studies received one or more of the following concomitant medications with ORENCIA: MTX, nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, TNF blocking agents, azathioprine, chloroquine, gold, hydroxychloroquine, leflunomide, sulfasalazine, and anakinra.

The most serious adverse reactions were serious infections and malignancies.

The most commonly reported adverse events (occurring in ≥10% of patients treated with ORENCIA) were headache, upper respiratory tract infection, nasopharyngitis, and nausea.

The adverse events most frequently resulting in clinical intervention (interruption or discontinuation of ORENCIA) were due to infection. The most frequently reported infections resulting in dose interruption were upper respiratory tract infection (1.0%), bronchitis (0.7%), and herpes zoster (0.7%). The most frequent infections resulting in discontinuation were pneumonia (0.2%), localized infection (0.2%), and bronchitis (0.1%).

Infections

In the placebo-controlled trials, infections were reported in 54% of ORENCIA-treated patients and 48% of placebo-treated patients. The most commonly reported infections (reported in 5-13% of patients) were upper respiratory tract infection, nasopharyngitis, sinusitis, urinary tract infection, influenza, and bronchitis. Other infections reported in fewer than 5% of patients at a higher frequency (>0.5%) with ORENCIA compared to placebo, were rhinitis, herpes simplex, and pneumonia [see *Warnings and Precautions (5.3)*]

Serious infections were reported in 3.0% of patients treated with ORENCIA and 1.9% of patients treated with placebo. The most common (0.2-0.5%) serious infections reported with ORENCIA were pneumonia, cellulitis, urinary tract infection, bronchitis, diverticulitis, and acute pyelonephritis [see *Warnings and Precautions (5.3)*].

Malignancies

In the placebo-controlled portions of the clinical trials (1955 patients treated with ORENCIA for a median of 12 months), the overall frequencies of malignancies were similar in the ORENCIA- and placebo-treated patients (1.3% and 1.1%, respectively). However, more cases of lung cancer were observed in ORENCIA-treated patients (4, 0.2%) than placebo-treated patients (0). In the cumulative ORENCIA clinical trials (placebo-controlled and uncontrolled, open-label) a total of 8 cases of lung cancer (0.21 cases per 100 patient-years) and 4 lymphomas (0.10 cases per 100 patient-years) were observed in 2688 patients (3827 patient-years). The rate observed for lymphoma is approximately 3.5-fold higher than expected in an age- and gender-matched general population based on the Surveillance, Epidemiology, and End Results Database [1]. Patients with RA, particularly those with highly active disease, are at a higher risk for the development of lymphoma. Other malignancies included skin, breast, bile duct, bladder, cervical, endometrial, lymphoma, melanoma, myelodysplastic syndrome, ovarian, prostate, renal, thyroid, and uterine cancers [see *Warnings and Precautions (5.6)*]. The potential role of ORENCIA in the development of malignancies in humans is unknown.

Infusion-Related Reactions and Hypersensitivity Reactions

Acute infusion-related events (adverse reactions occurring within 1 hour of the start of the infusion) in Studies III, IV, and V [see *Clinical Studies (14.1)*] were more common in the ORENCIA-treated patients than the placebo patients (9% for ORENCIA, 6% for placebo). The most frequently reported events (1-2%) were dizziness, headache, and hypertension.

Acute infusion-related events that were reported in >0.1% and ≤1% of patients treated with ORENCIA (abatacept) included cardiopulmonary symptoms, such as hypotension, increased blood pressure, and dyspnea; other symptoms included nausea, flushing, urticaria, cough, hypersensitivity, pruritus, rash, and wheezing. Most of these reactions were mild to moderate. Fewer than 1% of ORENCIA-treated patients discontinued due to an acute infusion-related event. In controlled trials, 6 ORENCIA-treated patients compared to 2 placebo-treated patients discontinued study treatment due to acute infusion-related events.

Of 2688 patients treated with ORENCIA in clinical trials, there were two cases of anaphylaxis or anaphylactoid reactions. Other events potentially associated with drug hypersensitivity, such as hypotension, urticaria, and dyspnea, each occurred in less than 0.9% of ORENCIA-treated patients and generally occurred within 24 hours of ORENCIA infusion. Appropriate medical support measures for the treatment of hypersensitivity reactions should be available for immediate use in the event of a reaction [see *Warnings and Precautions (5.2)*]

Adverse Reactions in Patients with COPD

In Study V [see *Clinical Studies (14.1)*], there were 37 patients with chronic obstructive pulmonary disease (COPD) who were treated with ORENCIA and 17 COPD patients who were treated with placebo. The COPD patients treated with ORENCIA developed adverse events more frequently than those treated with placebo (97% vs 88%, respectively). Respiratory disorders occurred more frequently in ORENCIA-treated patients compared to placebo-treated patients (43% vs 24%, respectively) including COPD exacerbation, cough, rhonchi, and dyspnea. A greater percentage of ORENCIA-treated patients developed a serious adverse event compared to placebo-treated patients (27% vs 6%), including COPD exacerbation (3 of 37 patients [8%]) and pneumonia (1 of 37 patients [3%]) [see *Warnings and Precautions (5.5)*].

Other Adverse Reactions

Adverse events occurring in 3% or more of patients and at least 1% more frequently in ORENCIA-treated patients during placebo-controlled RA studies are summarized in Table 2.

Table 2: Adverse Events Occurring in 3% or More of Patients and at Least 1% More Frequently in ORENCIA-Treated Patients During Placebo-Controlled RA Studies

Adverse Event (Preferred Term)	ORENCIA (n=1955)[a] Percentage	Placebo (n=989)[b] Percentage
Headache	18	13
Nasopharyngitis	12	9
Dizziness	9	7
Cough	8	7
Back pain	7	6
Hypertension	7	4
Dyspepsia	6	4
Urinary tract infection	6	5
Rash	4	3
Pain in extremity	3	2

[a] Includes 204 patients on concomitant biologic DMARDs (adalimumab, anakinra, etanercept, or infliximab)

[b] Includes 134 patients on concomitant biologic DMARDs (adalimumab, anakinra, etanercept, or infliximab).

Immunogenicity

Antibodies directed against the entire abatacept molecule or to the CTLA-4 portion of abatacept were assessed by ELISA assays in RA patients for up to 2 years following repeated treatment with ORENCIA. Thirty-four of 1993 (1.7%) patients developed binding antibodies to the entire abatacept molecule or to the CTLA-4 portion of abatacept. Because trough levels of abatacept can interfere with assay results, a subset analysis was performed. In this analysis it was observed that 9 of 154 (5.8%) patients that had discontinued treatment with ORENCIA for over 56 days developed antibodies.

Samples with confirmed binding activity to CTLA-4 were assessed for the presence of neutralizing antibodies in a cell-based luciferase reporter assay. Six of 9 (67%) evaluable patients were shown to possess neutralizing antibodies.

No correlation of antibody development to clinical response or adverse events was observed.

The data reflect the percentage of patients whose test results were positive for antibodies to abatacept in specific assays. The observed incidence of antibody (including neutralizing antibody) positivity in an assay is highly dependent on several factors, including assay sensitivity and specificity, assay methodology, sample handling, timing of sample collection, concomitant medication, and underlying disease. For these reasons, comparison of the incidence of antibodies to abatacept with the incidence of antibodies to other products may be misleading.

6.2 Clinical Studies Experience in Juvenile Idiopathic Arthritis

In general, the adverse events in pediatric patients were similar in frequency and type to those seen in adult patients [see *Warnings and Precautions (5), Adverse Reactions (6)*]

ORENCIA (abatacept) has been studied in 190 pediatric patients, 6 to 17 years of age, with polyarticular juvenile idiopathic arthritis. Overall frequency of adverse events in the 4-month, lead-in, open-label period of the study was 70%; infections occurred at a frequency of 36% [see *Clinical Studies (14.2)*]. The most common infections were upper respiratory tract infection and nasopharyngitis. The infections resolved without sequelae, and the types of infections were consistent with those commonly seen in outpatient pediatric populations. Other events that occurred at a prevalence of at least 5% were headache, nausea, diarrhea, cough, pyrexia, and abdominal pain.

A total of 6 serious adverse events (acute lymphocytic leukemia, ovarian cyst, varicella infection, disease flare [2], and joint wear) were reported during the initial 4 months of treatment with ORENCIA.

Of the 190 patients with juvenile idiopathic arthritis treated with ORENCIA in clinical trials, there was one case of a hypersensitivity reaction (0.5%). During Periods A, B, and C, acute infusion-related reactions occurred at a frequency of 4%, 2%, and 3%, respectively, and were consistent with the types of events reported in adults.

Upon continued treatment in the open-label extension period, the types of adverse events were similar in frequency and type to those seen in adult patients, except for a single patient diagnosed with multiple sclerosis while on open-label treatment.

Immunogenicity

Antibodies directed against the entire abatacept molecule or to the CTLA-4 portion of abatacept were assessed by ELISA assays in patients with juvenile idiopathic arthritis following repeated treatment with ORENCIA throughout the open-label period. For patients who were withdrawn from therapy for up to 6 months during the double-blind period, the rate of antibody formation to the CTLA-4 portion of the molecule was 41% (22/54), while for those who remained on therapy the rate was 13% (7/54).

The presence of antibodies was generally transient and titers were low. The presence of antibodies was not associated with adverse events, changes in efficacy, or an effect on serum concentrations of abatacept. For patients who were withdrawn from ORENCIA during the double-blind period for up to 6 months, no serious acute infusion-related events were observed upon re-initiation of ORENCIA therapy.

6.3 Postmarketing Experience

Adverse reactions have been reported during the post-approval use of ORENCIA. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to ORENCIA. Based on the postmarketing experience with ORENCIA in adult RA patients, the adverse event profile of ORENCIA does not differ from that listed/discussed above in Section 6.1 in adults.

7 DRUG INTERACTIONS

7.1 TNF Antagonists

Concurrent administration of a TNF antagonist with ORENCIA has been associated with an increased risk of serious infections and no significant additional efficacy over use of the TNF antagonists alone. Concurrent therapy with ORENCIA and TNF antagonists is not recommended [see *Warnings and Precautions (5.1)*]

7.2 Other Biologic RA Therapy

There is insufficient experience to assess the safety and efficacy of ORENCIA administered concurrently with other biologic RA therapy, such as anakinra, and therefore such use is not recommended.

7.3 Blood Glucose Testing

Parenteral drug products containing maltose can interfere with the readings of blood glucose monitors that use test strips with glucose dehydrogenase pyrroloquino-linequinone (GDH-PQQ). The GDH-PQQ based glucose monitoring systems may react with the maltose present in ORENCIA, resulting in falsely elevated blood glucose readings on the day of infusion. When receiving ORENCIA, patients that require blood glucose monitoring should be advised to consider methods that do not react with maltose, such as those based on glucose dehydrogenase nicotine adenine dinucleotide (GDH-NAD), glucose oxidase, or glucose hexokinase test methods.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C

There are no adequate and well-controlled studies of ORENCIA use in pregnant women. Abatacept has been shown to cross the placenta in animals, and in animal reproduction studies alterations in immune function occurred. ORENCIA should be used during pregnancy only if the potential benefit to the mother justifies the potential risk to the fetus.

Abatacept was not teratogenic when administered to pregnant mice at doses up to 300 mg/kg and in pregnant rats and rabbits at doses up to 200 mg/kg daily representing approximately 29 times the exposure associated with the maximum recommended human dose (MRHD) of 10 mg/kg based on AUC (area under the time-concentration curve).

Abatacept administered to female rats every three days during early gestation and throughout the lactation period, produced no adverse effects in offspring at doses up to 45 mg/kg, representing 3 times the exposure associated with the MRHD of 10 mg/kg based on AUC. However, at 200 mg/kg, 11 times the MRHD exposure, alterations in immune function were observed consisting of a 9-fold increase in T-cell dependent antibody response in female pups and thyroid inflammation in one female pup. It is not known whether these findings indicate a risk for development of autoimmune diseases in humans exposed *in utero* to abatacept. However, exposure to abatacept in the juvenile rat, which may be more representative of the fetal immune system state in the human, resulted in immune system abnormalities including inflammation of the thyroid and pancreas [see *Nonclinical Toxicology (13.2)*]

Pregnancy Registry: To monitor maternal-fetal outcomes of pregnant women exposed to ORENCIA (abatacept), a pregnancy registry has been established. Healthcare professionals are encouraged to register patients and pregnant women are encouraged to enroll themselves by calling 1-877-311-8972.

8.3 Nursing Mothers

It is not known whether ORENCIA is excreted into human milk or absorbed systemically after ingestion by a nursing infant. However, abatacept was excreted in rat milk. Because many drugs are excreted in human milk, and because of the potential for serious adverse reactions in nursing infants from ORENCIA, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

8.4 Pediatric Use

ORENCIA is indicated for reducing signs and symptoms in pediatric patients with moderately to severely active polyarticular juvenile idiopathic arthritis ages 6 years and older. ORENCIA may be used as monotherapy or concomitantly with MTX.

Studies in juvenile rats exposed to ORENCIA prior to immune system maturity have shown immune system abnormalities including an increase in the incidence of infections leading to death as well as inflammation of the thyroid and pancreas [see *Nonclinical Toxicology (13.2)*]. Studies in adult mice and monkeys have not demonstrated similar findings. As the immune system of the rat is undeveloped in the first few weeks after birth, the relevance of these results to humans greater than 6 years of age (where the immune system is largely developed) is unknown.

The safety and effectiveness of ORENCIA in pediatric patients below 6 years of age have not been established. Therefore, ORENCIA is not recommended for use in patients below the age of 6 years.

Safety and efficacy of ORENCIA in pediatric patients for uses other than juvenile idiopathic arthritis have not been established.

8.5 Geriatric Use

A total of 323 patients 65 years of age and older, including 53 patients 75 years and older, received ORENCIA in clinical studies. No overall differences in safety or effectiveness were observed between these patients and younger patients, but these numbers are too low to rule out differences. The frequency of serious infection and malignancy among ORENCIA-treated patients over age 65 was higher than for those under age 65. Because there is a higher incidence of infections and malignancies in the elderly population in general, caution should be used when treating the elderly.

10 OVERDOSAGE

ORENCIA is administered as an intravenous infusion under medically controlled conditions. Doses up to 50 mg/kg have been administered without apparent toxic effect. In case of overdosage, it is recommended that the patient be monitored for any signs or symptoms of adverse reactions and appropriate symptomatic treatment instituted.

11 DESCRIPTION

ORENCIA is a soluble fusion protein that consists of the extracellular domain of human cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) linked to the modified Fc (hinge, CH2, and CH3 domains) portion of human immunoglobulin G1 (IgG1). Abatacept is produced by recombinant DNA technology in a mammalian cell expression system. The apparent molecular weight of abatacept is 92 kilodaltons.

ORENCIA is supplied as a sterile, white, preservative-free, lyophilized powder for parenteral administration. Following reconstitution with 10 mL of Sterile Water for Injection, USP, the solution of ORENCIA is clear, colorless to pale yellow, with a pH range of 7.2 to 7.8. Each single-use vial of ORENCIA provides 250 mg abatacept, 500 mg maltose, 17.2 mg monobasic sodium phosphate, and 14.6 mg sodium chloride for administration.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Abatacept, a selective costimulation modulator, inhibits T cell (T lymphocyte) activation by binding to CD80 and CD86, thereby blocking interaction with CD28. This interaction provides a costimulatory signal necessary for full activation of T lymphocytes. Activated T lymphocytes are implicated in the pathogenesis of RA and are found in the synovium of patients with RA.

In vitro, abatacept decreases T cell proliferation and inhibits the production of the cytokines TNF alpha (TNFα), interferon-γ, and interleukin-2. In a rat collagen-induced arthritis model, abatacept suppresses inflammation, decreases anti-collagen antibody production, and reduces antigen specific production of interferon-γ. The relationship of these biological response markers to the mechanisms by which ORENCIA exerts its effects in RA is unknown.

12.2 Pharmacodynamics

In clinical trials with ORENCIA (abatacept) at doses approximating 10 mg/kg, decreases were observed in serum levels of soluble interleukin-2 receptor (sIL-2R), interleukin-6 (IL-6), rheumatoid factor (RF), C-reactive protein (CRP), matrix metalloproteinase-3 (MMP3), and TNFα. The relationship of these biological response markers to the mechanisms by which ORENCIA exerts its effects in RA is unknown.

12.3 Pharmacokinetics

Healthy Adults and Adult RA

The pharmacokinetics of abatacept were studied in healthy adult subjects after a single 10 mg/kg intravenous infusion and in RA patients after multiple 10 mg/kg intravenous infusions (see Table 3).

Table 3: Pharmacokinetic Parameters (Mean, Range) in Healthy Subjects and RA Patients After 10 mg/kg Intravenous Infusion(s)

PK Parameter	Healthy Subjects (After 10 mg/kg Single Dose) n=13	RA Patients (After 10 mg/kg Multiple Doses[a]) n=14
Peak Concentration (C_{max}) [mcg/mL]	292 (175-427)	295 (171-398)
Terminal half-life ($t_{1/2}$) [days]	16.7 (12-23)	13.1 (8-25)
Systemic clearance (CL) [mL/h/kg]	0.23 (0.16-0.30)	0.22 (0.13-0.47)
Volume of distribution (Vss) [L/kg]	0.09 (0.06-0.13)	0.07 (0.02-0.13)

[a] Multiple intravenous infusions were administered at days 1, 15, 30, and monthly thereafter.

The pharmacokinetics of abatacept in RA patients and healthy subjects appeared to be comparable. In RA patients, after multiple intravenous infusions, the pharmacokinetics of abatacept showed proportional increases of C_{max} and AUC over the dose range of 2 mg/kg to 10 mg/kg. At 10 mg/kg, serum concentration appeared to reach a steady-state by day 60 with a mean (range) trough concentration of 24 (1 to 66) mcg/mL. No systemic accumulation of abatacept occurred upon continued repeated treatment with 10 mg/kg at monthly intervals in RA patients.

Population pharmacokinetic analyses in RA patients revealed that there was a trend toward higher clearance of abatacept with increasing body weight. Age and gender (when corrected for body weight) did not affect clearance. Concomitant MTX, NSAIDs, corticosteroids, and TNF blocking agents did not influence abatacept clearance.

No formal studies were conducted to examine the effects of either renal or hepatic impairment on the pharmacokinetics of abatacept.

Juvenile Idiopathic Arthritis

In patients 6 to 17 years of age, the mean (range) steady-state serum peak and trough concentrations of abatacept were 217 (57 to 700) and 11.9 (0.15 to 44.6) mcg/mL. Population pharmacokinetic analyses of the serum concentration data showed that clearance of abatacept increased with baseline body weight. The estimated mean (range) clearance of abatacept in the juvenile idiopathic arthritis patients was 0.4 (0.20 to 1.12) mL/h/kg. After accounting for the effect of body weight, the clearance of abatacept was not related to age and gender. Concomitant methotrexate, corticosteroids, and NSAIDs were also shown not to influence abatacept clearance.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

In a mouse carcinogenicity study, weekly subcutaneous injections of 20, 65, or 200 mg/kg of abatacept administered for up to 84 weeks in males and 88 weeks in females were associated with increases in the incidence of malignant lymphomas (all doses) and mammary gland tumors (intermediate- and high-dose in females). The mice from this study were infected with murine leukemia virus and mouse mammary tumor virus. These viruses are associated with an increased incidence of lymphomas and mammary gland tumors, respectively, in immunosuppressed mice. The doses used in these studies produced exposures 0.8, 2.0, and 3.0 times higher, respectively, than the exposure associated with the maximum recommended human dose (MRHD) of 10 mg/kg based on AUC (area under the time-concentration curve). The relevance of these findings to the clinical use of ORENCIA is unknown.

In a one-year toxicity study in cynomolgus monkeys, abatacept was administered intravenously once weekly at doses up to 50 mg/kg (producing 9 times the MRHD exposure based on AUC). Abatacept was not associated with any significant drug-related toxicity. Reversible pharmacological effects consisted of minimal transient decreases in serum IgG and minimal to severe lymphoid depletion of germinal centers in the spleen and/or lymph nodes. No evidence of lymphomas or preneoplastic morphologic changes was observed, despite the presence of a virus (lymphocryptovirus) known to cause these lesions in immunosuppressed monkeys within the time frame of this study. The relevance of these findings to the clinical use of ORENCIA is unknown.

No mutagenic potential of abatacept was observed in the *in vitro* bacterial reverse mutation (Ames) or Chinese hamster ovary/hypoxanthine guanine phosphoribosyl-transferase (CHO/HGPRT) forward point mutation assays with or without metabolic activation, and no chromosomal aberrations were observed in human lymphocytes treated with abatacept with or without metabolic activation.

Abatacept had no adverse effects on male or female fertility in rats at doses up to 200 mg/kg every three days (11 times the MRHD exposure based on AUC).

13.2 Animal Toxicology and/or Pharmacology

A juvenile animal study was conducted in rats dosed with abatacept from 4 to 94 days of age in which an increase in the incidence of infections leading to death occurred at all doses compared with controls. Altered T-cell subsets including increased T-helper cells and reduced T-regulatory cells were observed. In addition, inhibition of T-cell-dependent antibody responses (TDAR) was observed. Upon following these animals into adulthood, lymphocytic inflammation of the thyroid and pancreatic islets was observed.

In studies of adult mice and monkeys, inhibition of TDAR was apparent. However, infection and mortality, altered T-helper cells, and inflammation of thyroid and pancreas were not observed.

14 CLINICAL STUDIES

14.1 Adult Rheumatoid Arthritis

The efficacy and safety of ORENCIA (abatacept) were assessed in five randomized, double-blind, placebo-controlled studies in patients ≥18 years of age with active RA diagnosed according to American College of Rheumatology (ACR) criteria. Studies I, II, III, and IV required patients to have at least 12 tender and 10 swollen joints at randomization. Study V did not require any specific number of tender or swollen joints. ORENCIA or placebo treatment was given intravenously at weeks 0, 2, and 4 and then every 4 weeks thereafter.

Study I evaluated ORENCIA as monotherapy in 122 patients with active RA who had failed at least one non-biologic DMARD or etanercept. In Study II and Study III, the efficacy and safety of ORENCIA were assessed in patients with an inadequate response to MTX and who were continued on their stable dose of MTX. In Study IV, the efficacy and safety of ORENCIA were assessed in patients with an inadequate response to a TNF blocking agent, with the TNF blocking agent discontinued prior to randomization; other DMARDs were permitted. Study V primarily assessed safety in patients with active RA requiring additional intervention in spite of current therapy with DMARDs; all DMARDs used at enrollment were continued. Patients in Study V were not excluded for comorbid medical conditions.

Study I patients were randomized to receive one of three doses of ORENCIA (0.5, 2, or 10 mg/kg) or placebo ending at week 8. Study II patients were randomized to receive ORENCIA 2 or 10 mg/kg or placebo for 12 months. Study III, IV, and V patients were randomized to receive a dose of ORENCIA based on weight range or placebo for 12 months (Studies III and V) or 6 months (Study IV). The dose of ORENCIA was 500 mg for patients weighing less than 60 kg, 750 mg for patients weighing 60 to 100 kg, and 1000 mg for patients weighing greater than 100 kg.

Clinical Response

The percent of ORENCIA-treated patients achieving ACR 20, 50, and 70 responses and major clinical response in Studies I, III, and IV are shown in Table 4. ORENCIA-treated patients had higher ACR 20, 50, and 70 response rates at 6 months compared to placebo-treated patients. Month 6 ACR response rates in Study II for the 10 mg/kg group were similar to the ORENCIA group in Study III.

In Studies III and IV, improvement in the ACR 20 response rate versus placebo was observed within 15 days in some patients. In Studies II and III, ACR response rates were maintained to 12 months in ORENCIA-treated patients. ACR responses were maintained up to three years in the open-label extension of Study II.

Table 4: ACR Responses in Placebo-Controlled Trials

	Percent of Patients					
	Inadequate Response to DMARDs		Inadequate Response to MTX		Inadequate Response to TNF Blocking Agent	
	Study I		Study III		Study IV	
Response Rate	ORENCIA[a] n=32	Placebo n=32	ORENCIA[b] +MTX n=424	Placebo +MTX n=214	ORENCIA[b] +DMARDs n=256	Placebo +DMARDs n=133
ACR 20						
Month 3	53%	31%	62%***	37%	46%***	18%
Month 6	NA	NA	68%***	40%	50%***	20%
Month 12	NA	NA	73%***	40%	NA	NA
ACR 50						
Month 3	16%	6%	32%***	8%	18%**	6%
Month 6	NA	NA	40%***	17%	20%***	4%
Month 12	NA	NA	48%***	18%	NA	NA
ACR 70						
Month 3	6%	0	13%***	3%	6%*	1%
Month 6	NA	NA	20%***	7%	10%**	2%
Month 12	NA	NA	29%***	6%	NA	NA
Major Clinical Response[c]	NA	NA	14%***	2%	NA	NA

* p<0.05, ORENCIA vs placebo.
** p<0.01, ORENCIA vs placebo.
*** p<0.001, ORENCIA vs placebo.
[a] 10 mg/kg.
[b] Dosing based on weight range [see *Dosage and Administration (2.1)*]
[c] Major clinical response is defined as achieving an ACR 70 response for a continuous 6-month period.

The results of the components of the ACR response criteria for Studies III and IV are shown in Table 5. In ORENCIA (abatacept)-treated patients, greater improvement was seen in all ACR response criteria components through 6 and 12 months than in placebo-treated patients.

Table 5: Components of ACR Response at 6 Months

	Inadequate Response to MTX				Inadequate Response to TNF Blocking Agent			
	Study III				Study IV			
	ORENCIA +MTX n=424		Placebo +MTX n=214		ORENCIA +DMARDs n=256		Placebo +DMARDs n=133	
Component (median)	Baseline	Month 6	Baseline	Month 6	Baseline	Month 6	Baseline	Month 6
Number of tender joints (0-68)	28	7***	31	14	30	13***	31	24
Number of swollen joints (0-66)	19	5***	20	11	21	10***	20	14
Pain[a]	67	27***	70	50	73	43**	74	64
Patient global assessment[a]	66	29***	64	48	71	44***	73	63
Disability index[b]	1 75	1 13***	1.75	1 38	1.88	1 38***	2.00	1.75
Physician global assessment[a]	69	21***	68	40	71	32***	69	54
CRP (mg/dL)	2 2	0.9***	2 1	1 8	3 4	1.3***	2 8	2 3

** p<0 01, ORENCIA vs placebo, based on mean percent change from baseline

*** p<0.001, ORENCIA vs placebo, based on mean percent change from baseline

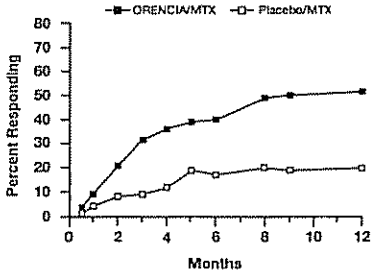
[a] Visual analog scale: 0 = best, 100 = worst.

[b] Health Assessment Questionnaire[2]: 0 = best, 3 = worst; 20 questions; 8 categories: dressing and grooming, arising, eating, walking, hygiene, reach, grip, and activities

The time course of ACR 50 response for Study III is shown in Figure 1. The time course for Study IV was similar.

Figure 1

Time Course of ACR 50 Response
Inadequate Response to MTX (Study III)



ORENCIA-treated patients experienced greater improvement than placebo-treated patients in morning stiffness.

Radiographic Response

In Study III, structural joint damage was assessed radiographically and expressed as change from baseline in the Genant-modified Total Sharp Score[3] (TSS) and its components, the Erosion Score (ES) and Joint Space Narrowing (JSN) score. ORENCIA/MTX slowed the progression of structural damage compared to placebo/MTX after 12 months of treatment as shown in Table 6.

Table 6: Mean Radiographic Changes in Study III[a]

Parameter	ORENCIA/MTX[b]	Placebo/MTX[c]	Differences	P-value[d]
First Year				
TSS	1 07	2 43	1 36	<0 01
ES	0 61	1 47	0 86	<0 01
JSN score	0 46	0 97	0 51	<0 01
Second Year				
TSS	0 48	0 74	–	–
ES	0 23	0 22	–	–
JSN score	0 25	0 51	–	–

[a] Based on radiographic reads following 2 years of treatment.

[b] Patients received 2 years of treatment with ORENCIA/MTX.

[c] Patients received 1 year of placebo/MTX followed by 1 year of ORENCIA/MTX.

[d] Based on ANCOVA model with treatment and site as factors and baseline score as covariate

In the open-label extension of Study III, 75% of patients initially randomized to ORENCIA (abatacept)/MTX and 65% of patients initially randomized to placebo/MTX were evaluated radiographically at Year 2. As shown in Table 6, progression of structural damage in ORENCIA/MTX-treated patients was further reduced in the second year of treatment.

Following 2 years of treatment with ORENCIA/MTX, 51% of patients had no progression of structural damage as defined by a change in the TSS of zero or less compared with baseline. Fifty-six percent (56%) of ORENCIA/MTX-treated patients had no progression during the first year compared to 45% of placebo/MTX-treated patients. In their second year of treatment with ORENCIA/MTX, more patients had no progression than in the first year (65% vs 56%).

Physical Function Response and Health-Related Outcomes

Improvement in physical function was measured by the Health Assessment Questionnaire Disability Index (HAQ-DI).[2,4] In Studies II-V, ORENCIA demonstrated greater improvement from baseline than placebo in the HAQ-DI. The results from Studies II and III are shown in Table 7. Similar results were observed in Study V. During the open-label period of Study II, the improvement in physical function has been maintained for up to 3 years.

Table 7: Mean Improvement from Baseline in Health Assessment Questionnaire Disability Index (HAQ-DI)

	Inadequate Response to Methotrexate			
	Study II		Study III	
HAQ Disability Index	ORENCIA[a] +MTX (n=115)	Placebo +MTX (n=119)	ORENCIA[b] +MTX (n=422)	Placebo +MTX (n=212)
Baseline (Mean)	0.98[c]	0.97[c]	1.69[d]	1.69[d]
Mean Improvement Year 1	0 40[c,***]	0.15[c]	0.66[d,***]	0.37[d]

*** p<0 001, ORENCIA vs placebo

[a] 10 mg/kg.

[b] Dosing based on weight range [see *Dosage and Administration (2 1)*]

[c] Modified Health Assessment Questionnaire[4]: 0 = best, 3 = worst; 8 questions; 8 categories: dressing and grooming, arising, eating, walking, hygiene, reach, grip, and activities

[d] Health Assessment Questionnaire[2]: 0 = best, 3 = worst; 20 questions; 8 categories: dressing and grooming, arising, eating, walking, hygiene, reach, grip, and activities

Health-related quality of life was assessed by the SF-36 questionnaire[5] at 6 months in Studies II, III, and IV and at 12 months in Studies II and III. In these studies, improvement was observed in the ORENCIA group as compared with the placebo group in all 8 domains of the SF-36 as well as the Physical Component Summary (PCS) and the Mental Component Summary (MCS).

14.2 Juvenile Idiopathic Arthritis

The safety and efficacy of ORENCIA were assessed in a three-part study including an open-label extension in children with polyarticular juvenile idiopathic arthritis (JIA). Patients 6 to 17 years of age (n=190) with moderately to severely active polyarticular JIA who had an inadequate response to one or more DMARDs, such as MTX or TNF antagonists, were treated. Patients had a disease duration of approximately 4 years with moderately to severely active disease at study entry, as determined by baseline counts of active joints (mean, 16) and joints with loss of motion (mean, 16); patients had elevated C-reactive protein (CRP) levels (mean, 3.2 mg/dL) and ESR (mean, 32 mm/h). The patients enrolled had subtypes of JIA that at disease onset included Oligoarticular (16%), Polyarticular (64%; 20% were rheumatoid factor positive), and Systemic (20%). At study entry, 74% of patients were receiving MTX (mean dose, 13.2 mg/m^2 per week) and remained on a stable dose of MTX (those not receiving MTX did not initiate MTX treatment during the study).

In Period A (open-label, lead-in), patients received 10 mg/kg (maximum 1000 mg per dose) intravenously on days 1, 15, 29, and monthly thereafter. Response was assessed utilizing the ACR Pediatric 30 definition of improvement,[6] defined as ≥30% improvement in at least 3 of the 6 JIA core set variables and ≥30% worsening in not more than 1 of the 6 JIA core set variables. Patients demonstrating an ACR Pedi 30 response at the end of Period A were randomized into the double-blind phase (Period B) and received either ORENCIA or placebo for 6 months or until disease flare. Disease flare was defined as a ≥30% worsening in at least 3 of the 6 JIA core set variables with ≥30% improvement in not more than 1 of the 6 JIA core set variables; ≥2 cm of worsening of the Physician or Parent Global Assessment was necessary if used as 1 of the 3 JIA core set variables used to define flare, and worsening in ≥2 joints was necessary if the number of active joints or joints with limitation of motion was used as 1 of the 3 JIA core set variables used to define flare.

At the conclusion of Period A, pediatric ACR 30/50/70 responses were 65%, 50%, and 28%, respectively. Pediatric ACR 30 responses were similar in all subtypes of JIA studied.

During the double-blind randomized withdrawal phase (Period B), ORENCIA-treated patients experienced significantly fewer disease flares compared to placebo-treated patients (20% vs 53%); 95% CI of the difference (15%, 52%). The risk of disease flare among patients continuing on ORENCIA was less than one third than that for patients withdrawn from ORENCIA treatment (hazard ratio=0 31, 95% CI [0 16, 0.59]). Among patients who received ORENCIA throughout the study (Period A, Period B, and the open-label extension Period C), the proportion of pediatric ACR 30/50/70 responders has remained consistent for 1 year.

15 REFERENCES

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16 HOW SUPPLIED/STORAGE AND HANDLING

ORENCIA® (abatacept) lyophilized powder for intravenous infusion is supplied as an individually packaged, single-use vial with a silicone-free disposable syringe. The product is available in the following strength: NDC 0003-2187-10, providing 250 mg of abatacept in a 15-mL vial.

Storage

Store in a refrigerator, 2°C to 8°C (36°F to 46°F). Do not use beyond the expiration date. Protect the vials from light by storing in the original package until time of use.

17 PATIENT COUNSELING INFORMATION

See FDA-Approved Patient Labeling (17 7)

17.1 Concomitant Use With Biologic Medications for RA

Patients should be informed that they should not receive ORENCIA treatment concomitantly with a TNF antagonist, such as adalimumab, etanercept, and infliximab because such combination therapy may increase their risk for infections [see *Indications and Usage (1.3)*, *Warnings and Precautions (5.1)*, and *Drug Interactions (7.1)*], and that they should not receive ORENCIA concomitantly with other biologic RA therapy, such as anakinra because there is not enough information to assess the safety and efficacy of such combination therapy [see *Indications and Usage (1 3)*, and *Drug Interactions (7.2)*].

17.2 Hypersensitivity

Patients should be instructed to immediately tell their healthcare professional if they experience symptoms of an allergic reaction during or for the first day after the administration of ORENCIA [see *Warnings and Precautions (5 2)*].

17.3 Infections

Patients should be asked if they have a history of recurrent infections, have underlying conditions which may predispose them to infections, or have chronic, latent, or localized infections. Patients should be asked if they have had tuberculosis (TB), a positive skin test for TB, or recently have been in close contact with someone who has had TB. Patients should be instructed that they may be tested for TB before they receive ORENCIA. Patients should be informed to tell their healthcare professional if they develop an infection during therapy with ORENCIA [see *Warnings and Precautions (5 3)*]

17.4 Immunizations

Patients should be informed that live vaccines should not be given concurrently with ORENCIA or within 3 months of its discontinuation. Caregivers of patients with juvenile idiopathic arthritis should be informed that the patient should be brought up to date with all immunizations in agreement with current immunization guidelines prior to initiating ORENCIA therapy and to discuss with their healthcare provider how best to handle future immunizations once ORENCIA therapy has been initiated [see *Warnings and Precautions (5 4)*]

17.5 Pregnancy and Nursing Mothers

Patients should be informed that ORENCIA has not been studied in pregnant women or nursing mothers so the effects of ORENCIA on pregnant women or nursing infants are not known. Patients should be instructed to tell their healthcare professional if they are pregnant, become pregnant, or are thinking about becoming pregnant [see *Use in Specific Populations (8.1)*]. Patients should be instructed to tell their healthcare professional if they plan to breast-feed their infant [see *Use in Specific Populations (8.3)*]

17.6 Blood Glucose Testing

Patients should be asked if they have diabetes. Maltose contained in ORENCIA can give falsely elevated blood glucose readings with certain blood glucose monitors on the day of ORENCIA infusion. If a patient is using such a monitor, the patient should be advised to discuss with their healthcare professional methods that do not react with maltose [see *Drug Interactions (7.3)*].

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17.7 FDA-Approved Patient Labeling

PATIENT INFORMATION

ORENCIA® (oh-REN-see-ah)

(abatacept)

Read this Patient Information before you start receiving ORENCIA (abatacept) and each time before you are scheduled to receive ORENCIA. The information may have changed. This leaflet does not take the place of talking with your doctor about your medical condition or your treatment.

What is ORENCIA?

ORENCIA is a prescription medicine that reduces signs and symptoms in:

- adults with moderate to severe rheumatoid arthritis (RA), including those who have not been helped enough by other medicines for RA. ORENCIA may prevent further damage to your bones and joints and may help your ability to perform daily activities
- children and adolescents 6 years of age and older with moderate to severe polyarticular juvenile idiopathic arthritis (JIA)

In RA and JIA, ORENCIA can reduce pain and joint inflammation, but it can also make your immune system less able to fight infection. ORENCIA can make you more likely to get infections or make any infection you have worse. It is important to tell your doctor if you think you have any infections.

ORENCIA has not been studied in children under 6 years of age.

What should I tell my doctor before treatment with ORENCIA?

Before you receive ORENCIA you should tell your doctor about all your medical conditions, including if you:

- have any kind of infection even if it is small (such as an open cut or sore), or an infection that is in your whole body (such as the flu). If you have an infection when taking ORENCIA, you may have a higher chance for getting serious side effects.
- have an infection that will not go away or a history of infections that keep coming back.
- have had tuberculosis (TB), a positive skin test for TB, or you recently have been in close contact with someone who has had TB. If you get any of the symptoms of TB (a dry cough that doesn't go away, weight loss, fever, night sweats) call your doctor right away. Before you start ORENCIA, your doctor may examine you for TB or perform a skin test
- have or have had viral hepatitis. Before you use ORENCIA, your doctor may examine you for hepatitis
- have a history of chronic obstructive pulmonary (lung) disease (COPD)
- are scheduled to have surgery.
- are allergic to any of the ingredients in ORENCIA. See the end of this leaflet for a list of the ingredients in ORENCIA.
- recently received a vaccination or are scheduled for any vaccination. If you are receiving ORENCIA, you should not take live vaccines
- have diabetes and use a blood glucose monitor to check your blood sugar (blood glucose) levels. ORENCIA contains maltose, a type of sugar that can give false high blood sugar readings with certain types of blood glucose monitors, on the day of ORENCIA infusion. Your doctor may tell you to use a different way to monitor your blood sugar levels.
- are pregnant or planning to become pregnant. It is not known if ORENCIA can harm your unborn baby.

  Bristol-Myers Squibb Company has a registry for pregnant women exposed to ORENCIA. The purpose of this registry is to check the health of the pregnant mother and her child. Patients are encouraged to call the registry themselves or ask their doctors to contact the registry for them by calling 1-877-311-8972.
- are breast-feeding. ORENCIA can pass into breast milk. Women who are breast-feeding should talk to their doctor about whether or not to use ORENCIA

Tell your doctor about all the medicines you take, including prescription and non-prescription medicines, vitamins, and herbal supplements. Do not start taking any new medicine without talking with your doctor.

Especially tell your doctor if you take other biologic medicines to treat RA or JIA that may affect your immune system, such as:

- Enbrel® (etanercept)
- Humira® (adalimumab)
- Remicade® (infliximab)
- Kineret® (anakinra)
- Rituxan® (rituximab)

You may have a higher chance of getting a serious infection if you take ORENCIA with other biologic medicines for your RA or JIA.

Know the medicines you take. Keep a list of your medicines and show it to your doctor and pharmacist when you get a new prescription.

How will I receive ORENCIA (abatacept)?

- You will be given ORENCIA by a healthcare provider through a needle placed in a vein (IV or intravenous infusion) in your arm. It takes about 30 minutes to give you the full dose of medicine.
- You will receive ORENCIA 2 weeks and 4 weeks after the first dose. You will then receive ORENCIA every 4 weeks.
- If you miss your appointment to receive ORENCIA, ask your doctor when to schedule your next dose.

What are the possible side effects of ORENCIA?

ORENCIA can cause serious side effects including:

- **Serious infections**. Patients receiving ORENCIA have a higher chance of getting infections including pneumonia, and other infections caused by viruses, bacteria, or fungi. Call your doctor right away if you feel sick or get any of the following symptoms of infection, which may be early signs of a serious infection:
  - a fever
  - feel very tired
  - have a cough
  - have flu-like symptoms
  - warm, red, or painful skin
- **Allergic reactions**. Allergic reactions can happen on the day of treatment or the day after receiving ORENCIA. Tell your doctor or get emergency medical help right away if you have hives, swollen face, eyelids, lips, tongue, throat, or trouble breathing.
- **Cancer (malignancies)**. Certain kinds of cancer have been reported in patients receiving ORENCIA. It is not known if ORENCIA increases your chance of getting certain kinds of cancer.
- **Vaccinations**. You should not receive ORENCIA with certain types of vaccines (live vaccines). ORENCIA may also cause some vaccinations to be less effective. Talk with your doctor about your vaccination plans.
- **Respiratory problems in patients with Chronic Obstructive Pulmonary Disease (COPD)**. You may get certain respiratory problems more often if you receive ORENCIA and have COPD, including:
  - worsened COPD
  - pneumonia
  - cough
  - trouble breathing

Common side effects of ORENCIA in both adults and children include:

- headache
- upper respiratory tract infection
- sore throat
- nausea

In children, other side effects may include:

- diarrhea
- cough
- fever
- abdominal pain

Tell your doctor if you have any side effect that bothers you or that does not go away. These are not all the possible side effects of ORENCIA. For more information, ask your doctor or pharmacist.

Call your doctor for medical advice about side effects. You may report side effects to FDA at 1-800-FDA-1088.

General information about ORENCIA

Medicines are sometimes prescribed for conditions that are not mentioned in patient information leaflets. Do not use ORENCIA for a condition for which it was not prescribed.

This patient information leaflet summarizes the most important information that you need to know about ORENCIA. If you would like more information, talk to your doctor.

You can ask your pharmacist or doctor for information about ORENCIA that is written for health professionals. For more information, go to www.ORENCIA.com or the company internet site at www.BMS.com or call 1-800-ORENCIA toll-free.

What are the ingredients in ORENCIA?

Active ingredient: abatacept

Inactive ingredients: maltose, monobasic sodium phosphate, sodium chloride for administration

Enbrel®, Humira®, Remicade®, Kineret®, and Rituxan® are trademarks of their respective companies.

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EXHIBIT 2

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EXHIBIT 3

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A new member of the immunoglobulin superfamily—CTLA-4

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The immunoglobulin superfamily is a group of proteins, each made of one or several domains sharing key structural features with either the variable (V) or the constant (C) immunoglobulin domains[1,2]. It includes such functionally important members as the immunoglobulins themselves, major histocompatibility complex (MHC) class I and class II and T-cell receptor (TCR) molecules. Several members of this superfamily are expressed on lymphocytes where they are membrane-bound and capable of interactions with other members of the family, thus taking part in cell–cell recognition. In screening mouse cytolytic-T-cell-derived cDNA libraries, we came across cDNA clones defining a sequence, CTLA-4, which could encode a 223-amino-acid protein clearly belonging to the immunoglobulin superfamily. It consists of one V-like domain flanked by two hydrophobic regions, one of which has a structure suggestive of membrane anchoring. CTLA-4 is mainly expressed in activated lymphocytes and is coinduced with T-cell-mediated cytotoxicity in inducible models of this process. The mouse *ctla-4* gene maps to band C of chromosome 1.

We previously reported the construction of a subtracted cytolytic-T-cell(Tc)-derived complementary DNA library of about 8,300 clones, from which three distinct cytolytic T-cell-associated sequences, CTLA-1, CTLA-2 and CTLA-3, were isolated by conventional differential screenings[3]. CTLA-1 and CTLA-3 encode distinct serine-esterases, also detected, using similar approaches, as CCP-1 (ref. 4) and H factor[5], respectively; CTLA-2 encodes a previously undescribed protein product (F.D. *et al.*, in preparation). Further screening of the same subtracted library with a subtracted (Tc−B) cDNA probe allowed the detection of 113 additional clones which had not detectably hybridized with a total Tc-derived cDNA probe. These clones were subjected to successive rounds of screening with several other Tc cDNA probes subtracted against messenger RNA derived from either the B lymphoma M12.4.1, or the EL4 thymoma, or thymocytes. The positive, thus still apparently Tc-specific, clones were then depleted of copies of CTLA-1, -2 and -3 by probing with a pool of inserts representative of these families. The inserts of the 17 cDNA clones left at that stage were used as probes on Northern blots of a panel of RNAs. Only one clone, M17G7, survived this ultimate screening for Tc specificity in that it detected a 2-kilobase (kb) transcript in RNA from the original KB5C20 Tc clone and not from other, non-Tc cells. The M17G7 cDNA insert defines CTLA-4.

A study of the tissue distribution of the CTLA-4 2-kb transcript (Fig. 1) revealed its presence in each of the Tc or Tc-containing populations tested (two clones: KB5C20 and A15.1.17, albeit at a low level for the latter; and three *in vitro* or *in vivo* primary populations: mixed leucocyte culture cells, concanavalin-A-activated blasts and allo-sensitized peritoneal exudate lymphocytes). In addition, like the CTLA-1, -2 and -3 transcripts[3], the CTLA-4 transcript was coinduced with cytotoxicity in two systems: on incubation of the constitutively growing cytolytic hybridoma PC60 (ref. 6) with interleukin-containing supernatants and on incubation of concanavalin-A-pulsed thymocytes with an interleukin-2-containing supernatant. A weaker signal was detected in lipopolysaccharide(LPS)-activated blasts (the significance of which is unclear due to the likely heterogeneity of this population) and non-induced PC60 cells, and a very weak one in non-activated thymocytes (Fig. 1) and in non-experimentally-activated peripheral lymphocytes, that is column-purified or unpurified normal spleen cells (data not shown). EL4, the T helper hybridoma T14-117, NK-cell-

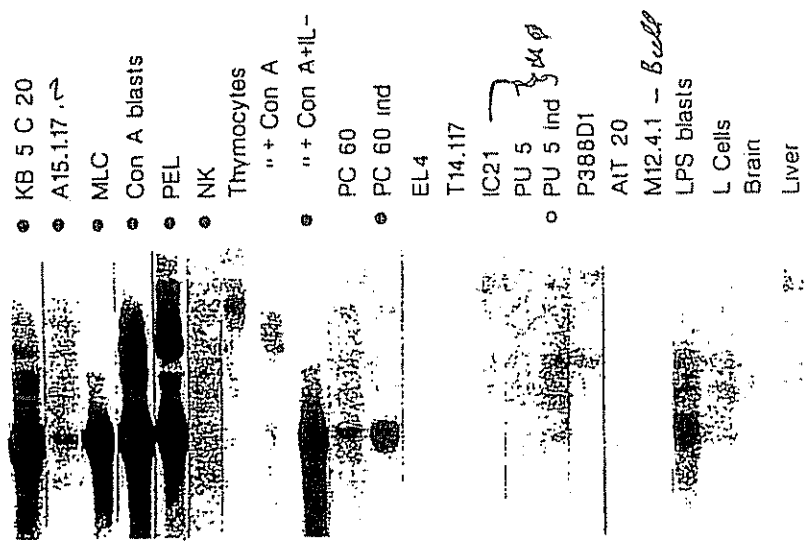

Fig. 1 Specificity pattern of CTLA-4 RNA transcripts. Northern blots were prepared with RNA from the following cells: KB5C20, a B10.BR anti-H-2K^b Tc clone[16]; A15.1.17, an A.Th anti-A.TL Tc clone[17]; MLC cells, A/J spleen cells stimulated for five days *in vitro* with C57BL/6 irradiated spleen cells; Con A blasts, a mixture of C57BL/6 spleen cells stimulated for two or three days *in vitro* with concanavalin A (1.5 μg ml^{-1}); PEL, nylon-wool purified BALB/c anti-EL4 peritoneal exudate cells[18]; NK, nylon-wool purified Swiss *nu/nu* spleen cells; thymocytes, from C57BL/6 mice; thymocytes plus Con A, the latter incubated for 24 h with Con A (5 μg ml^{-1}); thymocytes plus Con A and IL-2, the latter further incubated for five days in the presence of IL-2-containing supernatant from PMA-stimulated EL4 C116 cells; PC60, a mouse × rat hybridoma[6]; PC60ind, the latter induced with interleukin-containing supernatants[19]; EL4, a C57BL/6 thymoma; T14.117, a hybridoma between a T-helper cell clone and the BW5147 thymoma[20]; IC-21, PU5 and P388D1, macrophage cell lines; PU-5ind, PU-5 activated by a 24-h incubation with supernatant with supernatant from restimulated KB5C20 cells; AtT20, a neurosecretory cell line; M12.4.1, a B lymphoma[21]; LPS blasts, C57BL/6 spleen cells grown for three days with lipopolysaccharide (5 μg ml^{-1}); L cells, a C3H fibroblast line; brain and liver cells from C57BL/6 mice. The Northern blots (with about the same amount of RNA per lane according to ethidium bromide staining of gels, not shown) were probed with the radiolabelled 300-bp insert from the CTLA-4 cDNA clone M17G7, isolated from a first, subtracted, KB5C20 cDNA library[3]. Positive lanes show a main band corresponding to a transcript of about 2 kb, but often also higher bands corresponding to longer transcripts. In parallel, the same cells had been tested for cytotoxity on EL4, RDM4 and YAC target cells in the absence or in the presence of Con A (10 μg ml^{-1}); those found positive in a 4-h or in a 20-h ^{51}Cr release test are labelled ● or ○, respectively; the others were negative. All methods were exactly as described before[3].

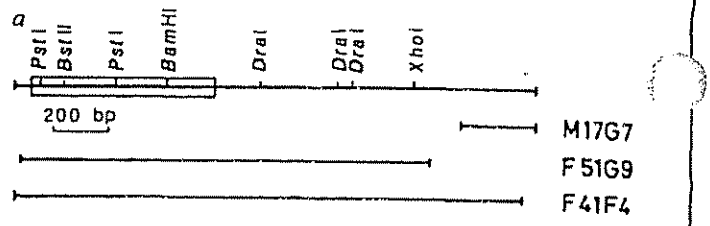

Fig. 2 *a*, Restriction map of CTLA-4 clones F41F4, F51G9 and M17G7 with restriction enzyme sites used for sequencing. Box: open reading frame. *b*, Nucleotide sequence of CTLA-4 with its predicted amino-acid translation. The nucleotide sequence includes, in its 5′ region, and for both F41F4 and F51G9 inserts, an in-frame TGA stop codon 30 residues upstream of the first ATG; the latter is part of a GCCATGG consensus sequence[22] for initiation of translation; the open reading frame, from nucleotide residue 58 to 726, is followed with a 1.16-kb long untranslated region; at its 3′ end, a polyadenylation signal (underlined) precedes a poly(A)stretch. Because of the uncertainties as to cleavage of the leader sequence (see text), the amino-acid sequence is numbered provisionally starting from the initial methionine residue of the conceptual sequence. The latter includes, from 5′ to 3′: a complex leader sequence (with, first, a few hydrophobic residues, the basic hydrophilic Arg-Arg-Tyr-Lys stretch at positions 7–10, and a long hydrophobic stretch); a possible first residue for the putative mature protein (the glutamine at position 36); the *V*-like domain (see Fig. 3*b* and *c*) with its two key cysteines (at positions 58 and 129) and three Asn-linked (Asn-X-Ser/Thr, where X is any amino acid) potential *N*-glycosylation sites (underlined); a long hydrophic, probably transmembrane stretch (residues 162–187); the basic stretch Lys-Met-Leu-Lys-Lys-Arg (residues 188–193); and the essentially hydrophilic 3′ end of the protein sequence. Curiously, a seven-residue stretch (residues 213–219) at the end of this cytoplasmic hydrophilic tail of mouse CTLA-4 is also found at residues 126–132 of the pol polyprotein of human adult T-cell leukaemia virus[23]; it will be interesting to find out whether this homology between a cellular gene transcribed in activated T cells and a virus causing T-cell leukaemia is more marked in the human equivalent of CTLA-4. Note that five cysteines are present in the central segment of CTLA-4; of the four belonging to the V-like domain, two can form the disulphide bond critical to the immunoglobulin fold and the other two can form another disulphide bond within this fold. The fifth cysteine, nearest to the putative transmembrane region, might be involved in forming dimeric structures, not unlike the situation in CD8 (ref. 24).

Methods. A second KB5C20-derived cDNA library was constructed according to Gubler and Hoffman[25] modified as described[3] except that it was size selected in the 1.6–2.2 kb range before it annealed to the pUC9 vector. The library was screened with the 300-bp insert of M17G7 which detected F41F4. The *Pst*I fragment of F41F4 was in turn used as a probe and detected clone F51G9. Sequencing was performed using a chemical cleavage method[26,27] on both strands of each of two clones except for stretches of the 3′ untranslated region.

containing nude spleen cells and also uninduced or induced macrophage lines (Fig. 1) and mast cells (not shown) were negative for the CTLA-4 transcript. Outside of the haematopoietic compartment, all the cells tested (liver, L fibroblasts, brain and the neurosecretory line AtT20) were also negative (Fig. 1). In view of this distribution a tentative statement can be made that CTLA-4 expression is restricted to the lymphoid lineage and is mainly found in activated cells.

Probing a second cDNA library with M17G7 led to the isolation of two more CTLA-4 clones with inserts of 1.85 kb and 1.5 kb respectively. The sequence of these clones (Fig. 2) revealed an open reading frame of 669 nucleotides encoding a putative protein of 223 amino acids. A search for similar sequences at the protein level in the NBRF sequence data bank showed a 22% overall similarity to the human $V\lambda^{VII}$ domain. The similarity was mainly around the two cysteine residues engaged in the disulphide bond crucial for the immunoglobulin fold. Sequence comparison was therefore extended to other members of the immunoglobulin superfamily. Most of the canonical features of V-like domains were indeed present (Fig. 3*b* and *c*) between residue 39 and residue 140 of CTLA-4. The main discrepancy was the absence of the tryptophan residue at position 52 (in the IgH numbering), which is also the case for instance for Thy-1. A local similarity of up to 70% was found between residue 116 and residue 131 with the third domain of the rabbit polyimmunoglobulin receptor around the corresponding cysteine, where several non-consensus residues are shared between both molecules.

The predicted protein sequence (Fig. 2) and its hydrophobicity pattern (Fig. 3*a*) reveal that CTLA-4 comprises the V-like domain described above and two flanking regions (Fig. 3*b*). The 3′ flanking region includes, in succession, a 26-residue-long hydrophobic stretch, very probably transmembrane (such hydrophobic regions are, however, replaced with another system of membrane anchoring in at least two members of the immunoglobulin superfamily, Thy-1 ref. 7 and N-CAM ref. 8); the basic stretch Lys-Met-Leu-Lys-Lys-Arg (positi 188–193) resembling that found at the transmembrane-cyt-

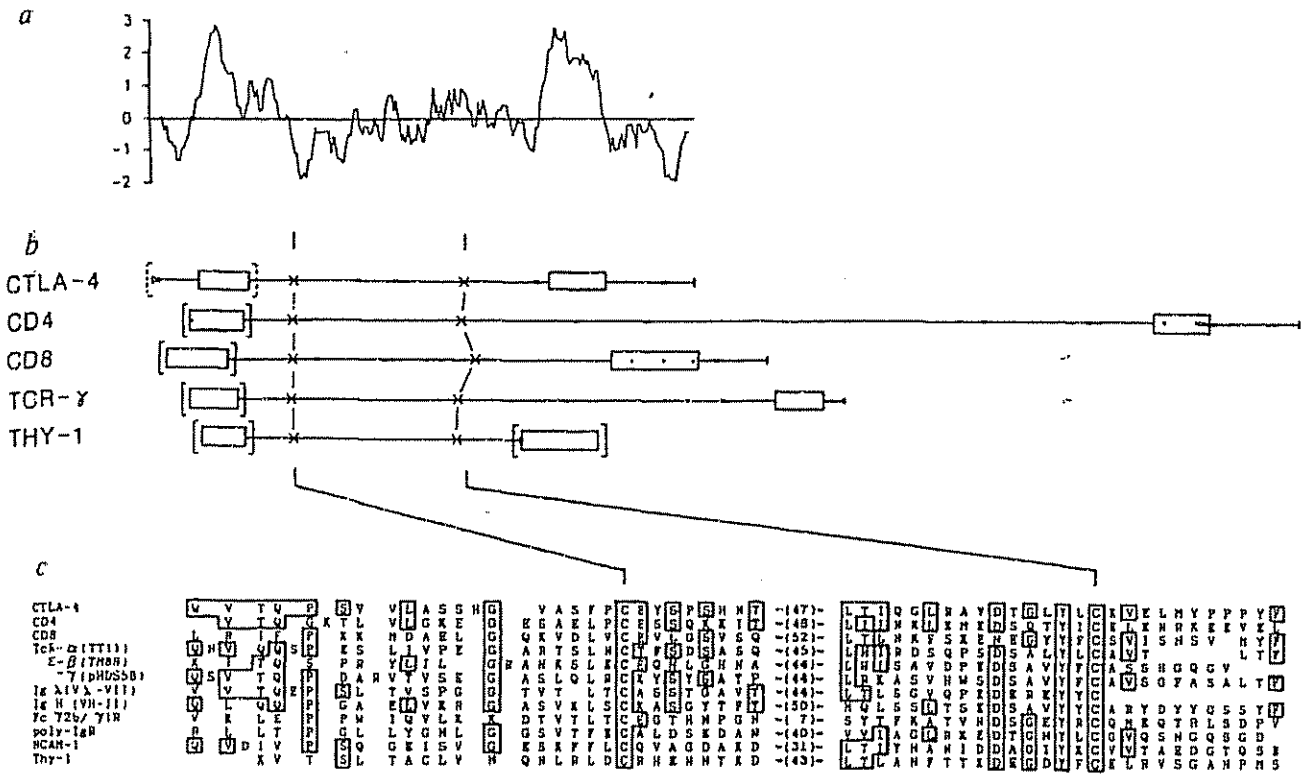

Fig. 3. *a*, Hydropathicity plot[28] of CTLA-4, with a window of 11 residues. *b*, Aligned overall primary protein structures of CTLA-4, CD4 (L3T4), CD8 (Lyt-2), γ-chain of the T-cell receptor (TcR)and Thy-1. Boxes, long hydrophobic residues; brackets, peptides known to be cleaved off the mature proteins; stippled brackets, putative CTLA-4 leader sequence; crosses, cysteine residues (note the two unusual extra cysteines in the V-like domain of CTLA-4). *c*, Alignment of the sequences, around the two cysteines involved in the immunoglobulin fold, of CTLA-4, CD4 (ref. 29), CD8 (ref. 30), T-cell receptor α-chain[31], β-chain[32] and γ-chain[33], human V_λ chain[34] (Ig λ), mouse V_H chain[35] (Ig H), Fcγ receptor[14,36] (Fc γ2b/ γ1R), polyimmunoglobulin (Ig) receptor[37], the first domain of murine N-CAM[38] and Thy-1 (ref. 1). Boxed residues are identified in CTLA-4 and three or more members of the immunoglobulin superfamily (conservative changes like Phe 56 are not boxed).

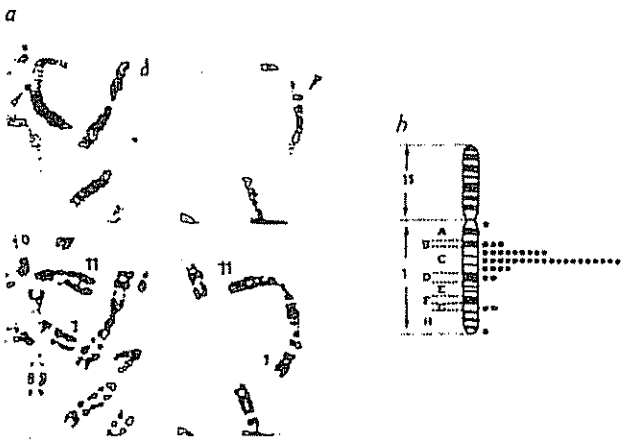

Fig. 4 Localization of the *ctla-4* gene to mouse chromosome 1 by *in situ* hybridization with the CTLA-4 probe F41F4. *a*, Two partial WMP mouse metaphases, showing the specific site of hybridization to chromosome 1. Top, arrowheads indicate silver grains on Giemsa-stained chromosomes, after autoradiography. Bottom, chromosomes with silver grains were subsequently identified by R-banding. Note the homogeneously staining region[39] in the C-D band interface of chromosome 1. *b*, Diagram of WMP mouse Rb(1; 11) chromosome, indicating the distribution of labelled sites. In the 123 metaphases examined, there were 250 silver grains associated with chromosomes and 41 of these (16.4%) were localized on chromosome 1. On this chromosome, 78% of the grains mapped to the C band (for banding nomenclature, see ref. 40), with a maximum in the middle part of this band.

Methods. *In situ* hybridizations with probe F41F4 were done using metaphase spreads from a male WMP mouse in which all the autosomes (except 19) are in the form of robertsonian translocations (given by J. L. Guénet, Pasteur Institute). All methods were as indicated before[3,41,42]. The specific activity of the probe was 6×10^7 d.p.m. μg^{-1}, its final concentration was 25 ng ml^{-1}, and the slides were exposed for 18 days at 4 °C.

plasmic hinge of many membrane receptors[9]; and the rest of a hydrophilic putative cytoplasmic tail. The 5′ flanking region has the location and hydrophobicity of a leader sequence, but is unusual in its overall length and also perhaps in the basic Arg-Arg-Tyr-Lys stretch at positions 7–10. This leader sequence could be cleaved off between the Ser at position 35 and the Glu at position 36 (taking into account the CTLA-4 hydrophobicity pattern and the site of cleavage for other members of the immunoglobulin superfamily); alternatively, it may stay uncleaved[10]. Antibodies should be of help in determining the location and mode of insertion into the membrane, the precise tissue distribution and possibly the functional (maybe interactive) role of the CTLA-4 protein product.

In a preliminary study of the corresponding gene(s), the Southern blot patterns (not shown) were consistent with there being only one copy of the *ctla-4* gene per mouse haploid

genome. Multiplicity of gene copies cannot therefore account for the several CTLA-4 transcripts longer than the 2-kb one, detected in Northern blots of, in particular, concanavalin A blasts and peritoneal exudate lymphocytes (Fig. 1). In agreement with the Southern blot hybridization patterns, by *in situ* hybridization only one main location was found for the *ctla-4* gene, on band C of mouse chromosome 1 (Fig. 4). The genes for the IgG1/IgG2b FcR-Ly-17 alloantigen and for M1s map apparently more distally on the same chromosome[11-14].

Altogether, compared to other currently known members[15] of the immunoglobulin superfamily, the CTLA-4 protein product has in common with Thy-1, CD4 (L3T4), CD8 (Lyt 2) and CD3 (T3) ε-chain a single V-like domain most probably expressed at the cell membrane in certain lymphocyte subpopulations. CTLA-4, however, is peculiar in that it is mainly found in activated lymphocytes. The regulation of transcription of the *ctla-4* gene, and also the function of the CTLA-4 protein, seem particularly interesting areas for future studies.

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Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung

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The guinea-pig perfused isolated lung, used in conjunction with the cascade superfusion system to measure the release of thromboxane A_2 (TXA_2), is a simple and convenient model for assessing the inhibition by glucocorticoids of eicosanoid formation[1–6]. Dexamethasone inhibits the release of TXA_2 from the lung when it is stimulated by agents such as RCS-RF[2] of leukotrienes,[4,8] but not when bradykinin or arachidonic acid are used[1–7]. Using this model we have shown that the glucocorticoids suppress eicosanoid generation by cells through the induction of a family of phospholipase A_2-inhibitory proteins[5,6] now termed the 'lipocortins'[9]. Recently the primary structure of one form of lipocortin has been elucidated and the human gene cloned[10]. Lipocortin 1 is a polar monomeric protein with anti-phospholipase properties *in vitro*[10,11] and we now report that when infused into guinea-pig lung preparations this protein has the same inhibitory profile as the glucocorticoids but with a more rapid onset of action. This is the first demonstration that eicosanoid formation can be inhibited by a recombinant phospholipase inhibitory protein applied extracellularly

Bolus injections of leukotriene C_4 (LTC_4), 1–10 ng, elicited a dose-dependent release of TXA_2 from the guinea-pig perfused lungs (equivalent to ~10–50 ng of the TXA_2 agonist U446) but had no direct action on the isolated tissues. In 32 out of experiments, in which lipocortin was infused into the lung concentrations of 0.4–4.0 μg ml^{-1} (10^{-8} M to 10^{-7}), inhibition of TXA_2 release was detected. The latency and final degree inhibition achieved depended upon the concentration of lipocortin, the duration of infusion and the dose of LTC_4. Table summarizes mean data obtained from 18 matched experiments in which different combinations of LTC_4 and lipocortin were tested. Despite variations in potency between batches of recombinant lipocortin, a general dose-response trend is apparent. The top two tracings in Fig. 1*a* show examples from two experiments where low and high doses of LTC_4 were used. Lipocortin at 0.4 μg ml^{-1} immediately and completely abolished the release of TXA_2 triggered with 2 ng of LTC_4. Recovery was complete ~70 min after infusion was terminated. With 10 ng of LTC_4, a more graded type of inhibition was observed. In the latter case infusions of 0.4 μg ml^{-1} lipocortin produced inhibition, but this was not as extensive in magnitude and was slower in onset.

In 4 experiments, a sham preparation of lipocortin was tested against 2, 3 and 10 ng of LTC_4 (see Table 1 with Fig. 1*a*). In no case was inhibition of more than 10% observed with the sham preparation (this is within the normal variation of the tissue response) and where small effects were seen, they were not sustained (Fig. 1*a*). A number of other controls were examined. Boiled lipocortin was without effect. Similarly, infusions of 10 μg ml^{-1} BSA (Sigma) or 1 μg ml^{-1} superoxide dismutase (Sigma, bovine kidney) were negative. *Escherichia*

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EXHIBIT 4

FORRESTER & BOEHMERT

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Date / Datum: 11th March, 1993

Dear Sirs,

re: European Patent Application No. 89100787.4-2105
ZymoGenetics, Inc.

With reference to the Communication of 4th September, 1992, we are filing herewith in triplicate an interlinearly amended set of claims, to replace the claims previously on file. Some amendments have been made to correct obvious clerical errors and others have been made to clarify the subject matter, the subject of the present Application. The latter amendments are clearly supported by the description as filed, for example at pages 11 and 17.

With regard to paragraphs 2 and 2.1 of the Communication, Claim 3 has been amended to recite a DNA construct containing a "DNA sequence encoding a non-immunoglobulin peptide requiring dimerization for biological activity.". This amendment has been made to claim more distinctly particular aspects which the Applicants regard as their invention. Support for this amendment may be found in the specification (see for example, page 17 and elsewhere). The T cell receptor is a disulfide-bonded heterodimer encoded by immunoglobulin-like genes that have exon structures analogous to immunoglobulin genes and which encode discrete domains structurally similar to immunoglobulins. Moreover, characterization of T cell receptor cDNA clones has shown significant sequence homology between T cell receptors and immunoglobulins (see Hedrick et al., Nature 308: 153-158, 1984). Thus, the T cell receptor is not a non-immunoglobulin peptide. In addition, the T cell receptor disclosed in reference D1 is biologically active as a disulfide-bonded heterodimer. The methods of the present invention allow the production of non-covalently associated dimers (see for example page 12). Thus, the T cell receptor does not consist of non-immunoglobulin peptides that are biologically active as non-covalently active dimers.

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Reference D1 does not disclose a peptide dimer comprising a non-immunoglobulin peptide requiring dimerization for biological activity fused to a first polypeptide chain capable of associating with a second polypeptide chain to form a dimer, wherein the second polypeptide chain is the same or different from the first polypeptide chain. Moreover, the Applicants wish to note that the immunoglobulin variable regions disclosed in reference D1 were not used as dimerizing proteins. In fact, the portion of the chimeric molecule of reference D1 responsible for dimerization is the T cell receptor portion, not the immunoglobulin region. Reference D1, therefore, does not teach or suggest the methods of producing biologically active peptide dimers of the present invention.

Regarding paragraph 3 of the Communication, it is noted that the present Application claims priority from a prior U.S. Application filed on January 22, 1988. In view of the earlier priority of the present Application, reference D2 is not citable.

With regard to paragraph 4, Claims 3, 9 and 10 have been amended to point out more particularly and distinctly the claimed invention. The claims have been amended to recite a DNA construct containing a "DNA sequence encoding a non-immunoglobulin peptide requiring dimerization for biological activity fused to a dimerizing protein.".

It is respectfully submitted that the amended claims do not cover the production of immunoglobulin chains and are novel.

With regard to paragraph 6, for the sake of clarity, Claims 19, 22, 24 and 25 have been amended to recite "wherein said polypeptide is capable of binding anti-PDGF antibodies, PDGF, PDGF isoforms, PDGF analogs or PDGF antagonists.". In addition, Claims 3, 19, 22, 24 and 25 have been amended to recite a peptide requiring dimerization for biological activity or a PDGF receptor analog fused to "a first polypeptide chain capable of associating with a second polypeptide chain to form a dimer, wherein the second polypeptide chain is the same or different from the first polypeptide chain.".

We trust that the Application is now in order to proceed to the grant stage. If this is not the case, we request the Primary Examiner to telephone our Mr. J.D. Brown with a view to discussing the outstanding objections. Mr. Brown is normally in our London Office (0044 - 81 - 889 6625) but can also be contacted via the secretariat in our Munich Office. If at any time refusal of the Application is contemplated, we request that Oral Proceedings be appointed before any refusal is issued.

Yours faithfully,
FORRESTER & BOEHMERT.
Alan L. Meddle

P.S. Please acknowledge safe receipt hereof by returning the enclosed copy letter, duly endorsed.

Claims

1. A method for producing a secreted human PDGF receptor analog, comprising:

introducing into a host cell a DNA construct capable of directing the expression and secretion of a human PDGF receptor analog, said DNA construct containing a transcriptional promoter operatively linked to a secretory signal sequence followed downstream in proper reading frame by a DNA sequence encoding at least a portion of the extracellular domain of a human PDGF receptor, said portion including a ligand-binding domain;

growing said host cell in an appropriate growth medium; and

isolating said PDGF receptor analog from said host cell.

2. The method of claim 1 wherein said DNA sequence encodes a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531.

3. A method for producing a secreted, biologically active peptide dimer, comprising:

introducing into a host cell a DNA construct capable of directing the expression and secretion of a biologically active peptide dimer, said DNA construct containing a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin peptide requiring dimerization for biological activity fused to a ~~dimerizing protein~~ first polypeptide chain capable of associating with a second polypeptide chain to form a dimer, wherein the second polypeptide chain is the same or different from the first polypeptide chain;

growing said host cell in an appropriate growth medium under conditions allowing the dimerization and secretion of the peptide dimer; and

isolating said peptide dimer from said host cell.

4. The method of claim 1 or claim 3 wherein said host cell is a yeast cell carrying a defect in a gene whose product is required for the addition of outer chain oligosaccharide moieties to glycoproteins.

5. The method of claim 1 or claim 3 wherein said host cell is a yeast cell containing a genetic defect in the MNN9 gene or a disruption of the MNN9 gene.

6. A method according to claim 1 or claim 3 wherein said secretory signal sequence is selected from the group consisting of the MFα1 pre-pro sequence, the PHO5 signal sequence, the BAR1 signal sequence, the SUC2 signal sequence, the PDGF receptor signal sequence and the mouse immunoglobulin V_H signal sequence.

7. The method of claim 3 wherein said dimerizing protein comprises at least a portion of a protein selected from the group consisting of an immunoglobulin light chain, an immunoglobulin heavy chain and yeast invertase, wherein said portion associates as a dimer in a covalent or a noncovalent manner.

8. The method of claim 3 wherein said dimerizing protein is selected from the group consisting of an immunoglobulin heavy chain hinge region and yeast invertase.

9. A method for producing a secreted, biologically active peptide dimer, comprising:

introducing into a host cell a first DNA construct containing a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and

non-immunoglobulin

in proper reading frame with a DNA sequence encoding a peptide requiring dimerization for biological activity joined to a dimerizing protein, said dimerizing protein comprising an immunoglobulin light chain constant region;

introducing into said host cell a second DNA construct containing a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, $C_\gamma1$, $C_\gamma2$, $C_\gamma3$, $C_\gamma4$, and μ joined to an immunoglobulin hinge region;

growing said host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of said biologically active peptide dimer; and

isolating said biologically active peptide dimer from said host cell.

10. A method for producing a secreted, biologically active peptide dimer, comprising:

introducing into a host cell a first DNA construct containing a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a peptide requiring dimerization for biological activity joined to a dimerizing protein, said dimerizing protein comprising at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, $C_\gamma1$, $C_\gamma2$, $C_\gamma3$, $C_\gamma4$, and μ joined to an immunoglobulin heavy chain hinge region;

introducing into said host cell a second DNA construct containing a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least an immunoglobulin light chain constant region;

growing said host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of said biologically active peptide dimer; and

isolating said biologically acitve peptide dimer from said host cell.

11. A method according to any of claims 3, 9 or 10 wherein said peptide requiring dimerization for biological activity is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441.

12. A method for producing a secreted, ligand-binding receptor analog, comprising:

introducing into a host cell a first DNA construct containing a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding receptor analog joined to a dimerizing protein, said dimerizing protein comprising an immunoglobulin light chain constant region;

introducing into said host cell a second DNA construct containing a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, $C_\gamma1$, $C_\gamma2$, $C_\gamma3$, $C_\gamma4$, and μ joined to an immunoglobulin heavy chain hinge region;

growing said host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of said ligand-binding receptor analog; and

isolating said ligand-binding receptor analog from said host cell.

13. A method for producing a secreted, ligand-binding receptor analog, comprising:

introducing into a host cell a first DNA construct containing a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding receptor analog joined to a dimerizing protein, said dimerizing protein comprising at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, $C_\gamma1$, $C_\gamma2$, $C_\gamma3$, $C_\gamma4$, and μ joined to an immunoglobulin hinge region;

introducing into said host cell a second DNA construct containing a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least an immunoglobulin light chain constant region;

growing said host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of said ligand-binding receptor analog; and

isolating said ligand-binding receptor analog from said host cell.

14. The method of claim 12 or claim 13 wherein said ligand-binding receptor analog is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441.

15. A method according to any of claims 9, 10, 12 or 13 wherein said first and second secretory signal sequences are selected from the group consisting of the MFα1 pre-pro sequence, the PHO5 signal sequence, the BAR1 signal sequence, the SUC2 signal sequence, the PDGF receptor signal sequence and the mouse immunoglobulin V_H signal sequence.

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first polypeptide chain capable of associating with a second polypeptide chain to form a dimer, wherein the second polypeptide chain is the same or different from the first polypeptide chain,

16. A method according to any of claims 1, 3, 8, 9, 11 or 12 wherein the host cell is a yeast cell or a cultured mammalian cell.

17. A human PDGF receptor analog consisting essentially of the extracellular domain of a human PDGF receptor, said portion being capable of binding human PDGF or isoforms thereof.

18. The human PDGF receptor analog of claim 17 wherein said receptor analog is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine,, number 29, to lysine, number 531.

19. A method for determining the presence of human PDGF or isoforms thereof in a biological sample, comprising:

incubating a polypeptide comprising a human PDGF receptor analog fused to a ~~dimerizing protein~~ with a biological sample suspected of containing human PDGF or an isoform thereof under conditions that allow the formation of receptor/ligand complexes; and

detecting the presence of the receptor/ligand complexes as an indication of the presence of human PDGF or an isoform thereof.

20. The method of claim 19 wherein said human PDGF receptor analog comprises a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441, and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531, fused to a dimerizing protein.

wherein said polypeptide is capable of binding anti-PDGF antibodies, PDGF, PDGF isoforms, PDGF analogs or PDGF antagonists;

(A) first polypeptide chain capable of associating with a second polypeptide chain to form a dimer, wherein the second polypeptide chain is the same or different from the first polypeptide chain,

21. The method of claim 19 wherein said dimerizing protein is selected from the group consisting of an immunoglobulin light chain constant region, an immunoglobulin heavy chain hinge region, at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, $C_\gamma1$, $C_\gamma2$, $C_\gamma3$, $C_\gamma4$, and μ joined to an immunoglobulin heavy chain hinge region and yeast invertase.

22. A pharmaceutical composition comprising a human PDGF receptor analog fused to a dimerizing protein in combination with a physiologically acceptable carrier or diluent.

23. The pharmaceutical composition of claim 22 wherein said PDGF receptor analog is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531.

24. A diagnostic composition comprising a polypeptide fusion comprising a human PDGF receptor analog fused to a ~~dimerizing protein~~ (A) (B), said polypeptide tagged with a label capable of providing a detectable signal.

25. A method for purifying human PDGF of an isoform thereof from a sample comprising:

immobilizing a polypeptide fusion comprising a PDGF receptor analog fused to a ~~dimerizing protein~~ (B) on a substrate;

contacting a sample containing human PDGF or an isoform thereof with the immobilized polypeptide fusion under conditions such that the human PDGF or isoform thereof binds to the polypeptide; and

(B) wherein said PDGF receptor analog is capable of binding anti-PDGF antibodies, PDGF, PDGF isoforms, PDGF analogs or PDGF antagonists,

eluting the human PDGF or isoform thereof from the polypeptide.

EXHIBIT 5

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EXHIBIT 6

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CTLA-4 Can Function as a Negative Regulator of T Cell Activation

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Summary

CD28 and CTLA-4 are related glycoproteins found on T cells. Ligation of CD28 following antigen receptor engagement provides a costimulatory signal required for T cell activation. Anti-CTLA-4 antibodies were generated to examine the role of the CTLA-4 receptor on murine T cells. Expression of CTLA-4 as a homodimer is up-regulated 2–3 days following T cell activation. Anti-CTLA-4 antibodies and Fab fragments augmented T cell proliferation in an allogeneic MLR. However, when optimal costimulation and Fc cross-linking were present, anti-CTLA-4 MAbs inhibited T cell proliferation. Together, these results suggest that the MAb may obstruct the interaction of CTLA-4 with its natural ligand and block a negative signal, or directly signal T cells to down-regulate immune function.

Introduction

The activation of T lymphocytes and subsequent generation of effector activity are dependent on at least two signals delivered by antigen-presenting cells (APC). The first signal is delivered when the T cell receptor (TCR) is ligated by the antigenic peptide–major histocompatibility complex (MHC) molecular complex. There is also a requirement for costimulatory signals mediated by the interaction of TCR-independent T cell surface molecules and ligands expressed on the APC. The interaction of CD28 on T cells with either B7-1 (CD80) or B7-2 (B70) on APC has been shown to provide one such costimulatory signal for helper T cells to produce interleukin-2 (IL-2) (June et al., 1990, 1994; Freeman et al., 1993a; Lenschow et al., 1993).

CTLA-4 is a molecule that was cloned from a subtractive library derived from a CTL clone (Brunet et al., 1987). The CTLA-4 gene colocalized to the same chromosome as CD28 in both mouse and human (Lafage–Pochitaloff et al., 1990; Harper et al., 1991). The predicted amino acid sequence of the gene was homologous to CD28, especially in the transmembrane domain (Harper et al., 1991). Most importantly, several structural motifs are shared between the two amino acid sequences, including a sequence, MYPPPY, which is predicted to allow binding of the CD28 molecule to the B7-1 molecule (Harper et al., 1991) and to B7-2 by a number of investigators (Lenschow et al., 1993; Freeman et al., 1993a, 1993b; Azuma et al., 1993). The binding of CTLA-4 to B7 was demonstrated by Linsley and colleagues (1991) using a soluble form of the molecule, made up of the extracellular domain of CTLA-4 and the Fc portion of a human immunoglobulin G1 (IgG1), CTLA-4Ig. In fact, binding studies have demonstrated that CTLA-4Ig has a 10- to 20-fold higher affinity for B7-1 than a similarly derived fusion protein with the extracellular domain of CD28 (Linsley et al., 1991). CTLA-4Ig is also capable of blocking in vitro proliferation in assays of alloreactivity (Linsley et al., 1991; Lenschow et al., 1993), as well as preventing xenograft rejection (Lenschow et al., 1992) and prolonging allograft rejection (Turka et al., 1992; Lin et al., 1993), presumably by preventing reactive T cells from receiving their required second signal. In addition, CTLA-4Ig also has inhibitory effects on immunoglobulin production (Linsley et al., 1992b; Lane et al., 1994).

However, in spite of the similarities between the extracellular binding domains of the CD28 and CTLA-4 glycoproteins, a number of differences suggest these molecules may participate in distinct ways during an immune response. First, CTLA-4 mRNA transcripts are virtually undetectable in resting human and murine T cells, and increase only after T cell activation. In activated human T cells, transcripts can be detected as early as 1 hr following stimulation by TCR- and CD28-mediated signals (Lindsten et al., 1993). CTLA-4 mRNA is also detected in activated murine T cells and T cell clones (Freeman et al., 1992). In fact, recent studies by Linsley et al. (1992a) have documented cell surface expression of CTLA-4 on activated human cells using a MAb specific for human CTLA-4. Expression of CTLA-4 on the surface of peripheral blood lymphocytes (PBLs) was restricted to the T cell blasts, 2–4 days following activation.

Although current research has increased our understanding of the regulation of CTLA-4 mRNA and surface expression, the functional role of CTLA-4 remains unclear. Initial studies have suggested that CTLA-4 may function as an additional costimulatory molecule. Linsley et al. (1992a) demonstrated that anti-human CTLA-4 MAb increased proliferation of T cells stimulated with anti-CD3 plus anti-CD28. However, these studies did not rule out other functional roles of CTLA-4 during natural immune responses. The present study was initiated to develop reagents specific for murine CTLA-4 to address further the role of this molecule in the regulation of T cell activation.

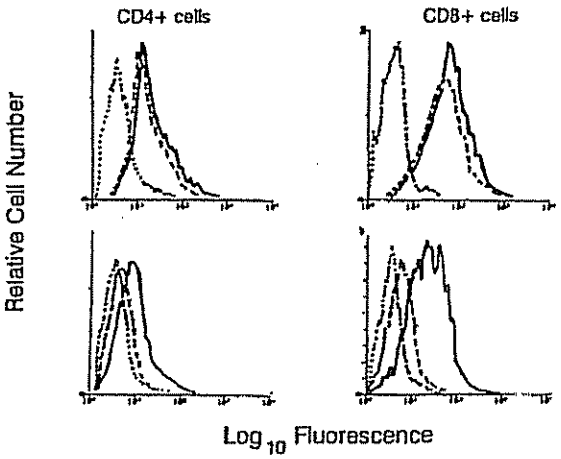

Figure 1. CTLA-4 Is Expressed on CD28-Deficient T Cells Stimulated with Anti-CD3 and rIL-2

Splenocytes from either CD28 (−/−) mice (bottom left and right) or B6 (wild-type) control mice (top left and right) were stimulated with complete media alone (dotted line), 10 μg/ml final concentration of anti-CD3 (dashed line), or 10 μg/ml anti-CD3 in the presence of 50 U/ml rIL-2 (solid line). Splenocytes were harvested 48 hr after the initiation of the culture and stained for either CD4 (top left and bottom left) or CD8 (top right and bottom right) versus CTLA-4. Data are expressed as histograms of 4F10 fluorescence following gating on the indicated T cell subsets. In all cases, unstimulated cells incubated in media alone expressed no CTLA-4 as determined by comparison of 4F10 staining to a negative control hamster antibody, GL3. Data are representative of two separate experiments.

Results

CTLA-4 Is Detected Only on Activated Murine T Lymphocytes Following Stimulation with Anti-CD3 or Antigen

To examine CTLA-4 protein expression on murine T lymphocytes, hamster MAbs were generated with a murine CTLA-4Ig fusion protein. Candidate hybridomas were screened by using the CTLA-4Ig molecule as a capture substrate for hybridoma supernatants in an enzyme-linked immunosorbent assay. The specificity of three MAbs, UC10-1B8, UC10-4B9-12, and UC10-4F10-11 (4F10) were confirmed on CHO cells transfected with a glycophosphatidylinositol (GPI)-linked form of the CTLA-4 extracellular domain (data not shown). All of the antibodies stained CTLA-4GPI–CHO transfectants, but not untransfected CHO cells. The 4F10 MAb routinely stained at the highest levels and was utilized for the majority of subsequent experiments.

Previous experiments have demonstrated that CTLA-4 mRNA transcripts are induced following activation of human or murine T lymphocytes. However, in the murine model, direct evidence of CTLA-4 protein expression has not been established. To examine murine T cells for CTLA-4 expression, splenocytes from B6 mice were incubated with soluble anti-CD3 at 10 μg/ml and examined for CTLA-4 expression on both the $CD4^+$ and $CD8^+$ T cell subsets at 48 hr (Figure 1, top left and top right, respectively). Resting cells, either $CD4^+$ or $CD8^+$, showed no evidence of cell surface CTLA-4 expression (data not shown). However, 48 hr following activation with anti-CD3, both $CD4^+$ and $CD8^+$ cells expressed CTLA-4. $CD8^+$ T cells expressed higher levels of CTLA-4 than $CD4^+$ cells. In both subsets, the large blast cells expressed the highest level of CTLA-4 (data not shown).

Previously, it has been shown that CTLA-4 mRNA transcripts are increased when human PBL are stimulated with both anti-CD3 and anti-CD28 as opposed to anti-CD3 alone (Lindsten et al., 1993). However, it has also been suggested, using human PBL, that CD28 ligation is not critical for CTLA-4 expression (Damle et al., 1994). To look at the dependence of CTLA-4 expression on CD28 signaling, splenocytes from CD28 (−/−) and B6 (wild-type) mice were activated either with anti-CD3 alone at 10 μg/ml or anti-CD3 in the presence of 50 U/ml rIL-2 (Figure 1, bottom left and bottom right). $CD4^+$ and $CD8^+$ T cells from the CD28 (−/−) mice expressed minimal levels of CTLA-4 in the presence of soluble anti-CD3 alone as compared with wild-type mice. However, CD28 (−/−) splenic T cells cultured in the presence of anti-CD3 plus IL-2 expressed significant levels of CTLA-4, albeit lower levels than observed on wild-type splenic T cells under either stimulation condition (Figure 1). These results suggest that CD28 expression is required for optimal CTLA-4 expression on the surface of T cells. The essential signals delivered through the CD28 receptor could be partially replaced by IL-2, implying that at least one important element of CD28-dependent CTLA-4 expression is the regulation of IL-2 through the CD28 pathway.

Kinetics of CTLA-4 Expression on Anti-CD3 and Antigen-Activated Murine T Lymphocytes

The functional role of CTLA-4 in T cell responses is likely to be a function of its time of expression, therefore, the kinetics of CTLA-4 expression following exposure to soluble anti-CD3 MAb or nominal antigen stimulation was examined (Figure 2). CTLA-4 expression is first detected at 24 hr following stimulation of T cells with anti-CD3 MAb. Peak expression was observed at 48 hr. By 96 hr, little, if any, CTLA-4 was detected on the surface of activated cells (Figure 2A). To determine whether stimulation of T cells with a nominal antigen resulted in a similar time course of CTLA-4 expression, $H\text{-}2L^d$-reactive T cells from the 2C TCR transgenic mice were stimulated with the $H\text{-}2L^d$-bearing P815 mastocytoma cells transfected with murine B7-1 (P815–B7-1). Up-regulation of CTLA-4 on clonotype-positive T cells, stimulated with the $H\text{-}2L^d$ alloantigen, followed a time course similar to that previously observed with anti-CD3 (Figure 2B). Maximal expression of CTLA-4 was observed at 48 hr following activation. These

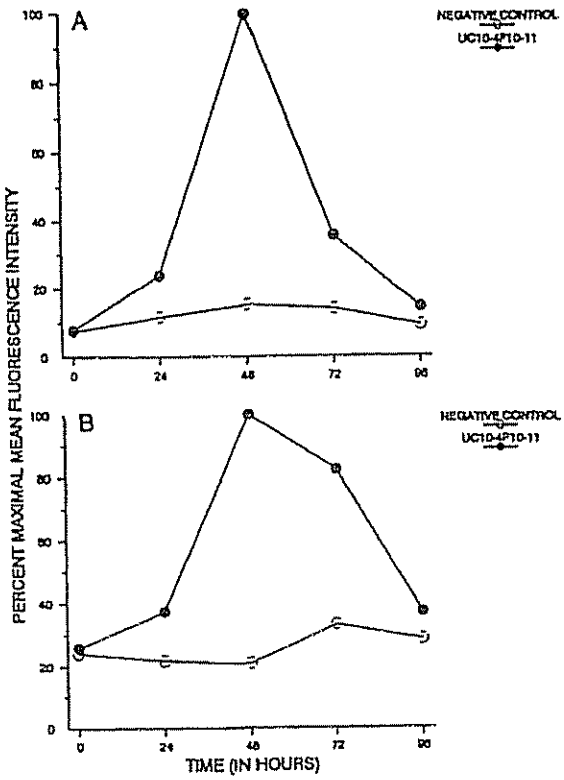

Figure 2. Kinetics of CTLA-4 Expression following Activation of Splenocytes with Anti-CD3 or Antigen

(A) B6 splenocytes were incubated with anti-CD3 MAb at 10 μg/ml final concentration for 0, 24, 48, 72, or 96 hr. Cells were stained with phycoerythrin-coupled anti-Thy1.2 versus FITC-coupled 4F10 or negative control MAb GL3. For histogram analysis, cells were gated for positive staining with the anti-Thy1.2 MAb.

(B) 2C TCR transgenic splenocytes (2×10^6) were incubated with 1×10^4 mitomycin C-treated P815-B7-1 mastocytoma cells for either 0, 24, 48, 72, or 96 hr. Cells were stained with biotin-coupled anti-clonotypic MAb 1B2 versus FITC-coupled 4F10 or negative control GL3. For histogram analysis, cells were gated for positive staining with the anti-clonotype reagent, 1B2. In both (A) and (B), data are expressed as percent maximal mean fluorescence intensity of FITC-coupled 4F10 staining. CTLA-4 expression is presented as closed circles; background staining by the negative control MAb is represented by the open circles.

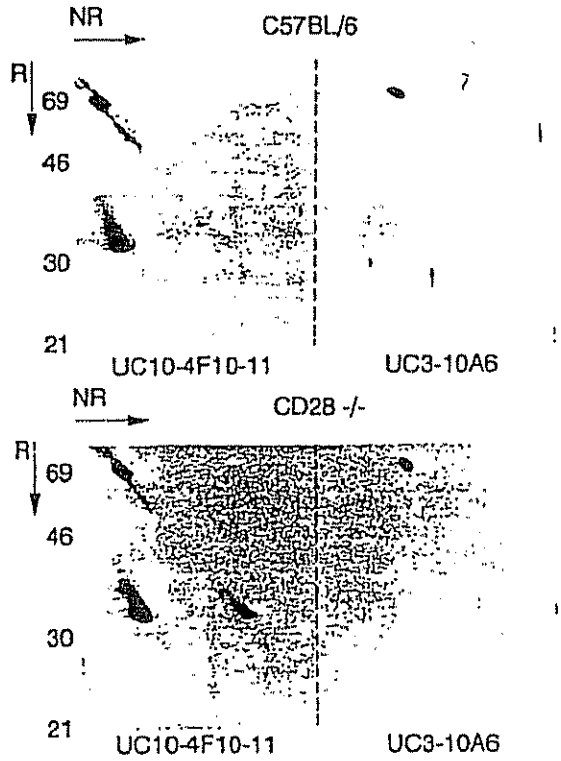

Figure 3. CTLA-4 Is Expressed as both a Nondisulfide-Linked Monomer and a Disulfide-Linked Dimer on the Surface of Activated Splenocytes

B6 splenocytes (top) or CD28 (−/−) splenocytes (bottom) were activated in the presence of 10 μg/ml soluble anti-CD3 and 50 U/ml IL-2. At 72 hr, cells were processed (as described in Experimental Procedures) and cell surface-labeled with biotin followed by solubilization in 0.5% NP40 lysis buffer. Immunoprecipitations were carried out with either anti-CTLA-4 MAb 4F10 or negative control hamster MAb UC3-10A6 and subjected to 2D SDS-polyacrylamide gel electrophoresis. The first dimension was electrophoresed under nonreducing conditions, while the second dimension was electrophoresed under reducing conditions in the presence of 2-mercaptoethanol. Proteins were transferred to nitrocellulose, the membranes were blocked with milk proteins, and developed with streptavidin-horseradish peroxidase and chemiluminescent substrate.

findings demonstrated that CTLA-4 is expressed on antigen-reactive T cells following activation by MHC-restricted nominal antigen in the presence of a costimulatory signal.

CTLA-4 Exists Predominantly as a Homodimer on the Surface of Activated T Cells

To examine the biochemical nature of the CTLA-4 glycoprotein expressed on activated T cells, splenocytes from B6 mice were stimulated using anti-CD3 and IL-2, cultured for 72 hr, harvested, passed over Ficoll to remove dead cells, and cell surface labeled with biotin. Labeled cell lysates were immunoprecipitated with either a negative control hamster antibody (UC3-10A6) or the anti-CTLA-4 MAb, 4F10, and analyzed by two-dimensional nonreduced/reduced polyacrylamide gel electrophoresis as described in the Experimental Procedures. Figure 3 illustrates that the CTLA-4 glycoprotein is expressed on activated murine T cells as a dimer composed of one of two molecules, either 33–34 kDa or 36–37 kDa. Treatment of the anti-CTLA-4 immunoprecipitate with N-glycanase resulted in a size reduction of both glycoprotein species to the same core size of approximately 21 kDa (data not shown). These results suggest that CTLA-4 can exist as a homodimer of differentially glycosylated forms. A nondisulfide-linked form of CTLA-4 was observed migrating on the diagonal at the same glycosylated and core molecular mass as the smaller of the two species of CTLA-4 dimer. This nondisulfide-linked dimeric or monomeric form was observed on activated normal and CD28-deficient T cells.

It has been hypothesized that CTLA-4 may exist on the cell surface dimerized with CD28, given the amino acid

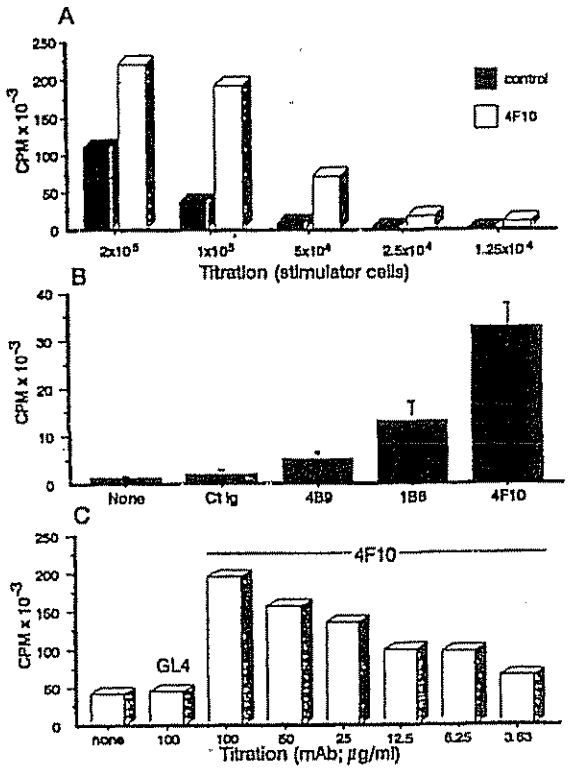

Figure 4. Anti-CTLA-4 MAbs Can Augment Proliferation in an Allogeneic MLR.

(A) Purified 2C T cells (2 × 10^5 cells/well) were cultured with the indicated numbers of allogeneic γ-irradiated DBA/2 splenocytes. Cells were cultured either with 4F10 MAb at 100 μg/ml final concentration (open bars) or without added MAb (closed bars). Cells were harvested 96 hr following the initiation of culture. Data is representative of three experiments.

(B) Purified 2C TCR transgenic T cells (2 × 10^5/well) were cultured in the presence of 5 x 10^4/well γ-irradiated (2000 rads) BALB/c splenocytes. All reagents were added at a 50 μg/ml final concentration. In no case was augmentation seen in the presence of syngeneic (B6) feeder cells. This figure is representative of data from four experiments.

(C) Purified 2C T cells (2 × 10^5 cells/well) were cultured with 1 × 10^5 allogeneic irradiated DBA/2 splenocytes in the presence of either negative control hamster MAb, GL4 (100 μg/ml) or the indicated concentration of 4F10. Assays were harvested at 96 hr following initiation of culture. Data is representative of five experiments.

homology shared between the two molecules. However, the data shown here provide no evidence for this association. First, the molecular mass of murine CD28 monomers is approximately 45 kDa, while CTLA-4 monomers appear to migrate between 33–37 kDa. Second, the pattern of CTLA-4 molecule expression is similar if not identical when activated T cells from normal and CD28 (−/−) mice were compared (Figure 3). Therefore, CTLA-4 does not need to heterodimerize with CD28 to be expressed on the surface of activated murine T cells. However, this does not preclude the fact that CTLA-4 and CD28 may heterodimerize under activation conditions not explored in this study.

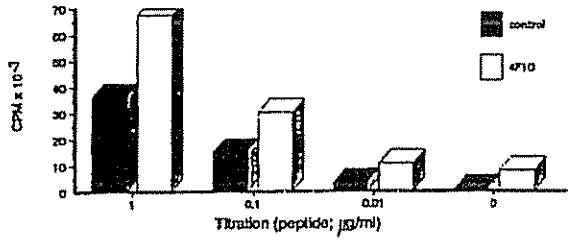

Figure 5. 4F10 Can Augment Proliferation in the Presence of Increased TCR Stimulation.

Allogeneic irradiated BALB/c stimulators (2.5 × 10^4 cells/well) were cultured with the octapeptide (LSPFPFDL) at the indicated concentrations for 1 hr at 37°C. 4F10 or a control hamster immunoglobulin were added at a final concentration of 50 μg/ml and 2 × 10^5 peptide-specific purified 2C TCR transgenic T cells were added. Assays were harvested 96 hr after the initiation of culture. Data are representative of three experiments.

Antibodies Against CTLA-4 Can Augment an Allogeneic Response

The anti-CTLA-4 antibodies were examined functionally in allogeneic in vitro T cell proliferative responses. T cells were purified from the lymph nodes of 2C TCR transgenic mice and stimulated with graded doses of irradiated allogeneic DBA/2 splenocytes. The anti-CTLA-4 MAb, 4F10, had a significant augmenting effect on T cell proliferation over a wide range of stimulation conditions (2- to 10-fold; Figure 4A). However, the greatest fold augmentation of proliferation was observed at the lower concentrations of stimulators. Therefore, further experiments were performed using reduced stimulators to examine the augmented proliferative response. As seen in Figure 4B, several different anti-CTLA-4 MAbs augmented the proliferative responses between 2- to 5-fold above responses observed in cultures treated with control hamster immunoglobulin (Figure 4B) or other hamster MAbs (data not shown). The most dramatic augmentation was observed following the addition of the 4F10 MAb, perhaps reflecting its epitope specificity, immunoglobulin subclass or relative affinity as compared with the other anti-CTLA-4 MAbs. The ability of anti-CTLA-4 MAb to augment T cell proliferation depended on an initial signal, presumably delivered via the TCR, inasmuch as the MAbs had no effect on the proliferative response of purified responder T cells to syngeneic B6 APCs (data not shown). Similar levels of enhanced proliferation were observed in allogeneic mixed lymphocyte reaction (MLR) assays using purified B6 T cells as the responder population (data not shown). In Figure 4C, the 4F10 MAb was titrated and examined for its ability to augment proliferation over a range of MAb concentration. Significant augmentation of the T cell proliferative response was observed upon the addition of as little as 6.25 μg/ml. Thus, anti-CTLA-4 MAbs were able to augment T cell proliferation over a wide range of stimulatory conditions and MAb concentrations.

Antibodies Against CTLA-4 Can Augment Proliferation in the Presence of Increased TCR Stimulation

The variation in the ability of 4F10 to augment proliferation

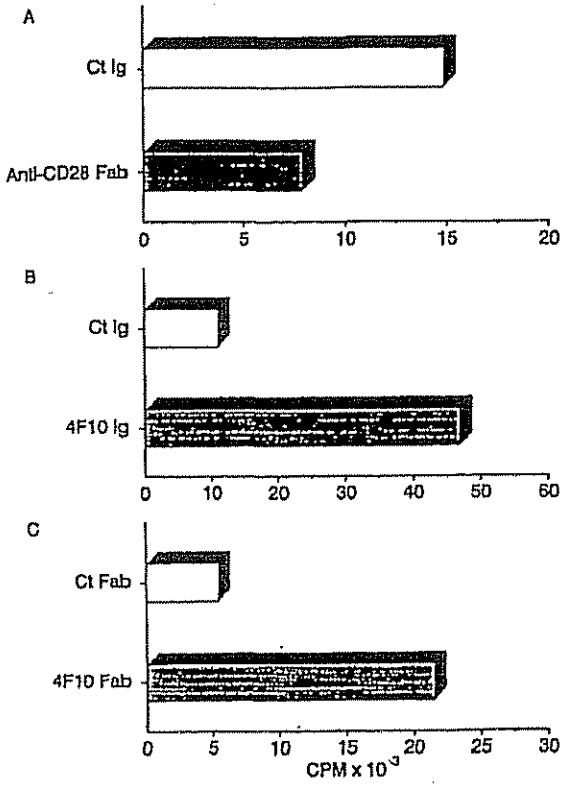

Figure 6. 4F10 and 4F10 Fab Fragments Can Augment Proliferation in an Allogeneic MLR

(A) Purified B6 T cells (4×10^5 cells/well) were cultured with 4×10^5 allogeneic irradiated BALB/c splenocytes. Control hamster immunoglobulin (Ct Ig) and anti-CD28 Fab fragments (anti-CD28 Fab) are present at 100 μg/ml final concentration. SEM of Ct Ig = 1952 and of anti-CD28 Fab = 960.

(B) Purified B6 T cells (4×10^5/well) were cultured in the presence of 5×10^4/well γ-irradiated (2000 rads) BALB/c splenocytes. Control hamster immunoglobulin (Ct Ig) and 4F10 are present at a final concentration of 50 μg/ml. SEM of Ct Ig = 2830 and of 4F10 = 6783.

(C) Control hamster Fab fragments from anti-B7-1 MAb, 16-10A1, and 4F10 Fab fragments (4F10 Fab) are present at a final concentration of 16.7 μg/ml. SEM of Ct Fab = 1100 and of 4F10 Fab = 4065. Whole MAb reagents and Fab reagents were compared here at equimolar concentrations. Control reagents are represented by open bars. Anti-CD28 Fab fragments, 4F10 and 4F10 Fab fragments are represented by the shaded bars. This figure is representative of three separate experiments.

may reflect the degree of initial activation mediated by TCR engagement with relevant antigen. Therefore, an antigen dose titration was performed (Figure 5). The 2C TCR is reactive to a known peptide (LSPFPFDL) in the context of the MHC class I molecule, H-2L^d (Udaka et al., 1992). Addition of this peptide to allogeneic H-2L^d-expressing stimulator cells increases the number of H-2L^d–peptide complexes on the cell surface of the H-2L^{d+} stimulator cells (Smith et al., 1992), such that there is increased ligation of the TCR on the surface of the transgenic T cells without increasing the level of costimulatory molecules available to the T cells. Under these conditions, 4F10 augmented proliferation when peptide was added over a range of concentrations from 1 μg/ml to 0.01 μg/ml. As seen in the dose titration of allogeneic stimulators (see Figure 4A), the greatest augmentation was observed under suboptimal TCR stimulation. However, even at high concentrations of peptide (i.e., 1 μg/ml), anti-CTLA-4 MAb was able to augment T cell proliferation significantly in the allogeneic MLR as compared with control hamster immunoglobulin (Figure 5).

4F10 Fab Fragments Augment Proliferation in an Allogeneic MLR

The costimulatory function of anti-murine CD28 MAb in an allogeneic MLR requires MAb cross-linking on FcR-bearing APC or plastic (T. L. W. et al., unpublished data). In fact, Fab fragments of anti-CD28 severely inhibit T cell proliferative responses (Figure 6A). This is consistent with the anti-CD28 Fab fragments blocking the costimulatory interaction of the CD28 receptor with its natural ligands B7-1, B7-2, or both. Therefore, in the present study, the functional effects of Fab fragments of the anti-CTLA-4 MAb were compared with whole immunoglobulin to distinguish between anti-CTLA-4 costimulation of T cell proliferation and the anti-CTLA-4 MAbs preventing the down-regulation of T cell activation following the interaction of CTLA-4 with its natural ligands. Pure 4F10 Fab fragments were prepared and added to an allogeneic MLR. The Fab fragments significantly augmented T cell proliferation (4-fold above control Fab fragments of 16-10A1) (Figure 6C). The augmented proliferation was 50% of that observed using the whole MAb at the same molar concentration (Figures 6B and 6C). The difference between the whole Ab and Fab fragments may reflect a decreased avidity of the monovalent Fab fragments. Most importantly, the augmentation of proliferation observed with the anti-CTLA-4 Fab fragments is distinctly different from the effects of adding anti-CD28 Fab fragments to the allogeneic MLR (Figures 6A and 6C). These results suggest that anti-CTLA-4 MAb and Fab fragments may augment the allogeneic MLR by inhibiting the interaction of CTLA-4 with its natural ligand. Thus, CTLA-4 may be involved in the down-regulation of T cell responses following the initial activation event (Figure 6).

Anti-CTLA-4 MAbs Inhibit T Cell Proliferation in the Presence of Anti-CD3 and Anti-CD28

To examine directly the inhibitory effects of anti-CTLA-4 MAbs, the 4F10 MAb was added to T cell cultures suboptimally stimulated with anti-CD3 MAb and maximally costimulated with anti-CD28 MAbs. Under these conditions, 4F10 inhibited proliferation of purified B6 and 2C TCR transgenic T cells to anti-CD3 and anti-CD28 in the presence of syngeneic APC (Figures 7A and 7B, respectively). As observed previously (see Figures 4A, 5, and 6B), the 4F10 MAb under suboptimal TCR stimulation (in these experiments, 0.1 μg/ml anti-CD3) augments T cell proliferation in the absence of anti-CD28. However, the addition of the anti-CTLA-4 MAb to cultures containing anti-CD28 resulted in the inhibition of T cell proliferation even at concentrations as low as 6.3 μg/ml 4F10 MAb (see Figure 5B). Interestingly, in contrast with the effects observed

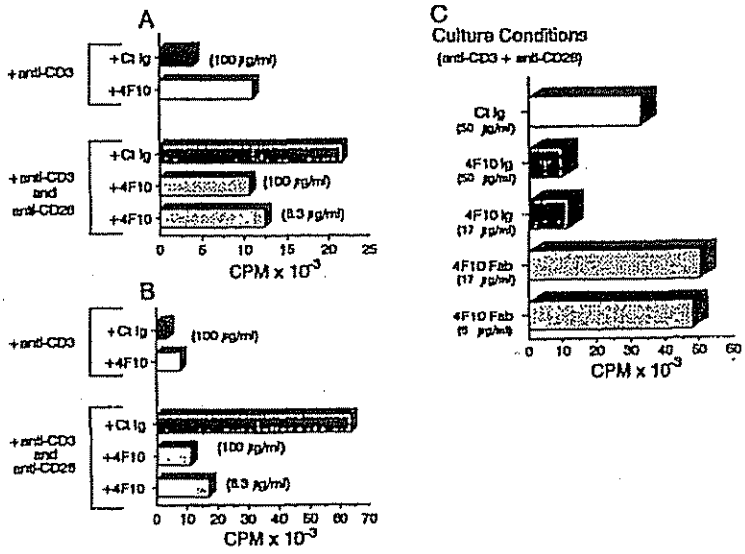

Figure 7. Anti-CTLA-4 MAbs Can Inhibit the Proliferation of Anti-CD3- and Anti-CD28-Stimulated T Cells

T cells (2 × 10^5) purified from B6 mice (A) or 2C TCR transgenic mice (B) were cultured in the presence of 1 × 10^5 syngeneic APCs and 0.1 μg/ml anti-CD3 alone or anti-CD3 at 0.1 μg/ml and 1:1000 anti-CD28 ascites. Anti-CTLA-4 MAbs were added at 100 μg/ml and 6.3 μg/ml final concentration. In (C), the 2 × 10^5 purified 2C TCR transgenic T cells were cultured with 1 × 10^5 syngeneic B6 splenocytes and 0.1 μg/ml anti-CD3 and a 1:1000 dilution of anti-CD28 ascites in the presence of whole 4F10 or 4F10 Fab fragments. Whole anti-CTLA-4 is shown at 50 μg/ml and 17 μg/ml final concentration; anti-CTLA-4 Fab fragments are shown at 17 μg/ml and 6 μg/ml final concentration. This represents equimolar concentrations of whole and Fab fragments of 4F10. These experiments are representative of seven experiments.

using the whole anti-CTLA-4 MAb, Fab fragments of 4F10 did not inhibit proliferation (Figure 7C); in fact, the addition of Fab fragments augmented proliferation. The inhibition is not due to blocking FcR binding by the anti-CD3 or anti-CD28 MAbs, because high concentrations (100 μg/ml in Figures 7A and 7B and 50 μg/ml in Figure 7C) of control hamster immunoglobulin had no inhibitory effect. In fact, in Figure 7A, the addition of the 4F10 MAb (100 μg/ml) to anti-CD3-stimulated (0.1 μg/ml) cultures augmented T cell proliferation. Thus, anti-CD3-mediated T cell activation that is FcR-dependent is not inhibited in this setting.

These results suggest that the addition of anti-CTLA-4 MAbs to these T cell stimulation assays has two distinct effects. Under conditions of maximal costimulation, the cross-linking form of 4F10 (whole immunoglobulin) inhibits T cell proliferation. In contrast, a soluble form of MAb (4F10 Fab fragments or whole MAb under conditions of limiting FcR cross-linking) results in augmentation (see Figure 6; Figure 7).

Discussion

During the past several years, it has become increasingly apparent that CD28 plays an important role in the regulation of T cell activation. Ligation of the CD28 receptor in the presence of TCR ligation results in IL-2 gene transcription and mRNA stabilization (June et al., 1990) and, under certain conditions, anti-CD28 MAb are capable of blocking anergy induction in T cell clones (Harding et al., 1992). CTLA-4 is homologous to CD28. CTLA-4 shares approximately 30% amino acid identity with CD28 and, like CD28, binds the B7-1 and B7-2 molecules. However, while the role of CD28 in T cell activation is becoming more clear, the function of CTLA-4 is still uncertain. To develop a better understanding of the fundamental role of CTLA-4 in T cell function, a panel of MAbs specific for murine CTLA-4 were developed and studied.

Several observations have been made with these reagents that confirm previous studies in the human model. First of all, CTLA-4 is only expressed on activated murine T lymphocytes. The kinetics of CTLA-4 expression on activated murine T cells closely parallels that previously reported for activated human T cells (Linsley et al., 1992a): CTLA-4 expression appears maximal at approximately 48 hr and becomes almost undetectable by 96 hr after T cell activation. Second, CTLA-4 is expressed on both $CD4^+$ and $CD8^+$ T cell subsets, although $CD8^+$ T cells appear to express higher levels of CTLA-4 during the course of activation. Whether this differential level of expression relates to differential functions of the two subsets remains to be determined. Third, both TCR ligation and CD28 receptor ligation are important in the regulation of CTLA-4 expression. CTLA-4 expression is CD28-dependent inasmuch as activation of CD28-deficient T cells with anti-CD3 alone did not up-regulate CTLA-4. The defect in the T cells from these mice may be due, in part, to a lack of IL-2 production, because the addition of IL-2 reconstituted CTLA-4 expression. However, other CD28-dependent costimulatory events are likely to be involved, given that CTLA-4 expression on the CD28-deficient activated T cells did not reach wild-type levels, even in the presence of exogenous IL-2.

In addition, several observations have been made with the MAbs that suggest that the expression and biological function of CTLA-4 may be more complicated than previously suggested. It appears that the human and murine CTLA-4 molecule may exist in multiple forms on the cell surface. Although the majority of CTLA-4 on activated murine T cells exists as a disulfide-linked dimer composed of two 33–37 kDa monomeric units, a nondisulfide-linked, perhaps monomeric, form also exists. Previous studies have suggested that alternative forms of CTLA-4 may exist on human cells as well. Anti-CTLA-4 antisera was shown to immunoprecipitate a nondisulfide-linked form of CTLA-4 from activated human PBL (Lindsten et al., 1993), whereas, an anti-human CTLA-4 MAb was shown to immunoprecipitate predominantly a disulfide-linked dimer of

CTLA-4 from transfectants. These discrepant results may represent differences in the serological reagents used. In parallel studies, immunoprecipitation of CTLA-4 from CTLA-4GPI-transfected CHO cells yield three different patterns of immunoprecipitation depending on the MAb used to immunoprecipitate the glycoprotein (data not shown). One set of MAbs, including 4B9-12, precipitated predominantly the higher molecular mass disulfide-linked form; others, including 4F10 and 1B8, precipitated both the disulfide-linked form and the lower molecular mass form; while the remaining MAbs precipitate predominantly the nondisulfide-linked form. Thus, the studies with the CHO transfectants suggest that the pattern of CTLA-4 immunoprecipitation depends on the antibody chosen for immunoprecipitation. In fact, the presence of both a nondisulfide- and disulfide-linked form of CTLA-4 on the cell surface of activated murine T cells is not unprecedented; CD28 has also been demonstrated to exist in both a dimeric and monomeric form on T lymphocytes (Lesslauer et al., 1986). Finally, despite the homology between CD28 and CTLA-4, these molecules do not form heterodimers on activated murine T cells that express both glycoproteins, since the electrophoretic mobility of CTLA-4 dimers immunoprecipitated from activated T cells isolated from CD28-deficient mice is identical to the patterns seen from B6 mice.

Most importantly, the present studies examined the role of CTLA-4 in the regulation of T cell function. Anti-CTLA-4 MAb augmented T cell proliferative responses to alloantigen. However, rather than delivering a positive signal through the CTLA-4 receptor, the anti-CTLA-4 MAb appear to block transduction of a negative signal. First, Fab fragments of the 4F10 MAb augmented the allogeneic MLR. The inability of Fab fragments to be cross-linked by FcR on the allogeneic APCs argues strongly against initiation of a costimulatory signal through CTLA-4. In fact, similar studies performed using anti-CD28 MAbs showed that costimulation could only be observed using whole Ab. Fab fragments of anti-CD28 profoundly inhibited the allogeneic MLR. Furthermore, when T cells were given suboptimal signal 1 stimulation with anti-CD3 but costimulated optimally using anti-CD28 MAb, the whole 4F10 but not the Fab fragments of 4F10 inhibited T cell proliferation. This down-regulation of T cell proliferation required both increased costimulatory signals and optimal cross-linking of the MAb. Thus, our data suggests that rather than playing an analogous role to CD28, CTLA-4 may be an antagonist of T cell function. In this model, resting T lymphocytes first encounter MHC–antigen complexes in the presence of a costimulatory signal mediated by CD28. The activated T cells produce IL-2 to drive T cell proliferation and up-regulate CTLA-4. At the time point of maximal CTLA-4 expression, CD28 function is diminished, since signaling through CD28, as evidenced by Ca^{2+} flux upon CD28 cross-linking, is depressed (Linsley et al., 1993). Thus, CTLA-4 can preferentially ligate with its counterreceptor, either B7-1 or B7-2, resulting in the transmission of a down-regulatory signal.

Although a number of cell surface molecules are involved in the costimulation or augmentation of T cell activation, molecules whose function it is to dampen or down-regulate T cell activation events to control T cell reactivity have not been extensively examined. CTLA-4 may represent one such molecule. The monoclonal reagents described here will be integral to study the cellular and biochemical behavioral consequences of CTLA-4 regulation of T cell activation in both in vitro and in vivo models of T cell immune response.

Experimental Procedures

Animals

Male DBA/2, BALB/c, and C57BL/6 (B6) mice (6- to 12-week-old) were purchased from Frederick Cancer Research Institute Laboratories and housed in a specific pathogen-free rodent facility at the University of Chicago. CD28 gene disrupted (CD28 (−/−)) mice (4–16 weeks of age) were bred in the University of Chicago specific-pathogen free mouse facility. CD28 gene expression was disrupted in these animals through the replacement of exon 2 of the CD28 gene with a neo gene sequence as previously described (Shahinian et al., 1993). 2C TCR transgenic mice were developed as previously described. These animals were bred in University of Chicago specific-pathogen free mouse facility and used at 6–16 weeks of age. The 2C TCR transgenic mouse was generated using the 2C CTL clone, which has a TCR reactive with the class I MHC molecule L^d. The presence of the transgenic TCR on the $CD8^+$ T cells in these mice is determined using the 1B2 anti-clonotypic MAb (Sha et al., 1988).

CTLA-4GPI Construct

Stable transfection of CTLA-4 cDNA expression constructs into commonly used cells such as CHO and NIH 3T3 does not lead to expression of cell surface CTLA-4 protein, as judged by the failure to bind B7–immunoglobulin (G. J. F., unpublished data). CTLA-4 was successfully expressed by linking the extracellular domain of CTLA-4 to a GPI anchor. The extracellular domain of CTLA-4 was amplified from a murine CTLA-4 cDNA by polymerase chain reaction using as sense primer, GAGTAAGCTTGCCGCCACCATGGCTTGTCTTGGA, containing a HindIII site, a strong start site, and the first 15 nt of the CTLA-4 coding sequence; an antisense primer, GAGAATTCTAGACTAGCTTAAGTCAGAATCTGGGCACGGT, containing the last 19 nt of the CTLA-4 extracellular domain and an Af1 II site. Polymerase chain reaction conditions were 94°C for 1 min; 43°C for 1 min; 72°C for 1 min for 35 cycles followed by one cycle of 72°C for 10 min. The polymerase chain reaction product was digested with HindIII and Af1 II, gel purified, and ligated into a HindIII- and AF1II-digested pCDMm8 vector containing the GPI anchor of human CD58 (Staunton et al., 1992; provided by Dr. D. Staunton, Center for Blood Research, Boston, Massachusetts). Plasmid containing the CTLA-4–GPI insert were transiently transfected into COS cells and strongly expressed cell surface CTLA-4, as judged by binding of B7–immunoglobulin fusion protein (G. J. F., data not shown).

Antibodies and Cell Lines

The P815 mastocytoma line, transfected with murine B7-1 was provided by L. Lanier (DNAX, Palo Alto, California) (Azuma et al., 1992). CTLA-4GPI-transfected CHO cells were generated using the CTLA-4GPI construct. 145-2C11 (anti-CD3) (Leo et al., 1987), UC3-10A6 (anti-V 2) (Houlden et al., 1989), 2.4G2 (rat anti-mouse Fc receptor) (Unkeless, 1979), 1B2 (anti-2C TCR) (Kranz et al., 1984), J11d (anti-heat-stable antigen) (Bruce et al., 1981), and the anti-$I\text{-}A^b$ MAbs 25-9-3 and 25-9-17 (Ozato and Sachs, 1981) were prepared in our laboratory. (anti-$V\gamma 2$) (Houlden et al., 1989), 2.4G2 (rat anti-mouse Fc receptor) (Unkeless, 1979), 1B2 (anti-2C TCR) (Kranz et al., 1984), J11d (anti-MAb (16-10A1) (Razi-Wolf et al., 1992) and anti-CD28 Fab fragments were provided by Repligen Corporation (Cambridge, Massachusetts). Purified GL4 (anti-Vδ4) was provided by L. Lefrancois (University of Connecticut, Farmington, Connecticut). Both GL4 and purified hamster immunoglobulin (purchased from Cappel Research Products, Durham, North Carolina) were used as negative control hamster antibodies in in vitro cultures. Biotin-coupled anti-CD4 MAb (RM-4-4), Fluorescein isothiocyanate (FITC)-coupled anti-$\gamma\delta$ TCR (GL3) and phy-

coerythrin-coupled anti-Thy1.2 (53-2.1) were purchased from PharMingen (San Diego, California); biotin-coupled anti-CD8 (53-6.7) was purchased from Becton-Dickinson (Mountain View, California). FITC-coupled 145-2C11 was provided by Boeringer Mannheim (Indianapolis, Indiana). Phycoerythrin-coupled streptavidin was purchased from Southern Biotechnology (Birmingham, Alabama).

Production of Anti-Murine CTLA-4 MAbs

Anti-murine CTLA-4 antibodies were generated by immunizing Armenian hamsters with the extracellular portion of murine CTLA-4 fused to a murine IgG_{2a} (mCTLA-4Ig, Wallace et al., 1994) by a protocol described previously (Leo et al., 1987). Subclones were generated by limiting dilution and tested for reactivity to murine CTLA-4Ig by an enzyme-linked immunosorbent assay. Subclones reactive to IgG_{2a} Fc were eliminated by testing for cross-reactivity on a murine IgG_{2a} MAb, 25-9-17, in an enzyme-linked immunosorbent assay.

T Cell Activation Cultures

Spleens were harvested, minced, and spleen cell suspensions were depleted of erythrocytes with ammonium chloride–potassium lysing buffer. Cells were cultured in DMEM containing 10% fetal calf serum, in the presence of 10 μg/ml soluble 145-2C11 in the presence or absence of 50 U/ml rIL-2 as indicated. For allogeneic reactions T cells were activated with P815–B7-1 stimulator cells that had been treated with 50 μg/ml mitomycin C for 2–3 hr at 37°C and washed 3 times in complete media before use. Responder LN or splenic T cells (2×10^6) were cultured with an equal number of P815–B7-1 transfectants in 2 ml macrowells over a 96 hr culture period.

Flow Cytometric Analysis

Cells to be examined were washed in FACS buffer (0.1% bovine serum albumin and 0.01% sodium azide in 1× phosphate-buffered saline [pH 7.4]) and incubated with anti-FcR MAb, 2.4G2, prior to staining. Directly FITC-coupled reagents and biotin-coupled reagents were added, incubated for 30 min at 4°C, washed, and incubated with phycoerythrin-coupled streptavidin for 15 min at room temperature. Two-color flow cytometry was performed using a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, California) and analyzed using the Lysis II software packages. Logarithmically-amplified fluorescence data were collected on 1×10^4 viable cells as determined by forward scatter intensity and exclusion of dead cells based on staining with propidium iodide.

2D Polyacrylamide Gel Electrophoresis

Bulk CD28 (−/−) or B6 spleen cell cultures were stimulated as described above for 72 hr, harvested, passed over Ficoll–Hypaque, washed extensively in phosphate-buffered saline (pH 7.4), and surface-labeled with ImmunoPure NHS-LC–biotin (Pierce, Rockford, Illinois) for 30 min at 14°C. Cells were lysed in 0.5% NP40 lysing buffer containing 50 mM Tris, 50 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml chymostatin, and 1 μg/ml peptstatin for 1 hr at 4°C. Lysate precleaning was performed using hyperimmune rabbit sera and a control hamster antibody (UC3-10A6) culture supernatant in the presence of protein A–Sepharose beads (Pharmacia, Piscataway, New Jersey). MAbs used for immunoprecipitation were purified from tissue culture supernatant using protein A–Sepharose. SDS–polyacrylamide gel electrophoresis tube gels (10%) were electrophoresed under nonreducing conditions in the first dimension, transferred to a second 12.5% slab gel, and electrophoresed under reducing conditions in the second dimension. Proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, New Hampshire) and blots were developed using a streptavidin–horseradish peroxidase conjugate (Amersham Corporation, Arlington Heights, Illinois) in combination with the chemiluminescent LumiGlo substrate (Kirkegaard Perry Labs, Gaithersburg, Maryland).

T Cell Purification

B6 or 2C TCR transgenic lymph node cells were enriched for T cells by passage over nylon wool columns. Cells positive for MHC class II molecules were further depleted using a mixture of anti-heat-stable antigen (J11d) and anti-$I\text{-}A^b$ (25-9-9) culture supernatants plus rabbit complement (Pel-Freez, Brown Deer, Wisconsin). T cell purity was evaluated by flow cytometry using anti-CD3 MAb (145-2C11). In all cases, T cells used in these assay were >95% $CD3^+$.

Allogeneic MLR

Purified T cells were plated out at either 4×10^5 cells/well for B6 T cells and at 2×10^5 cells/well for 2C TCR transgenic T cells. γ-irradiated (2000 rads) erythrocyte-depleted allogeneic stimulator cells, prepared from DBA/2 or BALB/c spleens, were used at indicated number per well in the B6 T and transgenic T cell assays. After 96 hr of incubation at 37°C, individual wells were pulsed with 1 μCi/well [^{3}H]thymidine for the last 12–16 hr of culture. Counts are represented as the mean cpm of triplicate wells.

Allogeneic MLR in the Presence of Ld-Binding Peptide

Allogeneic MLR assays were set up as described above, using the 2C TCR transgenic T cells as responders and 2.5×10^4 irradiated BALB/c splenocyte feeders pulsed with indicated concentrations and L^d-binding peptide, LSPFPFDL (described by Udaka et al., 1992), concentrations for 1 hr at 37°C before purified T cells were added. The L^d peptide was prepared by the University of Chicago peptide synthesis facility.

Preparation of Fab Fragments

4F10 and 16-10A1 MAbs in purified form were cleaved into Fab fragments using papain-immobilized agarose beads (Sigma, St. Louis, Missouri) for 72 hr at 37°C. Following fragmentation, the reaction mixture was passed over a protein A–Sepharose column to remove the remaining whole MAb and Fc fragments. Fab fragments (50,000 MW) were further purified by successive filtration through Centriprep30 and Centricon100 filters to remove residual Fc fragments or whole MAb, respectively (Amicon Incorporated, Beverly, Massachusetts). The purity of the Fab fragments was confirmed by nonreducing SDS–polyacrylamide gel electrophoresis. The avidity of the Fab fragments was tested by comparing the ability of whole 4F10 MAb and Fab fragments to block the binding of FITC-coupled 4F10 MAb to transfected CHO cells expressing a GPI-linked form of the CTLA-4 molecule.

Anti-CD3-Induced Proliferation Assays

Purified B6 or 2C TCR transgenic T cells (2×10^5) were cultured in the presence of 0.1 μg/ml anti-CD3, anti-CD28 MAb, PV-1 (1:1000 dilution of ascites), and 1×10^5 syngeneic irradiated (2000 rads) B6 splenocytes. Counts are represented as the mean cpm of triplicate wells harvested at 72 hr following a 16 hr pulse with 1 μCi/well [^{3}H]thymidine.

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EXHIBIT 7

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Characterization of CTLA-4 Structure and Expression on Human T Cells[1]

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ABSTRACT. CTLA-4 is an adhesion receptor expressed on activated T cells. The amino acid sequence of CTLA-4 is related to CD28, and although the function of CTLA-4 remains unknown, it shares several features with CD28, including a common counter-receptor, B7, that is present on Ag-presenting cells. In a recent study we found that CD28 and CTLA-4 were coexpressed at the mRNA level on activated T cells but that only CD28 was expressed on resting T cells. Here we show that within the T cell population, CTLA-4 expression is restricted to the subset of T cells that also express cell surface CD28. CTLA-4 mRNA expression can be induced on quiescent T cells via phorbol ester-mediated activation of protein kinase C but not with calcium ionophore treatment alone. Phorbol ester-induced expression of CTLA-4 mRNA could be enhanced with calcium ionophore treatment, and treatment of cells in this manner resulted in a reciprocal decrease in expression of CD28 mRNA. Ligation of CD28 with monoclonal antibody also resulted in the specific and rapid induction of CTLA-4 mRNA. To study the expression of CTLA-4 at the protein level, a rabbit antiserum against a recombinant protein derived from CTLA-4 cDNA was generated. When activated T cells were labeled with [^{35}S]methionine, the rabbit antiserum precipitated a 41- to 43-kDa protein from whole cell lysates. Similar results were found when detergent-soluble lysates from ^{125}I surface-labeled resting and activated T cells were analyzed by SDS-PAGE. Surprisingly, under the conditions tested, CTLA-4 migrated primarily as a monomer at the cell surface, and could not be shown to exist as a disulfide-bonded homodimer or as a heterodimer consisting of CTLA-4 and CD28. These results suggest that B7 can bind to T cells via distinct receptor complexes consisting of either CD28 or CTLA-4, and that these complexes may potentially mediate distinct biologic functions. Further, the present results suggest that noncovalent interactions might mediate association of CTLA-4 and/or CD28 at the cell surface. *Journal of Immunology*, 1993, 151: 3489.

Normal T cells or T cell clones do not proliferate in response to Ag unless additional signals are provided by Ag presenting cells (APC) (1). In most cases, the costimulatory signal provided by the APC requires cell contact, although in some instances soluble factors have been shown to provide an efficient costimulatory signal (2). The B7 antigen is one molecule on APC that is capable of providing costimulatory signals to T cells via cell contact (3–5). The B7 receptor is a 44 to 54 kDa Ag (6, 7) that is expressed on all APC of lymphoid origin examined to date (6, 8, 9). Recent studies have shown that B7 is the counter-receptor for both the CD28 and CTLA-4

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receptors expressed on T cells (10, 11).

Within the Ig gene superfamily, CD28 and CTLA-4 are closely related adhesion molecules that are members of the subfamily that contain single V-like domains (12, 13). The genomic colocalization of CTLA-4 and CD28 within 50 kb of each other, on band q33 of human chromosome 2 (14 and T. Lindsten, unpublished data), and on mouse chromosome 1 band C (15), suggests that the receptors arose by gene duplication, and that this event occurred before speciation (16, 17).

In both the murine and human immune systems, CD28 seems to have an important role in the delivery of costimulatory signals (reviewed in Reference 18). Experiments using mAb as a ligand of the CD28 receptor have shown that CD28 can regulate lymphokine expression by both transcriptional and post-transcriptional mechanisms (19, 20). Anti-CD28 mAb prevents the development of clonal anergy in mouse T cell clones (21). Several laboratories have shown that the interaction of T cells with B7 expressed on lymphoid and nonlymphoid cells is capable of regulating T cell lymphokine production (3, 4, 22) and is able to induce T cell cytotoxicity (23). Recent studies in vivo suggest that the interaction of B7 with ligand is necessary for efficient cell-mediated rejection of allografts (24–26).

CTLA-4 was originally identified as the fourth cDNA during a search for genes that are specifically expressed in cytotoxic T cells, and hence the name "cytolytic T lymphocyte associated antigen," CTLA-4 (13). The genes for both human and mouse CTLA-4 share a similar exon and intron structure with each other and with human and mouse CD28 (17, 27), and exhibit extensive homology at the nucleotide level. The extended interspecies conservation of CTLA-4 is seen in the high degree of overall homology between the human and mouse proteins (>70%), and in the complete identity of the cytoplasmic domains (17). In human T cells, CD28 and CTLA-4 mRNA are coexpressed on activated $CD4^+$ and $CD8^+$ cells, and in nearly all T cell clones (28). In the mouse, CTLA-4 mRNA is not expressed in resting T cells, and is expressed in activated T cells, including $CD8^+$ CTL (13) and in $CD4^+$ T cell clones of both Th1 and Th2 subtypes (28). In contrast, CD28 is expressed on the surface of most resting T cells and thymocytes predominantly as a disulfide-bonded homodimer (29–32). Despite the similarities between the CD28 and CTLA-4 genes and their expression in T lymphocytes, a role for CTLA-4 in T cell activation has not been established. In the present study we report the requirements for CTLA-4 gene expression at the mRNA and protein levels. In T cells, CTLA-4 mRNA expression was found to be restricted to the $CD28^+$ subset. The binding of human CD28 mAb to T cells was found to increase CTLA-4 mRNA expression. An antiserum that specifically recognizes CTLA-4 immunoprecipitated a 41- to 43-kDa protein from the cell surface of activated T cells. Surprisingly, on SDS-PAGE analysis CTLA-4 isolated from the surface of cells migrated primarily as a monomer, and therefore, contrary to expectations, is not present as a disulfide-bonded dimer.

Materials and Methods

Antibodies

CD28 mAb 9.3 and CD3 mAb G19.4 were produced and purified as previously described (33). In cell culture experiments mAb G19.4 was cross-linked to the surface of plastic dishes at a concentration of 1 μg/ml, as previously described (34, 35). mAb 9.3 was used at final concentration of 1 μg/ml in cell cultures. Rabbit antiserum 1438 against the extracellular domain of hCTLA-4 was generated as follows: human CTLA-4 plasmid pRIV2/7914 was digested with *Pml*I and *Bam*HI and a ~385-bp fragment encoding most of the extracellular domain of CTLA-4 was purified by electrophoresis in low temperature gelling agarose. The fragment was ligated into the *Eco*RV/*Bam*HI sites of the expression vector pRDC2.2. The ligated product was transformed into *Escherichia coli* JM105, selected by ampicillin resistance, and the recombinant plasmids verified by restriction enzyme digestion. Expression vector pRDC2.2 directs the constitutive expression of fusion proteins in which the gene of interest is fused to the first ~25 amino acids of β-glucuronidase. Plasmid DNA containing the extracellular domain of CTLA-4 (pCTLA4–2) was transformed into *E. coli* expression strain RGN715.

The CTLA-4 expression strain (RGN715/pCTLA4–2) was grown in 2× Luria Broth overnight at 37°C with constant shaking. The cells were collected by centrifugation, suspended in lysis buffer (50 mM Tris, pH 7.6, 5 mM EDTA, 0.5% Triton X-100), and lysozyme (2 mg/g cell paste) was added. The cells were incubated for 1 h at 37°C, $MgSO_4$ was added to a final concentration of 15 mM, DNase was added to 500 U/100 g cell paste, and the cells were incubated for an additional 15 min. The cells were then homogenized and lysed using repeated combinations of polytron/sonication. The inclusion bodies containing recombinant human CTLA-4 were collected by centrifugation and washed twice with lysis buffer.

The inclusion bodies containing recombinant CTLA-4 at >50% purity were solubilized in extraction buffer (50 mM Tris, pH 7.6, 8 M urea, 5 mM EDTA, 10 mM dithiothreitol, 0.5% Triton X-100), buffer exchanged into 25 mM Tris, pH 7.6, 8 M urea, 5 mM EDTA, 10 mM β-ME and chromatographed on a DEAE column. Under these conditions, the CTLA-4 appears in the column flowthrough and most contaminating proteins were retained by the DEAE. The flowthrough fraction containing soluble human CTLA-4

(hsCTLA-4)[4] was then chromatographed on a Sephadex S-200 (Pharmacia, Piscataway, NJ) sizing column, and the fractions containing recombinant hsCTLA-4 (>90% pure) were pooled.

Polyclonal antisera to the recombinant hsCTLA-4 was prepared by immunizing rabbits with 250 μg hsCTLA-4 in complete Freund's adjuvant and boosting at 2-wk intervals with 125 μg in incomplete Freund's adjuvant. Rabbits were bled 2 wk after the final boost, and the titer of anti-CTLA-4 antibodies was determined by ELISA. Rabbit 1438 yielded a high titer anti-CTLA-4 antiserum. ES5.2D8 is an IgG2b monoclonal antibody that binds to activated T cells and was used as a control in some experiments. KT3 mAb was a gift from G. Walter (36).

Cells

Human $CD28^+$ T cells were purified by a previously described negative selection method (35). To obtain $CD28^-$ T cells, negative selection using immunobeads was preformed as previously described (37), taking advantage of the reciprocal expression of the cell surface Ag CD11b and CD28 on T cells. Cells were cultured at 2×10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES. Cells were stimulated with cross-linked anti-CD3 mAb G19–4 with or without anti-CD28 mAb 9.3 (34, 35). PMA or PDBU (Sigma, St. Louis, MO) was used at a final concentration of 10 or 100 ng/ml, and ionomycin (Calbiochem, La Jolla, CA) at 0.4 μg/ml.

Northern blot analysis

Cells were harvested after stimulation and total RNA was extracted and equalized for rRNA, as previously described (35, 38). Equalized RNA samples were separated on 1% agarose/formaldehyde gels and transferred to nitrocellulose. The following DNA probes, labeled by nick translation, were used: a 1.5-kb CD28 cDNA (12) and a 1.4-kb HLA-B7 cDNA (39). A CTLA-4 cDNA clone was obtained by the polymerase chain reaction (PCR) using RNA from $CD28^+$ T cells stimulated with anti-CD3 plus anti-CD28 for 24 h. The CTLA-4 cDNA sequence has been described previously (17). First strand cDNA synthesis of the predicted coding region was performed (27) utilizing a 3′ oligonucleotide primer (GGGC GGC CGC CAT AAT GGT TTC [TCA] ATT GAT GGG AAT) which spans the stop codon (brackets) in exon 4. PCR was then performed using the same 3′ oligonucleotide primer and 5′ primer (GG GGA AGC TTA GCC [ATG] GCT TGC CTT GGA TTT CAG CGG CA) which spans the predicted initiation codon (brackets). PCR was performed for 30 cycles (94°C for 1.5′, 56°C for 1′, 72°C for 4′, followed by 72°C × 10′ final extension step). PCR products were subsequently digested with *Hin*dIII and NotI (to cleave restriction enzyme sites contained in the oligonucleotide primers) and cloned into Bluescript SK plasmid vector (Stratagene, San Diego, CA). The veracity of the CTLA-4 cDNA clones was confirmed by DNA sequencing using the Sequenase kit according to supplier's protocol (U.S. Biochemical Corp., Cleveland, OH).

In vitro transcription and translation of CTLA-4 protein

To facilitate identification of the CTLA-4 protein, a genetic chimera was produced by fusing the epitope recognized by the KT3 mAB to the 3′ end of the CTLA-4 coding region (40). Using the CTLA-4 cDNA clones derived above as DNA templates, PCR was performed using the same 5′ oligonucleotide primer and a 3′ oligonucleotide primer (GGC GGC CGC [TCA] TGT TTC TGG TTC TGG TGG TGG TGT **ATT GAT GGG AAT AAA**) which overlaps the last 5 codons of the CTLA-4 coding region (bold) and the stop codon (brackets) and contains the epitope from the 3′ end of the SV40 large T antigen recognized by KT3 (underlined). PCR, subcloning, and sequence verification were performed as above. In vitro transcription and translation were then performed according to manufacturer's protocol (Promega Gemini System II Ribo probe p2020 and Promega Wheat Germ Lysate, Promega, Madison, WI) using the fusion construct. Translation was performed in the presence of 10 mM final concentration of sodium acetate and 0.8 mCi/ml [^{35}S]methionine (in vitro translation grade, specific activity >1000 Ci/mmol, ICN, Irvine CA). Immunoprecipitations were performed on 20% of the total translation mix in a final volume of 100 μl, as outlined below.

Metabolic labeling and immunoprecipitation

Purified $CD28^+$ T cells were washed twice in methionine-free, cysteine-free DMEM (Gibco-BRL, Grand Island, NY). The cells were then resuspended at 1×10^6/ml in 9 parts methionine-free, cysteine-free DMEM supplemented with 10% dialyzed fetal calf serum and 1 part RPMI supplemented with 10% fetal calf serum. To the cell suspension were added 200 μCi/ml [^{35}S]methionine (Translabel, ICN), PMA at a final concentration of 10 ng/ml, and ionomycin at 0.4 μg/ml. The cells were activated for 6 to 36 h to induce CTLA-4 expression. After biosynthetic labeling and cellular stimulation, the cells were solubilized in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 0.1 mM EGTA, 1 mM EDTA, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 0.01 mM *N*-tosyl-L-lysine chloromethyl ketone (TLCK), 0.4 mM phenylmethanesulfonyl fluoride (PMSF),

[4] Abbreviations used in this paper: hsCTLA-4, recombinant soluble human CTLA-4; PCR, polymerase chain reaction.

and 1.2 μM aprotinin) at 10×10^6 cells/ml. Immunoprecipitations were performed with an aliquot of lysate (250 μl) representing 2.5×10^6 cell equivalents. After preclearing the extracts with a 2% final solution of Pansorbin (Calbiochem, La Jolla, CA), 1 μl of the 1438 antiserum was added, followed by 2 h incubation at 4°C. In immunoprecipitations with the in vitro translated CTLA-4/SV40 large T antigen fusion protein, 25 μl or 50 μl of culture supernatant from the KT3 hybridoma, or 10 μl of ascites from the ES5.2D8 hybridoma was used. 50 μl of a 3% protein A Sepharose (Pharmacia) solution was then added, and the incubation was continued on a rocking platform at 4° for 2 h. The immunoprecipitates were then washed in 10 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1% deoxycholate, and 0.1% SDS, dissolved in SDS sample buffer in the presence of β-ME, boiled, and loaded on a 9.5% SDS-polyacrylamide gel. After electrophoresis, the gels were fixed in 40% methanol, 10% glacial acetic acid, enhanced with Amplify (Amersham, Arlington Heights, IL), dried, and exposed to x-ray film. In blocking experiments, 1 μl of the 1438 antiserum was diluted in 10 μl of phosphate-buffered saline and incubated with 10 μl of hsCTLA-4 at 0.9 mg/ml for 2 h at room temperature.

Cell surface labeling and immunoprecipitation

Purified T cell subsets were cultured as indicated, washed, and lactoperoxidase-catalyzed cell surface iodination performed as described (41). Cells were lysed in ice-cold buffer (50 mM Tris, pH 7.6, 0.5% Triton X-100 or 30 mM octylglucoside, 150 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin and leupeptin, 25 μg/ml *p*-nitrophenyl-guanidinobenzoate). 10 mM iodoacetamide was included in the lysis buffer to alkylate free sulfhydryl groups. Detergent soluble supernatants were precleared with preimmune rabbit serum and protein A agarose, followed by immunoprecipitation with 1438 serum or mAb 9.3 for 4 h. Protein A Sepharose CL-4B (Pharmacia) was used to precipitate the immune complexes. The beads were washed, bound proteins were eluted by boiling in sample buffer, followed by SDS-PAGE on 8.5% to 12.5% gels, and the results were assessed by phosphoimagery (Phosphorimager, Molecular Dynamics, Sunnyvale, CA).

Results

CTLA-4 gene expression is limited to the CD28$^+$ subpopulation of T cells

CD28 is expressed on the surface of thymocytes, a major subset of resting and activated T cells, and on plasma cells. In contrast, available studies indicate that expression of CTLA-4 mRNA is more limited and is restricted to activated T cells (17), HTLV-1 transformed T cell lines (28), and possibly activated murine splenic B cells (13). In agreement with previous studies, we did not detect CTLA-4 mRNA in purified resting peripheral blood T cells (Fig. 1).

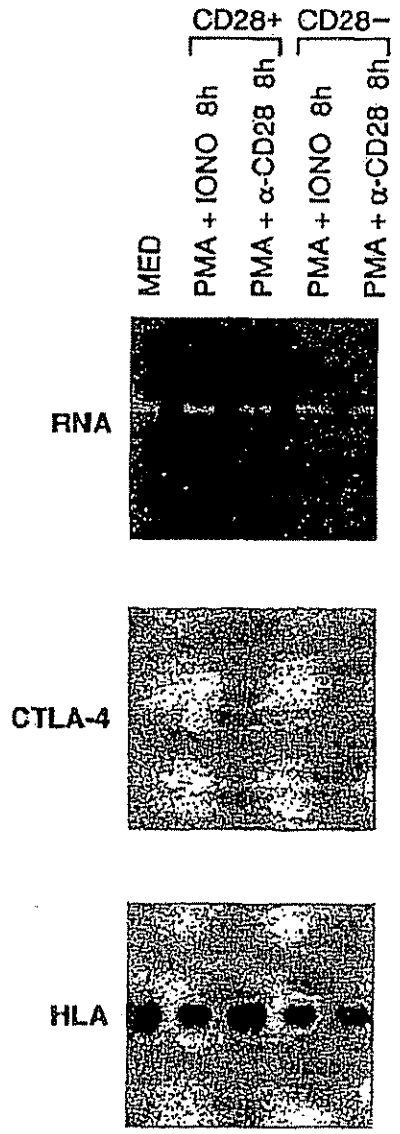

FIGURE 1. Expression of CTLA-4 mRNA in T cell subpopulations. Resting T cells were cultured in medium (*MED*) or fractionated into the CD28$^-$ and CD28$^+$ subsets using magnetic immunobead negative selection. The cells (2×10^6/ml) were cultured in the presence of PMA (3 ng/ml) plus ionomycin (0.4 μg/ml) (*PMA + IONO*), or PMA plus soluble anti-CD28 mAb 9.3 (1 μg/ml) for 8 h (*8 h*). RNA was isolated and equalized for rRNA, and the equalization confirmed by ethidium bromide staining (*upper panel*). Northern blots were prepared and the filters were hybridized sequentially with cDNA probes specific for CTLA-4 and HLA class I mRNA.

Previous studies have shown that CTLA-4 mRNA is expressed in activated CD4$^+$ and CD8$^+$ T cells (28). CD28 defines a subpopulation of T cells that includes ~95% of CD4$^+$ cells and about 50% of CD8$^+$ cells. To examine expression of CTLA-4 mRNA across this subset of T cells, peripheral blood T cells were separated into >95% pure

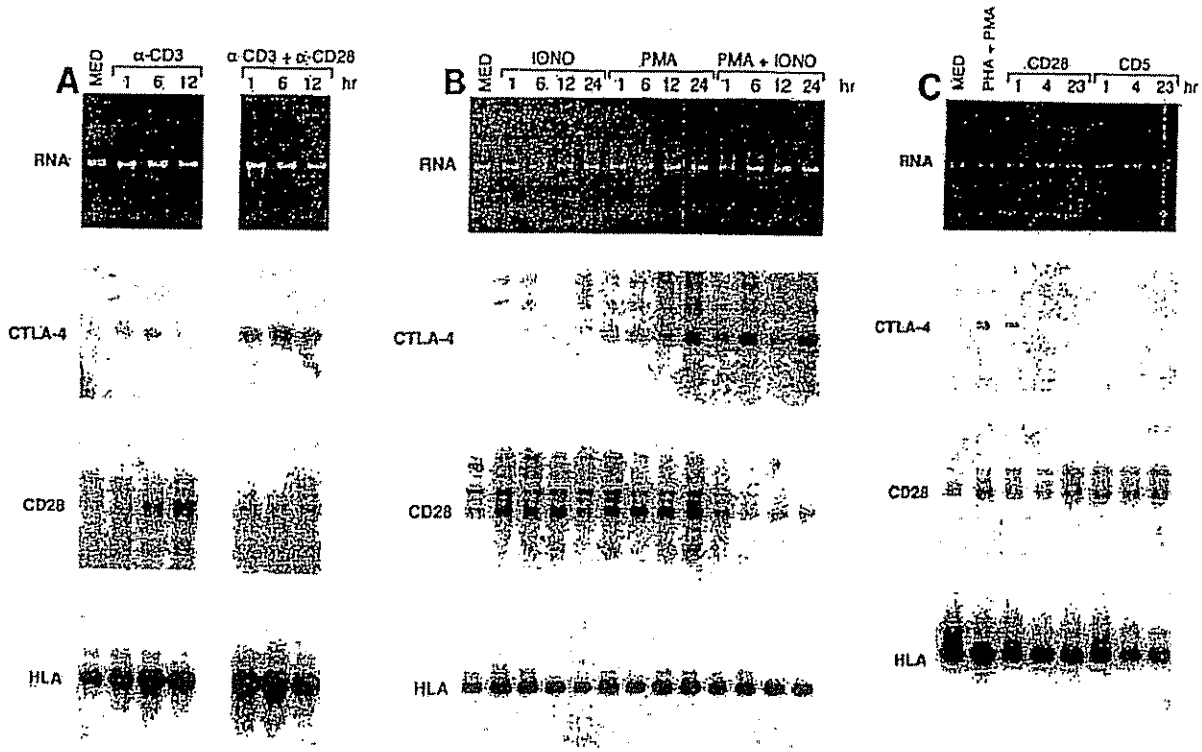

FIGURE 2. Requirements for induction of CTLA-4 gene expression. Peripheral blood $CD28^+$ T cells (2×10^6/ml) were cultured in the presence of (*A*) medium (*MED*), plastic immobilized anti-CD3 mAb G19–4, or anti-CD3 mAb plus soluble anti-CD28 mAb 9.3 (0.5 μg/ml) for 1, 6, or 12 h; (*B*) medium (*MED*), ionomycin (*IONO*) (0.4 μg/ml), PMA (3 ng/ml), or PMA plus ionomycin (*PMA + IONO*) for 1, 6, 12, or 24 h; or (*C*) medium (*MED*), PHA 20 μg/ml plus PMA 10 ng/ml for 23 h, or biotinylated anti-CD28 mAb 9.3 (*CD28*) or biotinylated anti-CD5 mAb 10.2 (*CD5*) plus avidin for 1, 4, or 23 h. In all experiments, the cells were collected after stimulation, lysed, and Northern blots prepared as described in Figure 1.

$CD28^+$ and $CD28^-$ populations (37). The cells were stimulated for 8 h with PMA and ionomycin, or with PMA and anti-CD28 mAb, and the expression of CTLA-4 was determined by Northern blot analysis. CTLA-4 expression was limited to the $CD28^+$ population of T cells (Fig. 1). Thus, these results confirm that CTLA-4 mRNA is only expressed in activated T cells. Further, a subpopulation of T cells has been identified that does not express CTLA-4 mRNA. These results also imply that all cells that express CTLA-4 mRNA coexpress CD28.

Stimulation requirements for CTLA-4 gene expression

To characterize the signals required for induction of CTLA-4 gene expression, purified $CD28^+$ T cells were subjected to a variety of stimuli. As expected from previous studies (17, 28), T cells expressed CTLA-4 mRNA after stimulation with the potent and relatively nonspecific mitogenic combination of PHA and PMA (Fig. 2*C*). CTLA-4 gene expression is also rapidly induced by cross-linking of the T cell receptor/CD3 complex with an anti-CD3 monoclonal antibody (Fig. 2*A*). CTLA-4 mRNA is present within 1 h after anti-CD3 stimulation and peaks at 6 h. CD28 receptor costimulation leads to an augmentation in CTLA-4 gene expression compared with that induced by anti-CD3 treatment alone (Fig. 2*A*). To further dissect the signals necessary to induce CTLA-4 gene expression, T cells were cultured in the presence of the pharmacologic agents ionomycin and PMA (Fig. 2*B*). Mobilization of intracellular calcium following stimulation with ionomycin alone did not induce CTLA-4 gene expression. In contrast, protein kinase C activation by PMA alone was sufficient to induce CTLA-4, with expression continuing to increase throughout the 24-h experiment. However, the combination of PMA and ionomycin stimulation had a synergistic effect, and resulted in earlier peak levels of CTLA-4 mRNA expression. A biphasic course of CTLA-4 mRNA expression was found after PMA plus ionomycin stimulation, with early and late peaks of mRNA levels. It is not yet known if this is a consistent finding, because further experiments to test this observation have not been performed. These early and late CTLA-4 peaks were specific, because the level of HLA class I mRNA remained relatively constant throughout the experiment.

The primary signal transduction pathways activated by

the CD28 receptor remain to be determined, but are known to involve calcium mobilization and protein tyrosine phosphorylation that is distinct from the T cell receptor (42, 43). Interestingly, cross-linking of the CD28 surface structure also leads to the rapid (≤1 h) induction of CTLA-4 mRNA as shown in Figure 2C. Induction of CTLA-4 expression by CD28 was somewhat specific, because cross-linking of CD5 resulted in transient, low-level CTLA-4 mRNA expression at the 1-h time point. Inhibition of anti-CD28-induced tyrosine phosphorylation with herbimycin C prevents the induction of CTLA-4 gene expression (data not shown). It should be noted that CD28 mRNA expression is present in resting cells, and in agreement with previous studies (44), is further induced by PMA or anti-CD3 stimulation. Interestingly, CD28 and CTLA-4 mRNA appear to be reciprocally expressed under some conditions. Combined treatment of T cells with PMA and ionomycin leads to the induction of CTLA-4 mRNA, and to a concomitant decrease in CD28 mRNA (Fig. 2*B*). The decrease in CD28 mRNA expression was most marked at 6 h after stimulation, and represented three- to fourfold decrease compared to baseline levels, as estimated by densitometric analysis of the autoradiogram (data not shown). Thus, CTLA-4 gene expression is induced after protein kinase C activation via PMA stimulation, and by cross-linking of the TCR/CD3 complex. CD28 costimulation, under conditions leading to substantial augmentation of T cell lymphokine production, also augments CTLA-4 mRNA expression. In addition, cross-linking of CD28 alone is sufficient to induce CTLA-4 mRNA accumulation.

CTLA-4 mRNA is not expressed nor induced in the Jurkat T cell line

We compared the expression of CTLA-4 in normal T cells to the Jurkat leukemia T cell line. Similar to normal, resting $CD28^+$ T cells, CTLA-4 is not expressed in unstimulated Jurkat T cells, while CD28 was constitutively expressed in both cell types, although at substantially higher levels in Jurkat cells (not shown). However, unlike normal T cells, treatment of Jurkat cells with PHA plus PMA did not induce CTLA-4 expression. In addition, treatment of Jurkat cells with PMA plus ionomycin or PMA plus ionomycin plus anti-CD28, conditions that induce IL-2 gene expression in Jurkat, did not induce CTLA-4 gene expression. Thus, induction of CTLA-4 expression is not required for induction of IL-2 synthesis, at least in transformed cell lines.

Characterization of rabbit anti-hsCTLA-4 antiserum

Previous studies have not identified or characterized CTLA-4 protein expression. To determine the structure of the CTLA-4 protein on the surface of the T cell and its possible relationship to CD28, rabbits were immunized with a bacterially derived fusion protein containing the extracellular domain of CTLA-4 (hsCTLA-4) as described in *Materials and Methods*. To establish specificity of the antiserum, a construct was made containing the full-length CTLA-4 cDNA to which eight amino acids from the C-terminus of the SV40 large T Ag were added as an epitope tag to the C-terminus of CTLA-4. The fusion protein containing this epitope tag is recognized by the KT3 mAb (36, 40) and was produced by in vitro transcription and translation. As can be seen in Figure 3*A*, one antiserum, 1438, precipitates material that migrates at 25 and 17 kDa from the in vitro translated CTLA-4/SV40 large T Ag construct. The 25-kDa band is assumed to be full-length CTLA-4 fusion protein, because it corresponds well to the predicted migration of the construct (24.9 kDa), whereas the 17-kDa material likely represents partially transcribed

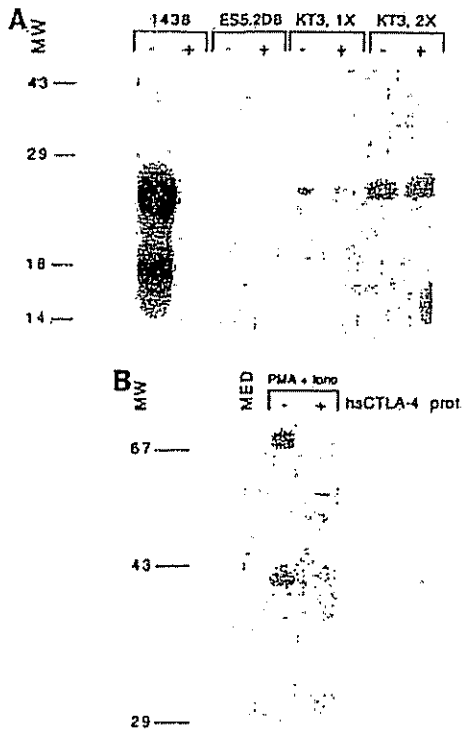

FIGURE 3. CTLA-4 protein expression in [^{35}S]methionine-labeled lysates from an in vitro translation mix or from activated T cells. *A*, In vitro-translated CTLA-4/SV40 large T antigen fusion protein was subjected to immunoprecipitation with rabbit anti-CTLA-4 antiserum 1438, KT3 mAb at two different concentrations (1× and 2×), or control mAb ES5.2D8 (see *Materials and Methods* for details). The antiserum or mAb were preincubated in the presence (+) or absence (−) of hsCTLA-4 before immunoprecipitation of the wheat germ lysates. *B*, Rabbit anti-CTLA-4 antiserum 1438 precipitates a 41-kDa protein (←) from activated T cells. $CD28^+$ T cells were labeled with [^{35}S]methionine during a 12-h stimulation with PMA plus ionomycin, and whole cell lysates subjected to immunoprecipitation. Antiserum 1438 was preincubated in the presence (+) or absence (−) of hsCTLA-4.

material. No immunoprecipitation was observed if the antiserum 1438 was preincubated with the immunogen (Fig. 3*A*). KT3 also was found to immunoprecipitate a 25-kDa protein, which was not inhibitable with the immunogen hsCTLA-4 (Fig. 3*A*). On longer exposures, the 17-kDa protein seen in the immunoprecipitations with 1438 was also present in the KT3 immunoprecipitates (not shown). Further, the effect of KT3 was specific because a control mAb did not precipitate the in vitro translated CTLA-4/SV40 large T antigen protein (Fig. 3*A*).

CTLA-4 is expressed as a 41- to 43-kDa protein in lysates of ^{35}S-labeled activated T cells

With the specificity of the 1438 antiserum established, we set out to identify and characterize the structure of the CTLA-4 protein in purified CD28$^+$ T cells. T cells were cultured for 12 h in the presence of PMA plus ionomycin to induce CTLA-4 mRNA expression, and were labeled with [^{35}S]methionine, as described in *Materials and Methods*. Postnuclear cell lysates were prepared and immunoprecipitated with the 1438 antiserum (Fig. 3*B*). The 1438 antiserum immunoprecipitates multiple bands from activated but not resting T cells, including material from T cell blasts that migrates at 41 to 43 kDa under reducing conditions. In contrast to the in vitro translated protein which has an apparent molecular mass of 25 kDa, the predominant cellular form of CTLA-4 recognized by antiserum 1438 has a migration of 41 to 43 kDa. This range is presumably caused by a variable amount of glycosylation or other processing. Similar results were obtained when T cell blasts were prepared by stimulation with cross-linked anti-CD3+IL-2 (data not shown). Complete inhibition of the 41- to 43-kDa bands and partial inhibition of the 34 kDa and 70- to 75-kDa bands was observed when the 1438 antiserum was preincubated with hsCTLA-4 (Fig. 3*B*), suggesting that these bands represent CTLA-4 in various stages of assembly or degradation. Alternatively, some of the bands could represent molecules associated with CTLA-4, or cross-reactive material of unknown identity.

CTLA-4 is expressed as a 41- to 43-kDa monomer on the surface of activated T cells

To study surface expression of CTLA-4, purified CD28$^+$ T cells were stimulated under a variety of conditions, and surface-labeled with lactoperoxidase-catalyzed radioiodination (Fig. 4). Consistent with previous reports (29–32), a wide band that was centered at 44 kDa was immunoprecipitated by CD28 mAb 9.3 under reducing conditions from both resting and PMA plus ionomycin-activated T cells. Approximately 30-fold more CD28 was precipitated from activated T cells than from resting T cells (Fig. 4*A*). In contrast, when lysates from T cell blasts were subjected to immunoprecipitation with the 1438 antiserum, a 41- to 43-kDa band was observed that was more sharply defined than CD28. Minor bands were also observed at 35 kDa and 90 kDa, but were observed less consistently and represented less than 10% of precipitated counts. Prolonged exposure of the gels from resting T cells revealed that small amounts of material were precipitated by 1438 (Fig. 4*A*, *left panel*). This material also migrated at 41- to 43-kDa, and was more than 300-fold less intense than the amount found in immunoprecipitates from activated T cells.

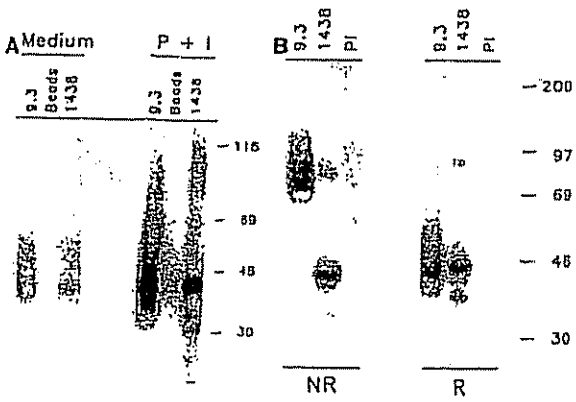

FIGURE 4. CTLA-4 is expressed as a 43-kDa monomer on the surface of activated T cells. CD28$^+$ peripheral blood T cells were isolated and surface labeled with ^{125}I by lactoperoxidase-catalyzed iodination. *A*, Cells were cultured in medium or PMA plus ionomycin (*P + I*) for 26 h, labeled, and membrane proteins solubilized in lysis buffer containing 30 mM octylglucoside. Immunoprecipitation of 10^6 cpm/sample was done with anti-CD28 mAb 9.3, anti-CTLA-4 antiserum 1438, or protein A beads alone (*Beads*), followed by 8.5% SDS-PAGE under reducing conditions. *B*, Cells were stimulated with PHA plus PMA for 24 h, labeled, and immunoprecipitations with mAb 9.3, rabbit antiserum 1438 or preimmune rabbit serum (*PI*) performed under reducing (*R*) or nonreducing (*NR*) conditions, followed by SDS-PAGE on an 8.5% gel.

CTLA-4 is not detected as a disulfide-bonded homo- or heterodimeric receptor

The predicted amino acid sequence of both human and mouse CTLA-4 and CD28 contains 5 cysteine residues in the extracellular domain (17). Four of these residues are expected to form the intrachain disulfide bonds required for the Ig-like V region, whereas a fifth membrane proximal cysteine exists. This unpaired cysteine is thought to be involved in the formation of a disulfide-bonded homodimer in the case of CD28, and has been predicted to function similarly in CTLA-4 (17). T cell blasts were prepared by stimulation with PHA plus PMA and were surface iodinated. When lysates from these cells were analyzed, 1438 again immunoprecipitated a band at 41 to 43 kDa under reducing conditions, while CD28 migrated as a more diffuse band (Fig. 4*B*). Surprisingly, material precipitated by

1438 also migrated at 41 to 43 kDa under nonreducing conditions, whereas, as expected, CD28 migrated as a broad smear centered at 90 kDa (Fig. 4*B*). The material precipitated by 1438 displayed somewhat less mobility under reducing than in nonreducing conditions, consistent with the presence of intrachain disulfide bonds. Preimmune serum from rabbit 1438 did not precipitate material from these lysates.

We considered that antiserum 1438 might recognize CD28, given the homology at the protein level. Although this was unlikely considering the scant amount of material precipitated by 1438 from CD28$^+$ resting T cells (Figs. 3*B* and 4*A*), immunoprecipitations were performed on Chinese hamster ovary cells transfected with CD28. The rabbit 1438 antiserum did not immunoprecipitate any protein from the CD28 transfectants, although anti-CD28 mAb 9.3 precipitated large amounts of CD28 (data not shown).

It was also possible that CTLA-4 might pair with CD28 and form a heterodimeric receptor, given the presence of an "unpaired" cysteine in CTLA-4 and CD28, and given that free subunits of CD28 can exist on the cell surface (29). This could potentially account for the small amount of material in the 90-kDa range that was immunoprecipitated with antiserum 1438. To test this possibility, T cell blasts were surface-labeled, and detergent lysates were prepared under nonreducing conditions. The lysates were precleared with anti-CD28 mAb 9.3 or anti-CTLA-4 serum 1438. The material precipitated by 1438 was not depleted in lysates precleared with anti-CD28, although CD28 was efficiently precleared (Fig. 5). Similarly, anti-CTLA-4 serum 1438 did not preclear the CD28 glycoprotein (Fig. 5).

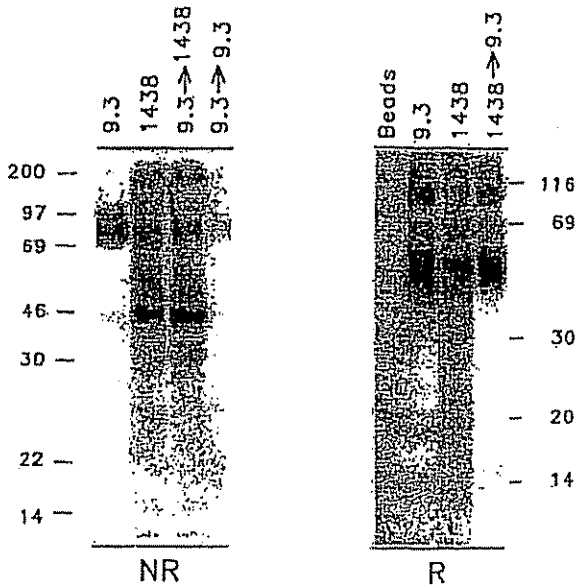

FIGURE 5. CTLA-4 and CD28 do not coprecipitate. CD28$^+$ peripheral blood T cells were stimulated in PHA plus PMA for 1 day (*left panel*) or for 4 days (*right panel*). After lysis and extraction in detergent buffer (0.5% Triton X-100), lysates were precleared with mAb 9.3 followed by a secondary immunoprecipitation with 1438 (9.3 → 1438) or 9.3 (9.3 → 9.3; *left panel*) or they were precleared with 1438 followed by immunoprecipitation with 9.3 (1438 → 9.3; *right panel*). Extracts were also immunoprecipitated with 9.3 and 1438 alone or with protein A beads (*Beads*) before SDS-PAGE on a 12.5% gel under reducing (*R*) or nonreducing (*NR*) conditions. The positions of molecular mass markers are indicated in kilodaltons.

Discussion

In the present study we have used T cells activated by a variety of stimuli to study CTLA-4 expression and structure. CTLA-4 mRNA expression was not detectable in quiescent T cells, although it was induced within 1 h after ligation of the TCR or CD28 receptor. The rapid expression of CTLA-4 mRNA was surprising because previous studies had not examined expression during the early phases of T cell activation. The significance of the early expression remains to be determined, but suggests that CTLA-4 protein is available on the surface of T cells to function in the G1 phase and in subsequent phases of the cell cycle.

Both protein kinase C activation and anti-CD3 stimulation were able to induce CTLA-4 gene expression. Surprisingly, stimulation by anti-CD28 mAb alone induced CTLA-4 mRNA expression, so that signals delivered by the T cell receptor, as well as costimulatory signals would be expected to induce CTLA-4 expression. Further studies will be required to determine the mechanism of CTLA-4 gene regulation, but the ability of anti-CD28 to augment anti-CD3-induced CTLA-4 expression is reminiscent of CD28-induced effects on lymphokine synthesis (19, 20, 45), and suggest that both transcriptional and post-transcriptional mechanisms may play a role. Indeed, recent studies have shown that anti-CD2 plus anti-CD28 stimulation is capable of transcriptional and post-transcriptional augmentation of IL-2R α (CD25) mRNA expression (46).

The rabbit antiserum 1438 raised against the extracellular domain of CTLA-4 has permitted the first identification and characterization of CTLA-4 at the protein level. In experiments with biosynthetically labeled T cells, we found that CTLA-4 protein was readily detectable during the first 12 h of T cell activation. In this respect, CTLA-4 seems to differ from CD28, because we and others (29) have had difficulty detecting CD28 in primary T cells by metabolic labeling, suggesting that the turnover of CD28 protein is quite slow as compared with CTLA-4.

Using SDS-PAGE analysis, we found that CTLA-4 is expressed primarily as a 41- to 43-kDa structure on the surface of activated T cells. It is likely that CTLA-4, like CD28, is heavily glycosylated because the apparent molecular mass of CTLA-4 greatly exceeds the 23.9-kDa

structure predicted by nucleic acid sequence, and the 25-kDa product observed following in vitro translation of our CTLA-4/SV40 construct. The characteristic "signature" of CD28 in immunoprecipitations is a poorly resolved band centered at 44 kDa under reducing conditions (29–32). This is observed in lysates of leukemic cell lines as well as primary T cells. In contrast, CTLA-4 migrates as a well resolved band, and did not seem to associate with CD28 on the cell surface. Detailed studies of expression of CTLA-4 on the cell surface will have to await the development of monoclonal antibodies, although our results from immunoprecipitation experiments of surface-labeled cells have shown that small amounts of CTLA-4 are detectable on resting T cells. Further studies will be required to determine whether the small amounts of CTLA-4 expressed on resting cells is present on all $CD28^+$ T cells, or on a subset of cells such as contaminating activated T cells. The later possibility is considered unlikely because lysates from resting T cells subjected to negative selection to remove T cells expressing MHC class II antigens fails to remove material that is immunoprecipitated by 1438 (not shown).

In previous work, we suggested that CTLA-4 might exist at the cell surface in both heterodimeric as well as homodimeric forms (28). Our present results do not exclude that possibility, and indicate that CTLA-4 migrates predominantly as a monomer upon SDS-PAGE analysis, with <10% of precipitated material displaying migration expected of a disulfide-bonded dimer. CTLA-4 displayed similar migration on SDS-PAGE analysis in both reducing and nonreducing conditions, whereas immunoprecipitates of CD28 displayed characteristics of a homodimer in the same experiments. It is likely that there are intrachain disulfide bonds in CTLA-4, because the migration of CTLA-4 was slightly retarded in reducing conditions compared with nonreducing conditions (Fig. 4*B*). Preclearing experiments followed by reciprocal immununoprecipitations failed to show any association between CTLA-4 and CD28. Peptide maps of CD28 and CTLA-4 immunoprecipitates are dissimilar (data not shown), further suggesting that a CTLA-4/CD28 disulfide-linked heterodimer is not a major species on the surface of T cell blasts. However, although our studies fail to provide evidence for homo- or heterodimeric CTLA-4, it is still possible that our antiserum fails to recognize dimerized CTLA-4 and is specific for a monomeric form of CTLA-4. Similarly, although it is known that mAb 9.3 recognizes monomeric and homodimeric CD28 (29), it is possible that the mAb does not immunprecipitate heterodimeric CD28/CTLA-4 receptors. Finally, it is possible that CTLA-4 and CD28 associate by a noncovalent process independent of disulfide bond formation.

It remains to be determined why CTLA-4 should exist predominantly as a non-disulfide-bonded molecule at the cell surface. Harper and co-workers previously have noted the presence of a fifth and presumably unpaired cysteine, Cys-92, just proximal to the putatative transmembrane region (17). This residue is conserved in both human and mouse CTLA-4, as well as in CD28. In the case of CD28, this unpaired cysteine is thought be involved in the disulfide linkage that pairs CD28 as a homodimer. There is precedence for an apparently reduced cysteine at this position, however, because human CD28 has been shown to exist in both monomeric as well as dimeric forms (29). Recent evidence suggests that MHC class I proteins may exist on the surface of cells in a monomeric form as well as a heterodimeric form (47).

It is possible that CTLA-4 exists as a homodimer or as a heterodimer, but that equilibrium favors the monomer. Given the probable, but still unproven assumption that ligand binding for B7 is a property confined to dimers or other higher order multimers of CTLA-4 and CD28, then it is likely that such disulfide exchange might be a regulated process. Thioredoxin or ADF (adult T-cell leukemia-derived factor) is a protein with T cell growth properties that could be a candidate for such a process (48). Finally, even though CTLA-4, and to a lesser extent CD28, appear as monomeric structures at the cell surface under the conditions that we have explored, it is likely that signal transduction through these molecules requires clustering or aggregation, if not dimerization (49). Indeed, "spontaneous" clustering of monomeric receptor ligand pairs can occur and appears to be thermodynamically favored, as first shown by Weis and co-workers (50).

To date our studies have not revealed a functional role for CTLA-4. Since submission of our manuscript, Linsley and co-workers have published studies demonstrating that anti-CTLA-4 mAb can be synergistic with anti-CD28 mAb in their ability to augment anti-TCR-induced proliferation of preactivated T cells (51). Based on the present observations concerning the independent pattern of expression of CD28 and CTLA-4, it remains possible that instead of potential synergistic roles in T cell activation, that they may instead play distinct and independent roles. Hypothetical functions of CTLA-4 must account for the perfect conservation at the protein level of the cytoplasmic domain of CTLA-4 (17) as well as the high affinity binding of the extracellular domain of CTLA-4 for B7 (11). Predictions concerning CTLA-4 function should also take into account the similarities to CD28 in terms of structure, sequence, tissue expression, and chromosomal location.

Acknowledgments

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EXHIBIT 8

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review

The B7 and CD28 receptor families

Carl H. June, Jeffrey A. Bluestone,
Lee M. Nadler and Craig B. Thompson

Current evidence suggests that T-cell receptor (TCR) recognition of antigen bound to the major histocompatibility complex (Ag–MHC) is insufficient to lead to T-cell proliferation or effector function. For a helper T cell to produce sufficient interleukin 2 (IL-2) to allow autocrine-driven clonal expansion, there is a requirement for so-called 'co-stimulatory' or 'accessory' signals in addition to TCR ligation by Ag–MHC. The interaction of the CD28 receptor on T cells with B7 on antigen-presenting cells (APCs) supplies one such co-stimulatory signal. However, the recent discovery that CD28 and B7 are each members of larger gene families suggests that the regulation of co-stimulation is more complex than previously imagined. Here, Carl June and colleagues highlight recent advances in the understanding of the CD28 and B7 receptor families.

A two-signal model of lymphocyte activation has been developed and refined by a number of investigators (reviewed in Ref. 1). The model was originally proposed to address the problem of self–nonself discrimination and, in the case of T cells, is particularly relevant to antigens that are not expressed or encountered in the thymus. The key observation of Lafferty and coworkers in the 1970s was that foreign antigens on tissues of non-hematopoietic origin were unable to induce a productive cellular immune response *in vivo* unless viable hematopoietic cells were also provided as a source of 'co-stimulatory activity'. In its simplest form, as originally proposed by Bretcher and Cohn, the model describes how an individual antigen-presenting cell (APC) can independently display either the antigen bound to the major histocompatibility complex (Ag–MHC) and/or the co-stimulatory ligand. Given the binary nature of this model, it is possible to predict three distinct outcomes consequent to the interaction between the APC and the T cell (Fig. 1). Activation of the T-cell receptor (TCR) in the presence of co-stimulatory signals results in T-cell clonal expansion and the induction of effector functions such as the production of lymphokines. By contrast, the interaction of T cells with cognate Ag in the absence of co-stimulatory ligand is not a neutral event but, rather, leads to induced unresponsiveness or to death by apoptosis. Finally, when resting T cells encounter co-stimulatory ligand in the absence of cognate antigen, no effects are predicted. Jenkins, Schwartz and others suggested in the 1980s that the co-stimulatory signal was fundamentally different from the signals delivered by the TCR (Ref. 2). This led to a search for T-cell surface molecules present on resting T cells that had signal-transducing properties distinct from the TCR.

Identification of CD28 as a co-stimulatory receptor on T cells

The first evidence that CD28 could initiate signal-transduction events that were distinct from those initiated through the TCR came following the observation that CD28, in conjunction with phorbol myristate acetate (PMA), induced cyclosporin (CsA)-independent T-cell proliferation and interleukin 2 (IL-2) production (reviewed in Refs 3,4). Although it is difficult to observe any effects of CD28 stimulation by itself on resting T cells, CD28 stimulation in conjunction with TCR stimulation can dramatically augment the production of multiple lymphokines. Augmentation of IL-2 production was observed both in $CD4^+$ and $CD8^+$ T-cell subsets. These properties of CD28 signaling bore

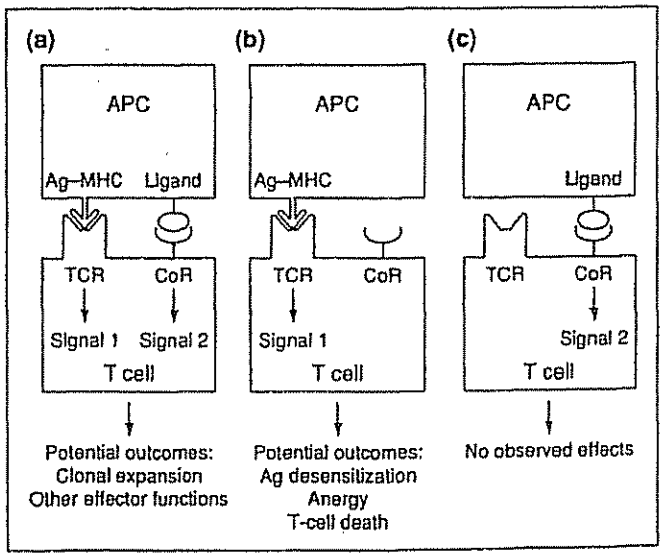

Fig. 1. *Model of potential outcomes of the interaction between T cells and antigen-presenting cells (APCs). Individual APCs express antigen bound to the major histocompatibility complex (Ag–MHC) and/or B7-family ligands. Encounter with resting T cells expressing T-cell receptor (TCR) and B7 co-receptors (CoR), such as CD28 and CTLA-4, results in either (a) clonal expansion, (b) anergy or apoptosis, or (c) no effect, depending on the combination of signals provided by the APC.*

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review

a striking similarity to the co-stimulatory signal that Schwartz and colleagues reported to be required for expansion of mouse T-cell clones[5]. Similarly, it was shown that CD28 activation could provide a co-stimulatory signal for IL-2 in antigen-specific T-cell clones[6]. Furthermore, prevention of CD28 activation with Fab fragments of an anti-CD28 antibody during the activation of a T-cell clone by antigen-primed APCs resulted in the failure of the clone to respond to a subsequent antigenic challenge[7]. An allogeneic mixed lymphocyte reaction (MLR) could also be blocked by Fab fragments of anti-CD28 mAb (Ref. 8), and an MLR could be prevented and anergy induced by the combination of anti-B7 antibody and CsA treatment[9]. Together, these data suggested that CD28 was an obligate co-stimulatory receptor for the activation both of resting T cells and T-cell clones.

Identification of B7 as a stimulatory ligand for the CD28 activation pathway

The first identification of a ligand for CD28 was reported by Linsley and co-workers when they demonstrated that antibodies to B7 could block the adhesion of B cells to Chinese hamster ovary (CHO) cells transfected with CD28 (reviewed in Ref. 4). Subsequently, it was shown that cells transfected with B7 could provide co-stimulatory signals to antigen- or mitogen-activated T cells[10,11]. Antibodies to B7 were also shown to block an allogeneic MLR induced by B cells transformed with Epstein–Barr virus (EBV) (Refs 6,9). These studies led to the conclusion that the B7 molecule was the natural ligand for CD28.

It is clear that B7–CD28 interactions provide secondary co-stimulatory signals to T cells since B7-induced CD28 ligation in the absence of cognate Ag interaction does not alter immune responses (as suggested in the model shown in Fig. 1). For instance, in transgenic mice that express the gene encoding B7 under the control of the insulin promoter, T-cell activation does not occur in the absence of foreign antigen and immune reactivity of islet cells is unaffected[12]. By contrast, self tolerance could be broken, and type 1 diabetes induced, when B7 was expressed in the pancreas in the context of self-reactive TCRs. Furthermore, ectopic B7 expression has proved to be effective in a number of tumor-vaccine models at triggering immune responses to tumor-specific Ag and at inducing an effective anti-tumor immune response (reviewed in Ref. 13).

B7- and CD28-knockout mice have distinct phenotypes

The relative importance of CD28 and B7 in mediating co-stimulation was clarified further through gene-knockout experiments in mice. Studies of CD28 receptor function using gene disruption to create a CD28-deficient mouse revealed pronounced and specific immune defects[14]. T-cell development was normal in the CD28-knockout mouse, but peripheral T cells had impaired lymphokine secretion after stimulation with concanavalin A (ConA) or other mitogens. This is consistent with previous studies in normal T cells that indicated a requirement for B7–CD28 interaction for mitogen activation. Surprisingly, generation of cytotoxic T lymphocytes (CTLs) was normal in the CD28-knockout mouse after infection with lymphocytic choriomeningitis virus (LCMV). However, there were defects in the humoral immune response of these mice, which is consistent with data demonstrating the inhibitory effect of a chimeric CTLA-4 immunoglobulin (CTLA-4–Ig) fusion protein on T-cell-dependent antibody formation[15]. This suggests that the CD28 receptor mediates regulation of T–B-cell interactions *in vivo*, although it is equally plausible that this indicates a direct role for CD28 in the development of plasma cells, consistent with the expression of CD28 on mouse and human plasma cells[16].

Further evidence for the existence of additional CD28 ligands was provided by B7-knockout mice, which were found to have virtually no immune defects[17]. A potential explanation for the immune competence of these B7-deficient mice was provided by the observation that their immune responses could be suppressed by CTLA-4–Ig. This suggested the presence of additional CD28 ligands. Independent evidence for this hypothesis was provided by the demonstration of an additional molecule that bound CTLA-4–Ig on the surface of activated mouse B cells[18,19], and by the discordant expression of B7 (BB-1) molecules on human cells[20–22].

B7 is also a ligand for CTLA-4

Another indication that the B7–CD28 activation pathway might be more complex than initially assumed came from the demonstration that B7 could also bind to the extracellular domain of CTLA-4. The gene for CTLA-4 has been cloned and has significant structural similarity to CD28 (Ref. 23). Furthermore, CTLA-4 has a similar genomic organization to CD28 and maps to the same chromosomal band. A CTLA-4–Ig fusion protein comprising the extracellular domain of CTLA-4 and the Fc domain of IgG1 was found to bind B7 with 20-fold higher affinity than a similar construct containing the extracellular domain of CD28 (Ref. 24). When tested *in vitro*, this construct was found to inhibit B7-dependent T-cell co-stimulation and allogeneic MLRs. Thus, the CTLA-4–Ig molecule has allowed investigators to test the role of the B7–CD28 activation pathway *in vivo* for the first time.

CTLA-4–Ig can inhibit immune responses not affected by anti-B7 antibody

Treatment of mice *in vivo* with CTLA-4–Ig strongly inhibits T-cell-dependent antibody responses[15], and permits the long-term acceptance of xenografted pancreatic islets[25]. In rats, cardiac allograft acceptance was prolonged by treatment with CTLA-4–Ig, although ultimate rejection was not prevented[26]. These findings were consistent with previously appreciated differences in the strength of the immune response to xenogeneic or allogeneic stimulation. However, long-term acceptance of rat cardiac allografts could be achieved if a donor-specific transfusion was administered at the time of transplantation, followed by a single dose of CTLA-4–Ig two days later[27]. Surprisingly, studies of the effects of anti-B7 antibody *in vivo* were not nearly as impressive. This raised the possibility that there might be additional CD28 ligands not recognized by B7 antibodies but recognized by CTLA-4–Ig.

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'Proliferation' of the B7 receptor family

The observations described above led to the recent independent isolation of a B7-related gene by two laboratories. The gene isolated by Freeman and co-workers was obtained from a human cDNA expression library made from activated B cells[28]. They have termed this molecule B7-2, and the original receptor B7 is now referred to as B7-1. In addition, Azuma and colleagues produced an antibody that inhibited lymphocyte co-stimulatory signals and yet did not bind B7 (Ref. 29). Direct expression cloning of this molecule isolated a gene they have termed B70. Comparison of the nucleotide sequences of B7-2 with B70 reveals that they are the same gene. However, a comparison of the published sequences indicates the presence of six additional residues at the amino terminus of the B7-2 sequence, perhaps due to the use of alternative initiation sites. Furthermore, there is an indication that alternative exons encode the putative cytosolic domain of the B7-2 receptor (G. Freeman and A. Sharpe, unpublished). The B7 family is likely to expand still further as there is evidence to indicate the existence of yet another ligand, tentatively identified as B7-3, based on differential binding of various anti-B7 monoclonal antibodies (mAbs) and CTLA-4–Ig (Ref. 30). It is equally plausible that alternative exon usage explains some of the diversity of B7 receptors.

B7-1 and B7-2 are both members of the Ig gene superfamily, and comprise a single Ig V-like and a single Ig C2-like extracellular domain (Fig. 2). Comparison of the predicted amino acid sequences reveals that B7-1 is distantly related to B7-2 (Table 1), although there are indications of structural similarities[31]. A search of the SWISS-PROT protein sequence database using the FASTA program indicates that B7-2 is a member of a subfamily of the Ig superfamily. Regions of the extracellular domain of B7-2 have significant homology to a number of molecules, including: lymphocyte activation gene 3; myelin-associated glycoprotein; contactin neural-cell recognition molecule; transient axonal glycoprotein (TAG-1), which is a cell adhesion molecule that promotes neurite outgrowth; and the axl/UFO protein, which has tyrosine kinase activity and has been shown to have transforming potential (C.H. June, unpublished). Mouse B7-2 cDNA has been isolated[31], and appears to be closely related to the human homolog. This homology is concentrated in the Ig-like domains, with little similarity in the cytoplasmic domains, suggesting that the B7 proteins may not have an important function in signal transduction and, rather, may function primarily as ligands (Fig. 2).

B7-1 was recently designated as CD80 at the Fifth Workshop on Leukocyte Differentiation Antigens[32], and was originally thought to be a receptor that is expressed primarily on B cells. More-recent studies from a number of laboratories indicate that activated monocytes, dendritic cells and activated T cells also express B7-1 and B7-2. Thus, neither B7 nor CD28 are now considered lineage-specific receptors. The interaction of CD40 with its ligand gp39, and of the TCR with MHC class II, have both been shown to induce B7-1 expression on B cells[33,34]. Cytokines also have a major role in the expression of B7. IL-2 and IL-4

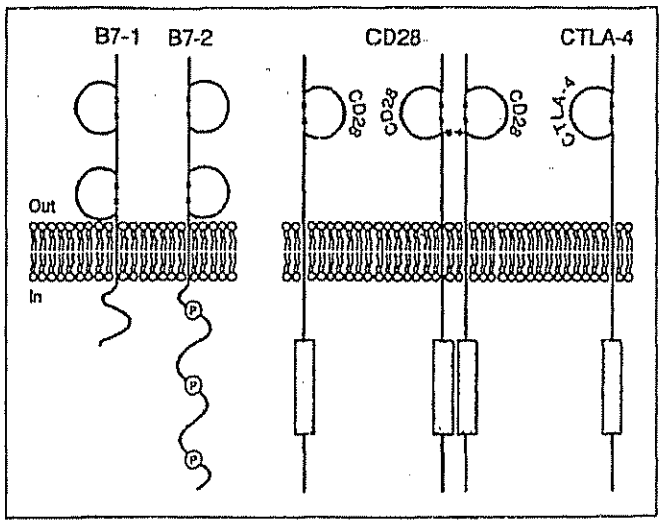

Fig. 2. The B7 and CTLA-4 receptor families. CD28 and CTLA-4 are expressed on the surface as monomeric or homodimeric forms. B7-1 and B7-2 have similar organization of extracellular domains but markedly different cytosolic domains. Three potential sites of phosphorylation by protein kinase C are indicated (P) in B7-2. Loops indicate disulfide-bonded (S-S) Ig-like folds, and gray rectangles indicate the presence of a potential binding site for phosphoinositide 3-kinase (PI 3-kinase).

enhance the induction of B7-1 expression on mitogen-stimulated tonsillar B cells[35], and it has recently been shown that IL-7 treatment of T cells results in the induction of B7-1 expression[36].

Many aspects of the function and surface expression of B7-2 are still being clarified. Surface expression of B7-2 was first studied indirectly by identifying cells that showed differential binding of B7-1 mAb and CTLA-4–Ig and, subsequently, with mAbs that bind specifically to human or mouse B7-2. B7-2 is expressed at extremely low levels on resting mouse B cells, and is rapidly upregulated after crosslinking of the B-cell antigen receptor or after stimulation with lipopolysaccharide

Table 1. Conservation amongst members of the B7 and CD28 receptor families

Receptor	Conservation (% amino acid identity) Overall	Signal	Ig-V	Ig-C	Tm	Cytoplasmic
hCD28						
mCD28	69	72	66		67	80
rCD28	70	67	68		67	83
cCD28	50	44	52		48	61
hCTLA-4						
mCTLA-4	74	65	67		83	100
hCD28	31	16	30		42	34
mCD28	27	16	27		42	26
hB7-1						
mB7-1	45	24	46	59	20	23
hB7-2	25	17	24	34	23	6
mB7-2	23	9	30	24	21	8
hB7-2						
mB7-2	50	56	66	59	17	10

Abbreviations: h, human; m, mouse; r, rat; c, chicken; Ig-V, immunoglobulin variable domain; Ig-C, immunoglobulin constant domain; Tm, transmembrane domain.

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(LPS) (Ref. 18). On human B cells, B7-2 expression peaks within 24 h of activation, whereas B7-1 expression peaks several days later[30]. The major ligand for soluble CTLA-4 on the surface of activated mouse B cells is B7-2 (Refs 19,37). Unlike B7-1, the cytoplasmic domain of B7-2 contains three potential sites for phosphorylation by protein kinase C (PKC), which may explain the distinct surface expression of B7-1 and B7-2. Alternatively, it is possible that the cytoplasmic domain of B7-2 has a function in signal transduction that would not be expected of B7-1 (Fig. 2).

The CD28 receptor family

Two members of the CD28 gene family, CD28 and CTLA-4, have been described to date. CD28 and CTLA-4 show 31% identity at the amino acid level, and both contain single Ig V-like extracellular domains, a single membrane-spanning domain and a short but highly conserved intracellular domain (Table 1). The cytosolic domains of both genes encode a consensus binding site that predicts binding to phosphoinositide 3-kinase (PI 3-kinase) (see below). The two genes have similar exon/intron structures and co-localize on band q33 of human chromosome 2, and on band C of mouse chromosome 1, suggesting that the receptors arose by gene duplication[23,38].

CD28 was originally identified as a 44 kDa homodimeric glycoprotein expressed on 80% of human peripheral blood T cells. A molecule genetically and functionally homologous to CD28 is expressed on the surface of primate, mouse, rat and avian lymphocytes. The chicken CD28 homolog has recently been cloned and the protein identified. The cDNA sequence shows a 50% overall sequence identity to human CD28, with complete identity in the extracellular domain thought to be involved in ligand binding, and complete conservation of the cytoplasmic motifs thought to be important for signal transduction. In contrast to mammalian CD28, avian CD28 is expressed as a non-disulfide-bonded receptor, presumably due to the loss of the membrane-proximal cysteine residue that is present in human and mouse CD28, and thought to be involved in the formation of disulfide bonds. In spite of this structural difference, chicken CD28 appears to have functional properties that are remarkably similar to mammalian CD28 (Ref. 93).

CTLA-4 was originally identified as the fourth cDNA during a search for genes that are specifically expressed in CTLs (CTLA-4 is an abbreviation of 'cytolytic T-lymphocyte-associated antigen')[39]. In common with CD28, CTLA-4 has been highly conserved in evolution with >70% overall homology between the human and mouse proteins, and complete conservation of the cytoplasmic domains. CTLA-4 also appears to be expressed as both a monomer[40] and a disulfide-linked dimer in mice (T. Walunus, unpublished) and in humans (C.H. June, unpublished). There is no evidence for heterodimeric receptors consisting of CD28 and CTLA-4.

The majority of human and mouse resting T cells express CD28. By contrast, CTLA-4 expression appears to be restricted to activated T cells. CD28 and CTLA-4 mRNA is coexpressed in activated $CD4^+$ and $CD8^+$ cells, and in nearly all T-cell clones[41,42]. In humans, activation-induced expression of CTLA-4 mRNA appears to be restricted to the $CD28^+$ subset[40]. In mice, CTLA-4 mRNA is detected in activated T cells, including $CD8^+$ CTLs (Ref. 39) and $CD4^+$ T-cell clones, both of the T helper 1 (Th1) and Th2 subtypes[42]. Only limited information is available on the surface expression of CTLA-4. Present evidence suggests CTLA-4 surface expression peaks three days after activation[41]. CD28 co-stimulation increases the expression of CTLA-4 mRNA in cells activated by anti-CD3 mAb, suggesting that the signals generated by antigen and co-stimulation might regulate CTLA-4 gene expression[40].

Despite the marked similarities between the CD28 and CTLA-4 cytosolic domains, a clear functional role for CTLA-4 has not been established, although a role for this molecule in T-cell activation has been suggested[41]. The phenotype of transgenic mice that overexpress a soluble form of mouse CTLA-4–Ig fusion protein has been studied recently[43]. The mice showed expanded populations of antigen-specific $CD4^+$ T cells after immunization with protein antigens, suggesting that CTLA-4–Ig prevented the delivery of a negative signal, or that the binding of CTLA-4–Ig itself delivered a positive signal. Furthermore, these mice had severely impaired Ig responses to T-cell-dependent antigens while T-cell-independent Ig responses were normal. Other potential roles for CTLA-4 are discussed below. A CTLA-4-deficient mouse has not yet been reported, and may prove to be a valuable tool in the study of the function of this receptor.

Signal transduction through CD28: signal 2?

The evolutionarily conserved but distinct cytoplasmic domains of CTLA-4 and CD28 (Table 1) suggest potentially non-overlapping roles in signal transduction. Similarities between CD28 signal transduction in human T cells and the nature of the co-stimulatory signal deduced by Schwartz and colleagues[5] prompted initial speculation that CD28 might provide the essential T-cell co-stimulatory signal[3]. A hallmark of CD28-mediated signal transduction is the production of IL-2, amongst other cytokines, that is resistant to CsA and FK506 (reviewed in Ref. 3). More-recent studies have shown that CHO cells transfected with mouse B7 can trigger CsA-resistant IL-2 production[44], and that P815 cells transfected with human B7 can trigger the CsA-resistant generation of CTL activity[45]. Thus, in the case of lymphokine production, the nature of the signal transmitted by the receptor is similar whether mAb or natural ligand is used to trigger T cells. As noted below, this agreeable situation does not always hold when biochemical signal-transduction events are examined.

While the precise nature of the co-stimulatory signal remains unknown, several criteria and predictions can be made based on the functional studies that established the two-signal model: (1) The biochemical characteristics of the co-stimulatory signal must differ from those provided by the TCR. (2) Co-stimulation in the absence of signal 1 must not activate resting T cells. (3) Given previously established differences concerning

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T-cell activation and CsA sensitivity, co-stimulation is unlikely to involve primarily the calcium-dependent calcineurin phosphatase pathway that signal 1 activates. (4) There may be more than one signal-transduction pathway coupled to CD28. This prediction is not based on the two-signal model but, rather, the increasing literature that links CD28 to the two distinct effector functions of lymphokine production and cytotoxicity. To date, there is no evidence that the two functions are linked to the same signal-transduction event, and signal transduction *vis-à-vis* CD28-induced cytotoxicity has yet to be addressed. (5) Transformed cell lines may be poor models of signal 2 because they have been selected to be independent of co-stimulatory signals. A prediction related to the fifth criterion is that results from studies of transformed cell lines will vary because different mechanisms would be expected to be 'selected' during the process of transformation in order to bypass the co-stimulatory signal-transduction requirement. Conceptually, co-stimulatory signal transduction can be divided into intra- and inter-molecular events at the cell surface, nuclear events that affect gene expression and intervening events that might link the cell surface to changes in gene expression.

Receptor interactions at the cell surface

No published results are available concerning details of structural interactions between B7-family ligands and CD28-family ligands. The MYPPPY hexamer motif, just proximal to the Ig V-like region of CD28 and CTLA-4, was first noted by Golstein and colleagues to be highly conserved, and represents a possible binding site for B7-family ligands. This prediction is based on the observation that this unusual motif does not appear in any other protein described to date, as well as the complete evolutionary conservation of the motif across the 300 million years that bridge the avian and mammalian immune systems. Domain-swapping experiments are underway in several laboratories to define regions involved in conferring specific binding of B7-1 and B7-2 to CD28 and/or CTLA-4. It is not yet known if all B7 ligands bind to the same sites on CD28 and CTLA-4. Another crucial piece of information that is missing is whether there is preferential binding of one ligand to another. Based on temporal patterns of receptor expression, we favor a model whereby B7-2 is the principal ligand for CD28 and B7-1 is the primary ligand for CTLA-4, although there are several alternative and equally plausible models. Noting conserved sequences in the Ig V- and C-like regions of the human and mouse B7 family, Freeman has made predictions regarding residues that are involved in the binding of B7 to the CD28 family[31].

CD28-mediated post-transcriptional effects

A major mechanism by which the CD28 receptor augments lymphokine secretion in mature T cells is by inhibiting the degradation of lymphokine mRNAs (reviewed in Ref. 3). As a result of the stabilization of mRNA, the steady-state levels of specific lymphokine mRNA increases, leading to enhanced translation and protein secretion. To date, the cytokine mRNAs that the CD28 signal has been shown to stabilize have been limited to lymphokines selectively produced by Th1 cells. However, given the recent demonstrations that signals delivered by CD28 can augment T-cell gene expression of a multitude of cytokines, including IL-1α (Ref. 46), IL-4 (Refs 47–49), IL-5 (Ref. 48), IL-6 (Ref. 50), IL-13 (Ref. 51) and colony-stimulating factor 1 (CSF-1) (Ref. 52), it is likely that CD28 signaling may affect multiple genes at the post-transcriptional level. In fact, there is recent evidence that CD28 can prevent the degradation of mRNA expressed by genes other than cytokines. Cerdan and co-workers have shown that CD28 co-stimulation in combination with CD2 signaling results in a pronounced stabilization of IL-2 receptor α chain mRNA (Ref. 53).

CD28-mediated transcriptional events

In addition to the post-transcriptional effects, IL-2 mRNA levels are enhanced late after stimulation of T cells with anti-CD28 (>6 hrs), and this appears to be due to an increase in transcription. These results are consistent with the observation that the magnitude of the increase in IL-2 production accompanying CD28 stimulation could not be accounted for simply by an effect on mRNA stability[54]. Several laboratories have studied the effects of CD28 ligation on IL-2 promotor activity. Fraser and co-workers found that CD28 stimulation of Jurkat cells transfected with a construct carrying the IL-2 promotor induced the binding of a protein complex to an element located between positions -164 and -154 relative to the transcription start site (reviewed in Ref. 55). They have called this binding site the CD28-response element (CD28RE), and termed the protein complex the CD28-responsive complex (CD28RC). Most recently, the same group has shown that CD28 also increases expression of constructs that contain the IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF) and interferon γ (IFN-γ) promoters, and have identified proteins of 35, 36 and 44 kDa as comprising the CD28RC.

Similar studies by Verweij and co-workers on Jurkat cells transfected with another IL-2 promotor construct have suggested that the CD28RC is similar to NF-κB (Ref. 56), which does not completely agree with the previous work by Fraser *et al.* The explanation for the differences in these studies is not yet clear, but may reflect differences in the characteristics of the Jurkat sublines. For example, the subline studied by Fraser *et al.* does not undergo IL-2 promotor expression after stimulation with PMA and anti-CD28, while the same treatment of the Jurkat subline studied by Verweij *et al.* results in the expression of the IL-2 construct as well as the native gene encoding IL-2. Furthermore, another Jurkat subline (J32) has been described that produces IL-2 after CD28 stimulation alone[57]. Thus, there appear to be major differences in the signal-transduction requirements for IL-2 expression in variants of the Jurkat line, so that caution should be exercised in the extrapolation of results to non-transformed T cells. To date, only one study of CD28-mediated transcriptional effects has been published using primary T cells. This study found that members of the NF-κB family bound to the CD28RE after stimulation of primary T cells with PMA and anti-CD28, or

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Table 2. Effects of ligating the CD28 receptor on early signal-transduction events

Response	Stimulus	Comment	Refs
Tyrosine phosphorylation	B7-1-CHO or CD28 mAb	CD28 crosslinking, or ligation by B7-1, causes increased tyrosine phosphorylation of cellular substrates in activated T cells	67,68,77
PLCγ1	B7-1-CHO or CD28 mAb	CD28 crosslinking, or ligation by B7-1, causes PLCγ1 phosphorylation in activated T cells	78
Ca^{2+}	CD28 mAb	CD28 crosslinking does not cause a Ca^{2+} response in resting T cells; CD28 crosslinking elevates Ca^{2+} in Jurkat cells; CD28 crosslinking does not synergize with TCR crosslinking	59,79–83
Ins(1,4,5)P_3	CD28 mAb	CD28 crosslinking causes appearance of PtdIns(4,5)P_2 metabolites in Jurkat or activated T cells	59,80,84
PI 3-kinase	B7-1-CHO or CD28 mAb	CD28 crosslinking causes appearance of PtdIns(3,4,5)P_3 metabolites in Jurkat cells; CD28 associates with the p85α subunit of PI 3-kinase in Jurkat cells	62–65 (D. Olive *et al.*, unpublished) (Y. Lu *et al.*, unpublished)
raf-1 kinase	CD28 mAb	CD28, TCR and CD4 crosslinking activate raf-1 kinase activity in resting T cells	72
cGMP	CD28 mAb	CD28 signal elevates [cGMP] in Jurkat cells	79
K^+ channel	CD28 mAb	CD28 signal is resistant to charybdotoxin, while TCR signal is sensitive	85
IL-2	CD28 mAb	CD28 signal synergizes with pervanadate to induce IL-2 secretion in primary T cells; pervanadate induces IL-2 secretion in absence of CD28 signal in Jurkat cells	70,71

Abbreviations: PLCγ1, phospholipase Cγ1; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate; PI 3-kinase, phosphoinositide 3-kinase; cGMP, guanosine 3',5'-cyclic monophosphate; IL-2, interleukin 2; B7-1-CHO, Chinese hamster ovary cells transfected with B7-1; mAb, monoclonal antibody; TCR, T-cell receptor; PtdIns(4,5)P_2, phosphatidylinositol (4,5)-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol (3,4,5)-trisphosphate.

with anti-CD3 and anti-CD28 mAbs (Ref. 58). Clearly, much remains to be learned concerning the mechanisms leading to CD28-mediated effects on expression of lymphokine genes.

Intervening signal-transduction events

Many second-messenger systems can be affected by crosslinking the CD28 receptor (Table 2). However, it has yet to be determined whether any of these events are required for the co-stimulatory pathway that involves IL-2 secretion and, presumably, stabilization of cytokine mRNA. It has previously been emphasized that at least two distinct signals can be delivered through the CD28 receptor, depending on the degree of receptor oligomerization and the state of cellular activation[1,59]. Of particular interest are the recent observations that signaling through the CD28 pathway *via* a natural ligand, B7-1, can increase cellular tyrosine phosphorylation[68] and activate PI 3-kinase[62].

Is the CD28 family a receptor for PI 3-kinase?

Recent studies from the laboratories of several investigators indicate that CD28 can bind to PI 3-kinase and activate lipid kinase activity[62–65]. The regulation of kinase activity is thought to occur through the p85 subunit (Box 1). Two isoforms of p85 (p85α and p85β) have been identified, and both are present in T cells. The regulation of PI 3-kinase is complex, and may involve serine phosphorylation in T cells after TCR stimulation[60] and tyrosine phosphorylation after IL-2 binding[61]. While the role of PI 3-kinase in cellular activation remains unknown, the consensus of many studies is that PI 3-kinase can activate an essential second-messenger system (Box 2). Ward and colleagues were the first to show that CD28 crosslinking by mAb or by CHO cells that express B7-1 could induce the appearance of phospholipids that indicate PI 3-kinase activation[62]. Independent findings from at least five laboratories have now confirmed this observation (Table 2). The laboratories of Imboden and Rudd have shown that the p85α subunit of PI 3-kinase can associate with the cytosolic domain of CD28 *via* a YMNM motif[63,64]. The stoichiometry of the association is high, since more than 25% of the PI 3-kinase molecules can be found in association with CD28 in several cell lines. Laboratories in New York[65], Marseille (D. Olive *et al.*, unpublished) and Texas (Y. Lu *et al.*, unpublished) have similar results and, in addition, have shown that the cytosolic tail of CD28 becomes tyrosine phosphorylated after CD28 crosslinking. Furthermore, Prasad *et al.* used site-directed mutagenesis to show that Tyr191 of human CD28 is essential for PI 3-kinase association[64].

Given these exciting data, some of the initial biochemical events can be incorporated in the two-signal

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model of T-cell co-stimulation (Fig. 3). This model incorporates the results from many laboratories, although the details remain speculative. A central feature of the model is that two primary signal-transduction pathways are coupled to CD28, one that is dominant in T-cell blasts and one in naive T cells. Ag–MHC binding to the TCR activates a group of protein tyrosine kinases (PTKs) comprising $p59^{fyn}$, $p56^{lck}$ and ZAP-70, forming a group of CD45-dependent signals that are collectively termed 'signal 1'. Some of the components of signal 1 include activation of phospholipase Cγ1 (PLCγ1) and Ras (Ref. 55). In the absence of TCR occupancy, the cytoplasmic domain of CD28 remains non-phosphorylated at the PI 3-kinase binding site. The identity of the kinase that phosphorylates CD28 after antigen stimulation remains unknown. One laboratory has found that $p56^{lck}$ is activated after CD28 crosslinking[66], while another laboratory has found that CD28-induced substrate tyrosine phosphorylation can occur in Jurkat cells that do not express $p56^{lck}$ (Y. Lu *et al.*, unpublished).

In T-cell blasts or Jurkat cells, a PTK is able to activate the CD28 receptor by phosphorylation of the Tyr191 residue. Such a pre-activated T cell enters a fully armed and activated state when antigen encounter occurs in the context of CD28 receptor occupancy. In T-cell blasts, the binding and activation of PI 3-kinase by CD28 initiates signal 2b. Tyrosine phosphorylation of PLCγ1 and calcium elevation have been observed after CD28 stimulation of activated T cells (Table 2), generating a CsA-sensitive component to signal 2b. It is presumed that this involves the *activation* of a tyrosine kinase, although it is equally plausible that this form of signal transduction is initiated by the *inhibition* of a tyrosine phosphatase. The CD28-associated PTK could associate *via* the *src*-homology region 2 (SH2) or SH3 domains of PI 3-kinase, although, to date, the identity of this putative PTK remains unknown. It is unlikely to be any of the PTKs associated with the TCR, since ligation of CD28 by natural ligand B7 can induce T-cell protein-tyrosine phosphorylation in activated peripheral blood T cells, whereas CD28-induced protein-tyrosine phosphorylation was nearly undetectable in naive T cells[67,68]. These observations suggested that the CD28-associated PTK, or an essential adaptor protein, is either not active or not present in resting T cells.

In naive T cells, it is proposed that CD28 predominately transmit signal 2a, a CsA-insensitive message. Since tyrosine phosphorylation of the cytosolic domain of human CD28 has not been demonstrated in resting T cells (Y. Ueda, M. Huang and C.H. June, unpublished), if PI 3-kinase is involved in signal 2a then it is likely to occur *via* a tyrosine-kinase-independent mechanism. The signal-transduction cascade in naive T cells is proposed to involve serine/threonine kinases and/or phosphatases. However, PKCζ is a PKC variant that is insensitive to phorbol ester[69]. Given that PI 3-kinase activation may lead to activation of PKCζ, this model could explain the observation that neither PMA nor CD28 stimulation alone activates naive T cells, whereas PMA plus anti-CD28 mAb treatment is capable of activating human T cells.

Box 1. PI 3-kinase: structure and binding motifs

Two catalytic PI 3-kinase p110 isoforms have been isolated, which are likely to be derived from distinct gene products[87]. Two regulatory p85 subunits for PI 3-kinase have been identified, also representing different gene products. The p85 subunit is an adaptor protein that contains an SH3 domain, two SH2 domains and a unique N-terminal region. SH3 domains mediate protein–protein interactions by binding to proline-rich sequences[88]. By contrast, SH2 domains bind to tyrosine-phosphorylated protein motifs. SH2 domains are approximately 100 amino acids in length and are specific for phosphotyrosines in a specific linear pYXXM motif. The recent description of the consensus SH2 motif specific for the PI 3-kinase p85α subunit[89], permitted protein sequence database searches that identified CD28 and CTLA-4 as potential receptors for PI 3-kinase (see below). Binding of ligand to a multitude of receptors induces activation of PI 3-kinase. These receptors include PDGFR, EGFR, and, in T cells or thymocytes, the TCR (Ref. 90) and the IL-2R, IL-4R and IL-7R. Examples of proteins that contain the pY(M/V/I/E)XM consensus motif that are predicted to bind to the SH2 domain of the p85α subunit of PI 3-kinase are shown below.

Protein	Sequence
PDGFR β chain	YMDMSKDE
Rat IRS-1	YMNMSP
c-kit	YMDMKP
CSF-1	YVEMRP
Human CD28	YMNMTPR
Rat CD28	YMNMTPR
Mouse CD28	YMNMTPR
Chicken CD28	YMNMTPR
Human CTLA-4	YVKMPP
Mouse CTLA-4	YVKMPP
Human CD7	YEDMSH
Human CD19	YEDMRG
Human IL-7R	YVTMSS

Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; SH3, *src*-homology region 3; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; TCR, T-cell receptor; IL-2R, interleukin 2 receptor; IRS-1, insulin receptor substrate 1; CSF-1, colony-stimulating factor 1.

A central feature of this model is that CD28 transmits two distinct signals. Signal 2a occurs in naive T cells after stimulation of T cells with low levels of CD28 crosslinking, provided *in vitro* by sub-saturating amounts of bivalent CD28 mAb, and perhaps *in vivo* by APCs that express low-affinity B7-family ligands. By contrast, signal 2b is transmitted in T-cell blasts and requires saturating amounts of crosslinked CD28 mAb and, *in vivo*, may require multiple and/or high-affinity B7-family ligands (Fig. 3). Based on previous studies (reviewed in Ref. 3), it is possible that the signals associated with cytokine production and mRNA stabilization are associated with signal 2a and, therefore, that this is the biochemical equivalent of the co-stimulatory signal described by Bretcher and Cohn.

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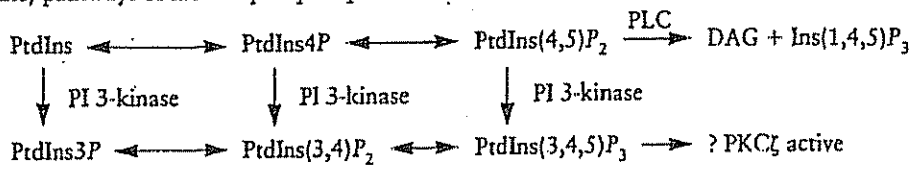
Box 2. Does PI 3-kinase mediate co-stimulatory signal transduction?

PI 3-kinase generates phosphoinositides in which the inositol ring is phosphorylated at the D-3 position, while phosphate additions occur at the D-1, D-4 and D-5 positions in the classical pathway. Unlike the classical pathway, in which PtdIns(4,5)P_2 is cleaved by PLC, the products of PI 3-kinase are not hydrolysed by any known phospholipase. Thus, the 3-phosphate-containing products of PI 3-kinase are unlikely to subserve signal-transduction functions as precusors of water-soluble second messengers, and it is thought that the lipids function as lipids *per se*. The role of PI 3-kinase in cellular activation remains unknown. However, the consensus from many studies is that PI 3-kinase can activate an essential second-messenger system that is independent of the classical pathway that is initiated by PLC. Evidence indicating the importance of PI 3-kinase includes the demonstration that the transforming activity of the polyoma virus middle T antigen is dependent on the binding of PI 3-kinase to $p60^{c\text{-}src}$. In normal cells, the induction of cell growth through the PDGFR requires the binding of PI 3-kinase (Ref. 91). A recent study indicated that a product of PI 3-kinase, PtdIns(3,4,5)P_3, could activate a PMA-insensitive isoform of protein kinase C, PKCζ (Ref. 92). The classical (upper line) and alternate (lower line) pathways of inositol phospholipid metabolism are shown below.



Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PLC, phospholipase C; PDGFR, platelet-derived growth factor receptor; PMA, phorbol myristate acetate; DAG, diacylglycerol; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate.

Particularly relevant to this are the recent studies of T cells using pervanadate, a pharmacological means to mimic the effects of PTK/phosphatase signal transduction. It has been shown that activation of PTKs in resting T cells by pervanadate did not result in expression of the gene encoding IL-2 unless CD28 co-stimulation was provided[70]. By contrast, in Jurkat cells, pervanadate treatment alone was sufficient for IL-2

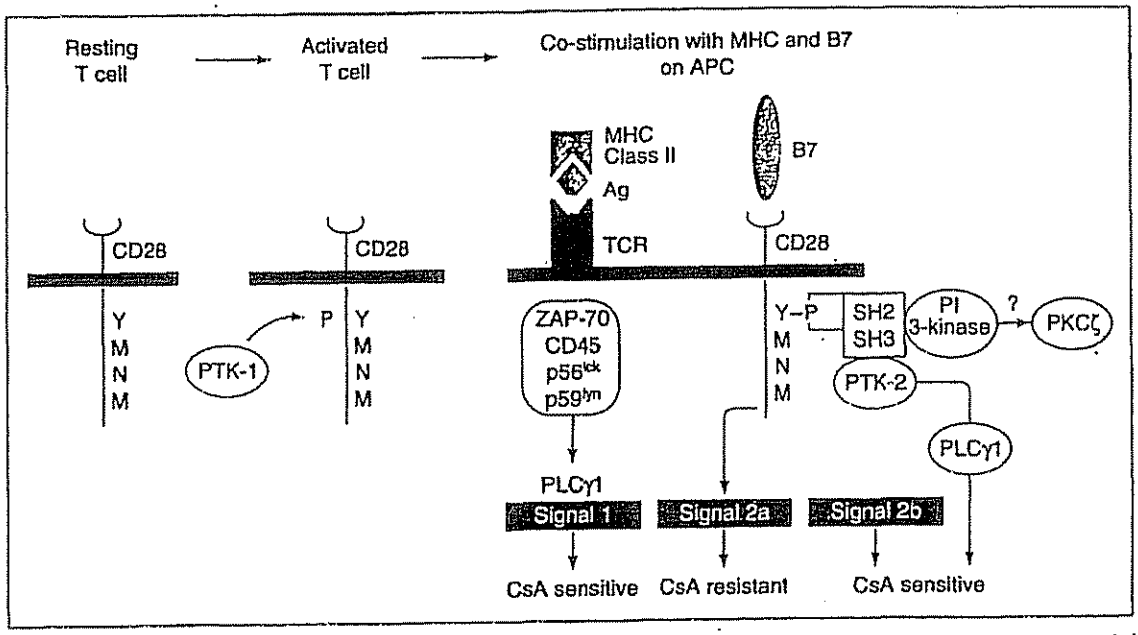


Fig. 3. *Proposed model of signal transduction mediated by the CD28 receptor. Two primary signal-transduction pathways are coupled to CD28, one that is dominant in activated T cells (signal 2b) and is cyclosporin A (CsA) sensitive, and one that occurs in naive T cells (signal 2a) and is CsA resistant. In activated T cells, antigen (Ag) bound to the major histocompatibility complex (MHC) class II on antigen-presenting cells (APCs) binds to the T-cell receptor (TCR) and results in the activation of a group of protein-tyrosine kinases (PTKs), including ZAP-70, $p56^{lck}$ and $p59^{fyn}$, as well as the CD45 phosphotyrosine phosphatase (PTP), thereby generating signal 1. This signal involves activation of phospholipase Cγ1 (PLCγ1). Also in activated T cells, binding of high-affinity B7-family ligands to the CD28 receptor results in phosphorylation (P) of the tyrosine residue in the CD28 YMNM motif by a PTK. Phosphoinositide 3-kinase (PI 3-kinase) binds to this site and initiates signal 2b via tyrosine phosphorylation of PLCγ1. In naive T cells, low levels of CD28 crosslinking, possibly by low-affinity B7-family ligands, results in transmission of signal 2a. The identity of PTKs 1 and 2 is presently unknown.*

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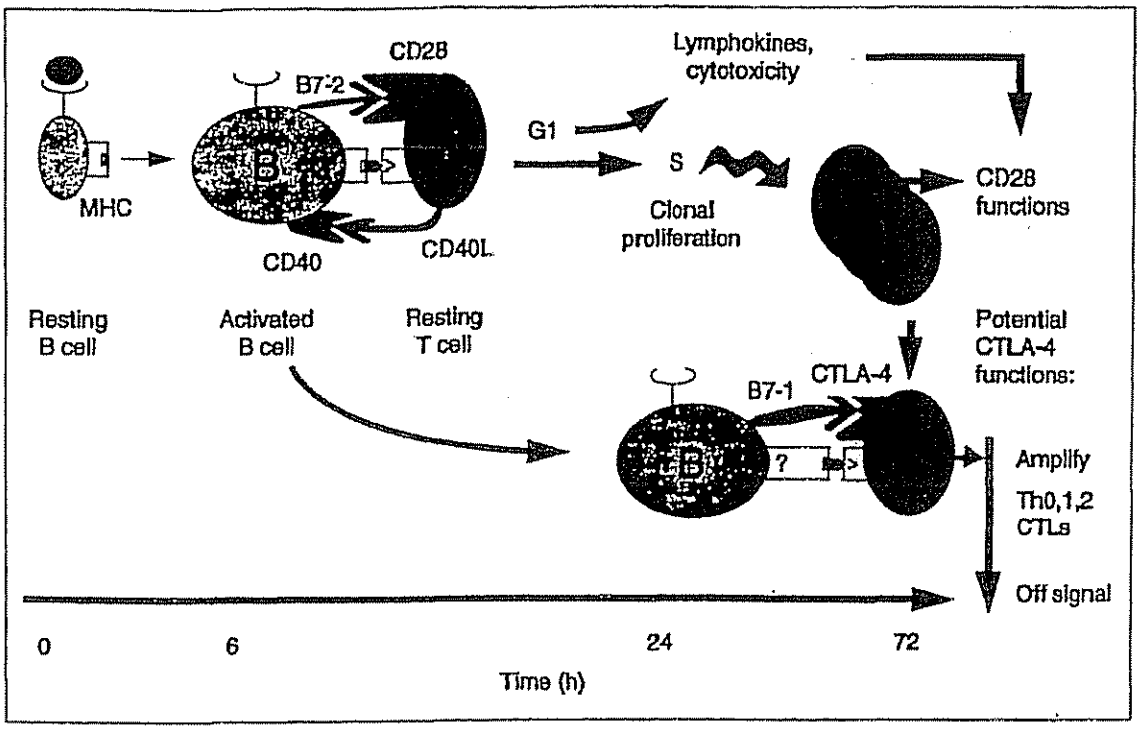

Fig. 4. *Proposed models of T–B-cell interaction. Early in the immune response, the binding and processing of antigen (Ag) by resting B cells induces expression of B7-2 and CD40. Subsequent cognate T–B interaction and co-stimulatory ligand–receptor interaction via CD28 and CD40L results in T- and B-cell effector functions. Later in the immune response, activated B cells interact with T cells, generating signals that may involve B7-1 and CTLA-4 interaction. This may or may not involve interaction between the T-cell receptor (TCR) and processed Ag bound to major histocompatibility complex (MHC) class II on the B cell. Ligation of CTLA-4 may result in amplification of the response, generation of T helper 0, 1 and 2 cells (Th0,1,2) and cytotoxic T lymphocytes (CTLs), and an 'off' signal.*

production[71]. These contrasting results highlight the difficulty of using Jurkat cells as a model for co-stimulatory signal transduction but, more importantly, suggest that signal 2a is independent of pervanadate-sensitive PTKs and tyrosine phosphatases.

Herbimycin A, a PTK inhibitor, can prevent CD28-induced tyrosine phosphorylation and CD28-induced IL-2 production in normal T cells[67,68]. In human T-cell blasts, the herbimycin-sensitive step(s) could be at the level of Tyr191 phosphorylation of the CD28 cytosolic domain, and/or at the level of the PTK that contributes to signal 2b. Crosslinked CD28 mAb can activate the serine/threonine raf-1 kinase[72] in normal T cells, and it is not yet known if this is part of signal 2a or signal 2b. Raf-1 appears to be required for IL-2 production in Jurkat cells[73]. Together, these results suggest that proximal events in CD28 signal transduction are likely to involve multiple protein kinases as well as lipid kinases.

There are many questions that remain to be answered regarding CD28 and signal transduction. For instance, how is specificity of the CD28 signal maintained given that the TCR as well as cytokine receptors on T cells can also activate PI 3-kinase? Is activation of PI 3-kinase necessary for co-stimulatory signal transduction? The recent availability of pharmacological inhibitors of PI 3-kinase should provide relevant information to address this issue. What is the role of CTLA-4 in signal 2, given the amazing degree of conservation in the cytoplasmic domain and the presence of a motif in CTLA-4 that predicts that it too may be a receptor for PI 3-kinase? Finally, what roles do the different members of the B7 family have in signal transduction by CD28 and CTLA-4? Rapid progress in these areas can now be expected given the potential discovery of a molecular switch in the tail of CD28.

Summary and conclusions

A model of T- and B-cell activation, incorporating available information on the CD28 and B7 families, is shown in Fig. 4. Given that CD28 and B7-2 exhibit similar temporal patterns of increased expression after activation of naive T and B cells, and that the expression of CTLA-4 and B7-1 also share similar kinetics of induction, it is likely that B7-2 is a principal ligand for CD28 and that B7-1 is a principal ligand for CTLA-4. The binding of native antigen to surface Ig on resting B cells induces rapid expression of B7-2, so that the presentation of processed Ag–MHC to T cells would be expected to be coincident with the interaction between B7-2 and CD28. Culture of T cells with fibroblasts transfected with B7-1 results in the upregulation of expression of CD40 ligand during the first 12 h of cell culture, presumably through CD28 and/or CTLA-4 interaction[48]. These events are sufficient for CD28-mediated effector T-cell functions that do not require cell division, such as cytokine production and the non-MHC-restricted cytotoxicity that has been described by Azuma and colleagues[74].

In vitro experiments show that the effects of the initial TCR and CD28 co-stimulation suffice for T-cell doublings approximately three times in the first 72 h of the immune response. At this time, expression of CTLA-4

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is maximal, and there is evidence that CD28 may not transmit signals during this phase of differentiation[40,75,76]. Similarly, signals transmitted through CD40 and MHC class II molecules induce maximal expression of B7-1 on B cells after 2–3 days, leading to preferential signaling through CTLA-4. It is possible that signals delivered through CTLA-4 on T-cell blasts do not require cognate Ag, as a number of studies have shown that CD28 mAb alone can activate T-cell blasts, in contrast to T cells at rest. A primary question concerns the nature of the putative CTLA-4 signal. Since CTLA-4 has higher affinity for B7-1 (and possibly B7-2) than CD28, it is possible that CTLA-4 may terminate CD28-mediated signal transduction by competition for shared counter ligands. This is compatible with the phenotype of the CTLA-4–Ig transgenic mouse, where increased numbers of antigen-specific T cells are generated after protein immunization[43]. Alternatively, Linsley has proposed that CTLA-4 may function to stabilize and prolong CD28-mediated signals and, thereby, amplify the response[41]. However, it is also possible that CTLA-4 acts to downregulate T-cell function by delivering signals that are unique from CD28. Furthermore, given the potential of combinatorial signals that could be provided by crossbinding ligands in the enlarging family of co-stimulatory receptors, it is possible that each receptor pair transmits distinct differentiation signals, for example by triggering differing patterns of cytokine production. Finally, it is possible that CTLA-4 has been aptly named, in that it could facilitate the differentiation of MHC-restricted CTLs. This idea would agree with recent observations that CTLA-4 is expressed at a higher level on $CD8^+$ cells (T. Walunus *et al.*, unpublished). In addition, activated T cells from the CD28-knockout mouse have at least partially preserved expression of CTLA-4, and have retained normal CTL development[14].

Many questions regarding the co-stimulatory family of receptors remain to be answered. Characterization of the CD28 signal at the molecular level will certainly lead to a more-complete knowledge of the regulation of cytokine production by T cells. In addition, the function of the mysterious CTLA-4 receptor requires clarification. Finally, it is clear that further progress in the development of selective immunosuppressive therapies and in the treatment of T-cell immunodeficiencies requires an increased understanding of the role these new receptor–ligand pairs mediate in the intact immune system.

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EXHIBIT 9

A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells

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CTLA-4, expressed by activated T cells, transduces an inhibitory signal. We show here that PCR amplification of the coding sequence of CTLA-4 in nonstimulated human T lymphocytes results in the amplification of two transcripts of 650 and 550 bp. Sequencing shows that the larger form codes for membrane CTLA-4 and the 550-bp transcript is a spliced variant in which exon 2 coding for the transmembrane region is deleted. This spliced cDNA has been named CTLA-4delTM. The splicing induces a frame shift which results in the addition of 22 extra amino acids before a translational termination. Activation of T cells with phorbol 12-myristate 13-acetate plus ionomycin or anti-CD3 plus anti-CD28 monoclonal antibodies induces a suppression of CTLA-4delTM mRNA expression associated with a preferential expression of the membrane CTLA-4 mRNA, showing that CTLA-4delTM mRNA expression is restricted to nonactivated T cells. A soluble immunoreactive form of CTLA-4 was detected in the serum of 14/64 healthy subjects. These results suggest that nonstimulated T cells may constitutively produce a soluble form of CTLA-4 which may have an important role in the regulation of immune homeostasis.

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1 Introduction

The generation of an efficient antigen-specific T cell response is initiated by signals delivered via the CD3/TCR complex and requiers co-stimulatory signals via cell surface molecules expressed by T cells [1]. Among the co-stimulatory signals, the interaction between CD80 and/or CD86 expressed by APC and CD28 constitutively expressed by T cells is crucial [2]. It is responsible for the proliferation, cytokine production and acquisition of effector functions by T cells [3–5]. Blocking this interaction using neutralizing anti-CD28 or anti-CD86 mAb induces T cell anergy [6] and inhibits the development of helper and cytotoxic T cells [7–9]. Once activated, T cells also express another ligand for CD80/CD86, CTLA-4 or CD152 [10–12]. CTLA-4 and CD28 are homologous and belong to the Ig superfamily. Their binding motifs to CD80 are located in the complementarity-determining region (CDR)1- and CDR3-like domains [13, 14] and involve at least the MYPPPY motif but CTLA-4 binds to CD80/CD86 with 20- to 50-fold higher affinity than does CD28 [15]. In contrast to CD28 which delivers an activation signal to T cells, CTLA-4 transduces an inhibitory signal to T cells [16–18]. The role of CTLA-4 was clearly evidenced in CTLA-4-deficient mice which present massive lymphoproliferation and multiorgan tissue destruction [19, 20]. Anti-CTLA-4 mAb increased *in vivo* the antigen-specific T cell proliferation [21] and allowed rejection of established tumors [22].

In this study, reverse transcriptase (RT)-PCR analysis shows that nonactivated human peripheral blood T lymphocytes express an alternatively spliced form of CTLA-4 mRNA. The splicing induces the deletion of the transmembrane region, resulting in the production of a soluble form of CTLA-4 in transfected cells. Interestingly, a soluble form of CTLA-4 was detected in the sera of healthy individuals.

[I 19836]

The two first authors constibuuuted equally to this work

Abbreviations: CDR: Complementarity-determining region **RT:** Reverse transcriptase

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2 Results

2.1 Identification of spliced variants of CTLA-4 mRNA

The amplification by PCR of the human CTLA-4 coding sequence revealed the constitutive expression by non-activated T cells of two transcripts whose sizes are approximatively 650 and 550 bp (Fig. 1 A). Both cDNA fragments were subcloned in the TA cloning vector and sequenced. The larger form (650 bp) showed complete homology with the published CTLA-4 sequence (data not shown) [23]. The smaller cDNA fragment has a deletion from nucleotide 456 (starting from ATG codon) to nucleotide 563. According to the genomic organization of the human CTLA-4 gene [24], this cDNA is generated by alternative splicing of the CTLA-4 mRNA of which the exon encoding the transmembrane region (exon 2) is spliced out (Fig. 1 b). The deletion causes a frame shift resulting in 22 extra amino acids before a translational termination at position 523 (Fig. 1 b). This spliced transcript has been named CTLA-4delTM. The expression of the two CTLA-4 mRNA variants has been observed in 10/10 healthy subjects tested. CTLA-4delTM has a predicted molecular mass of 20 kDa.

2.2 CTLA-4delTM mRNA is expressed only in nonactivated T cells

CTLA-4 mRNA expression is increased following stimulation [25–27]. Thus, we have evaluated the influence of T cell activation on CTLA-4 *vs.* CTLA-4delTM mRNA expression. An 8-h stimulation with PMA plus ionomycin or anti-CD3 plus anti-CD28 mAb resulted in the inhibition of CTLA-4delTM mRNA (Fig 2). As expected [25–27], activation increased membrane CTLA-4 mRNA expression. These data suggest that CTLA-4delTM mRNA expression is down-regulated by activation.

2.3 Recombinant CTLA-4delTM is produced in a soluble monomeric form

Membrane CTLA-4 is expressed as a homodimer; the covalent dimerization is mediated by a disulfide bond at cysteine residue 120 [28]. The alternative splicing of CTLA-4 mRNA results in the loss of this cysteine residue, suggesting that CTLA-4delTM could be produced as monomer. This hypothesis has been confirmed by Western blotting analysis showing that recombinant CTLA-4delTM expressed in COS cells has an apparent molecular mass of 23 kDa under non-reducing conditions (Fig. 3 a). As previously reported, recombinant membrane CTLA-4 has an apparent molecular mass of 45

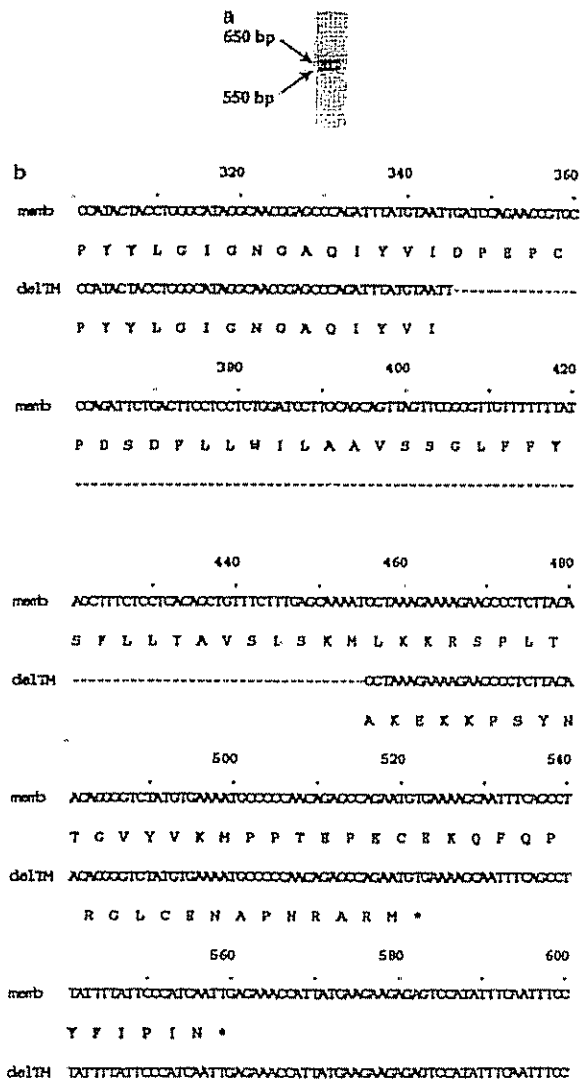
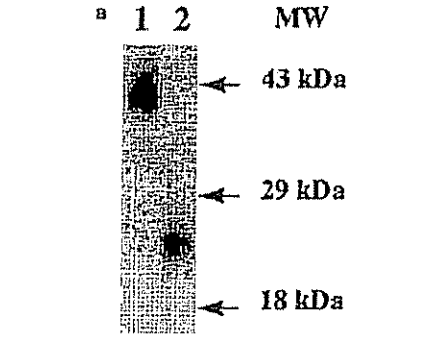


Figure 1. Identification of an alternatively spliced variant of CTLA-4. (a) RT-PCR amplification of CTLA-4 mRNA. RT-PCR was performed as described in Sect. 4.2. (b) Alignment of 3' nucleic acid sequences of membrane CTLA-4 and CTLA-4delTM. The deduced amino acid sequence of CTLA-4delTM is also shown. The stop codon is indicated by an asterisk.

kDa under nonreducing conditions (Fig. 3 a). The deletion of exon 2 results in the lack of the transmembrane domain, thereby suggesting that CTLA-4delTM could be produced as a soluble protein by transfected cells. A soluble protein reactive with anti-CTLA-4 mAb was detected by ELISA in the cell-free supernatant of CTLA-4delTM-transfected cells but not in the supernatants of membrane CTLA-4-transfected cells and mock-transfected cells (Fig. 3 b).



b

	Soluble CTLA-4
Mock	< 1 ng/ml
Membrane CTLA-4	< 1 ng/ml
CTLA-4delTM	55 ± 8 ng/ml

Figure 2. Recombinant CTLA-4delTM is produced in a soluble monomeric form. (a) Recombinant CTLA-4delTM is produced in a monomeric form. COS cells were transfected with membrane CTLA-4 and CTLA-4delTM cDNA ligated into the pCDNA3.1* vector. CTLA-4 expression was evaluated by Western blotting under nonreducing conditions. (b) Recombinant CTLA-4delTM is produced as a soluble molecule. The production of an immunoreactive form of CTLA-4 was evaluated by ELISA in the concentrated culture supernatant of mock-transfected cells or COS cells transfected with membrane CTLA-4 or CTLA-4delTM. Results are expressed in ng/ml (mean ± SD, $n = 5$).

These data suggest that CTLA-4delTM could be expressed in a soluble monomeric form by nonactivated peripheral blood T cells.

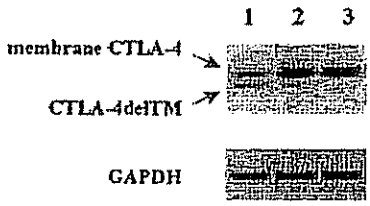


Figure 3. Regulation of CTLA-4 mRNA expression in activated T cells. CTLA-4 mRNA expression was evaluated in human T cells either unstimulated (lane 1) or stimulated with PMA plus ionomycin for 6 h (lane 2) or anti-CD3 plus anti-CD28 mAb for 18 h (lane 3). RNA integrity and cDNA synthesis was verified by amplifying GAPDH cDNA.

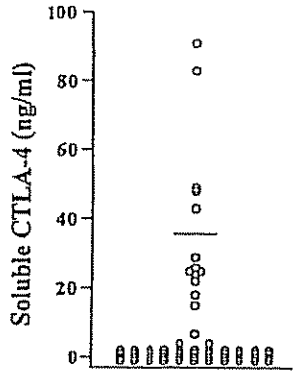


Figure 4. A soluble immunoreactive form of CTLA-4 is detected in the serum of healthy subjects. The concentration of soluble CTLA-4 was evaluated by ELISA using recombinant CTLA-4-Fc as a standard. Results are expressed in ng/ml.

2.4 A soluble immunoreactive form of CTLA-4 is detected in the serum of healthy subjects

We then evaluated whether soluble CTLA-4 could be detected by ELISA in the serum of healthy subjects. A soluble immunoreactive form of CTLA-4 was detected in the freshly isolated serum of 14 out of 64 healthy subjects (36 ± 8 ng/ml, mean ± SEM, ranging from 3 to 90 ng/ml) (Fig. 4).

3 Discussion

Nonstimulated human T cells constitutively express membrane CTLA-4 transcript [29]. Here we describe the identification of an additional spliced variant named CTLA-4delTM which lacks both transmembrane and intracellular domains. The splicing, resulting in the deletion of the exon 2 which encodes the transmembrane domain and the cytoplasmic tail of CTLA-4 [24], occurs as for the Ig genes [30]. The recombinant molecule is expressed as a soluble monomeric protein. Interestingly, a soluble immunoreactive form of CTLA-4 can be found in the serum of healthy subjects.

The Ig-like structure of CTLA-4 is maintained by two intrachain disulfide bonds between Cys^{21}–Cys^{92} and Cys^{48}–Cys^{66} [28]. These cysteine residues are conserved in CTLA-4delTM, suggesting that the extracellular conformation is maintained. The binding domains of CTLA-4 to CD80/CD86 are located in the CDR1- and CDR3-like domains [14, 15]. As these domains are conserved in CTLA-4delTM, it is tempting to speculate that its ability to bind CD80/CD86 could be unaffected. Membrane CTLA-4 is a homodimer [28]. The dimerization implies

the formation of a disulfide bond at C120 and requires both transmembrane and intracellular domains [28]. Moreover, the intracytoplasmic tail of membrane CTLA-4 presents the motif TVLM which is reponsible for its accumulation in the endosomes of activated T cells [31, 32]. The deletion of exon 2 results in the loss of both transmembrane and intracellular domains (including C120), suggesting that CTLA-4delTM could be produced as a soluble monomer. This hypothesis was confirmed by Western blotting and ELISA showing that recombinant CTLA-4delTM is a soluble monomeric molecule. The monomeric CTLA-4-Fc has reduced binding affinity and shows rapid dissociation from CD80/CD86 compared with dimeric CTLA-4 [28, 33]; therefore one can assume that the affinity of soluble CTL4-4delTM could be reduced compared with membrane CTLA-4.

A soluble immunoreactive forms of CTLA-4 was detected in the serum of healthy subjects. This unexpected results suggests a potentially important role for soluble CTLA-4 in the regulation of immune homeostasis. This role may be dependent on the time when soluble CTLA-4 is present. Indeed, blocking the interaction of B7 molecules with their ligands could interfere either at the initiation [34–36] or during the outcome of the immune response [22]. The administration of CTLA-4-Ig fusion protein before the onset of disease prevents the initiation of Th-mediated pathologies such as lupus [37], EAE [38], celiac disease [39] or collagen-induced arthritis [40]. On the contrary, blocking the CTLA-4-B7 interaction using neutralizing anti-CTLA-4 mAb has been shown useful to induce tumor rejection in a mouse model [23] while it exacerbates EAE when injected during the development of the pathology [41]. As a consequence, soluble CTLA-4 could either inhibit or exacerbate the immune response if present during the initiation (blocking of CD28-B7 interaction) or at latter stages (blocking the CTLA-4-B7 interaction) of the immune response, respectively. Upon activation, T cells selectively express membrane CTLA-4, showing that soluble CTLA-4 may be produced only by nonactivated T cells. This result suggest that soluble CTLA-4 could act essentially in a paracrine manner to regulate the immune response.

4 Materials and methods

4.1 Isolation and activation of human T cells

T cells were isolated from heparinized venous blood from healthy subjects. Briefly, PBMC were isolated by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and T cells were purified by rosetting with sheep erythrocytes. The purity of T cells was > 95 %, as assessed by FCM analysis using a FITC-labeled anti-CD3 mAb (Immunotech, Marseille, France) (data not shown). T cells were cultured in complete medium [RPMI 1640 medium supplemented with 5 % FCS and antibiotics (all from Life technologies, Cergy Pontoise, France)] and were either unstimulated or stimulated with 10 ng/ml anti-CD3 mAb (clone OKT3; ATCC, Manassas, VA) plus 10 ng/ml immobilized anti-CD28 mAb (Immunotech) for 24 h or stimulated for 6 h with 10 ng/ml PMA plus 1 µg/ml ionomycin (both from Calbiochem, La Jolla, CA).

4.2 Cloning and sequencing of the CTLA-4 mRNA variants

Total RNA was extracted using Trizol reagent (Life Technologies) and polyA$^+$ RNA was purified using oligo-dT-coated magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's recommendations. The single-stranded cDNA was synthesized using 1 µg of polyA$^+$ RNA by reverse transcription using an oligo-dT primer (Pharmacia). PCR reactions were performed with cDNA corresponding to 10 ng of polyA$^+$ RNA, Taq polymerase (Clontech, Palo Alto, CA) and primers designed to amplify the entire coding sequence of CTLA-4: 5'-ATGGCTTGCCTTGGATTTC-AGCGGCACAAGG-3' and 5'-TCAATTGATGGGAATAAAA-TAAGGCTGAAATTGC-3' (predicted size: 672 bp). PCR reaction was as follows: 94 °C for 5 min, 30 cycles 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min followed by a final extension at 72 °C for 5 min. The amplified fragments were separated on 1 % agarose gel and visualized by ethidium bromide. After excision from the gel, each amplified cDNA fragment was subcloned into a TA cloning vector (pCRII; In Vitrogen, Leek, The Netherlands). Sequencing was performed using the ABI-PRISM Dye Terminator Cycle Ready Reaction kit (Perkin Elmer, Foster City, CA). The expression of CTLA-4 mRNA in activated *vs.* nonstimulated human T cells was evaluated by PCR using the primers mentioned above. RNA integrity and cDNA synthesis was verified by amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (5'-TCCACCACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3').

4.3 Production of a recombinant soluble CTLA-4 protein

The membrane CTLA-4 and CTLA-4delTM cDNA were subcloned in the vector and used to transfect COS cells. COS cells (ATCC) were cultured in DMEM medium supplemented with 10 % FCS, L-glutamine and antibiotics (all from Life Technologies) and transfected with the vectors using the calcium phosphate method (5prime-3prime, Boulder, CO). The production of a soluble form of CTLA-4 was evaluated by ELISA in the cell-free supernatants of transfected cells. Briefly, 96-well plates (Nunc) were coated with the anti-CTLA-4 mAb (clone BNI3; Pharmingen, San Diego, CA) at 1 ng/100 µl/well in 0.1 M phosphate buffer, pH 4.0 (16 h at 4 °C) before incubation for 2 h at room temperature with PBS/BSA 1 %. After washing, plates were incubated for

16 h at 4 °C with concentrated culture supernatant (200 µl/well). Bound CTLA-4 was detected with biotin-labeled anti-CTLA-4 mAb 8H5 (Pharmingen) followed by incubation with streptavidin-biotinylated-horseradish peroxidase (used at 1/5000; Amersham, Amersham, GB). The specificity and concentration of soluble CTLA-4 was determined using commercially available recombinant CTLA-4-Ig (Ancell, Bayport, MN).

4.4 Western blotting analysis

Western blotting analysis was performed with COS cells transfected with CTLA-4 or CTLA-4delTM cDNA. Cells were washed in ice-cold PBS before lysis in 10 mM phosphate buffer, pH 7.4 containing 0.5 % NP40 (Sigma) and protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Proteins from 5×10^6 cells were electrophoretically separated on a 10 % polyacrylamide gel under nonreducing conditions and then transferred onto a nitrocellulose membrane (Bio-Rad, Ivry sur Seine, France). After saturation, membranes were incubated with an anti-CTLA-4 mAb (Pharmingen). After washing, membranes were incubated with peroxidase-labeled anti-mouse IgG Ab (DAKO) and bound Ab were detected using the enhanced chemiluminescence (ECL) system (Amersham).

4.5 Detection of soluble CTLA-4 in human serum

Soluble CTLA-4 was evaluated in the serum of healthy volunteers by ELISA, as described above. The concentration of soluble CTLA-4 was estimated by using chimeric CTLA-4-Ig as standard.

4.6 Statistical analysis

Statistical significance was determined using the Student's *t*-test.

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EXHIBIT 10

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A Native Soluble Form of CTLA-4[1,2]

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CTLA-4 is an immunoregulatory receptor expressed on the surface of activated T and B lymphocytes. The counterreceptors for CTLA-4 are the B7 family molecules. We describe alternatively spliced mRNAs expressed in hematolymphoid tissues of humans, mice, and rats that lack the transmembrane domain coded by exon 3 of the CTLA-4 gene. These alternate transcripts were detected by RT-PCR in B cells and resting T cells of both the CD4 and the CD8 phenotype. Activation of human blood mononuclear cells with PHA or anti-CD3 + anti-CD28 monoclonal antibodies appears to effect a decrease in the amount of the alternative transcript relative to the full-length transcript. Recombinant sCTLA-4 is a B7-binding protein and has immunomodulatory effects as measured by inhibition of the mixed leukocyte response. Human serum contains immunoreactive material consistent with a native soluble form of CTLA-4. © 2000 Academic Press

Key Words: T lymphocytes; comparative immunology; mRNA.

INTRODUCTION

CTLA-4[4] is a lymphocyte cell surface receptor originally discovered in a search for molecules having a role in T cell cytotoxicity (1, 2). Although originally identified as a cytolytic T-cell-associated molecule, CTLA-4 expression has been detected in both activated CD4 and CD8 T cell populations as well as in B cells with the use of monoclonal antibodies (3, 4). CTLA-4 is a member of the immunoglobulin superfamily and is highly homologous to another T cell surface receptor, CD28 (1, 2, 5, 6). Both CD28 and CTLA-4 bind the same ligands, namely, members of the B7 family of molecules expressed on APC (7, 8). Expression of CTLA-4 is highly activation-dependent. CTLA-4 is not detected on resting T cells but is induced during T cell activation (3, 9, 10). With activated T cells, maximal surface CTLA-4 protein expression is ~2–3% of surface CD28 (9). Recombinant forms of CTLA-4 bind CD80 (B7.1) and CD86 (B7.2) with higher avidity (>20-fold) than soluble CD28 (9, 11). Thus, CTLA-4 is a high-avidity, low-abundance receptor for B7 molecules.

Much of the study of the interactions of CTLA-4 and its B7 counterreceptors has relied on the use of a recombinant soluble form of CTLA-4 (CTLA4-Ig). CTLA4-Ig is a fusion protein consisting of the extracellular domain of CTLA-4 and the human IgG heavy chain constant region gene (8). Numerous reports have documented the usefulness of CTLA4-Ig as an immunoregulatory agent. For example, CTLA4-Ig inhibits alloreactivity both in the context of the mixed leukocyte reaction (8, 12, 13) and *in vivo* in experimental cardiac allografts (12, 14) and pancreatic islet xenografts (15). CTLA4-Ig is also reported to modify the course of several animal models of human disease including murine lupus (16), experimental autoimmune encephalomyelitis (17), allergic contact dermatitis (18), and autoimmune anti-glomerular basement membrane glomerulonephritis (19). In addition, CTLA4-Ig also inhibits cellular and humoral responses to conventional antigens such as keyhole limpet hemocyanin and sheep red blood cells (20) and pigeon cytochrome c (21).

We have reported the molecular cloning and the nucleotide sequence of the full-length rat CTLA-4 gene (22). During the course of these experiments, in addition to the expected target CTLA-4 transcript, distinct smaller amplification products were also observed following RT-PCR of RNA from a variety of lymphoid

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[2] The nucleotide and predicted amino acid sequence data reported in this paper have been submitted to the GenBank sequence database and have been assigned the following accession numbers: Mouse sCTLA-4, U90270; Rat sCTLA-4, U90271; Rat intron/exon boundaries, AF012711, AF012712, and AF012713; Human sCTLA-4, U90273.

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[4] Abbreviations used: APC, antigen presenting cells; BMC, bone marrow cells; CTLA-4, cytotoxic T lymphocyte-associated molecule-4; EIA, enzyme immunoassay; KLH, keyhole limpet hemocyanin; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; SA, splice acceptor; SD, splice donor; sCTLA-4, soluble CTLA-4; RT-PCR, reverse transcriptase-PCR; TM, transmembrane.

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tissues from humans, mice, and rats. This report describes the nucleotide and the predicted amino acid sequence of these alternative transcripts. In addition, we show that the variant transcript is most abundant in cells from bone marrow, blood, and lymph nodes, whereas the transmembrane form of the molecule is the predominant, and perhaps the only species, expressed in the adult thymus. We also report the expression and immunoregulatory properties of recombinant sCTLA-4 and present data that suggest that a native soluble form of sCTLA4 is present in human serum.

MATERIALS AND METHODS

Tissue samples and cells. Female ACI rats (150–200 g) and Balb/c mice were obtained from Harlan Sprague–Dawley (Indianapolis, IN). Human blood samples were obtained from healthy volunteers. Human adult lymph node and spleen samples and neonatal thymus were obtained at autopsy. Mononuclear cells were obtained from spleen and blood by standard Ficoll–Hypaque density gradient centrifugation. Other organs and tissues were frozen and stored in liquid nitrogen until used for RNA extraction. Human PBMCs were activated for 48 h *in vitro* by culture in RPMI 1640 medium containing 10% FBS, 5 μg/mL Con A, and 10 nM PMA (from Sigma Chemical Co., St. Louis, MO). Rat $CD4^+$ and $CD8^+$ splenic lymphocytes were isolated by dual rounds of affinity chromatography (Cellect immunocolumns, Biotex Labs, Edmonton, Alberta) according to the manufacturer's instructions. B cells were negatively selected by dual passage over a T cell chromatography column followed by immunomagnetic positive selection with the use of anti-CD45RA/B (clone OX-33). The purity of these suspensions was examined by flow cytometry using anti-CD4 and anti-CD8 monoclonal antibodies (clones OX-35 and OX-8, respectively). Fewer than two fluorescence events per 200,000 ungated cells were present in these enriched populations (not shown) demonstrating the high enrichment of the cells used for RT-PCR amplification. The antibodies used in these studies were purchased from Pharmingen (San Diego, CA).

RT-PCR. Total cellular RNA was extracted by the guanidine thiocyanate method using kit-supplied reagents (RNAgents, Promega Biotec, Madison, WI). Single-strand cDNA was generated from 1 μg total cellular RNA with the use of Superscript II preamplification reagents (Life Technologies, Bethesda, MD) according the manufacturer's instructions. PCR was carried out in 25-μL volumes of 1× PCR buffer (60 Tris–HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$) containing 1/10 the contents of the reverse transcription reaction, 0.2 mM each dNTP, 0.5 μM each primer, and 0.05 U/μL Taq DNA polymerase (Promega, Madison, WI). The reactions were subjected to 20–35 amplification cycles on a Perkin–Elmer Cetus thermal cycler. The amplification cycle profile was 95°C denaturation for 1 min, followed by primer annealing at 60°C and extension at 72°C for 1 min each. PCR products were separated by gel electrophoresis and analyzed with a Gel-Doc 1000 system equipped with Molecular Analyst software (Bio-Rad Instruments).

Oligonucleotide primers. Primers were designed with the aid of commercially available software (Primer Designer; S&E Software, State Line, PA) from previously published sequences of rat (22), mouse (1), and human (5) CTLA-4 genes. The following are the 5′ → 3′ sequences of the sense (s) and antisense (as) primers used in these studies: RCTLA4-57s = CATGGCTTGTCTTGGACTCC; RCTLA4-781as = GCCTCAGCTTTTAGAGACTG; HCTLA4-4s = TTCCTGAAGACCTGAACACC; HCTLA4-766as = AGAATTGCCTCAGCTCTTGG; RCTLA4-366s = TGACACCGGACTGTACTTCT; HCTLA4-341s = TCACTATCCAAGGACTGAGG; RHCTLA4-635as = TCACATTCTGGCTCTGTTGG. The size of the amplification products from cDNA derived from the transmembrane (TM) and the variant transcript described in this communication are as follows. 57s/781as rat primer set yields a 724-bp TM fragment and a 614-bp sCTLA-4 fragment; 4s/766as human primer set yields a 762-bp TM fragment and a 652-bp sCTLA-4 fragment. The 366s/635as primer set yields TM and sCTLA-4 fragments of 269 and 159 bp, respectively, using rat or mouse cDNA as a template. The 341s/635as primer set yields a TM product of 294 bp and a sCTLA-4 product of 184 bp with human cDNA as a template. Note that the 635as oligonucleotide is used as a common anti-sense primer for rat, mouse, and human cDNAs, as this sequence is derived from the highly conserved cytoplasmic domain of CTLA-4. All primers were synthesized by Operon Technologies (Alameda, CA).

Cloning and sequencing. PCR product was cloned into the pCR3 vector using a TA cloning kit (Invitrogen Corp., CA). PCR products as well as cloned inserts were sequenced bidirectionally by dideoxy cycle sequencing using the above-described PCR primers and vector primers, respectively.

Antibodies. Rabbit anti-human CTLA-4 antibodies were generated by standard methods in New Zealand White rabbits immunized with KLH-conjugated peptides. The peptides were synthesized and conjugated by Research Genetics (Huntsville, AL). Antiserum 8K was raised to the novel carboxyl-terminal sequence of the predicted sCTLA-4 protein (KPSYNRGLCENAPNRARM). This amino acid sequence shares no significant similarity with proteins cataloged on the GenBank database, and the 8K antiserum is not reactive with commercially available CTLA4-Ig. The anti-CTLA4 monoclonal antibody (clone BNI3) used in inhibition experiments was obtained from Pharmingen.

sCTLA-4 expression. Recombinant baculovirus clones containing the human sCTLA-4 gene were generated by standard cloning methods. Viral stocks were generated by cotransfection of recombinant sCTLA-4 in pBlueBacHis expression vector with the pBlueBac transfer vector (Invitrogen, Carlsbad, CA) into the SF9 insect cell line. The High-Five insect cell line was used as the host for expression experiments. Control baculovirus containing the chloramphenicol acetyl transferase gene (CAT) was prepared and tested in parallel in all baculovirus expression experiments. His fusion proteins (CAT and sCTLA-4) were purified by Ni column chromatography before use in functional assays.

Fusion proteins. B7.1 and B7.2 immunoglobulin fusion proteins were generated by cloning the extracellular domains of these cDNAs into the signal-pIG vector (Novagen, Madison, WI). For this purpose, we used reverse transcription PCR of Raji cell (ATCC, Rockville, MD) RNA using the following sense and antisense primers:

B7.1s = AAGCTTGGTCTTTCTCACTTCTGTTCAG;
B7.1as = GGATCCGCATCAGGAAAATGCTCTTGC;
B7.2s = GCTAGCGCTCCTCTGAAGATTCAAGCTT;
B7.2as = GGATCCGCCGGGGGAGGCTGAGGGTCCTCA.

The antisense primers contain 3′ *Bam*HI sites, and the B7.1 and B7.2 sense primers contain a 5′ *Hin*dIII and *Nhe*I site, respectively, to provide for directional cloning into the signal pIG vector. Cos-7 cells were transfected with the use of Cellfectin (Life Technologies, Bethesda, MD), and neomycin-resistant cell lines were generated by stepwise selection in G418 up to 1 mg/mL of active drug. Cell culture supernatants were collected in serum-free medium and tested in a sandwich ELISA for B7 and human IgG1 epitopes. Positive culture supernatants were pooled and purified by protein A chromatography (Pierce Immunochemical, Rockville, IL). Each fusion protein was reactive with CTLA4-Ig (Ancell, Bayport, MN) as determined by an enzyme immunoassay (not shown). SDS–PAGE analysis of the fusion proteins showed major products of approximately 150 kDa under nonreducing conditions and products of 74 and 83 kDa for B7.1–Ig and B7.2–Ig, respectively, when analyzed under reducing conditions. Each fusion protein demonstrated T cell costimulation in conjunction with fluid phase OKT3 as measured by a PBMC proliferation assay (not shown). The Muc–Ig fusion protein was expressed from a commercially available (Novagen) plasmid following transfection in CHO cells as described above.

Enzyme Immunoassays. A modified sandwich EIA was used for determination of binding of B7–Ig fusion proteins to recombinant sCTLA-4. For this purpose, wells of a 96-well microtiter plate were coated overnight with approximately 200 ng/well of the appropriate B7–Ig fusion protein. The wells were washed with 0.5% Tween 20 in PBS, pH 7.6, and then blocked with Superblock (Pierce Immunochemical). One hundred microliters of the test samples was applied to the wells, and the plates were incubated for 30 min at room temperature and then washed to remove the unbound sample. One hundred microliters of a 1:500 dilution of the rabbit anti-human sCTLA-4 (8K) antiserum was then added, and the plates were incubated for 1 h, washed, and reacted for 30 min with 100 μL of a 1:50,000 dilution of affinity-purified HRP-conjugated goat anti-rabbit IgG. After being washed, the reactions were developed with the use of 3, 3′, 5, 5′-tetramethyl benzidine substrate (Turbo TMB ELISA, from Pierce). Absorbance was read at 450 nm on a Molecular Devices microplate spectrophotometer.

Mixed leukocyte reactions (MLR). Two-way MLR was used to test recombinant sCTLA-4 for functional activity. The cells used in these experiments were from a panel of previously HLA typed laboratory volunteers and were selected to provide two HLA-DR mismatches. PBMCs were isolated by standard Ficoll–Hypaque density gradient centrifugation, and equal numbers of cells (100,000) were cocultured in tissue culture medium (RPMI 1640 supplemented with 10% human AB serum, 10 μg/mL gentamicin, and 50 μM 2-mercaptoethanol) containing graded dilutions of sCTLA-4 test sample. Triplicate cultures were set up in 0.2-mL volumes in round-bottom 96-well microculture plates and were incubated for 5 days at 37°C and 5% CO_2 in air. The cultures were pulsed with 0.5 μCi tritiated thymidine and harvested 18 h later. Isotope incorporation was measured on a Packard Tri-Carb 2000 scintillation counter.

Western blotting. Western blotting was used to detect recombinant and native (serum) sCTLA-4. Proteins were separated by 10–20% gradient PAGE in a discontinuous buffer system on a Mini-Protean system (Bio-Rad). The separated components were electroblotted onto PVDF membranes. The blots were washed with 0.15 M NaCl, 0.05 M Tris (TBS), pH 7.5, with 0.3% Tween 20 and reacted with a 1:100 dilution of the 8K antiserum for 1 h at room temperature, washed, and then reacted with reporter antibody (HRP-conjugated anti-rabbit IgG) for 1 h. The blots were then developed with the use of a commercially available chemiluminesence detection kit (BMB, Indianapolis, IN) according to the manufacturers instructions. Control blots were stained as described above but with the addition of a preimmune serum from the individual rabbit used for antibody production.

RESULTS

In order to characterize the rat and human CTLA-4 mRNAs, we isolated cellular RNA from splenocytes of ACI rats and lymph nodes from humans. The CTLA-4 mRNA transcripts were then analyzed by RT-PCR am-

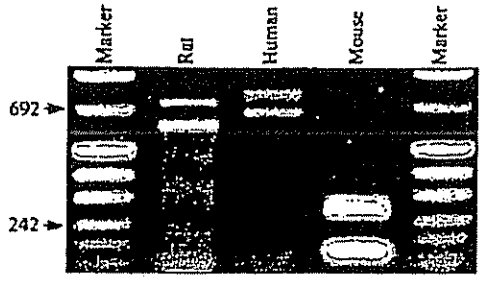

FIG. 1. Gel electrophoresis of RT-PCR products of rat, human, and mouse mRNAs with CTLA-4 primers. Rat and human PCR products are derived from spleen cDNAs amplified with primers designed to detect the coding region of CTLA-4. Mouse products are from spleen cDNA amplified with primers that flank the CTLA-4 TM region. The upper bands in each lane represent amplification products of the CTLA-4 TM transcript, the lower bands are amplifications of the sCTLA-4 transcript.

plification with the use of oligonucleotide primers that flanked the ATG initiation codons and the translation termination codons. The amplified products were expected to encompass the entire translation region as well as a portion of the 5′ untranslated region of the CTLA-4 gene and to be 724 and 762 bp for rat and human, respectively. In addition to the anticipated full-length CTLA-4 cDNA, a distinct smaller 614-bp amplification product was also observed in rats, and a 652-bp fragment was detected in humans (Fig. 1). We designated these transcripts *sCTLA-4* and refer to them as such hereafter. Screening of the cloned PCR product also revealed recombinant clones of similar sizes, and restriction enzyme analysis showed the absence of two restriction enzyme sites (based on the nucleotide sequences of full-length CTLA-4) within the transmembrane region of CTLA-4 (data not shown). Similar findings were also observed upon RT-PCR of RNA from mouse splenocytes when amplified with primers that flank the transmembrane region of CTLA-4. These data suggest that these molecules represent alternative transcripts of the CTLA-4 molecule that lack a transmembrane domain.

Nucleotide sequencing of these cDNAs proved this assumption to be correct. The nucleotide and predicted amino acid sequence of the mature human sCTLA-4 molecule is presented in Fig. 2. The predicted sCTLA-4 molecule (minus the leader peptide) is a 137 amino acid polypeptide and is 49 amino acids shorter than the full-length molecule. When compared to their full-length counterparts, rat and human molecules have identical nucleotide and predicted amino acid sequences spanning their entire extracellular domains, but the sCTLA-4 form represents a deletion of the entire transmembrane domain. The nucleotide sequences of the "cytoplasmic" domains of each species are identical to their full-length counterparts with the exception of the first codon. The net effect of this is that the deletion changes the reading frame of the molecule and, thus, the amino acid sequence of the vestige of the cytoplasmic domain of the sCTLA-4. A comparison of the rat sCTLA-4 molecule to its transmembrane counterpart is presented in Fig. 3. This alternative transcript is also found in mouse splenocyte RNA and has

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  M  H  V  A  Q  P  A  V  V  L  A  S  S  R  G  I  A  S  F  V -20
ATGCACGTGGCCCAGCCTGCTGTGGTACTGGCCAGCAGCCGAGGCATCGCCAGCTTTGTG
************************************************************

  C  E  Y  A  S  P  G  K  A  T  E  V  R  V  T  V  L  R  Q  A -40
TGTGAGTATGCATCTCCAGGCAAAGCCACTGAGGTCCGGGTGACAGTGCTTCGGCAGGCT
************************************************************

  D  S  Q  V  T  E  V  C  A  A  T  Y  M  M  G  N  E  L  T  F -60
GACAGCCAGGTGACTGAAGTCTGTGCGGCAACCTACATGATGGGGAATGAGTTGACCTTC
************************************************************

  L  D  D  S  I  C  T  G  T  S  S  G  N  Q  V  N  L  T  I  Q -80
CTAGATGATTCCATCTGCACGGGCACCTCCAGTGGAAATCAAGTGAACCTCACTATCCAA
************************************************************

  G  L  R  A  M  D  T  G  L  Y  I  C  K  V  E  L  M  Y  P  P -100
GGACTGAGGGCCATGGACACGGGACTCTACATCTGCAAGGTGGAGCTCATGTACCCACCG
************************************************************

  P  Y  Y  L  G  I  G  N  G  T  Q  I  Y  V  I  A  K  E  K  K -120
CCATACTACCTGGGCATAGGCAACGGAACCCAGATTTATGTAATTGCTAAAGAAAAGAAG
************************************************

  P  S  Y  N  R  G  L  C  E  N  A  P  N  R  A  R  M  -
CCCTCTTACAACAGGGGTCTATGTGAAAATGCCCCCAACAGAGCCAGAATGTGA
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FIG. 2. The nucleotide and predicted amino acid sequence of the human sCTLA-4 transcript. (***), "extracellular" V domain; (—), coding region of remnants of cytoplasmic domain. Bold amino acids represent predicted N-linked glycosylation sites.

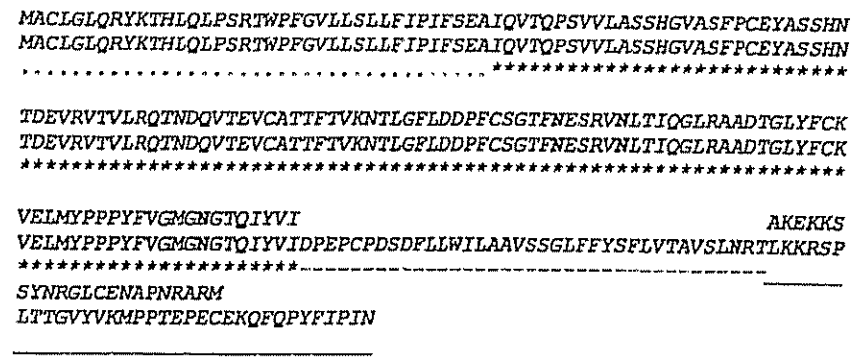

FIG. 3. Comparison of the predicted amino acid sequences of the sCTLA-4 and transmembrane forms of rat CTLA-4. The sCTLA-4 sequence is shown in the top line; the transmembrane form is shown below. (. . .), Signal sequence; (***), V-like extracellular domain; (- - -), transmembrane region; (—), cytoplasmic tail. Bold amino acids represent predicted sites for N-linked glycosylation.

identical splice sites when compared to the rat and human sCTLA-4 molecules (not shown).

There is a high degree of nucleotide and amino acid sequence similarity among the three species examined in this work. Figure 4 is a comparison of the amino acid sequences of the predicted sCTLA-4 polypeptides of human, mouse, and rat. Note that there is 100% conservation in the amino acid sequence of the vestige of the cytoplasmic tails of mouse and rat sCTLA-4 and 95% conservation between human and mouse or rat. The sCTLA-4 molecules from all three species contain the MYPPPY peptide motif found in the extracellular domains of full-length CTLA-4 of rat, mouse, and human. The conservation of this motif is important because it is critical to the binding of CTLA-4 to B7 family molecules (23); thus, its preservation predicts that the putative sCTLA-4 protein would have B7 binding properties. The human and rat sCTLA-4 molecules are predicted to have two and three N-linked glycosylation sites, respectively, based upon the presence of the Asn-X-Ser/Thr motifs. The membrane proximal cysteine (amino acid 120 in the human numbering system) which appears to be responsible for the formation of homodimers in the TM CTLA-4 protein (24) is absent in the sCTLA-4 molecule, although a novel cysteine residue is created within the vestige of the cytoplasmic domain. Thus, it is possible that the predicted sCTLA-4 molecule also exists as a dimer.

In order to determine whether sCTLA-4 arose by translation of an alternatively spliced transcript, genomic DNA surrounding the TM-encoding regions of rat CTLA-4 was amplified by PCR and cloned. Restriction enzyme analysis and sequencing of the cloned genomic DNA revealed two introns. Intron A is approximately 500 bp and lies between the extracellular and TM exons; intron B is approximately 1100 bp and resides between the TM and cytoplasmic domain exons. DNA sequencing of the splice donor/acceptor (SD and SA, respectively) sites adjacent to the intron/exon boundaries predicts two possible transcripts of the CTLA-4 molecule based on consensus sequences of SD\SA sites (Fig. 5). The transmembrane form of the molecule arises via two RNA splicing events between the SD\SA sites of intron A as well as between the SD\SA sites of intron B. By application of the same guidelines for SD\SA splicing (25), splicing of the in-

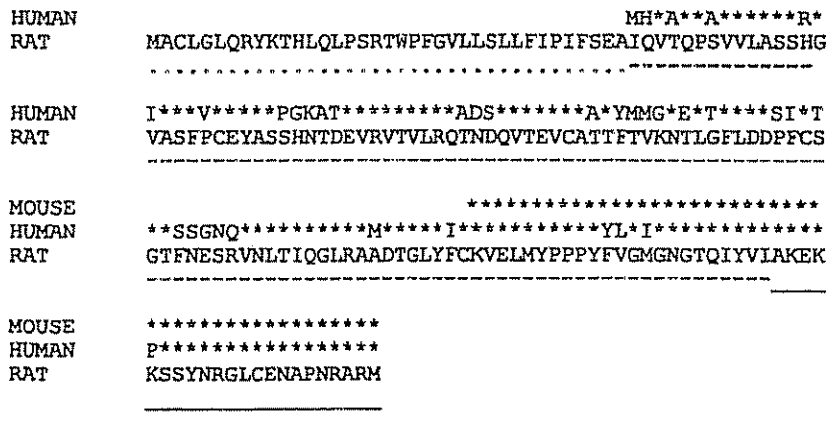

FIG. 4. Comparison of the predicted amino acid sequences of the rat, human, and mouse sCTLA-4 molecules. (*) Indicates amino acid identity when compared to the rat sequence. (. . .) Signal peptide; (- - -), Immunoglobulin V-like region; (—), vestige of cytoplasmic tail.

```
   I  Y  V  I ↓                (≈0.5 Kb)              ↓ D  P  E  P
ATTTATGTCATCGgtaagcagagccattcc.........ttttgtgttgcagATCCAGAACCA
   I  Y  V  I                   INTRON A

  C  P  D  S  D  F  L  L  W  I  L  A  A  V  S  S  G  L  F  F  Y
TGCCCAGATTCAGACTTCCTCCTTTGGATCCTTGCTGCAGTTAGTTCGGGGTTGTTTTTCTAC

  S  F  L  V  T  A  V  S  L  N  R  T↓        (≈1.1 Kb)
AGTTTCCTGGTCACCGCTGTTTCTTTGAACAGGACGgtgagtgt.........tcttttgtg
                                              INTRON B
          ↓ L  K  K  R  S  P.............
ttttttggcagCTAAAGAAAAGAAGTCCT
            A  K  E  K  K  S.............
```

FIG. 5. The Intron\exon boundaries of genomic DNA of the rat CTLA-4 gene. ↓ Indicates location of nucleotide at intron\exon boundary. Uppercase nucleotides denote exons; lowercase denotes introns. Splices between the SD\SA sites of both introns A and B produces the transcript encoding the transmembrane form of the molecule (amino acid sequence shown above nucleotide sequence). A splicing event between the SD site of intron A and the SA site of intron B produces the soluble form of CTLA-4. The amino acid sequence of sCTLA-4 is shown below the nucleotide sequence.

tron A donor site to the intron B acceptor site would accurately yield the sCTLA-4 transcript. The net effect of this splicing event is a 110-bp deletion corresponding to the entire transmembrane domain of the CTLA-4 molecule. The A → B splicing event also introduces a frameshift mutation within the "cytoplasmic" domain of sCTLA-4 (the alternative reading frames of each are illustrated in Fig. 5). Thus, although the nucleotide sequences of these molecules are identical downstream of the splice sites, the change in reading frame produces an amino acid tail that is unique to the sCTLA-4 molecule. This tail is 22 amino acids long and 12 amino acids shorter than that of the transmembrane form of CTLA-4 due to the introduction of a TGA termination codon at nucleotide position 523 of the rat sCTLA-4 gene. The genomic structure of the human CTLA-4 gene is highly similar (5), and examination of the sequences of the SD\SA sites predicts the possible presence of the described sCTLA-4 transcript.

In order to examine the tissue distribution of the TM and sCTLA-4 transcripts, we used RT-PCR with primers that flank the TM region of CTLA-4. Because the two transcripts compete for the same oligonucleotide primers during PCR, the relative levels of each transcript can be estimated from their fluorescent intensities on an ethidium-bromide-stained gel. The results of a typical experiment are shown in Fig. 6. Both the TM and the sCTLA-4 transcripts were detected in lymph node, spleen, and peripheral blood in both humans and rats (Fig. 6A). The TM transcript appears to be the only transcript expressed in the thymus of adult rats, whereas the sCTLA-4 transcript is the only mRNA species detected in bone marrow cells. The expression of both CTLA-4 transcripts appears to be exclusive to the hematolymphoid system: neither the TM nor the sCTLA-4 transcripts were detected in a wide variety of nonlymphoid tissues including adrenal, brain, eye, heart, kidney, liver, lung, ovary, pancreas, salivary gland, seminal vesicles, skeletal muscle, testes, and thyroid (data not shown).

We next attempted to determine the distribution of the TM and sCTLA-4 transcripts within lymphocyte subsets. As demonstrated in Fig. 6B, both the TM and the sCTLA-4 transcripts are expressed in the CD4 and CD8 subsets of T cells, as well as B lymphocytes. The relative amounts of the TM and sCTLA-4 transcripts appear to differ between the CD4 and CD8 T cell subsets. For example, nearly identical amounts of the TM and sCTLA-4 products are obtained following RT-PCR of $CD4^+$ T cells, whereas there appears to be nearly 2.5-fold more TM product than sCTLA-4 product among $CD8^+$ T cells. This skewed distribution of CTLA-4 transcripts among the CD4 and CD8 populations produces a TM:sCTLA-4 ratio of approximately 1.5:1 when analyzed as unfractionated T cells. The TM

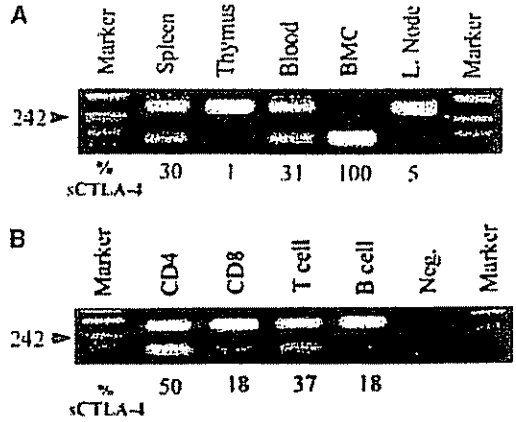

FIG. 6. The tissue distribution of the sCTLA-4 transcript in rats. Gels of RT-PCR products of RNA from various rat hematolymphoid tissues using primers that surround the transmembrane region of rat CTLA-4. (A) Unfractionated tissues; (B) fractionated splenic mononuclear subsets. The upper band is approximately 269 bp and corresponds to products of the transcript that encodes the TM form of CTLA-4; the lower band is approximately 159 bp, which is consistent with products of the sCTLA-4 transcript. Arrow indicates a marker band of 242 bp; "Neg" lane is product from RT-PCR reaction mix minus cDNA.

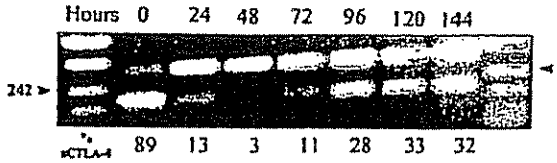

FIG. 7. Effects of activation status on the relative levels of TM vs sCTLA-4 mRNA. Human PBMCs were activated for the indicated time periods as described under Materials and Methods and harvested for RT-PCR analysis using primers that span the TM region of the CTLA-4 gene. The arrow indicates a 242-bp marker; the upper band represents the product of the TM CTLA-4 transcript; the lower band indicates a 184-bp product of the sCTLA-4 transcript. The results are representative of three separate experiments on different blood donors.

CTLA-4 species is the predominant transcript among B cells, representing 71% of the total CTLA-4 amplification product in this experiment. By contrast, the relative amount of TM and sCTLA-4 transcripts among unfractionated T cells is 59% vs 41%, respectively. Similar data were obtained in a duplicate set of experiments.

Because the mRNA and protein product of the CTLA-4 gene are reported to be expressed on activated but not resting T cells, we attempted to determine the relative levels of CTLA-4 TM vs sCTLA-4 on resting and activated human PBMCs. The results of a representative experiment are shown in Fig. 7. Although both transcripts are detected in resting cells, the sCTLA-4 transcript appears to be the predominant one. This ratio is reversed by 24 h postactivation, and by 48 h full-length CTLA-4 is almost the exclusive mRNA transcript. From 72 to 120 h following activation, the sCTLA-4 transcript appears to increase relative to CTLA-4 TM, although it does not exceed about 2/3 of the total CTLA-4 mRNA. Similar results were obtained upon activation of either rat or human PBMC with anti-CD3 + anti-CD28 (not shown). Several human and mouse T and B cell lines (including EL-4, EL-4-IL2, Jurkat, HPB-MLT, Hut-78, and SP2-0/Ag-14) were negative when tested for expression of the sCTLA-4 transcript.

Recombinant sCTLA-4 was expressed in a baculovirus system for the purpose of determining functional activity. Recombinant material obtained by infection of the High-Five insect cell line with the baculovirus expression plasmid contained immunoreactive sCTLA-4 which demonstrated specific binding to each of the B7.1 and B7.2 immunoglobulin fusion proteins, but minimal binding to a control protein (Fig. 8). Partial inhibition of B7:CTLA-4 interactions could be demonstrated when the binding assays were performed in the presence of monoclonal antibody to CTLA-4. Recombinant sCTLA-4 showed specific binding to Raji and Daudi cells (known to express B7 molecules) as well as CHO transfectants of the transmembrane forms of B7.1 and B7.2 by an indirect immunofluoresence assay (not shown).

We next examined the immunoregulatory properties of recombinant sCTLA-4 by determining its effect on the MLR. The data presented in Fig. 9 show that sCTLA-4 inhibits the MLR in a dose-dependent manner and is capable at high concentrations of complete inhibition of cellular proliferation in this system. The inhibition of the MLR by sCTLA-4 could be partially reversed by the addition of anti-CTLA-4 monoclonal antibody, and recombinant sCTLA4 also inhibited the costimulatory properties of a CHO cell line that expresses a full-length B7.1 gene in a manner similar to CTLA4–Ig (data not shown). Taken together, the data support the notion that sCTLA-4 encodes a receptor with functional properties including its ability to bind the B7 family of molecules and, as a consequence, inhibit B7:CD28 interactions that enhance cellular immune responses to alloantigen.

Finally, we attempted to determine whether a circulating form of CTLA-4 was present in human serum. For this purpose, Western blots of human serum were probed with the antiserum (8K) generated against the carboxyl-terminal fragments of the sCTLA-4 polypeptide. As shown in Fig. 10, this antiserum reacts predominantly with a polypeptide species of approximately 23 kDa, which is consistent with the predicted size based on the amino acid sequence and predicted N-linked glycosylation pattern of native sCTLA-4. This immunoreactive species was identified in 11 of 20 serum samples from patients with endocrine autoimmune disease. A more detailed description of these findings is published elsewhere (26). Some serum samples revealed a larger immunoreactive species of ap-

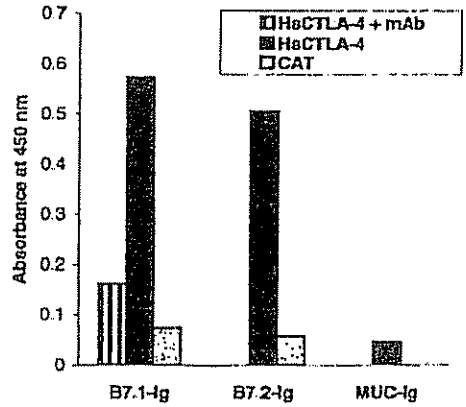

FIG. 8. Recombinant sCTLA-4 is a B7-binding protein. B7.1–, B7.2–, or Muc18–immunoglobulin fusion proteins were immobilized to microplate wells, recombinant proteins were added, and CTLA-4 immunoreactive material was detected as outlined under Materials and Methods. CAT is chloramphenicol acetyl transferase expressed in a baculovirus system and is used here as a negative control. The data represent five replicates in two separate experiments. Muc18–Ig is an irrelevant fusion protein was used to determine the specificity of B7–Ig:CTLA-4 binding. In select experiments, we added anti-CTLA4 monoclonal antibody (500 ng/mL final concentration) and sCTLA-4 simultaneously in order to examine inhibition of the B7:sCTLA-4 interaction.

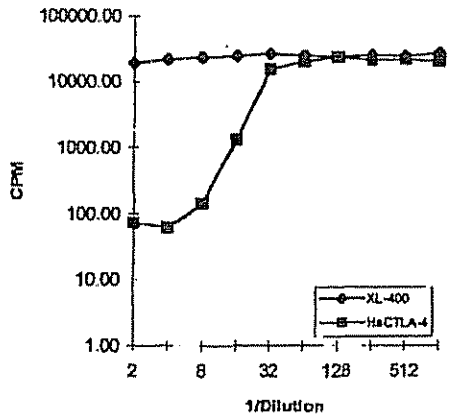

FIG. 9. Immunoregulatory properties of sCTLA-4. Inhibition of two-way MLR. HsCTLA-4 is recombinant CTLA-4 derived from baculovirus expression and partially purified by Ni-resin chromatography; XL-400 culture supernatant was derived from mock-infected insect cells. The data represent the mean of quadruplicate samples. Initial dilutions of proteins used in these assays are approximately 250 ng/mL. Similar results were obtained in three separate experiments from a variety of donor/recipient combinations.

proximately 75–80 kDa that was observed under both reducing and nonreducing conditions. The nature of this species is not known and was not investigated further. No immunoreactive material was detected on blots probed with preimmune sera from individual animals used for the immunization procedure, even when used 50-fold more concentrated than that from immune rabbits. Immunoprecipitation experiments using a monoclonal antibody (BNI3 described under Materials and Methods section) to capture CTLA-4 immunoreactive material from serum and developed with the 8K antiserum also showed the same 23-kDa species (not shown). Thus, the material present in human serum appears to have epitopes derived from the extracellular domain of the classic CTLA-4 protein as well as those believed to be unique to the sCTLA-4 polypeptide. Western analysis of recombinant sCTLA-4 shows two major immunoreactive species of approximately 25 and 19 kDa. The 25-kDa species is consistent with the expected size of the fully glycosylated recombinant protein. The origin of the 19-kDa species is not clear, but is consistent with a hypoglycosylated form of the recombinant protein. This smaller species was not observed following expression of sCTLA-4 in CHO cells (not shown) and is thought to represent a variant due to expression in insect cells.

DISCUSSION

Soluble forms of receptors are produced through proteolytic cleavage of membrane-bound receptors, as in the case of the tumor necrosis factor (27) and the interleukin-2 receptors (28), or by translation of alternative spliced mRNA as found in the IL-4 (29), IL-7 (30), and Fas (31) receptors. Secreted receptors represent truncated forms of the membrane-bound receptors because of the introduction of a frameshift or stop codons. These soluble receptors can bind ligand in a similar manner as their membrane-bound counterparts and as a result may play an important role in the regulation of receptor activity (31, 32). The data presented in this report suggest that, like many other receptors with immunoregulatory functions, CTLA-4 exists in a truncated form. When compared to the transmembrane form of the CTLA-4 molecule, the sCTLA-4 transcript appears to be more predominant in resting cells than in activated cells. This finding may explain why this mRNA species has not been previously identified, as CTLA-4 is widely considered an activation antigen and, thus, most studies of CTLA-4 expression are performed on activated cells.

We think it unlikely that the sCTLA-4 mRNA species represents an artifact that was amplified during PCR. First, cellular activation appears to regulate the relative levels of each CTLA-4 transcript, as the sCTLA-4 variant appears to be the predominant transcript in resting peripheral blood mononuclear cells, whereas the TM form is the predominant species by 24–48 h postactivation (see Fig. 7). Second, the relative levels of each transcript appear to vary by lymphocyte subset. For example, among T cells the relative levels of each CTLA-4 mRNA species appear to be similar; whereas the TM species is the predominant one within B cells. Third, no other variant transcripts or clones (such as transcripts lacking an extracellular region) were identified in numerous PCR experiments and during the screening of several hundred recombinant plasmids. Perhaps more importantly, lymphoid tissues appear to express varying relative amounts of each transcript. For example, the TM form appears to be the only transcript expressed in the thymus, but the sCTLA-4 transcript is the predominant species in bone marrow cells. It is thus difficult to envision a scenario by which errors in splicing would be differentially reg-

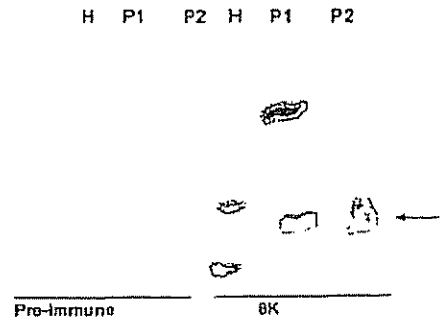

FIG. 10. Western blot of recombinant sCTLA-4 and human serum proteins. Lane marked H indicates the recombinant His-sCTLA-4 fusion protein. P1 and P2 refer to different samples used in these experiments. The left side of the blot was probed with a preimmune serum from the same animal used to produce the 8K antiserum used on the right side of the blot as underlined. The arrow indicates an ~23-kDa band of CTLA-4 immunoreactive material.

ulated in different lymphoid compartments as well as by activation status.

Our data suggest that a native soluble form of CTLA-4 is expressed in humans. Western blotting experiments with an anti-peptide antiserum designed to be reactive with the polypeptide encoded by the variant transcript described here (but not the transmembrane form of the molecule) reveal immunoreactive material in human serum consistent with the size of the predicted sCTLA-4 molecule. Our observation that the sCTLA-4 transcript was not detectable in T cell lines is perhaps not surprising given that it appears to be downregulated in activated cells. The fact that this transcript lacks the entire membrane-spanning domain of the classical CTLA-4 molecule suggests that it encodes a native soluble form of the CTLA-4 molecule. Because the amino acid residues that are thought to be critical to B7 molecule binding (namely the MYPPPY motif) are intact, we predict that expression of this molecule should encode a protein capable of binding the B7 family of molecules. In support of this, we show that recombinant sCTLA-4 is a B7 binding molecule and demonstrate that it has immunoregulatory properties *in vitro*. Furthermore, we show that human serum contains circulating material that is immunoreactive with an antibody raised against an epitope within the putative sCTLA-4 molecule, but not the transmembrane species.

Our understanding of the functional consequences of the interactions of the B7 family of molecules with the coreceptors CD28 and CTLA-4 has become clearer over recent years. The consequences of B7 molecule binding of the membrane forms of CD28 and CTLA-4 appear to be opposing (33). It is well established that B7:CD28 interactions provide a major costimulatory signal to T cell activation (34, 35). The emerging notion of B7:CTLA-4 binding is that CTLA-4 triggering provides a negative signal to T cells (36–38) and in fact may lead to apoptosis and/or a state of immunologic anergy (39–42). In this context, one can envision a dual role for the candidate sCTLA-4 molecule. On one hand, sCTLA-4 may bind B7 expressed on APC and thus interfere with B7:CD28 mediated costimulation of T cell responses. On the other hand, sCTLA-4 may also be capable of interfering with B7:CTLA-4 interactions, thereby blocking the negative signal imparted via the TM form of CTLA-4. In this regard, it is interesting that sCTLA-4 does not appear to be expressed within the thymus, an organ in which negative selection of autoreactive T cells occurs.

Our data also raise the possibility that recombinant sCTLA-4 could be used to regulate B7:CD28 interactions. For example, CTLA4–Ig has been highly successful in regulating the *in vivo* and *in vitro* immune response to a wide variety of antigens (20, 21, 43). Expression of the sCTLA-4 gene may produce a natural soluble form of CTLA-4 with similar useful immunoregulatory properties.

ACKNOWLEDGMENTS

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EXHIBIT 11

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Soluble CTLA-4 in autoimmune thyroid diseases: Relationship with clinical status and possible role in the immune response dysregulation

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KEYWORDS
CTLA-4;
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Abstract CTLA-4 molecule, expressed by activated T and B lymphocytes, transduces an inhibitory signal. Increasing evidence showed *CTLA-4* gene as an important susceptibility locus for autoimmune endocrinopathies and other autoimmune disorders. The aim is to evaluate the augmented sCTLA-4 serum levels in different autoimmune thyroid diseases when compared with normal donors or with non-autoimmune hyperthyroidism and to investigate the functional activities and suggest the possible pathogenetic role of sCTLA-4. We demonstrate the presence of a soluble form of CTLA-4 in 59/90 sera from patients with autoimmune thyroid diseases (both Graves' disease and autoimmune thyroiditis). sCTLA-4 levels were not related to specific clinical manifestations, such as clinical thyroid status (hypo- or hyperthyroidism), circulating thyroid hormones, or other clinical features (ophthalmopathy). sCTLA-4 production does not seem to be affected by disease evolution during time. We showed that sCTLA-4 from sera of patients with thyroid autoimmunity is able to bind its physiological ligands CD80/CD86 and displays functional activities on different *in vitro* systems (T-cell proliferation induced by specific soluble antigens, bi-directional mixed lymphocyte reaction). In conclusion, we demonstrate an increment of sCTLA-4 in serum of patients with autoimmune thyroid diseases. Its possible pathogenetic role during autoimmune processes can be speculated: sCTLA-4 can specifically inhibit the early T-cell activation by blocking the interaction of CD80/CD86 with the co-stimulatory receptor CD28. Conversely, higher levels of sCTLA-4 could compete with membrane-bound CTLA-4 for CD80/CD86, in later T lymphocytes activation phase, causing a reduction of inhibitory signaling.
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Introduction

Effective T-cell activation requires a primary signal delivered by the antigenic peptide presented by major histocompatibility complex molecules and a non-specific signal (co-stimulation signal) mediated by CD28 interaction with B7

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family members on antigen-presenting cells [1]. Cytotoxic T lymphocyte associated gene-4 (CTLA-4) was initially described as a classical type I glycoprotein on the surface of activated T cells [2]. CTLA-4 is a member of the Ig gene superfamily and along with its homologue, CD28, which is a B7 binding protein [3,4]. The emerging opinion about the function of CTLA-4 is that it represents a negative regulator of T-cell activation. Thus, ligation of CTLA-4 on the T-cell surface initiates a cascade of biochemical events that attenuate an ongoing immune response [5]. CTLA-4 specific antibodies, which were cross-linked by a second antibody or immobilized on beads, have been found to prevent activation [6–8]. The most convincing data that support such a role for CTLA-4 come from experiments in which the *CTLA-4* gene is inactivated by a construction of CTLA-4 knockout mice [9,10]. These mice demonstrate profound polyclonal lymphoproliferative disorders that infiltrate most major organ systems and die a few weeks after birth. The majority of animals have increased levels of IgG: this fact illustrates the role of CTLA-4 on humoral immune responses as well [11,12].

A role for CTLA-4 in autoimmune diseases is suggested by the observation that blockade of B7/CTLA-4 interaction via administration of anti-CTLA-4 mAb exacerbates animal models of autoimmune diseases such as experimental autoimmune encephalomyelitis [13] and type-1 diabetes (T1D) [14].

A search for genetic markers that segregate with Graves' disease revealed an association with CTLA-4 polymorphisms [15]. Such association was further substantiated in various ethnic populations, as well as in other endocrine autoimmune disease [16–20]. More recently, Ueda et al. [21] analyzed the fine polymorphism of *CTLA-4* gene in patients with various autoimmune disease, including ATD and they found some possible associations.

Oaks and Hallett [22] submitted the nucleic acid sequences of alternate transcripts of *CTLA-4* in man, mouse, and rat that lacked transmembrane encoding regions. More recently, Magistrelli et al. [23] described the same transcript and detected immunoreactive material in human serum demonstrating the presence of a native soluble form of CTLA-4 (sCTLA-4). In addition, the presence of high concentration of sCTLA-4 was observed in sera patients with ATD (mainly Graves' disease) [24].

Finally, recent reports show that sCTLA-4 levels are augmented in patients with autoimmune diseases, such as type-1 diabetes [25], diffuse cutaneous systemic sclerosis [26], systemic lupus erythematosus [27], and myasthenia gravis [28]. In addition, plasma levels of sCTLA-4 are increased in patients with allergic asthma [29].

The present study has been conceived to further evaluate the immunopathological roles of sCTLA-4. In particular, we have verified the increasing of sCTLA-4 in different autoimmune thyroid diseases, analyzing the possible differences related to clinical manifestations. Finally, we have investigated the functional activities of sCTLA-4 in the serum of these patients.

Materials and methods

Serum samples

Serum samples were obtained from 90 patients with autoimmune thyroid diseases (ATD) (16 males, 74 females, age range 22–60) after informed consent. Fifty-nine of them had Hashimoto's thyroiditis (HT), 31 Graves' disease (GD). The very large majority of ATD patients had detectable levels of autoantibodies to thyroid peroxidase (86 out of 90), 51 out of 90 had also autoantibodies to thyroglobulin. The diagnosis was formulated on the basis of commonly accepted clinical and laboratory criteria, including thyroid ultrasonography, serum TSH and free thyroid hormone levels, anti-thyroid peroxidase antibodies, and anti-TSH receptor antibodies and thyroid scintigraphy (for GD). Patients with HT were either mild hypothyroid (raise TSH with low–normal free thyroxine) or euthyroid under L-thyroxine replacement. Patients with GD were hyperthyroid (from mild to severe) and were studied before or immediately after starting anti-thyroid therapy: 26 of them had clinically apparent Graves' ophthalmopathy.

From some GD patients serum samples collected at different time points, namely before, 1, and 3 months after radioiodine therapy, were available. In these patients euthyroidism or post-treatment hypothyroidism was documented, as well as thyroid gland volume reduction (by ultrasonography).

For control, we collected sera from 20 patients with non-autoimmune hyperthyroidism (toxic adenoma or multinodular toxic goiter) and from 45 age- and sex-matched blood donors.

Free thyroid hormone concentrations were assayed by routine ELISA methods, as well as TSH (3rd generation) and thyroid peroxidase and thyroglobulin autoantibodies.

ELISA

Specific ELISA kits were used for measuring serum sCTLA-4 levels (Bender Med System, Milano, Italy), according to the manufacturer's protocol. Each sample was diluted 1:10 and tested in triplicate. Deviation between triplicates was <10% for any reported value. The lowest sensitivity threshold is 0.1 ng/ml.

The analytical response was linear approximately between 0.162 and 1.200 of absorbance values (corresponding to 0.1–50 ng/ml) as assessed by serial dilution test using a strongly positive serum (data not shown).

As control, for a subgroup of patients ELISA kits specific for sCD28, sCD80 and sCD86 were also used (Bender manufacturer's protocol).

Western blotting

Western blotting was used to detect serum sCTLA-4. Proteins were separated by 10–20% gradient PAGE in a discontinuous buffer system on a Mini-Protean system (Bio-Rad, Segrate, Milano, Italy). The separated components were electroblotted onto PVDF membranes. The blots were washed with 0.15 M NaCl, 0.05 M Tris (TBS), pH 7.5, with 0.3% Tween 20 and reacted with a 1:100 dilution of the anti-CTLA-4 mAb (clone 14D3, IgG2a, eBiosciences, San Diego, CA, USA) for 1 h at room temperature, washed, and then reacted with reporter antibody (HRP-conjugated anti-rabbit IgG) for 1 h. The blots were then developed by the use of a commercially available chemiluminescence detection kit (BMB, Indianapolis, IN) according to the manufacturer's instructions.

Evaluation of immunophenotype

A B-cell line transformed by Epstein–Barr virus (B-EBV) (5×10^5), obtained as previously described [8], was preincubated with different sera from patients selected for their sCTLA-4 positivity or negativity. In addition, B-EBV cells were incubated with Fc-blocking mAb (KD1, IgG1, anti-CD16) to prevent non-specific binding of labeled mAb and then stained with FITC-conjugated anti-CD80 (clone HIB19, Becton Dickinson, Milano, Italy) and anti-CD86 (clone G18–145). Cells not pretreated with serum were used as positive control of CD80/CD86 expression. After staining, cells were analyzed, and fluorescence signals were collected in log mode using a FACScalibur (Becton Dickinson). CTLA-4 Ig (provided by A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland) was utilized as a positive control of molecule reacting with CD80 and CD86 ligands.

Mixed leukocyte reactions (MLR)

Two-way MLR was used to test sCTLA-4 for functional activity. The cells used in these experiments were from a panel of previously HLA typed laboratory volunteers and were selected to provide two HLA-DR mismatches. PBMCs were isolated by standard Ficoll-Hypaque density gradient centrifugation, and equal numbers of cells (100,000) were cocultured in tissue culture medium (RPMI 1640 supplemented with 10% human AB serum, 10 mg/ml gentamicin, and 50 mM 2-mercaptoethanol) containing graded dilutions of sCTLA-4 test sample. Sera were added at different time points as indicated. Triplicate cultures were set up in 0.2-ml volumes in round-bottom 96-well microculture plates and were incubated for 5 days at 37 °C and 5% CO_2 in air. The cultures were pulsed with 0.5 mCi ^{3}H-thymidine and harvested 18 h later. Isotope incorporation was measured on a Beckman β-counter. For tests with *Candida* bodies (Ca), Ca-specific CD4$^+$ T-cell lines were generated from total PBMCs by repeated stimulation with autologous irradiated PBMCs pulsed with the appropriate antigen, as previously described [30].

Depletion of sCTLA-4 protein from sera

In order to understand the effective role played by serum sCTLA-4 avoiding the effect related to other serum proteins, we performed an MLR assay using sera previously depleted of sCTLA-4. To this end we incubated the sera from different patients (2 positive and 2 negative after ELISA testing) for 3 h at room temperature on ELISA plates coated with the capture mAb anti-sCTLA-4 (included in the ELISA kit). In order to avoid any specific binding to the plastic, plates were blocked (with PBS buffer containing 5 g/l of bovine serum albumin and 0.5 ml/l of Tween 20) and fixed (with PBS buffer containing 15% sucrose) before adding the sera. This procedure (suggested by the manufacturer) was repeated twice before utilizing these sera in an MLR assay as specified before. As a control we used sera not subjected to the depletion procedure.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test for comparison of sCTLA-4 levels by using the GraphPad Prism4 software 4.0 (GraphPad Software Inc., CA, USA). Differences in cell proliferation between control and mAb-treated cells were observed. To assess their statistical significance, the Student's *t* test was used with a level of $p<0.05$.

Results

Serum sCTLA-4 levels in ATD patients

To determine whether a circulating form of CTLA-4 was present in human serum, we utilized a sensitive ELISA. Fig. 1A shows combined data from the ELISA on patients with ATD and normal apparently healthy volunteers. sCTLA-4 was virtually undetectable in the very large majority of healthy volunteers as defined by the detection limit of 0.1 ng/ml for this assay. Six serum samples out of 45 from healthy controls

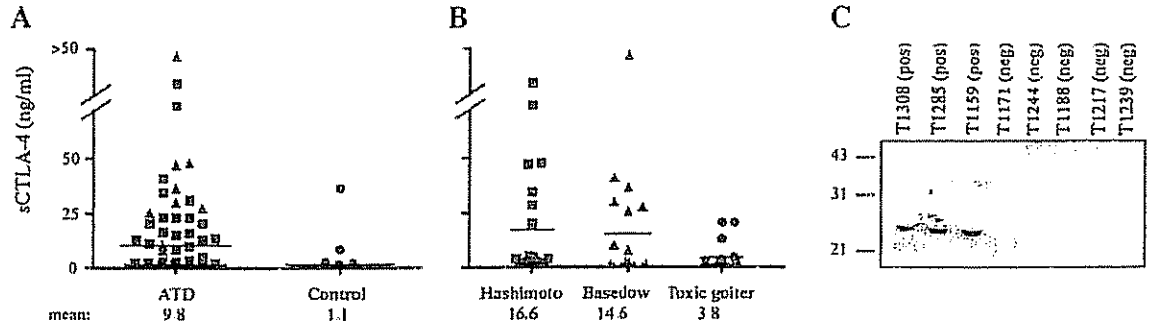

Figure 1 A soluble form of CTLA-4 is found in serum of patients with ATD. (A) The concentration of sCTLA-4 was evaluated by ELISA on sera collected from ATD patients ($n=90$; squares HT, triangles GD) and a healthy donor group as a control ($n=45$). Results are expressed as ng/ml. Each sample was diluted 1:10 and tested in triplicate. Deviation between triplicates was <10% for any reported value. (B) Results of sCTLA-4 ELISA on 24 selected sera of untreated hyperthyroid GD, 20 sera of HT patients, and 20 control sera of patients with non-autoimmune hyperthyroidism (toxic adenoma or multinodular toxic goiter). Results are expressed in ng/ml. Each sample was diluted 1:10 and tested in triplicate. Deviation between triplicates was <10% for any reported value. (C) Immunoprecipitation of sCTLA-4 was performed on sera from a representative group of ATD patients. T1308, T1285, and T1159 were resulted positive during ELISA analysis; T1171, T1244, T1188, T1217, and T1239 were sera negative for sCTLA-4. Arrow marks 23-kDa species. Positive or negative sera are indicated by an alphanumeric code followed by (+) and (−) respectively.

Table 1 Sera collected from the same patients at different time points after therapy showed a comparable amount of sCTLA-4

Time points	Patient sCTLA-4 evaluation (ng/ml)					
	T105	T77	T56	T52	T23	T155
T_0	37.6	36.4	27.4	<0.1	<0.1	<0.1
T_1	27.0	46.9	9.4	<0.1	0.3	0.6
T_2	30.6	47.1	16.1	0.2	0.3	0.3

ELISA was performed on sera collected from the same patients at different time points after radioiodine therapy (T_0: before radioiodine therapy, T_1: 1 month and T_2: 2 months after therapy). Of note, patients that were positive for the soluble spliced form (T105, T77 and T56) maintain the ability to produce it over time, irrespective of the kinetic of thyroid function. On the other hand, patients unable to produce sCTLA-4 at the beginning of the therapy (T52, T23, and T155) have virtually no detection of this protein at any time points.

had detectable sCTLA-4 (from 0.1 to 36.1 ng/ml). By contrast, 59 out of 90 patients (65%) with ATD had detectable circulating CTLA-4 levels in the range of 0.1–>50 ng/ml ($p<0.05$) (Fig. 1A). Of note, there were no differences between patients with HT and GD. In addition, sCTLA-4 concentration was significantly higher in patients with hyperthyroid GD than in controls with non-autoimmune hyperthyroidism ($p<0.05$) (Fig. 1B). There was no obvious relationship between the sex and age of the patient and levels of sCTLA-4. Moreover, no relationship was found in GD patients between sCTLA-4 levels and degree of hyperthyroidism (free T4 or T3 concentrations) or severity of ophthalmopathy. Finally, there was no relationship between thyroid autoantibodies and sCTLA-4 levels (data not shown).

In addition, Western blot of human serum was probed with specific anti-CTLA-4 mAb. As shown in Fig. 1C, the three different samples resulted positive by ELISA analyses (T1308, T1285, and T1159) were characterized by a band of approximately 23 kDa, which is consistent with previous reports [22–24]. These species were not evident when sera which resulted negative during ELISA were plotted (T1171, T1244, T1188, T1217, and T1239).

Of interest, the analysis of the sera collected from the same patient at different time points after radioiodine therapy showed that the ability to produce sCTLA-4 is not related to thyroid functions. In particular, patients who were positive for the soluble spliced form maintained their ability to produce it over time. On the other hand, patients unable to produce sCTLA-4 at the time of therapy resulted negative during the follow-up (Table 1).

sCTLA-4 from serum of autoimmune patients is able to bind CD80/CD86

In order to verify the capability of sCTLA-4 to recognize its specific ligands (CD80/CD86) and to avoid the possibility that the soluble molecule is the result of a degradation or shedding process, we attempted to block the binding of specific mAb to CD80/CD86 present on B-EBV cell targets. Pretreatment of B-EBV cell lines with sera from autoimmune patients characterized by the presence of sCTLA-4 led to a significant reduction of staining by anti-CD80 or anti-

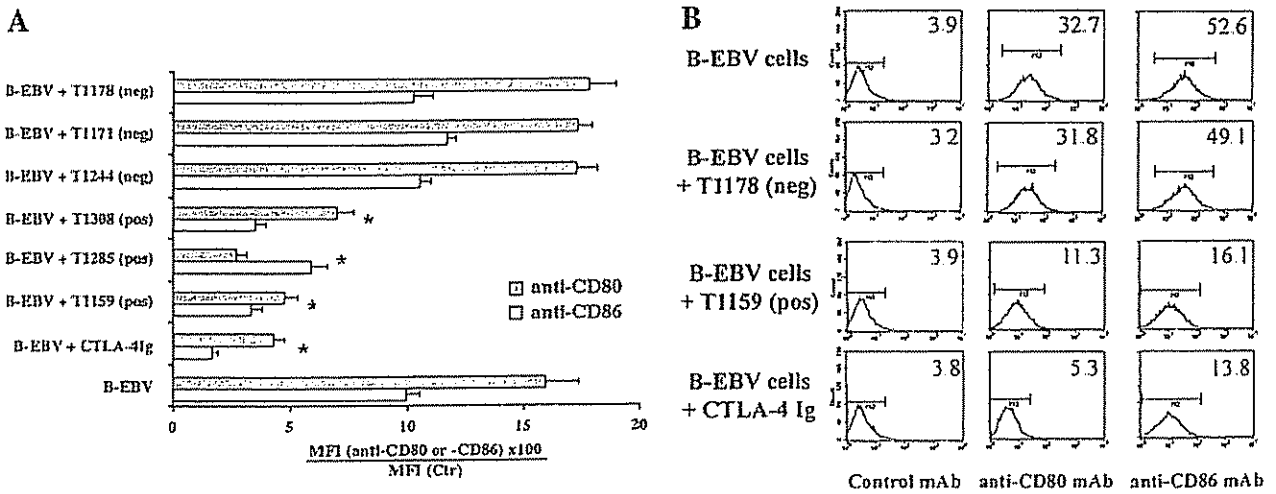

Figure 2 sCTLA-4 from sera of ATD patients is able to interact with its natural ligands. (A) Data from three independent experiments showing the capability of sCTLA-4 to inhibit anti-CD80/anti-CD86 mAb immunoreactivity. The test was performed utilizing a B-EBV cell line expressing both CD80 and CD86 molecules. Six different sera from patients with ATD (T1308, T1285, and T1159 positive and T1171, T1244, and T1188 negative for sCTLA-4) were preincubated with B-EBV cells prior to stain cells with mAb to CD80 and CD86. The addition of CTLA-4 Ig fusion protein was utilized as positive control of interference of specific mAb binding to CD80 and CD86. The graphic plots the ratio between the Mean Fluorescence Intensity (MFI) of the cells stained with specific mAb (anti-CD80 or -CD86) and the MFI of the negative control (B-EBV no treated with sera). The averages±standard deviations (error bars) of three different experiments are shown. * Indicates samples that are significantly different ($p<0.05$) from the control (B-EBV). (B) Representative data from an experiment to test whether sCTLA-4 was capable of inhibiting anti-CD80/anti-CD86 mAb immunoreactivity. The test was performed as previously described.

CD86 mAb (T1308, T1285, and T1159). These results were obtained on three independent serum samples and ranged from 17 to 61% and from 56 to 85% of inhibition, respectively, for CD80 and CD86 (Fig. 2). A negligible inhibition (<5%) was observed when sera negative for sCTLA-4 were tested. CTLA-4 Ig, which reduced the staining of B-EBV cell lines by anti-CD80 or anti-CD86 mAb, was utilized as a positive control. These data show that the CTLA-4 immunoreactive material present in human serum represents an intact functional receptor for CD80/CD86 ligands.

sCTLA-4 from serum of autoimmune patients is able to inhibit both mixed leukocyte reactions (MLR) and proliferation of antigen-specific T-cell clones in a dose dependent fashion

We next examined the immunoregulatory properties of sCTLA-4 by determining its effect on two-way MLR. The data presented in Fig. 3A show that sCTLA-4 had significant inhibitory effect, similarly to that one obtained by the addition of CTLA-4 Ig. Moreover, we verified the dose effect

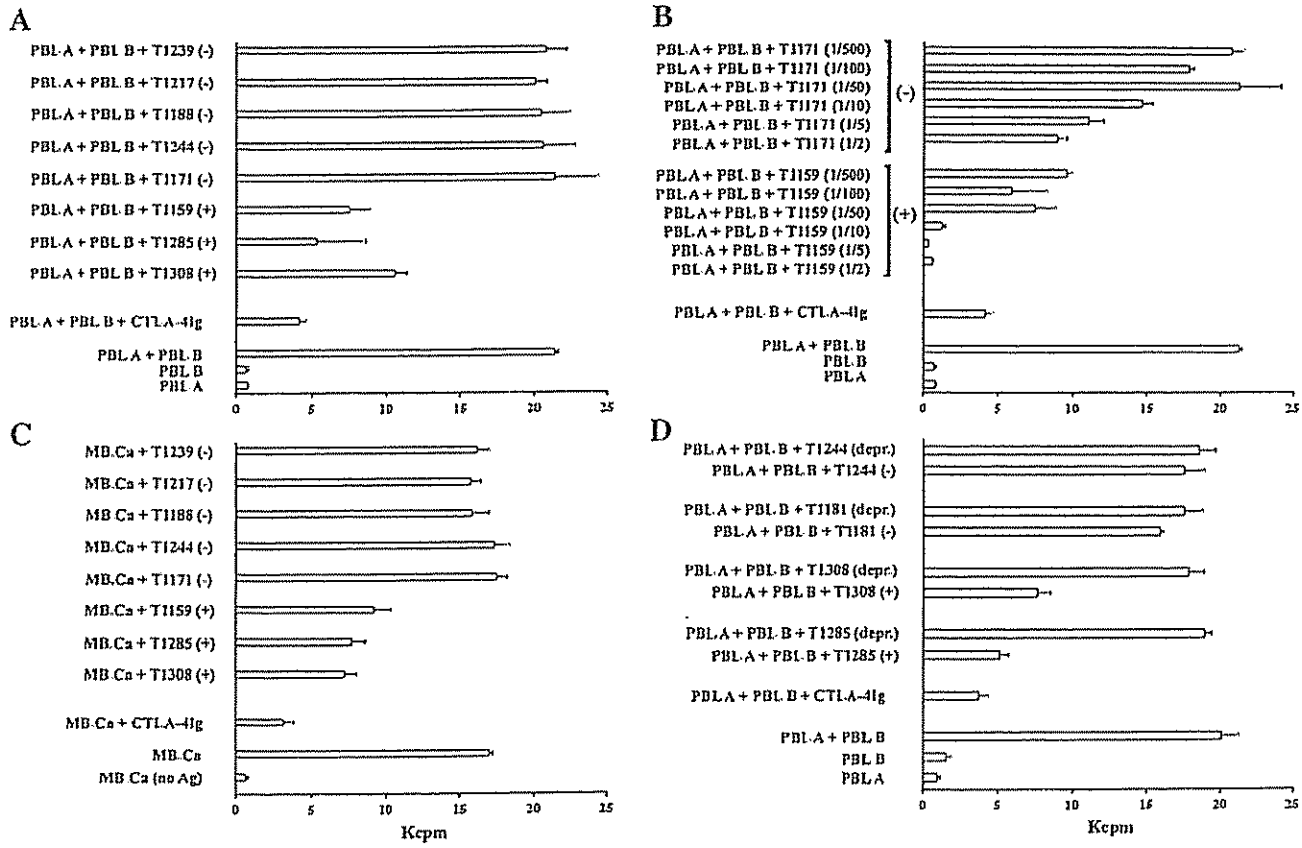

Figure 3 Immunoregulatory properties of sCTLA-4. (A) sCTLA-4 containing sera from ATD patient (T1308, T1285 and T1159) are able to inhibit significatively a two-way MLR. CTLA-4 Ig fusion protein is a positive control of inhibition of proliferation. Sera from ATD patients negative for sCTLA-4 (T1171, T1244, T1188, T1244, and T1239) do not affect the two-way MLR. The data represent the mean of quadruplicate samples. Initial dilutions of proteins used in these assays are approximately 0.1 ng/ml. The averages ± standard deviations (error bars) of three different experiments are shown. Similar results were obtained in three separate experiments from a variety of donor/recipient combinations. Positive or negative sera are indicated by a alphanumeric code followed by (+) and (−) respectively. (B) Analysis of the dose effect of 2 different sera (T1159 and T1171) respectively positive and negative for sCTLA-4. The inhibition rate depends on serum concentration of sCTLA-4. CTLA-4 Ig fusion protein is a positive control of inhibition of proliferation. The data represent the averages ± standard deviations (error bars) of quadruplicate samples. Positive or negative sera are indicated by a alphanumeric code followed by (+) and (−) respectively. (C) Similar inhibitory effect of sCTLA-4 was observed in an antigen-specific ^{3}H-thymidine incorporation assay. The proliferation of an antigen-specific T-cell clone (specific for *Candida albicans* bodies, MB.Ca) induced by PBMCs pulsed with the antigen was reduced by the addition of sera which resulted positive for sCTLA-4, whereas the negative ones had no effect. The averages ± standard deviations (error bars) of three different experiments are shown. Positive or negative sera are indicated by an alphanumeric code followed by (+) and (−) respectively (D) The inhibitory effect on T lymphocytes proliferation is related to sCTLA-4 and is not due to a different serum protein. sCTLA-4-deprived sera (T1285 and T1308) are ineffective on MLR ^{3}H-thymidine incorporation. Controls are the same sera before the treatment with the capture mAb specific for sCTLA-4 (see Materials and methods). The averages ± standard deviations (error bars) of three different experiments are shown. Positive or negative sera are indicated by an alphanumeric code followed by (+) and (−) respectively. The abscissa indicate the incorporation of ^{3}H-thymidine during the last 18 h of culture. Isotope incorporation was measured on a Beckman β-counter (Kcpm = 1000 cpm).

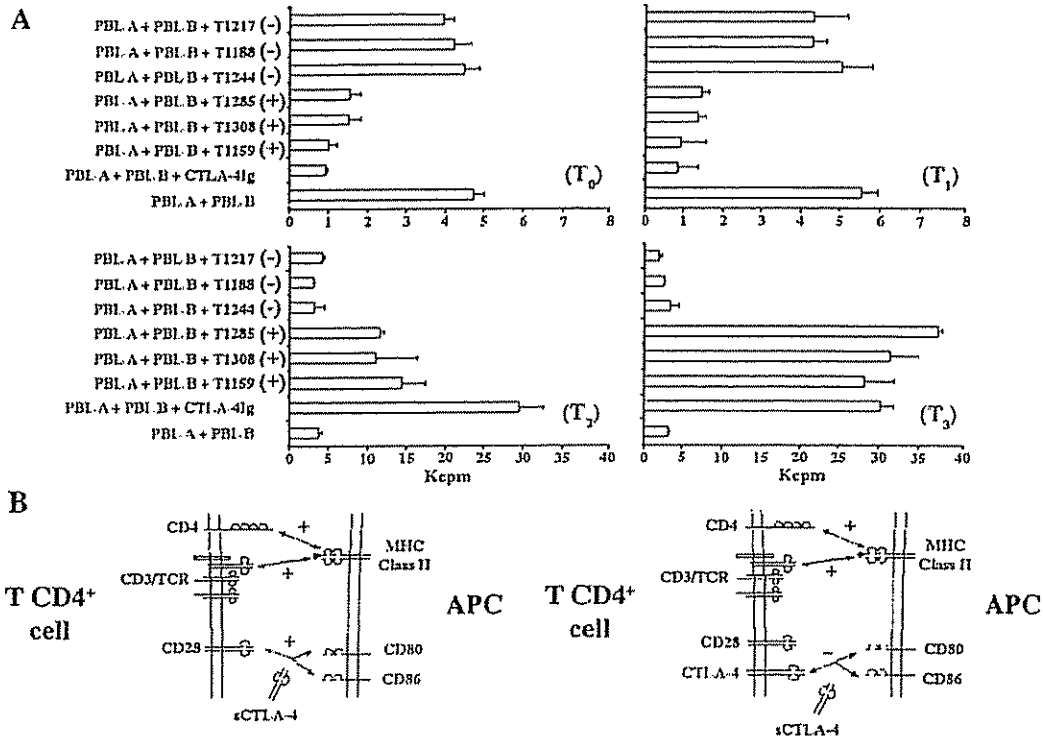

Figure 4 sCTLA-4 from serum of autoimmune patients is able to control MLR in a dynamic way. (A) The inhibitory effect on T lymphocytes proliferation due to sCTLA-4 is related to the activation state at which the soluble protein is added. When sCTLA-4-positive sera or CTLA-4 Ig fusion protein are added to the two-way MLR at time 0 (T_0, from the beginning of the T lymphocyte activation) or at time 1 (T_1, 24 h later) the result is a decrease of proliferative ability. On the contrary, when sCTLA-4 and CTLA-4 Ig were added at times 2 and 3 (T_2, T_3, respectively after 2 and 3 days from the beginning of the test) ^{3}H-thymidine incorporation rate is increased. The averages ± standard deviations (error bars) of three different experiments are shown. (B) A model explaining the different role played by sCTLA-4 on T-cell activation. T lymphocyte activation requires two signals: signal 1, provided by antigen–TCR engagement and signal 2 (co-stimulatory), mainly provided by the interaction of the co-stimulatory molecule, CD28, with its ligands CD80/CD86, on antigen-presenting cells. CTLA-4 is another co-stimulatory molecule also binding to CD80/CD86 but providing a negative signal to T-cell activation. The altered levels of sCTLA-4 could lead to either blockade of available CD80/CD86 molecules leading to a decreased stimulatory signal from CD28 engagement during the priming of immune response (left) or to an inability of membrane-bound CTLA-4 to engage with CD80/CD86 and hence less negative signaling (right) later during T lymphocyte activation. The abscissa indicates the incorporation of ^{3}H-thymidine during the last 18 h of culture. Isotope incorporation was measured on a Beckman β-counter (Kcpm = 1000 cpm).

of 2 different sera (namely T1159 and T1171) respectively positive and negative for sCTLA-4 ELISA. As shown in Fig. 3B, the inhibition rate depends on serum concentration of sCTLA-4. A similar inhibitory effect of sCTLA-4 was observed in an antigen-specific ^{3}H-thymidine incorporation assay. The proliferation of an antigen-specific T-cell clone (specific for *Candida* bodies, MB.Ca) induced by PBMCs pulsed with the antigen was reduced by the addition of sCTLA-4-positive sera, whereas the negative ones had no effect (Fig. 3C).

The inhibitory effect on T lymphocytes proliferation is related to sCTLA-4 and is not due to a different serum protein

In Fig. 3D we show an assay similar to that one described in Fig. 3A, where the sera utilized were previously deprived of sCTLA-4 proteins as described in Materials and methods section. Controls were the same sera before depletion. The results show that the depleted sera (T1285 and T1308) are ineffective on MLR ^{3}H-thymidine incorporation. This strongly suggests a direct role of the soluble form of CTLA-4 in regulating lymphocytes proliferation and rules out the intervention of putative different serum molecules.

sCTLA-4 from serum of autoimmune patients is able to control MLR in a dynamic way

It remains unknown how alterations of sCTLA-4 levels may contribute in the development of autoimmunity. It has been speculated that a reduction in sCTLA-4 level could lead to the reduction in the blocking of CD80/CD86 molecules and sustained T-cell activation through CD28 [26]. However, higher levels of sCTLA-4 could compete with membrane-bound CTLA-4 for CD80/CD86-binding sites and cause a reduction of inhibitory signals. In an attempt to clarify this point we planned experiments of two-way MLR where the ATD sera were added at different time points after the activation. As shown in Fig. 4A, the addition of sCTLA-4-

Table 2 Sera collected from the ATD patients and controls showed a comparable amount of sCD28

	Patient sCD28 evaluation (ng/ml)	
	Range	Mean±SD
Hashimoto patients (n=11)	4.1–315.3	153.9±120.62
Basedow patients (n=11)	5.7–292.8	108.3±111.88
Toxic goiter (n=10)	1.3–239.1	81.7±88.24
Normal donors (n=9)	0.4–172.6	103.2±76.47

ELISA was performed on selected sera collected from ATD patients (Hashimoto's thyroiditis, and Graves' disease) and from the control groups (non-autoimmune hyperthyroidism — toxic adenoma or multinodular toxic goiter- and age- and sex-matched blood donors).

positive sera (T1159, T1308, and T1285), as well as the control CTLA-4 Ig, resulted in a reduction of proliferation when added at the beginning (T_0) and after 24 h (T_1) of the test. The opposite effect was measurable when sera were added after 48 h (T_2) and 72 h (T_3). Sera negative for sCTLA-4 protein (T1244, T1188 and T1217) were ineffective on thymidine incorporation.

In our model (Fig. 4B), altered levels of soluble CTLA-4 could lead to either blockade of available CD80/CD86 co-stimulatory molecules with decreased co-stimulatory signal from CD28 engagement (if sCTLA-4 is increased during the early activation process) or to an inability of membrane-bound CTLA-4 to engage with CD80/CD86 and hence to reduced inhibitory signals (if sCTLA-4 levels are increased during the late activation phase).

Evaluation of other soluble molecules belonging to the B7 family in sera from ATD patients

In order to better understand the pivotal role of sCTLA-4 and to investigate the possible involvement of other soluble B7 family members, we performed specific ELISA tests. We found undetectable amount of sCD80 and sCD86 both in the ATD patient group and in the control ones (data not shown). On the other hand, sCD28 was detectable in both groups (Table 2). However, the concentrations of sCD28 were widely dispersed in both ATD and control groups. Statistical analyses for sCD28 did not evidence any significative correlation when comparing the amount of sCD28 detected by ELISA and the pathological state of the donors.

Discussion

The regulation of immune responses is the outcome of a balance between positive signals that trigger them and inhibitory mechanisms that prevent excessive clonal expansion and autoimmunity. Therefore, a prevalence of activation should render T cells responsive to antigens, whereas a prevalence of inhibition should lead to T-cell anergy. The majority of the data on the inhibitory function exerted by CTLA-4 have been gathered by studies of proliferation or cytokine production of T lymphocytes analyzing the interaction between CTLA-4 and CD80/CD86 at the membrane level.

Several papers [22–24] have recently described an alternate transcript of the *CTLA-4* gene that encodes a protein that lacks a transmembrane region and likely represents a native soluble form of CTLA-4. The data herein reported confirm and extend the original findings of Oaks et al. [24], who detected sCTLA-4 in the serum of about half of a group of ATD patients. Consistently, we found detectable serum sCTLA-4 in 59 out of 90 ATD patients. Furthermore, we evaluated the possible relationship of sCTLA-4 levels with single ATD, GD, or HT, but no differences were found: accordingly, no relationships were observed between sCTLA-4 and specific clinical features (namely, severity of ophthalmopathy) among GD patients. Finally, there was no correlation between sCTLA-4 and clinical thyroid status (eu-, hypo-, or hyperthyroidism) or circulating thyroid hormones or TSH levels. It is likely that the presence of sCTLA-4 in the serum in the majority of ATD patients is linked to autoimmunity in a broad sense and not to specific clinical manifestations. As well, the levels of sCTLA-4 in a single patient do not seem related to disease evolution during time: this is suggested by our findings on sera of GD patients withdrawn before and after radioiodine treatment. In fact the ability to produce sCTLA-4 persists after therapy, despite profound changes in thyroid function, volume, and structure. On the other hand, radioiodine *per se* does not induce sCTLA-4 release in patients that were negative before treatment. It is known that radioiodine treatment of GD may transiently result in an increased activation of the autoimmune process (specifically, TSH-receptor antibodies increase), probably due to massive self-antigens release [31]. This further supports that the ability to produce sCTLA-4 is genetically determined, and not merely dependent to immune activation. This would be consistent with the hypothesis that increased sCTLA-4 levels in ATD are "constitutive", possibly related to the genetic control of sCTLA-4 synthesis [21,24]: however no correlation between sCTLA-4 levels and the well known CT-60 A/G SNP was observed [25]. Definitive evidence for, or against the genetic regulation of sCTLA-4, might derive from longitudinal studies starting from disease onset.

The present work provides evidence about the possible functional role of sCTLA-4. The fact that CTLA-4 immunoreactivity can be blocked by its known ligands (namely, CD80 and CD86) and by the synthetic receptor (CTLA-4 Ig) supports the notion that sCTLA-4 is a functional receptor. Its ability to interfere with T-cell proliferation in MLR or following specific soluble antigen stimulation in a dose-dependent manner supports its possible immunoregulatory action *in vivo*, like other soluble receptors [32–37]. In addition, the absence of the soluble form of CD80 and CD86 and the lack of correlation with the pathological state of the amount of soluble CD28 in the analyzed sera support the hypothesis that the functional effects of serum from autoimmune patients are principally due to the presence of sCTLA-4. The effect of sCTLA-4 binding to CD80/CD86 molecules might depend on the activation status of the cells involved. For example, on resting cells, sCTLA-4 may block CD80/CD86–CD28 interactions, thereby interfering with T-cell co-stimulation. On the other hand, inhibition of CD80/CD86–CTLA-4 interactions on activated T cells (in these conditions the transmembrane form of the molecule is selectively expressed) may prevent down-regulation of T-cell responses.

The experiments performed in the present study suggest that sCTLA-4 present in ATD sera is able to specifically enhance T-cell proliferation induced by MLR when added after the initial phase, i.e. in the presence of already activated T cells. Thus, sCTLA-4 might indirectly act as an enhancer of the autoimmune response considering that in ATD intrathyroid T lymphocytes are mainly composed by activated cells [38] and that, as shown in previous studies, molecules of the B7 family are expressed within the thyroid by different cell types, including follicular cells, in ATD [39].

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EXHIBIT 12

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Binding Stoichiometry of the Cytotoxic T Lymphocyte-associated Molecule-4 (CTLA-4)

A DISULFIDE-LINKED HOMODIMER BINDS TWO CD86 MOLECULES*

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CD28 and CTLA-4 are homologous T cell receptors of the immunoglobulin (Ig) superfamily, which bind B7 molecules (CD80 and CD86) on antigen-presenting cells and transmit important costimulatory signals during T cell activation. Here we have investigated the subunit structure of CTLA-4 and the stoichiometry of its binding to B7 molecules. We demonstrate CTLA-4 is a homodimer interconnected by one disulfide bond in the extracellular domain at cysteine residue 120. Each monomeric polypeptide chain of CTLA-4 contains a high affinity binding site for B7 molecules; soluble CTLA-4 and CD86 form complexes containing equimolar amounts of monomeric CTLA-4 and CD86 (*i.e.* a 2:2 molecular complex). Thus, CTLA-4 and probably CD28 have a receptor structure consisting of preexisting covalent homodimers with two binding sites. Dimerization of CTLA-4 and CD28 is not required for B7 binding, nor is it sufficient to trigger signaling.

Intercellular interactions between T lymphocytes and antigen-presenting cells (APC)[1] generate T cell costimulatory signals which regulate T cell responses to antigen in an antigen nonspecific manner (1). Costimulatory signals determine the magnitude of T cell responses, and whether an encounter with antigen activates or inactivates subsequent responses to antigen (2). T cell activation in the absence of costimulation results in aborted or anergic T cell responses (3). A key costimulatory signal(s) is provided by engagement of T cell receptors CD28 and CTLA-4 with B7 molecules on APC (4). CD28 and CTLA-4 are both members of the immunoglobulin superfamily (IgSF) having single variable-like domains in their extracellular regions (5). These molecules share sequence identity, most notably clustered in their CDR3-like regions (6). Both CD28 and CTLA-4 bind the same natural ligands, CD80 (formerly known as B7-1) and CD86 (formerly known as B7-2 or B70) (7, 8). Engagement of CD28 by B7 molecules delivers a strong costimulatory signal (4, 9), whereas engagement of CTLA-4 in different systems triggers either a weak costimulatory signal (10, 11) or a negative signal (12).

Binding interactions between CD28, CTLA-4, and B7 molecules have been studied with recombinant-soluble immunoglobulin fusion proteins of these molecules. CTLA4Ig bound to CD80 with ~20-fold higher avidity than did the analogous CD28Ig (13). Amino acid residues responsible for the increased binding activity of CTLA4Ig to CD80 were localized to the CDR-1 and COOH-terminally extended CDR3-like regions (6). CD80 and CD86 share only ~25% amino acid sequence identity in their extracellular domains, but show very similar equilibrium binding to CD28Ig or CTLA4Ig (14).

A fundamental uncertainty regarding the binding interactions of CTLA-4·CD28 and B7 molecules is their binding stoichiometry. CD28 is a disulfide-linked homodimer, whereas the subunit structure of CTLA-4 has been controversial. Some studies have suggested that CTLA-4 is also a disulfide-linked homodimer (10, 13, 15), but another suggested that it exists as a monomer (16). It is currently unknown whether dimerization is required for binding activity of CD28 and or CTLA-4, as it is for many IgSF members (17). In this study, we have determined the binding stoichiometry of CTLA-4 for B7 molecules. We show that CTLA-4 is a disulfide-linked homodimer containing two binding sites for its ligands.

MATERIALS AND METHODS

mAbs, Ig Fusion Proteins, and Flow Cytometry—Murine mAbs 9.3 (anti-CD28), G10-1 (anti-CD8), and G17-2 (anti-CD4) and F(ab')$_2$ fragments of mAb G19-4 (anti-CD8) were provided by Dr. J. Ledbetter of Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle. Murine mAb L6 (anti-human lung tumor antigen) and its chimeric derivative (human Fc) were from Drs. K. E. and I. Hellström also of Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle. mAb 11D4 (anti-CTLA-4) has been described previously (10). mAb 7H4 (anti-CTLA-4) was prepared in the same manner as other anti-CTLA-4 mAbs (10); mAb 7H4 binds CTLA4Ig, but does not block its binding to CD80. Human CTLA4Ig, CD28Ig, CD80Ig, CD86Ig, CD80 mIg, CD86 mIg fusion proteins were prepared as described (14). For immunostaining, cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. CHO cells ($1–10 \times 10^5$) were first incubated with mAbs or Ig fusion proteins in DMEM containing 10% fetal bovine serum (FBS), then washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse or anti-human Ig second step reagents (Tago, Burlingame, CA). Cells were washed and analyzed on a FACScan (Becton Dickinson).

Cell Culture—Anti-CD3-activated T cell blasts were prepared essentially as described (10), except that culture flasks were coated with F(ab')$_2$ fragments (10 μg/ml) of anti-CD3 mAb G19-4 instead of intact mAb. Peripheral blood mononuclear cells were isolated from heparinized blood and cultured on anti-CD3 F(ab')$_2$-coated flasks for 3 days at 37 °C in RPMI 1640 containing 10% FCS, 2 mM glutamine, and pen-strep (100 units/ml penicillin, 75 units/ml streptomycin). Retroviral vectors encoding CD28 and C4–28 (extracellular and transmembrane

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[1] The abbreviations used are: APC, antigen-presenting cell; IgSF, immunoglobulin superfamily; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; Ig, immunoglobulin; FBS, fetal bovine serum; IPA, immobilized protein A; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Ch, chymotrypsin.

regions of human CTLA-4 fused to the cytoplasmic domain of CD28) will be described elsewhere.[5] 3T3 cells were transduced with these vectors, selected for neomycin resistance, and cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and pen-strep. COS cells were maintained in DMEM containing 10% FBS.

Iodination and Immunoprecipitation—Cells were harvested and washed with PBS. Cell surface iodination using lactoperoxidase was performed as described (18). Surface-labeled cells were washed three times with PBS and solubilized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1% Nonidet P-40, 0.25% deoxycholate, 10 μg/ml each of aprotinin and leupeptin) for 30 min at 4 °C. Detergent-insoluble material was then removed by centrifugation (13,000 × *g* for 15 min at 4 °C) and the supernatant was used for immunoprecipitation. Solubilized membrane proteins were also iodinated using lactoperoxidase. Cells (2 × 10^8 for COS cells and 150 × 10^6 for peripheral blood lymphocytes) were resuspended in (5 or 10 ml, respectively) hypotonic buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 10 μg/ml each of aprotinin and leupeptin) and incubated on ice for 10 min. Cells were then lysed (>95% lysis) using a tight fitting Dounce homogenizer. The homogenate was transferred to a clean tube containing 0.1 volume of 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 1.5 M NaCl, and debris was removed by centrifugation at 800 × *g* for 5 min. The supernatant was removed, and EDTA was added to a final concentration of 5 mM. Membranes were collected by centrifugation at 125,000 × *g* for 45 min. The membrane pellet was solubilized in lysis buffer on ice for 30 min, and detergent-insoluble material was removed by centrifugation. Soluble proteins were then iodinated using lactoperoxidase, and unreacted ^{125}I was removed by passage over a column of Sephadex G25 (Pharmacia Biotech Inc.), equilibrated with lysis buffer. The labeled protein extract was then used for immunoprecipitation analysis.

Immunoprecipitation Analysis—^{125}I-Labeled lysates were incubated at 4 °C with an irrelevant antibody (2 μg) and 50-μl packed volume immobilized protein A (IPA, r-protein A-Sepharose, Repligen, Cambridge, MA). IPA was incubated before use for ~1 h in a solution of 5% bovine serum albumin in PBS and washed in lysis buffer. Sepharose beads were removed by centrifugation, and aliquots of the supernatant ($1–2 \times 10^6$ cpm) were mixed with mAbs or Ig fusion proteins and incubated at 4 °C for 1–2 h. Bovine serum albumin-blocked IPA was added (50 μl packed volume), and incubation at 4 °C was continued for an additional 1–2 h. Samples treated with mAb 11D4 or its isotype controls (mouse IgG1) were incubated with a "bridging" antibody (rabbit anti-mouse IgG, Sigma) at 50 μg/ml for 1–2 h at 4 °C before addition of protein A-Sepharose. Beads were collected by centrifugation, washed four times with PBS containing 0.5% Nonidet P-40, twice with PBS alone, and immunoprecipitated proteins solubilized in SDS-PAGE sample buffer. When labeled lysates from anti-CD3-stimulated T lymphocytes were used, CD28 was removed from solution by immunoprecipitation with anti-CD28 mAb 9.3 before addition of CD80Ig. For immunoblotting analysis, aliquots of conditioned medium or samples of purified protein (1 μg) were incubated with 5 μg of human Ig (negative control), CD80Ig, or CD86Ig for 1 h at 4 °C. Ig-containing proteins were then precipitated with 50 μl of IPA. Precipitates were washed three times with PBS, bound proteins were eluted by addition of concentrated SDS-PAGE sample buffer, and samples were analyzed by SDS-PAGE.

Preparation of Recombinant Fragments of CTLA-4—Soluble extracellular domain of CTLA-4 was expressed in COS cells by introducing a stop codon into CTLA-4 cDNA after Asp^{124} by polymerase chain reaction-directed mutagenesis (5). For CTLA4X, the OMCTLA-4 (18) expression plasmid was used as template, the forward primer GAGGTGATAAAGCTTCACCAATGGGTGTACTGCTCACAGAG was chosen to match sequences in the vector, and the reverse primer, GTGGTGTATTGGTCTAGATCAATCAGAATCTGGGCACGGTTC, corresponded to the last seven amino acids in the extracellular domain of CTLA-4 followed by a stop codon (TGA). $CTLA4X_{C120S}$ was constructed in similar fashion, except that the reverse primer specified a C120S mutation. Polymerase chain reaction products were digested with *HindIII/XbaI* and directionally subcloned into the expression vector αLN (a gift of Dr. A. Aruffo, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle). Each construct was verified by DNA sequencing.

Expression and Purification of CTLA-4 Fragments—CTLA4X and $CTLA4X_{C120S}$ were produced by transiently transfected COS cells (18). Recombinant proteins were purified by CTLA-4 mAb affinity chromatography from transfected COS cell conditioned medium. Anti-CTLA-4 mAb 11D4 was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (8 mg of mAb/ml of resin) as suggested by the manufacturer (Pharmacia Biotech Inc.). COS cell conditioned medium was collected, filter-sterilized, and applied to the affinity resin by gravity flow. Following application of conditioned medium, the resin was washed with 5 bed volumes of PBS. Bound CTLA-4 fragments were eluted with 0.05 M trisodium phosphate, pH 11.5. Fractions (0.5 ml) were collected and immediately neutralized by addition of 0.2 ml of 2 M Tris-HCl, pH 6.8. CTLA-4-containing fractions were identified by capture immunoassay (10) and/or A_{280}. Peak fractions were pooled, concentrated using a Centriprep 10 (Amicon, Beverly, MA), and filter-sterilized. Recoveries of recombinant proteins were ~50–90% based on immunoreactivity in the capture immunoassay. Extinction coefficients of 1.0 and 1.1 ml/mg were experimentally determined by amino acid analysis for purified CTLA4X and $CTLA4X_{C120S}$ preparations, respectively. M_r = 13,400, corresponding to the predicted peptide mass of CTLA4X, was assumed for calculation of molar concentrations. Where indicated, concentrated conditioned medium from transfected COS cells was used as a source of recombinant CTLA-4X. This was concentrated ~30-fold using a Centriprep 10 before fractionation.

Preparation of Thrombin Fragments—CTLA4Ig or CD86Ig fusion proteins (0.5 mg/ml in Tris-HCl buffer, pH 7.4–7.5) were digested with purified bovine thrombin (Armour Pharmaceutical Corp., Kankakee, IL) at a final concentration of 100 units/ml, at 37 °C for 16–24 h. After cleavage, thrombin was removed by twice incubating the protein solution with 0.10 packed bed volume of washed Affi-Gel Blue (Bio-Rad). Ig Fc-containing fragments were removed by twice incubating the protein solution with 0.20 packed bed volume of washed IPA. The resulting solution contained protein fragments CTLA4t and CD86t. The final recovery of purified fragments was ~10–40%. Extinction coefficients of 1.3 and 1.2 ml/mg were experimentally determined by amino acid analysis for purified CTLA4t and CD86t preparations, respectively. M_r values of 13,900 and 25,800, corresponding to the predicted peptide mass of a single chain of CTLA4t and CD86t, respectively, were assumed for calculation of molar concentrations.

Gel Permeation Chromatography—Two columns were used for gel permeation chromatography. CTLA4X preparations were analyzed using a TSK-GEL G2000 SW_{XL} column (7.8 × 300 mm, Toyohaas, Montgomeryville, PA). The column was equilibrated in PBS containing 0.02% NaN_3 at a flow rate of 1 ml/min. Concentrated COS cell medium containing CTLA4X (0.2 ml) was fractionated by gel permeation chromatography, and fractions of 0.5 ml were collected and used for analysis. In other experiments, purified protein fragments were analyzed on a Waters 800/SW column equilibrated with PBS, containing 10 mM Tris-HCl, pH 7.4, and 0.01% NaN_3, at a flow rate of 0.35 ml/min. Essentially the same results were obtained with both systems.

Protein Sequencing—Protein sequencing methods have been described previously (21). CTLA4t was first cleaved with cyanogen bromide (CNBr), and the peptides were purified. CNBr peptides were further digested with trypsin or chymotrypsin in the presence of 1 mM iodoacetamide and purified by reversed-phase HPLC. Automated sequence analysis was performed in a pulsed-liquid protein sequencer, and mass spectra were recorded on a Bioion 20 plasma desorption mass spectrometer (Applied Biosystems, Inc., Foster City, CA).

SDS-PAGE and Immunoblotting—SDS-PAGE was performed on Tris/glycine gels. In some cases, performed gels (Novex, San Diego, CA) were used. Analytical gels were stained with Coomassie Blue, and images of wet gels were obtained by digital scanning. Gels containing ^{125}I-labeled proteins were dried and subjected to autoradiography. For immunoblotting, separated proteins were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with Specimen Diluent (Genetic Systems Corp., Redmond, WA) and probed with anti-CTLA-4 mAb 7H4 diluted in Specimen Diluent. Bound mAb was detected using horseradish peroxidase-conjugated goat anti-mouse Ig (Biosource International, Camarillo, CA) and ECL reagents (Amersham Corp.).

Competitive Binding Assays—Binding assays were performed on immobilized recombinant B7Ig fusion proteins (14). Individual wells in 96-well plates (Immulon 2; Dynatech laboratories, Inc., Chantilly, VA) were coated for 16–24 h with 50 ng/well goat F(ab')$_2$ anti-mouse immunoglobulin antibodies (Tago). Wells were then washed, blocked with Ig binding medium (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10% FBS, 0.1% NaN_3), and coated with CD80 mIg or CD86 mIg at 1 μg/ml. CTLA4Ig was radiolabeled with ^{125}I using Bolton-Hunter reagent (14), diluted in Ig binding medium, and mixed with solutions of competitor proteins diluted in PBS. Aliquots of the mixtures (50 μl) were then added to duplicate B7Ig-coated wells. Binding was allowed to proceed for ~4 h at 23 °C, wells were washed, and bound radioactivity was measured by γ counting.

[5] H. Leung and P. S. Linsley, manuscript in preparation.

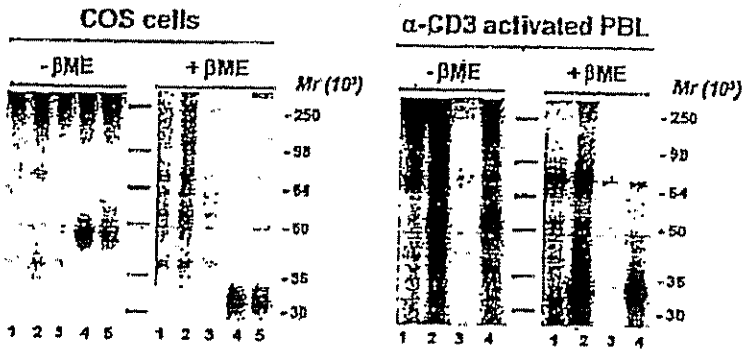

FIG. 1. CTLA-4 contains an intermolecular disulfide bond. *Left panel*, immunoprecipitation from COS cells. COS cells were transfected with the OMCTLA-4 expression plasmid. Forty-eight h post-transfection, cells were removed from their culture dishes and cell surface-labeled with ^{125}I using lactoperoxidase. ^{125}I-Labeled cells were then solubilized with non-ionic detergent solution, subjected to immunoprecipitation analyses with: CD5Ig (4 μg) (*lanes 1*), control mAb G17–2 (anti-CD4, 2 μg) (*lanes 2*), control mAb G10–1 (anti-CD5, 2 μg) (*lanes 3*), CD80Ig (4 μg) (*lanes 4*), and mAb 11D4 (anti-CTLA-4, 2 μg) (*lanes 5*). Samples were analyzed by SDS-PAGE (10% gel) under nonreducing (−β-mercaptoethanol (*βME*)) or reducing +β-mercaptoethanol (+*βME*) conditions. Migration positions of standards are indicated. *Right panel*, immunoprecipitation from anti-CD3-activated peripheral blood T cells. Membranes were prepared from anti-CD3-activated human mononuclear peripheral blood cells. Detergent extracts of these membranes were labeled with ^{125}I and subjected to immunoprecipitation analysis. Labeled extracts were precleared of CD28 by immunoprecipitation with mAb 9.3 before addition of the following (4 μg each): control mAb L20 (*lanes 1*); mAb 11D4 (anti-CTLA-4) (*lanes 2*); control Ig, chimeric mAb (lanes 3); CD80Ig (lanes 4). SDS-PAGE (10% gel) was run under nonreducing (−*βME*) or reducing (+*βME*) conditions.

RESULTS

Cell Surface CTLA-4 Is a Disulfide-linked Homodimer—Various studies on the subunit structure of CTLA-4 have employed different serological reagents and different cell types (10, 13, 35, 16). It was unclear how these differences contributed to the reported variation in the subunit structure of CTLA-4. We wished to determine the subunit structure of biologically active CTLA-4 transfected COS cells and of activated T cells. As shown in Fig. 1, both specific anti-CTLA-4 mAb and CD80Ig immunoprecipitated from surface-labeled COS cells a M_r ~ 46,000 protein under nonreducing conditions and a M_r ~ 30,000 protein under reducing conditions. Similar results were obtained with activated T cells; a M_r ~ 50,000 protein was obtained under nonreducing conditions and a M_r ~ 33,000 protein under reducing conditions. Thus, regardless of the cell source, CTLA-4, as recognized by specific mAb or soluble natural ligand, migrated more rapidly under reducing conditions. This was consistent with CTLA-4 being a disulfide-linked homodimer or being linked to an unlabeled protein. Evidence supporting the first of these possibilities was provided in subsequent experiments (see Table I).

Preparation and Characterization of Soluble Recombinant Fragments of the Extracellular Domains of CTLA-4 and CD86—Since biologically active CTLA-4 was a homodimer, we wished to determine whether one or two CTLA-4 chains comprise a binding site for B7 molecules. We therefore prepared soluble fragments of CTLA-4 and CD86 extracellular domains. Two strategies were employed, as shown schematically in Fig. 2. The characterization of these fragments is presented below (Fig. 3 and Table I).

We first prepared proteolytic fragments of CTLA4Ig and CD86Ig which retained binding activity. Preliminary examination of the Ig hinge sequence showed that it contained the sequence 126E-P-K128 which is similar to a thrombin cleavage site in actin (22). We determined that this nonclassical thrombin site was suitable for producing active fragments of CTLA-4, CD86, CTLA4t, and CD86t. As a second strategy, we introduced an in frame stop codon into a cDNA construct encoding CTLA-4, proximal to the transmembrane domain. The resulting cDNA was then expressed in COS cells and, yielding a mixture of monomeric and dimeric proteins, CTLA4X. These proteins were purified on an anti-CTLA-4 mAb affinity column as described under "Materials and Methods." Analysis of purified preparations by gel permeation chromatography indicated that purified preparations contained ~80% monomeric CTLA4X and ~20% dimeric CTLA4X. To improve the yield of monomeric CTLA4X, we later mutated the cysteine residue which mediates the interchain disulfide bond in CTLA-4 to a serine (see below). This mutant protein, CTLA4X$_{C120S}$, was expressed in COS cells and purified.

TABLE I

Disulfide-linked peptides from CTLA4t

CTLA4t was fragmented with CNBr and peptides were isolated by gel permeation chromatography and identified by amino acid sequence analysis. CNBr peptide M-(88–128) was subfragmented with chymotrypsin (Ch) and the peptides were purified by reversed-phase HPLC. An intermolecular disulfide bond (C120-C120) in one of these fragments was confirmed by mass spectral analysis as described in the text. A high molecular weight CNBr fragment was subfragmented with trypsin (T), cystine-containing peptides were isolated and identified by amino acid sequence analysis. Peptide T-(55–89) was sequenced only as indicated. These analyses permitted determination of two intramolecular disulfide bonds (Cys^{87}–Cys^{95} and Cys^{73}–Cys^{80}) as described in the text. Numbers are from the amino terminus of mature CTLA4t.

Designation	Peptide sequence	Disulfide bond assignment
Ch-(113–128)	^{124}VIDPEPKPDSDQEPK^{128}	Interchain, Cys^{120}–Cys^{120}
T-(15–28)T-(86–97)	^{15}GIASFVXEYASPGK^{28}/ ^{86}DTGLYXCEVELM^{97}	Intrachain, Cys^{87}–Cys^{95}
T-(39–53)T-(65–88)	^{39}QADSQVTEVXAATYM^{53}/ ^{65}GNELTFLDDSICTGT . . .	Intrachain, Cys^{73}–Cys^{80}

These fragments of CTLA-4 and CD86 were characterized by SDS-PAGE (Fig. 3A). Under nonreducing conditions, CTLA4Ig gave a major M_r ~ 100,000 protein and a minor protein of M_r ~ 50,000 (*lane 1*). The minor protein was not seen in all preparations and comigrated with the M_r ~ 50,000 protein found under reducing conditions (13). CD86Ig migrated as a diffuse M_r ~ 75,000 protein (*lane 2*) which was not affected by reduction (data not shown). Thus, as shown previously, CTLA4Ig migrated during SDS-PAGE as a disulfide-linked homodimer of M_r ~ 50,000 subunits, whereas CD86Ig did not contain an interchain disulfide bond. Since the Ig hinge disulfides were mutated to serine residues, dimerization of CTLA4Ig results from a disulfide bond in CTLA-4 (13). CD86 does not contain an interchain disulfide bond, hence, CD86Ig was not disulfide linked.

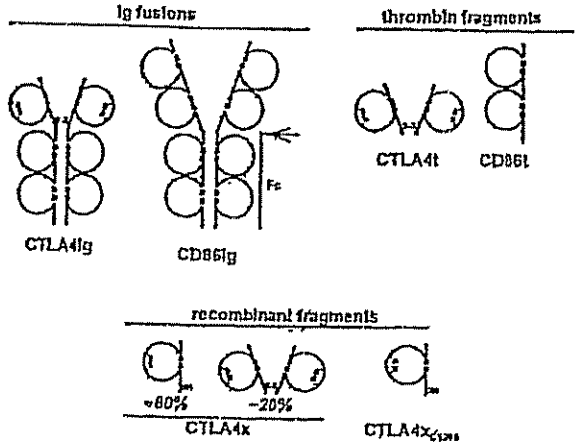

FIG. 2. Schematic representation of Ig fusions and proteolytic and recombinant fragments used in this study. Shown are cartoon structures of CTLA4Ig, CD86Ig, CTLA4t, CD86t, CTLA4X, and CTLA4X$_{C120S}$. Preparation of these molecules and elucidation of their subunit structures are discussed in the text. The relative positions of inter- and intracellular disulfide bonds in CTLA-4-related structures was determined as described in Table I; the disulfide bonds in CD86 were inferred from homology to other members of the IgSF. The Fc domains of CTLA4Ig and CD86Ig are indicated. The *arrow* indicates the site of thrombin cleavage in CTLA4Ig. The relative proportions of monomeric and dimeric CTLA4X were estimated from gel permeation chromatography profiles of purified preparations.

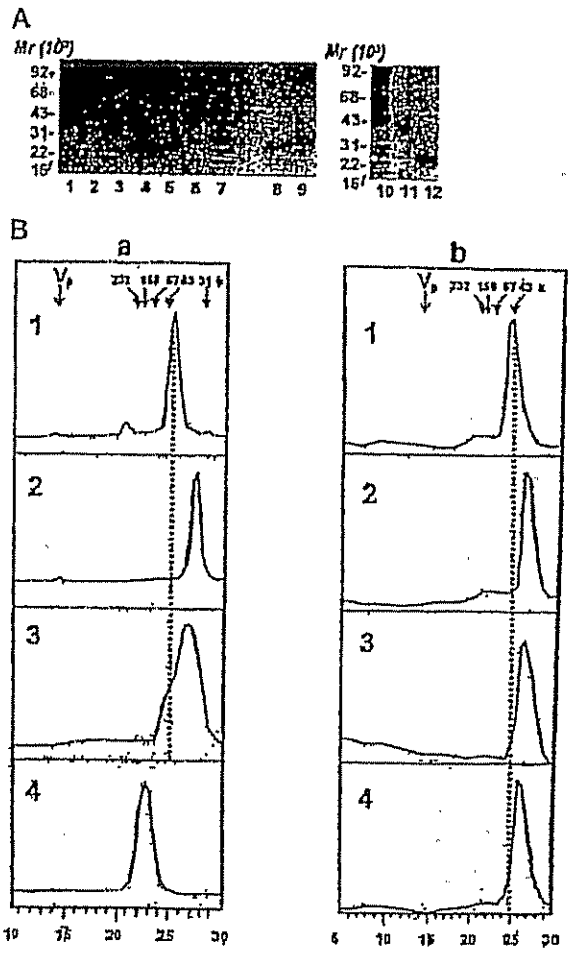

FIG. 3. Characterization of Ig fusions and proteolytic and recombinant fragments. Purified recombinant Ig fusion proteins, thrombin fragments and soluble receptors were analyzed by SDS-PAGE (*A*) or gel permeation chromatography (*B*). *A*, SDS-PAGE analysis. Samples (~1 μg) of each of the indicated proteins were analyzed on 4–20% (*lanes 1–9*) or 5–15% (*lanes 10–12*) acrylamide gels under nonreducing (*lanes 1–7, 10–12*) or reducing (*lanes 8, 9*) condition: *lane 1*, CTLA4Ig; *lane 2*, CD86Ig; *lane 3*, CTLA4t; *lane 4*, CTLA4X; *lane 5*, CTLA4X, which had been enriched for monomeric protein by gel permeation chromatography; *lane 6*, CD86t; *lane 7*, molecular weight markers; *lane 8*, CTLA4t; *lane 9*, CTLA4X; *lane 10*, molecular weight markers; *lane 11*, CTLA4t (a different preparation from that shown in *lane 1*); and *lane 12*, CTLA4X$_{C120S}$. *B*, gel permeation chromatography. Samples (~3–4 μg each) of the indicated proteins were analyzed by gel permeation chromatography on a Waters 300 SW column. *Panel a1*, CTLA4t; *panel a2*, CTLA4t, following reduction with 10 mM dithiothreitol at 23 °C for 30 min; *panel a3*, CTLA4X; *panel a4*, CD86t. *Panel b1*, CTLA4t (a different preparation than *panel a1*); *panel b2*, CTLA4t following reduction with dithiothreitol; *panel b3*, CTLA4X$_{C120S}$; *panel b4*, CTLA4X$_{C120S}$ following reduction. *Arrows* indicate elution positions of standards having the indicated molecular weights: V_o, void volume; catalase, M_r = 232,000; aldolase, M_r = 158,000; bovine serum albumin, M_r = 67,000; ovalbumin, M_r = 43,000; carbonic anhydrase, M_r = 31,000.

An intermolecular disulfide bond was also present in CTLA4t. CTLA4t (prepared from CTLA4Ig analyzed in lane 1) contained a major dimeric M_r ~ 88,000 protein and a minor monomeric M_r ~ 22,000 protein (*lane 3*). The presence of monomeric CTLA4t reflects the presence of monomeric CTLA4Ig in the starting preparation (*lane 1*). Another preparation of CTLA4t contained only the dimeric M_r ~ 88,000 protein when analyzed under nonreducing conditions (*lane 11*). Analysis of CTLA4t preparations after reduction revealed only the monomeric M_r ~ 22, 000 protein (*lane 8*). CTLA4X preparations contained a minor dimeric M_r ~ 88,000 protein and a major monomeric M_r ~ 22,000 protein (*lane 4*); reduction of this preparation gave a M_r ~ 22,000 protein (*lane 9*). The proportion of monomeric CTLA4X in these preparations could be enriched by gel permeation chromatography (*lane 5*). This enriched preparation was stable for several weeks when reanalyzed, indicating that monomeric CTLA4X did not readily reform dimeric molecules. CTLA4X$_{C120S}$ migrated as a monomeric M_r ~ 22,000 protein (*lane 12*). CD86t migrated as a M_r ~ 57,000 protein (*lane 6*); as with the parental molecule, CD86Ig, the mobility of CD86t was unaffected by reduction (data not shown).

Fragments of CTLA-4 and CD86 were further characterized by gel permeation chromatography (Fig. 3*B*). The elution times of CTLA-4 fragments corresponded closely to M_r values obtained by SDS-PAGE. The CTLA4t preparation analyzed by SDS-PAGE in Fig. 3*A*, *lane 1*, gave a major protein of M_r ~ 40,000 and a minor high molecular weight protein when analyzed by gel permeation chromatography (Fig. 3*B*, *panel a1*). The minor high molecular weight protein was not observed in another preparation which had not been concentrated after final purification (*panel b1*); reduction of both preparations before chromatography gave a M_r ~ 30,000 protein (*panels a2* and *b2*). CTLA4X eluted primarily as a M_r ~ 30,000 peak, with a shoulder (*panel a3*). CTLA4X$_{C120S}$ eluted as a M_r ~ 30,000 protein (*panel b3*) whose elution time was unaffected by reduction (*panel b4*). Thus, CTLA4t behaved as a disulfide-linked dimer in solution, whereas CTLA4X$_{C120S}$ and the major protein in CTLA4X were monomeric. As shown in *panel a4*, CD86t eluted larger (M_r ~ 140,000) than its M_r determined by SDS-PAGE (M_r ~ 57,000). The elution time of CD86t was unaffected

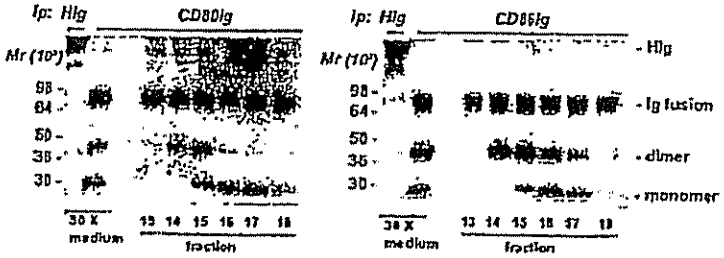

FIG. 4. Monomeric extracellular domain of CTLA-4 binds CD80 and CD86. COS cells were transfected with the CTLA4x expression plasmid, conditioned medium was collected and concentrated approximately 30-fold. A sample of concentrated conditioned medium (0.2 ml) was fractionated by gel permeation chromatography on a TSK-Gel G2000 SW_{XL} column at 1 ml/min and fractions (0.5 ml) were collected. In parallel column runs, aldolase, M_r = 158,000, eluted at 6.5 min. (*fraction 13*); bovine serum albumin, M_r = 67,000, at 6.8 min. (*fraction 14*); and ovalbumin, M_r = 43,000, at 7.4 min (*fraction 15*). Aliquots of concentrated conditioned medium (0.02 ml) or the indicated fractions (0.25 ml) were then subjected to immunoprecipitation analysis with human Ig (*Hlg*, negative control), CD80Ig, or CD86Ig. Precipitates were analyzed by SDS-PAGE (8–16% gel) under nonreducing conditions and CTLA-4-related proteins were detected by immunoblotting analysis. Migration positions of molecular weight (M_r) standards are indicated on the *left* and on the *right*, migration positions of Hlg, CD80Ig and/or CD86Ig, and CTLA4x dimer and monomer. Hlg, CD80Ig, and CD86Ig reactivities in this assay represent cross-reactions of the horseradish peroxidase conjugate with human Ig. This figure is representative of three experiments using concentrated culture medium and two experiments using affinity-purified CTLA4x.

by reduction and denaturation with 4 M guanidine HCl (data not shown and Fig. 6). This treatment disrupted the high avidity interaction between CTLA-4 and CD86, suggesting that it likewise would have disrupted interactions between subunits of CD86. Therefore, the larger apparent size of CD86t in solution is probably not due to oligomerization, but to aberrant migration during gel permeation chromatography, possibly resulting from its high degree of glycosylation (19, 20).

Determination of the Disulfide Bonding Pattern in CTLA4t—CTLA4t was further characterized by protein sequencing to identify amino and carboxyl termini and disulfide bonding patterns. The amino terminus of CTLA4t was determined by automated Edman degradation (21). Sequence analysis revealed an amino terminus corresponding to M1 (M27 of the precursor sequence), in agreement with the predicted site of signal peptide processing (13). Carboxyl-terminal sequence analysis was performed to determine the site of thrombin cleavage. CTLA4t was first fragmented with CNBr, and the peptides were purified by gel permeation chromatography. The carboxyl-terminal CNBr fragment was identified by sequence analysis. This peptide CNBr-(98-X) was subsequently subfragmented with chymotrypsin (Ch), and the amino acid sequence of the most carboxyl-terminal fragment (Ch-(114-X)) was determined. The sequence of this peptide terminated at residue Lys^{128}, suggesting that this residue was the carboxyl terminus. Fragment Ch-(114–128) was subjected to mass spectral analysis which confirmed the carboxyl-terminal residue as K128. This was the predicted thrombin cleavage site.

The disulfide bonding pattern of CTLA4t was determined (Table I). The position of the intermolecular disulfide bridge in CTLA4t was determined by further analysis of fragment Ch-(114–128). Mass spectral analysis of the peptide was performed in the presence or absence of dithiothreitol. This analysis revealed that the mass $((M + H)^+)$ of Ch-(114–128) under nonreducing and reducing conditions was 3336.4 and 1669.2, respectively. The change in mass of the peptide following reduction demonstrates that this peptide contained one intermolecular bond at Cys^{120}. The experimental mass values were in good agreement with the predicted masses of this peptide under nonreducing and reducing conditions, 3336.6 and, 1668.6, respectively.

The intramolecular disulfide bonding pattern was determined by Edman degradation of cystine-containing peptides. Sequence analysis located the positions of the purified CNBr peptides in the CTLA-4 sequence. Edman degradation of a large molecular weight CNBr fragment gave simultaneously three amino-terminal sequences beginning at Met^1 (or His^2), Gly^{50}, and Asp^{80} in equimolar amounts. These results suggested that the large molecular weight CNBr fragment consisted of three peptides interlinked by two disulfide bonds: CNBr-(1(2)-58)-CNBr-(55–85)-CNBr-(86–97). To confirm this, the large molecular weight peptide was subfragmented with trypsin, and the disulfide-containing peptides were purified by reversed-phase HPLC and identified by Edman degradation. Two major cystine-containing tryptic peptides (T) were obtained: T-(15–28)-T-(86–97) and T-(59–63)-T-(65–88). These results demonstrate the existence of two intramolecular disulfide bonds between Cys^{23}–Cys^{92} and Cys^{49}–Cys^{65}. The first of these pairs is the disulfide bond characteristic of the Ig fold. The C65-C88 disulfide bond is unusual in the Ig superfamily, but did not appear to be artifactual, since disulfide interchange during purification of fragments could be excluded by the lack of carboxamidomethylated cysteines in the fragments. This bond was also predicted in molecular modeling studies of CTLA-4, since these residues were adjacent in the model (6).

Monomeric CTLA-4 Binds CD80 and CD86—To determine if monomeric CTLA-4 contained an intact binding site for B7 molecules, we compared B7 binding properties of monomeric and dimeric forms of CTLA-4. Concentrated conditioned medium from CTLA4X-transfected COS cells was subjected to immunoprecipitation analysis with CD80Ig and CD86Ig. Immunoprecipitated proteins were separated by SDS-PAGE and different forms of CTLA-4 were identified by immunoblotting analysis. As shown in Fig. 4, CD80Ig and CD86Ig immunoprecipitated both monomeric and dimeric forms of CTLA-4 from solution. Fractionation of CTLA4X preparations by gel permeation chromatography separated monomeric and dimeric forms of CTLA-4. Both forms were immunoprecipitated with CD80Ig and CD86Ig. Identical results were obtained when purified CTLA4X was used for analysis. Thus, both monomeric and dimeric forms of CTLA-4 bound CD80 and CD86. However, the relative amounts of monomeric and dimeric forms of CTLA-4 precipitated by CD80Ig and CD86Ig were different (Fig. 4). CD80Ig precipitated more monomeric CTLA-4, whereas CD86Ig precipitated more dimeric CTLA-4.

The relative avidities of monomeric and dimeric CTLA-4 were compared by competitive binding assay in the experiment shown in Fig. 5. CTLA4Ig was labeled with ^{125}I and bound to CD80Ig and CD86Ig which had been immobilized on plastic wells. CTLA4t competed similar to CTLA4Ig (EC_{50} ~4 *versus* ~8 nM for CTLA4Ig *versus* CTLA-t inhibition of CD80Ig binding and ~8 *versus* ~10 nM for CD86Ig). $CTLA4X_{C120S}$ competed for ^{125}I-CTLA4Ig binding with an EC_{50} ~300 nM for CD80Ig and ~900 nM for CD86Ig. Monomeric CTLA-4 was a more

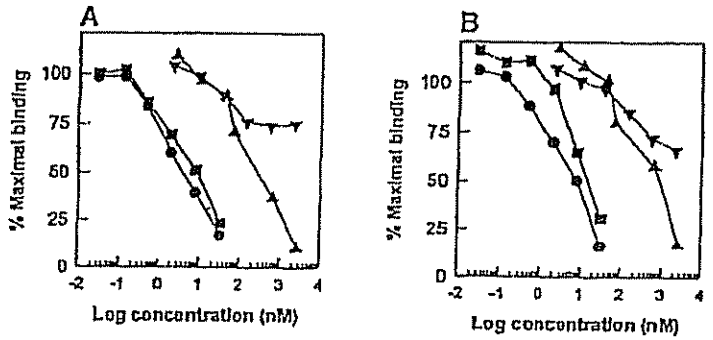

FIG. 5. Comparison of the avidities of dimeric and monomeric CTLA-4. Ninety-six-well plates were coated with CD80Ig (*A*) or CD86Ig (*B*). ^{125}I-Labeled CTLA4Ig (final concentration, 0.5 nM) was mixed with unlabeled CTLA4Ig (*circles*), CTLA4t (*squares*), CTLA4X$_{C120S}$ (*triangles*), or CD28Ig (*inverted triangles*), to the indicated final concentrations. The mixtures were then added to B7-coated wells and binding was measured. Binding in the presence of competitor is expressed as a percentage of binding in wells containing no competitor (30,000 cpm for CD80Ig and 16,000 cpm for CD86Ig). Concentrations of competitors were determined assuming the following M_r values per chain or binding site): CTLA4Ig, M_r = 80,000; CTLA4t, M_r = 13,900; CTLA4X$_{C120S}$, 13,400; CD28Ig, M_r = 42,000; and chimeric L6 mAb, M_r = 75,000. Addition of chimeric mAb L6 inhibited ^{125}I-CTLA4Ig binding by <10% over a concentration range of 5–13,000 nM. This experiment was repeated twice with identical results, and similar results were also observed using partially purified monomeric preparations of CTLA4X.

effective competitor than multimeric (18) CD28Ig (EC_{50} >~10,000 nM). The apparent plateau in binding inhibition by CD28Ig was not seen in other experiments. Control Ig did not show significant competition in this assay at concentrations >13,000 nM. Thus, monomeric CTLA-4 was a less avid competitor for ^{125}I-CTLA4Ig binding than was dimeric CTLA-4, but was more effective than multivalent CD28Ig.

CTLA-4 Homodimer Binds Two CD86 Molecules—To determine the stoichiometry of CTLA-4·B7 complexes, we analyzed the size of complexes formed with monomeric and dimeric CTLA-4 and the composition of the complex formed with dimeric CTLA-4 (Fig. 6). Addition of increasing concentrations of CD86t to a fixed concentration of CTLA4X monomer (*A*) or CTLA4t (*B*) resulted in formation of larger complexes. Higher concentrations of CD86t were required to force complete complex formation with monomeric CTLA-4X than with dimeric CTLA4t, consistent with the lower avidity of monomeric CTLA-4 (Fig. 5). Complexes formed with monomeric CTLA-4X eluted ~1 min later than complexes formed with dimeric CTLA4t. The elution times of these complexes were faster than the largest molecular weight standard, so it was not possible to accurately estimate their molecular mass; however, a change in elution time of 1 min in the range of standards analyzed corresponded to M_r ~ 70,000. To determine the composition of complexes between dimeric CTLA4t and CD86t, larger amounts of these molecules were mixed and complexes were subjected to preparative gel permeation chromatography (*C*). The elution profile of this mixture gave a more clearly resolved complex peak than seen in the experiment shown in *B*, and in addition, another late eluting peak. The identity of the late eluting peak was not determined (it was not seen when smaller amounts of these proteins were analyzed, see Fig. 3), although it eluted similar to monomeric CTLA4t. A peak corresponding to free CD86t was observed, indicating that it was present in excess. The leading edge (19–20 min elution time) of the high molecular weight complex peak was collected and analyzed for the relative amounts of CD86t and CTLA4t in two ways. In the first, an aliquot of the complex was dissociated with 4 M guanidine HCl and 10 mM dithiothreitol and reanalyzed by gel permeation chromatography (*D*). Peaks corresponding to CD86t and monomeric CTLA-4 were clearly resolved. Absorbance at 214 nm is proportional to the number of peptide bonds, and hence, to peptide mass. Thus, comparison of integrated peak areas divided by calculated molecular masses permits an estimate of the relative molecular ratios of CD86t and CTLA4t in the complex. This analysis gave a ratio of CD86t to CTLA4t of 1.2:1. The same value was obtained when a separate aliquot of the complex was fractionated and analyzed.

The composition of the CD86t·CTLA-4 complex was also determined by subjecting it to NH_2-terminal amino acid sequencing. An aliquot corresponding to ~⅛ of the total isolated CD86t·CTLA4t complex was subjected to automated Edman degradation. Two sequences were obtained simultaneously, corresponding to the NH_2 termini of CD86 and CTLA-4. The initial yield of M1 from CTLA-4 was 38 pmol and L1 from CD86; 41 pmol. At position 3, 34 pmol of valine (CTLA-4) and 37 pmol of isoleucine (CD86) were determined. Thus, the average ratio of CD86t to CTLA4t determined by amino acid sequence analysis was 1.1:1, similar to the ratio determined by comparison of absorbance peaks.

It was important to determine if some of the CD86t present in the complex peak was due to contamination with free CD86, which was present in excess. Extrapolation of the free CD86t A214 peak (*C*; see also Fig. 3B) indicated that free CD86t in the 19–20-min fraction contributed <~3% of initial CD86t. Since ~1700 pmol of CD86t were used, then ~<50 pmol were present in fraction 19–20. The total amount of CD86t recovered was ~320 pmol (40 pmol at position 1 in ~1/8 total sample). Thus, <~16% (<50/320) of recovered CD86t could be attributed to excess free CD86t based on sequence analysis. This was similar to the 10–20% of CD86t recovered in excess of a 1:1 ratio, as measured by both absorbance and NH_2-terminal sequencing.

DISCUSSION

CTLA-4 expressed in transfected COS cells or in activated T cells exists primarily as a protein of M_r ~ 45,000–50,000 under nonreducing conditions and M_r ~ 30,000–33,000 under reducing conditions. The native dimeric form of CTLA-4 is preserved in soluble fragments of its extracellular domain by an intermolecular disulfide bond at C120. Mutation of this cysteine to serine abolished residual dimeric protein in CTLA4X preparations. Our data thus show that CTLA-4, like its homologue CD28, exists primarily as a disulfide linked homodimer.

Residue Cys^{120} is conserved in most CTLA-4·CD28 family homologues (6) but a recent report (23) showed that chicken CD28 lacks this cysteine residue and does not contain an intermolecular disulfide bond. This led to the proposal that chicken CD28 differs from other family members in being monomeric. However, it cannot be ruled out that chicken CD28 exists as a non-disulfide-linked dimer. Our data show that

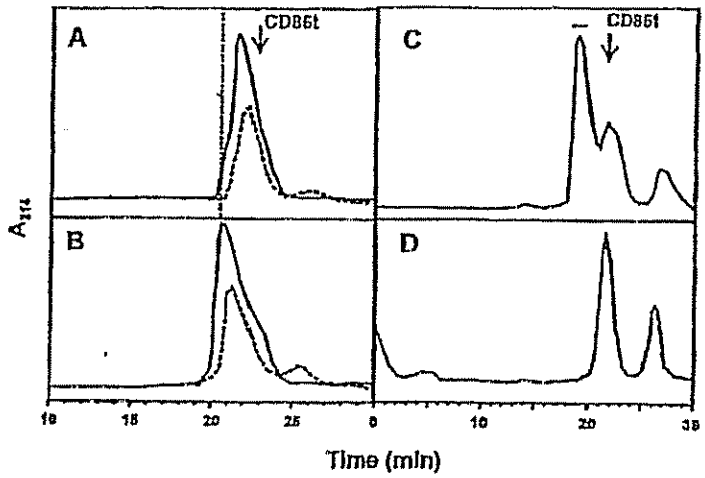

FIG. 6. CTLA-4 homodimer binds two CD86 molecules. *A*, formation of complexes between monomeric CTLA4X and CD86t. Aliquots (~3 μg, ~200 pmol) of CTLA4X, which had been enriched for monomeric protein by preparative gel permeation chromatography (see Fig. 3A), were incubated in a final volume of 0.1 ml with CD86t (7.4 μg, ~300 pmol, *dotted line*, or 15 μg, ~600 pmol, *solid line*) for 30 min at 23 °C before fractionation by gel permeation chromatography as in Fig. 3. The *arrow* indicates the elution position of CD86t and the *vertical dotted line* the elution position of CTLA4/CD86t complexes. Monomeric CTLA4X elutes at 26 min. *B*, formation of complexes between CTLA4t and CD86t. Aliquots of CTLA4t (3.4 μg, ~250 pmol) were incubated in a final volume of 0.1 ml with CD86t (3.7 μg, ~140 pmol, *dotted line*, or 7.4 μg, ~300 pmol, *solid line*) for 30 min at 23 °C before fractionation by gel permeation chromatography. CTLA4t elutes at 25 min. *C*, preparative chromatography of complexes between CTLA4t and CD86t. Aliquots of CTLA4t (22 μg, ~1600 pmol) were incubated in a final volume of 0.2 ml with CD86t (44 μg, ~1700 pmol) for 30 min at 23 °C before fractionation by gel permeation chromatography. Complexes eluting with the *horizontal bar* (19–20 min) were collected and used in *D*. The *arrow* indicates the elution position of CD86t. The peak eluting at >28 min was not observed in other experiments in which lower concentrations were used. *D*, composition of complexes between CTLA4t and CD86t. An aliquot (~½ the total) of complexes eluting at the position of the *horizontal bar* in *C* was dissociated by treatment for ~30 min at 23 °C with 4 M guanidine HCl and 10 mM dithiothreitol and reanalyzed as in *B*. This experiment was repeated twice with identical results.

although full-length CTLA-4 is predominantly homodimeric, removal of the transmembrane and cytoplasmic domains (CTLA4X) leads to predominantly monomeric protein. Thus, the presence of C120 does not ensure dimerization. This suggests that dimerization of CTLA-4 and formation of the disulfide bond at Cys^{120} requires the transmembrane and/or cytoplasmic domains of the two chains.

Each chain of a CTLA-4 homodimer has a binding site for CD80 and CD86. Monomeric CTLA-4X has binding activity for CD80Ig and CD86Ig in immunoprecipitation analysis and monomeric $CTLA4X_{C120S}$ competes for binding of ^{125}I-labeled CTLA4Ig to CD80Ig and CD86Ig. Monomeric CTLA-4 had reduced binding activity when compared with dimeric CTLA-4. $CTLA4X_{C120S}$ was 30–90-fold less active at competing for ^{125}I-CTLA4Ig than dimeric CTLA4t. CD80Ig and CD86Ig differed in their preferences for monomeric and dimeric forms of CTLA-4. Monomeric CTLA-4 was preferentially immunoprecipitated by CD80Ig, whereas dimeric CTLA-4 was preferentially precipitated by CD86Ig. In competitive binding assays, dimeric CTLA4t was approximately equivalent at competing for ^{125}I-CTLA4Ig binding to CD80Ig and CD86Ig (EC_{50} ~8 *versus* 10 nM, respectively), whereas monomeric $CTLA4X_{C120S}$ was ~3-fold less effective at competing for binding to CD86Ig than to CD80Ig (EC_{50} ~300 *versus* 900 nM). Thus, CD86 binds less well to monomeric CTLA-4.

This difference may be due to differences in binding kinetics of CD80 and CD86. We previously showed that the equilibrium dissociation constants for CTLA4Ig binding to CD80 and CD86 were very similar (within 2-fold) but that CTLA4Ig dissociated more rapidly from CD86 than from CD80. We proposed that CD86 is a faster on/faster off counter receptor for CTLA-4 than is CD80. Since monomeric CTLA-4 binds less avidly than dimeric CTLA-4, dissociation differences between CD80 and CD86 may be more pronounced with monomeric CTLA-4. *In vivo*, this difference may be important where CD80 or CD86 expression levels are a limiting factor for interaction with CTLA-4. Occupancy of CTLA-4 would be suboptimal, and on average, less than two binding sites per molecule occupied. Since complexes of CD86 with a single CTLA-4 binding site would dissociate faster, the time of CTLA-4 receptor occupancy by CD86 would be less, and hence, effects on T cell costimulation would also be less. Thus, differences in costimulatory signals by CD80 and CD86 would be exaggerated at low expression levels. We previously noted that CHO cells transfected with CD80 and CD86 differed more in their adhesion to CD28 and in T cell costimulation when added at low numbers (14).

The complex of CD86t·CTLA4t formed in the presence of excess CD86t was isolated and determined to contain 1.1–1.2 mol of CD86t/mol of CTLA-4. Thus, one molecule of CD86t fragment bound per chain of CTLA4t or two molecules of CD86t per CTLA4t homodimer (*i.e.* a 2:2 stoichiometry). Since CD28 is homologous to CTLA-4, and is homodimeric, it likely also binds two B7 molecules.

The Ig domains of CD28 and CTLA-4 display little sequence similarity with other members of the IgSF, limited to a few conserved residues characteristic of the Ig fold (24). Despite sharing a common structural scaffold, members of the IgSF have diverse structures, utilize different combinations of Ig domains to form binding sites, and have distinct binding stoichiometries per molecule. In antibodies, variable regions from two different Ig domains combine to form an antigen binding site (25), and on per molecule basis, each Ig molecule contains two antigen binding sites (a 2:2 stoichiometry). With human growth hormone receptor, two molecules (two Ig domains) bind a single growth hormone molecule (human growth hormone receptor); the complex has a 2:1 stoichiometry (26). Thus, the number of Ig domains per binding site of human growth hormone receptor is the same as a Fab fragment, but the stoichiometries of the intact molecules is different. A single domain of CD2 binds its CD58 ligand and the binding stoichiometry on a

per molecule basis is thought to be 1:1 (27, 28). Finally, with CD8, a single Ig domain of each chain of a homodimer is believed to bind a major histocompatibility complex class I molecule; thus, on a per molecule basis, CD8/MHC class II complexes have a 2:2 stoichiometry (29, 30). The stoichiometry of CD8 has not been directly demonstrated, but is inferred from mutagenesis studies (29).

The binding stoichiometries of CTLA-4 and CD28 most closely resemble CD8. With both molecules each chain of a homodimer contains a binding site and the intact molecule has a 2:2 stoichiometry. However, there are significant differences in the way CD8 and CTLA-4·CD28 bind ligand. Different areas of the V(ariable)-like domains are utilized for binding, with the CDR-1 and CDR-2-like loops and β-strands A and B being involved in the binding site of each CD8 subunit (29), and the CDR-1 and CDR-3-like loops and β-strand G, at least in part, being involved in binding by CTLA-4 (26). Also, CD8 forms an Fv-like homodimer, which is stable even in the absence of a disulfide bond (30). In contrast, the two subunits of a homodimer of CTLA-4 have no particular affinity for each other, and monomeric CTLA-4 is stable in solution.

Ligand-induced receptor dimerization is a common theme of many signaling pathways. Dimerization of growth factor receptors permits receptor transphosphorylation (31) and facilitates recruitment of SH2-containing effector molecules (32, 33). The CD28 signaling pathway also involves tyrosine kinase activation and binding of SH2-containing effector molecules (9). However, CTLA-4 or CD28 signaling differ from growth factor receptor signaling in two ways: their dimeric states are preformed and they are inactive. Thus, with CTLA-4·CD28, dimerization *per se* is not sufficient to trigger signal transduction.

Why then are CTLA-4 and CD28 covalent homodimers having two binding sites? One possible clue to the answer is the fast kinetic off rates of B7 binding (14). Recent data[3] indicate that the $t_{1/2}$ of CTLA4Ig occupancy by CD80Ig and CD86Ig is ~11 and ~8 s, respectively, and that the $t_{1/2}$ of CD28Ig occupancy is also very short. These off rates are rapid compared with those known for growth factor/growth factor receptor interactions (34–36). Rapid receptor kinetic off rates favor disengagement of CD28/CTLA-4/B7 intercellular interactions, but discourage productive intracellular signaling. Homodimerization of CTLA-4 and CD28 should enhance signaling by increasing the time of receptor occupancy by ligand. Homodimeric CTLA-4 has increased binding avidity for B7 molecules, since the probability of rebinding ligand after its dissociation is increased. The effective time of receptor-ligand interactions would thereby be prolonged and receptor activation may be increased.

It is currently unknown whether signal transduction requires occupancy of one or both subunits of CTLA-4 and/or CD28 by B7 molecules. If occupancy of both subunits is required for signal transduction, monomeric B7 molecules on the APC must be brought into proximity such that both subunits of CTLA-4·CD28 can be occupied. However, unlike CTLA-4 and CD28, B7 molecules are not disulfide-linked. Thus for full occupancy of CTLA-4·CD28 to occur, B7 molecules must either preexist as noncovalent oligomers on the APC, or they must oligomerize as the result of binding to CTLA-4·CD28. Evidence for the former possibility was provided by immunofluorescence microscopy studies showing that that B7 molecules on human Langerhans cells assume a non-uniform distribution (37).

[3] W. Cosand, J. Emswiler, G. Leytze, J. Greene, and P. S. Linsley, unpublished data.

It is also possible that signaling through CTLA-4·CD28 requires occupancy of only one receptor subunit by a B7 molecule. If so, oligomers of B7 on APC (37) could cross-link different CTLA-4·CD28 homodimers. Early studies (4) showed that CD28 receptor cross-linking with mAbs enhanced signaling through this receptor, but that Fab fragments did not, suggesting that cross-linking of CTLA-4·CD28 receptors is an essential feature of their signaling pathways. The binding stoichiometry of CTLA-4·CD28 receptors may enhance CTLA-4·CD28 receptor cross-linking by B7 aggregates on APC.

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EXHIBIT 13

Abatacept Binds to the Fc Receptor CD64 But Does Not Mediate Complement-Dependent Cytotoxicity or Antibody-Dependent Cellular Cytotoxicity

PATRICIA M. DAVIS, RALPH ABRAHAM, LINDA XU, STEVEN G. NADLER, and SUZANNE J. SUCHARD

ABSTRACT. ***Objective.*** To assess the ability of abatacept to mediate complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC) of antigen-presenting cells, and to characterize the binding of abatacept to the 3 Fc receptor classes.

Methods. CDC was measured *in vitro* using rabbit, baby rabbit, guinea pig, or human complement with human B cell line PM-LCL as the target. ADCC was also measured with PM-LCL target cells, but with human peripheral blood mononuclear cells from 12 healthy blood donors as effectors. Fc receptor binding was analyzed *in vitro* by flow cytometry and surface plasmon resonance (SPR).

Results. In contrast to unmodified CTLA4-Ig, abatacept did not mediate CDC or ADCC of target B cells. While abatacept was found to bind its target receptor, CD80/86, it did not appreciably bind the low-affinity Fc receptors CD16 and CD32 as measured by flow cytometry and SPR. Abatacept was found to minimally bind the high-affinity Fc receptor CD69 as measured by flow cytometry and SPR with a K_d of 3×10^{-7} M as measured by SPR.

Conclusion. Abatacept does not mediate CDC or ADCC of target B cells *in vitro* and has limited Fc receptor binding. These data support the concept that abatacept therapeutic activity is primarily due to the binding to CD80/86 through the CTLA4 extracellular domain and not through activities mediated by the modified Fc domain. (First Release Sept 1 2007; J Rheumatol 2007;34:2204–10)

Key Indexing Terms:
ABATACEPT CYTOTOXICITY Fc RECEPTOR

Abatacept, a selective T cell costimulation modulator, is the first in a class of agents for the treatment of rheumatoid arthritis (RA) that selectively modulates the CD80/CD86:CD28 costimulatory signal required for full T cell activation[1]. Abatacept has demonstrated efficacy in patients with active RA and an inadequate response to methotrexate, and also in those patients with an inadequate response to anti-tumor necrosis factor therapy[1,2]. Recently, clinical trials have also demonstrated that abatacept therapy provides significant improvements in the health related quality of life scores in these same patient populations[2,3].

In studies in mice, abatacept or related T lymphocyte-associated antigen 4 immunoglobulin (CTLA4-Ig) molecules demonstrated safety and efficacy in murine models of lupus[4], suppression of a primary antibody response[5], cardiac allograft rejection[6], human pancreatic islet cell xenograph rejection[5], graft versus host disease[7], and skin allograft rejection[8].

Abatacept is a genetically engineered fusion protein that consists of the extracellular domain of human CTLA4 linked to the modified Fc (hinge, CH2, and CH3 domains) portion of human immunoglobulin G1 (IgG1)[9]. The addition of a modified Fc region from human IgG1 increases the *in vivo* stability of abatacept in this soluble form, which has a half-life comparable to native human IgG1[10].

The Fc region of human IgG binds to multiple receptors that function in the activation or inhibition of immune responses such as antibody-mediated inflammation, phagocytosis, maturation of dendritic cells, antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), regulation of cytokines, and B cell proliferation[11,12]. In abatacept, however, a series of directed, select mutations in the hinge region were introduced to improve protein production and reduce Fc-mediated binding to abrogate the effects normally associated with the binding of human IgG1 heavy chain, such as CDC and ADCC. The mutations in abatacept include the replacement of cysteine with serine at amino acid positions 130, 136, and 139 of the IgG1 constant region, and replacement of proline with serine at amino acid position 148 (Figure 1).

The Fc IgG receptors have been divided into 3 classes based on close relationships in their extracellular domains:

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CTLA4 Region:

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Aba   -MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAA
C-Fc  AMHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAA

Aba   TYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPP
C-Fc  TYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPP

Aba   PYYLGIGNGTQIYVIDPEPCPDSD
C-Fc  PYYLGIGNGTQIYVIDPEPCPDSD
```

Fc Region:

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Aba   QEPKSSDKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
C-Fc  --PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
IgG1  KEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED

Aba   PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
C-Fc  PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
IgG1  PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK

Aba   ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES
C-Fc  ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES
IgG1  ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES

Aba   NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS
C-Fc  NGQPENNYKATPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS
IgG1  NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS

Aba   LSLSPGK------
C-Fc  LSLSPGKHHHHHH
IgG1  LSLSPGK------
```

Figure 1. Amino acid sequence comparison of abatacept (Aba), CTLA4-Ig (C-Fc), and the human IgG1 heavy chain constant region. IgG1 sequence begins at amino acid 125.

receptor I (here referred to as CD64), receptor II (here referred to as CD32), and receptor III (here referred to as CD16)[12]. The CD64 proteins are high-affinity receptors capable of binding monomeric IgG, whereas the CD16 and CD32 proteins bind IgG with lower affinities, usually restricted to recognizing IgG aggregates surrounding multivalent antigens[11].

This report describes studies that assess the Fc receptor binding of abatacept using surface plasmon resonance (SPR) binding to purified CD16, CD32, or CD64. Also assessed, by flow cytometry, was the binding of abatacept to cells expressing the Fc IgG receptors. Additionally, *in vitro* assays were used to determine the effector function of the Fc domain of abatacept using experiments to determine ADCC and CDC. The results of this study give further insight into the specificity of abatacept binding, functionality, and mechanism of action.

MATERIALS AND METHODS

Abatacept and CTLA4-Fc materials. Abatacept was manufactured at the facilities of Bristol-Myers Squibb in East Syracuse, New York. CTLA4-Fc was purchased from commercial suppliers (Biosource, Camarillo, CA, or R&D Systems, Minneapolis, MN, USA). Recombinant human CTLA4-Fc reagent is a fusion protein of CTLA4 fused to a nonmutated human IgG1 Fc tail. A comparison of the amino acid sequences between abatacept protein and CTLA4-Fc is provided in Figure 1.

Complement-dependent cytotoxicity. The effect of abatacept on CDC of target cells was measured *in vitro* using PM-LCL cells (an Epstein-Barr virus-transformed, CD80/86-positive human B cell line) as target cells. PM-LCL cells (1×10^6) were incubated at room temperature with antibody, abatacept at 30 µg/ml or 100 µg/ml, or CTLA4-Fc (Biosource) in 500 µl of RPMI medium containing 10% fetal calf serum (FCS). Positive controls used in the experiment were complement-fixing anti-human CD20 murine mAb as described[13], murine anti-CD86 mAb (BD Pharmingen), or CTLA4-Fc. After 30 min incubation, 500 µl of 2× concentration of various types of complement were added to the cells and incubated an additional 60 min at 37°C. PM-LCL cells were preincubated in RPMI medium plus 10% FCS (in the absence of abatacept, or control mAb) and then exposed to complement to determine the effect of complement alone. The types of complement used were low-tox-H rabbit complement at 1:32 dilution, baby rabbit complement at 1:16 dilution, guinea pig complement at 1:4 dilution (CedarLane Laboratories, Hornby, ON, Canada), and human complement at 1:8 dilution (Quidel Corp., Santa Clara, CA, USA). Each type of complement had been previously titrated to determine the optimal dilution for the detection of cell lysis. Following the incubation with complement, 50 µl propidium iodide (PI, 1 mg/ml) was added to detect dead cells. The PI-stained cells were subsequently analyzed by flow cytometry (Becton Dickinson FACScan) for uptake of PI. PI intercalates into the DNA of dead or dying cells; thus the percentage of cells that stain with PI indicates the extent of cell lysis.

Antibody-dependent cellular cytotoxicity. Target cells used in the assay were logarithmically growing PM-LCL cells labeled with sodium chromate (100 µCi/10^6 cells; NEN/Perkin Elmer) for 1 h at 37°C and washed extensively. Effector cells were peripheral blood mononuclear cells (PBMC) isolated by Ficoll-gradient separation from the whole blood of 12 different healthy blood

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donors. Effector cells were combined with target cells in microwell plates at a ratio of 100:1 (optimized ratio), together with 30 μg/ml abatacept, CTLA4-Fc, ChiL6 control Ig (isotype-matched chimeric protein Ig), or buffer (effectors alone). The wild-type IgG1-Fc tail fused to CTLA4 is capable of facilitating complement fixation and serves as a positive control for these assays. Wells containing target cells with media only or with 1% Triton X-100 were assayed for spontaneous and maximal release of the target cells, respectively. All treatments were done in quadruplicate. Microplates were incubated 6 h at 37°C and 5% CO_2, then supernatants were removed and analyzed for chromium release by direct counting on a Cobra gamma counter. Percentage cytotoxicity was calculated according to the following formula: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100.

Fc receptor binding assayed by flow cytometry. Before evaluation of abatacept binding, the relevant expression of CD80, CD86, and Fc IgG receptors CD16, CD32, and CD64 on Raji and U937 cell lines was evaluated using fluorescence flow cytometry. Logarithmically growing Raji and U937 cells were washed in staining buffer [1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)] and stained (1×10^6 cells/sample in 0.1 ml) with FITC-labeled murine IgG1 isotype control; with FITC-labeled murine anti-human CD80 and CD86 antibodies or phycoerythrin (PE)-labeled murine IgG1 isotype control (BD Biosciences, San Jose, CA, USA); and with PE-labeled murine anti-human CD16, CD32, and CD64 (Ancell Corp., Bayport, MN, USA) at 4°C for 30 min. Cells were washed and analyzed on a FACScan flow cytometer.

The specific binding of abatacept to CD80, to CD86, and to the Fc IgG receptors on U937 and Raji cell lines was examined. Logarithmically growing Raji and U937 cells were washed in staining buffer (1% BSA in PBS) and preincubated (1×10^6 cells/sample in 0.1 ml) with 750 μg/ml of whole mouse IgG (Sigma, St. Louis, MO, USA) to block Fc IgG receptor binding, or with saturating levels (300 μg/ml each) of purified murine anti-human CD80 and CD86 antibodies (BD Biosciences) to detect blocking of CD80/CD86 binding, or with no added antibodies for 30 min at 4°C. Following this preincubation, 30 μg/ml abatacept, or a ChiL6 control immunoglobulin (an irrelevant isotype-matched chimeric immunoglobulin), or no immunoglobulin was added, and then incubated an additional 30 min at 4°C. Cell samples were washed and incubated with FITC-conjugated donkey anti-human IgG $F(ab)'_2$ (Jackson Immunoresearch, West Grove, PA, USA) for 30 min at 4°C. Cells were washed and analyzed on a FACScan flow cytometer.

Surface plasmon resonance. Specific binding of abatacept or CTLA4-Fc (R&D Systems) to the 3 recombinant human Fc IgG receptor surfaces (CD16, CD32, and CD64) was compared by SPR detection using a BIAcore 3000 instrument. Recombinant human CD16b (Fcγ RIII B, NA2 allele), CD32b/c (Fcγ RII B/C), and CD64 (Fcγ RI) receptors (R&D Systems; catalog numbers 1597-FC, 1875-CD, 1257-FC, respectively) were immobilized covalently at low densities onto the surfaces of 3 flow cells on a CM5 sensorchip. Abatacept and CTLA4-Fc at a concentration of 1000 nM and a negative control were injected simultaneously over the 3 human Fc receptor surfaces at a flow rate of 25 μl/min, allowing for a 10 min association time and a 2 h dissociation time. Additionally, equilibrium binding of abatacept to the immobilized CD64 fusion protein was evaluated by SPR. CD64 was immobilized covalently via primary amino groups at a low density (~1000 resonance units, RU) on the surface of the flow cells of CM5 sensor chips. Abatacept was diluted to concentrations ranging from 25 nM to 25 μM and then injected over the human Fc receptor surface to generate binding sensorgrams. Equilibrium binding of these concentrations in RU was measured and plotted against sample concentration. These plots were fitted with a 4-parameter curve-fit model using XL Fit software (Excel). The dissociation constant K_d for the abatacept/Fc receptor interaction was determined at the inflection points of the individual fitted curves. The following experimental parameters and techniques were used to perform and evaluate the SPR experiments; diluent and running buffer: HBS-EP (BIAcore, HEPES-buffered saline with 0.05% P20 surfactant and 0.01% EDTA), flow rate 25 μl/min, temperature 25°C; abatacept concentrations: 25, 100, 250, 1000, 2500, 10,000, 25,000 nM; equilibrium binding measurements: BIAcore 3000 control software, report points set at 595 seconds after injection start; 4-parameter curve-fit: XL Fit (Excel, version 2 build 30).

RESULTS

Abatacept does not mediate complement-dependent cytotoxicity. A human B cell line, PM-LCL, was exposed to complement alone or in combination with anti-CD20, anti-CD86, CTLA4-Fc or abatacept at 30 and 100 μg/ml. Each component was tested for activity with rabbit, baby rabbit, guinea pig, and human complement. The background level of B cell lysis in the presence of complement alone ranged from 0% with rabbit complement to 4.2% with guinea pig complement (Figure 2). As positive controls, CTLA4-Fc, anti-CD86, and anti-CD20 were tested, and induced significant CDC in the presence of both rabbit complement (24.2%, 22.7%, and 34.6%, respectively) and baby rabbit complement (43.5%, 56.7%, and 61.9%). Only anti-CD20 demonstrated significant activity in the presence of guinea pig and human complement (67.4% and 53.8%). In contrast, abatacept had minimal effect on CDC, with a range of activity from 0.2% to 3.5% with rabbit complement, 0.3%–4.3% with baby rabbit complement, 3.9%–5.8% with guinea pig, and 0–4.1% with human complement. These effects were similar to complement alone and demonstrated no dose-dependence (Figure 2).

Abatacept does not induce antibody-dependent cellular toxicity. The ability of abatacept to induce ADCC was evaluated in separate PBMC preparations derived from 12 healthy blood donors. Figure 3 shows ADCC data expressed as percentage cytotoxicity (± SD) above the value of effectors alone. CTLA4-Fc-mediated ADCC was observed in all 12 donors with a range of 7.4% to 38.7%. In contrast, abatacept at 30 μg/ml demonstrated no significant ADCC in any donor, with values similar to those of control Ig fusion protein.

Fc IgG receptor and CD80/86 expression on U937 and Raji cell lines. Analysis of the U937 monocyte cell line by flow cytometry revealed that these cells express significant levels of the Fc IgG receptors CD32 and CD64, but insignificant levels of detectable CD80 and CD86 (Figure 4).

In contrast, flow cytometry analysis of the Raji B lymphoblastoid cell line revealed significant expression only of Fc IgG receptor CD32, as well as both CD80 and CD86 (Figure 4C, 4D). Neither cell line expressed measurable levels of Fc IgG receptor CD16 (Figure 4A, 4C). The cell-surface expression of CD64 and CD32 in the presence or absence of CD80 and CD86 expression, and the ability to compete for binding with anti-CD80/86 or murine IgG, enabled a direct comparison of the relative binding of abatacept to 2 of the 3 receptor subclasses.

Abatacept binding to U937 and Raji cell lines. Binding of control Ig to U937 cell lines was blocked with excess murine IgG, but not with anti-CD80/86, thus demonstrating that the binding of control Ig was specific for the Fc IgG receptor CD64 on the surface of the U937 cell line (Figure 5A). In this

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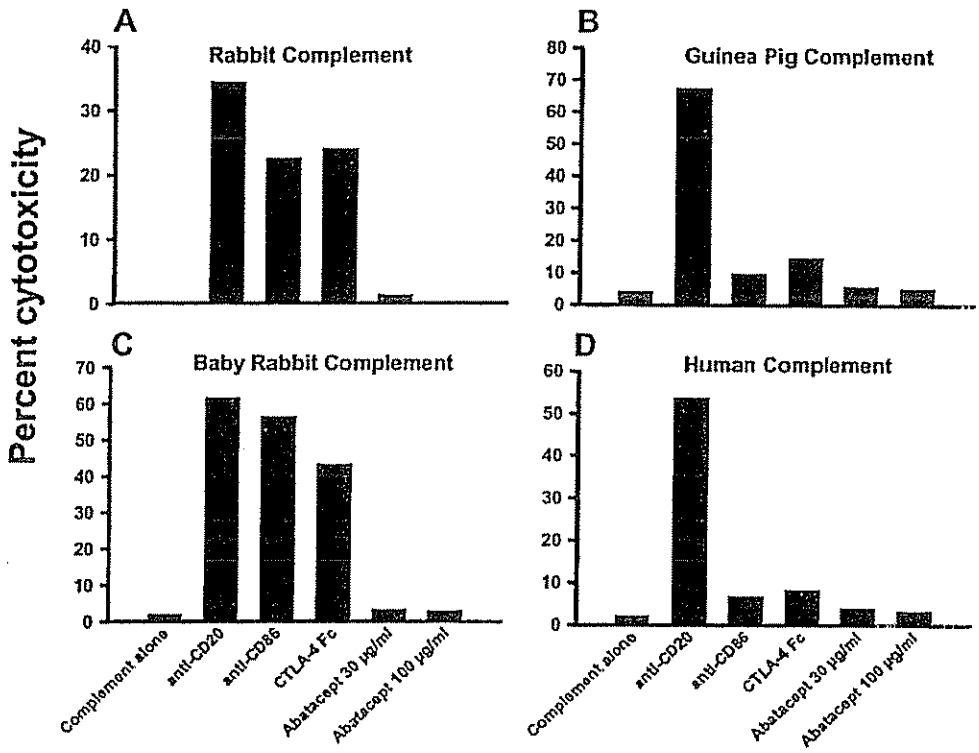

Figure 2. Abatacept does not mediate CDC of target B cells *in vitro*. Percentage toxicity was evaluated with rabbit, baby rabbit, guinea pig, and human complement.

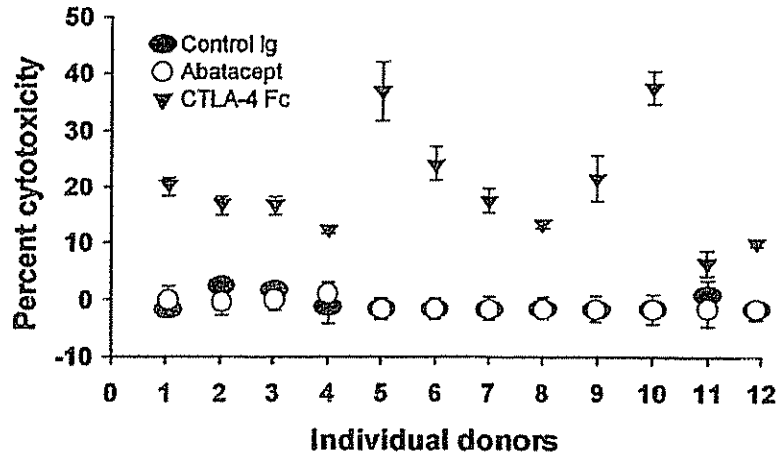

Figure 3. Abatacept does not induce target cell ADCC *in vitro*. Percentage cytotoxicity of PBMC preparations from 12 normal donors (± standard deviation) using abatacept, CTLA-4 Fc, or control Ig is expressed as the value above effectors alone.

same cell line, decreased Fc IgG receptor binding was observed with abatacept (Figure 5B). Similar to Ig control, the lower level of abatacept binding to these cells was ablated by excess murine IgG, but not anti-CD80/86, demonstrating that abatacept was binding specifically to the Fc IgG receptor CD64 on these cells.

In contrast, there was much less binding of control Ig to the CD32-expressing Raji cell lines (Figure 5C). Abatacept demonstrated strong binding to Raji cells, consistent with the high level of CD80 and CD86 on these cells (Figure 5D). Anti-CD80 and anti-CD86 blocked the binding of abatacept, whereas murine IgG had little effect, thus demonstrating that

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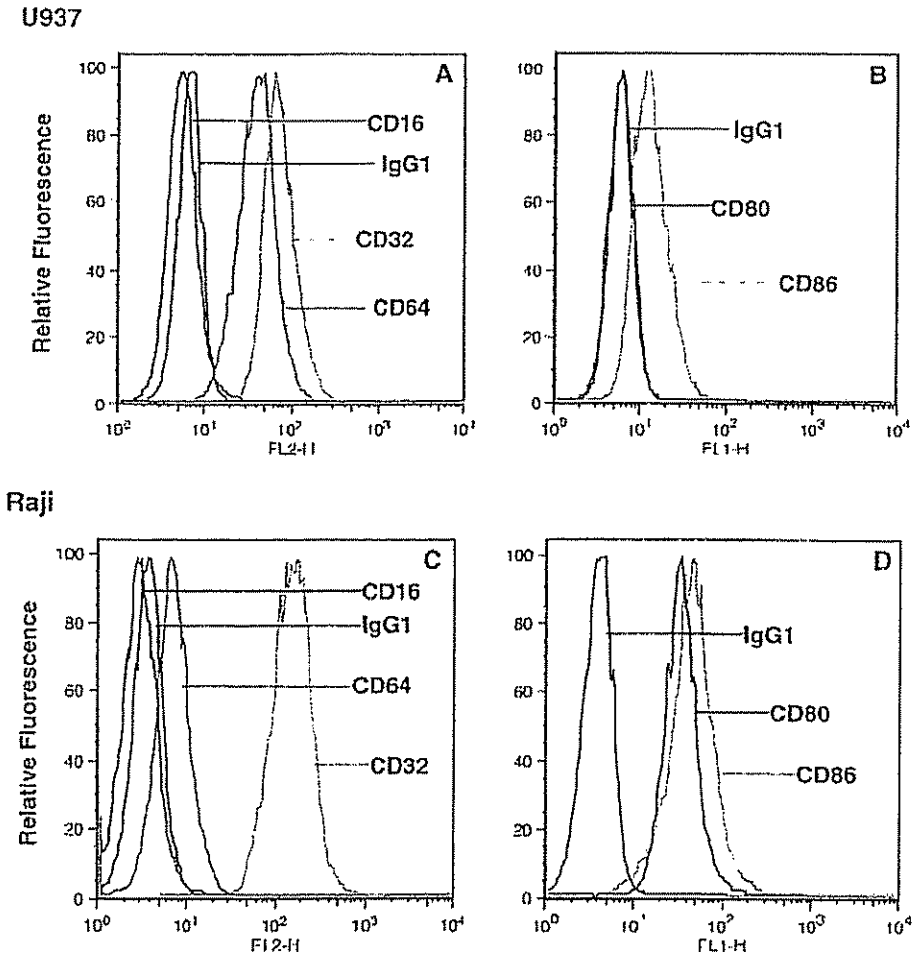

Figure 4. IgG Receptor and CD 80/86 expression on the U937 and Raji cell lines as measured by flow cytometry. A. U937 cells express CD32 and CD64 and not CD16 FcγR. B. U937 cells express insignificant levels of CD80/86. C. Raji cells express CD32 and not CD64 or CD16 FcγR. D. Raji cells express significant levels of CD80/86.

abatacept was specifically binding to CD80/86 on the surface of these cells mediated by the CTLA4 domain. The combined binding results from the U937 and Raji cell lines also demonstrate that abatacept specifically binds through the modified Fc region to the high-affinity Fc receptor CD64, but not to the low-affinity Fc receptor CD32 on the surface of these cells.

Surface plasmon resonance. Abatacept or CTLA4-Fc-positive control was injected at a concentration of 1 μM over an immobilized CD64 surface (described above). Figure 6A displays the combined sensorgrams for binding and dissociation of abatacept and CTLA4-Fc. Consistent with results from flow cytometric analysis, both molecules bound to CD64; however, CTLA4-Fc bound with higher affinity relative to abatacept. Additionally, abatacept demonstrated complete dissociation from the CD64 receptor approximately 2000 seconds after binding, while CTLA4-Fc did not completely dissociate from the CD64 receptor after binding. Table 1 summarizes the SPR binding results for abatacept and CTLA4-Fc to the 3 Fc receptors, CD64, CD32, and CD16. The data confirm the results from the cell-surface binding assays, and demonstrate that abatacept binds specifically only to CD64, and not to CD16 or CD32. Further, the binding of abatacept to CD64 was lower than the binding observed with CTLA4-Fc.

Because abatacept did not require the use of surface regeneration reagents (which caused loss of binding capacity with all 3 Fc IgG receptor surfaces) due to complete dissociation from CD64 after binding, it was possible to evaluate the equilibrium binding and dissociation constant (K_d) of abatacept to the CD64 high-affinity Fc receptor. A 4-parameter curve-fit of abatacept binding in concentrations from 25 nM to 25 μM is shown in Figure 6B. The K_d for abatacept binding to CD64 was calculated to be 3×10^{-7} M.

DISCUSSION

The results from these studies demonstrate that abatacept containing a modified Fc domain was determined to have no cyto-

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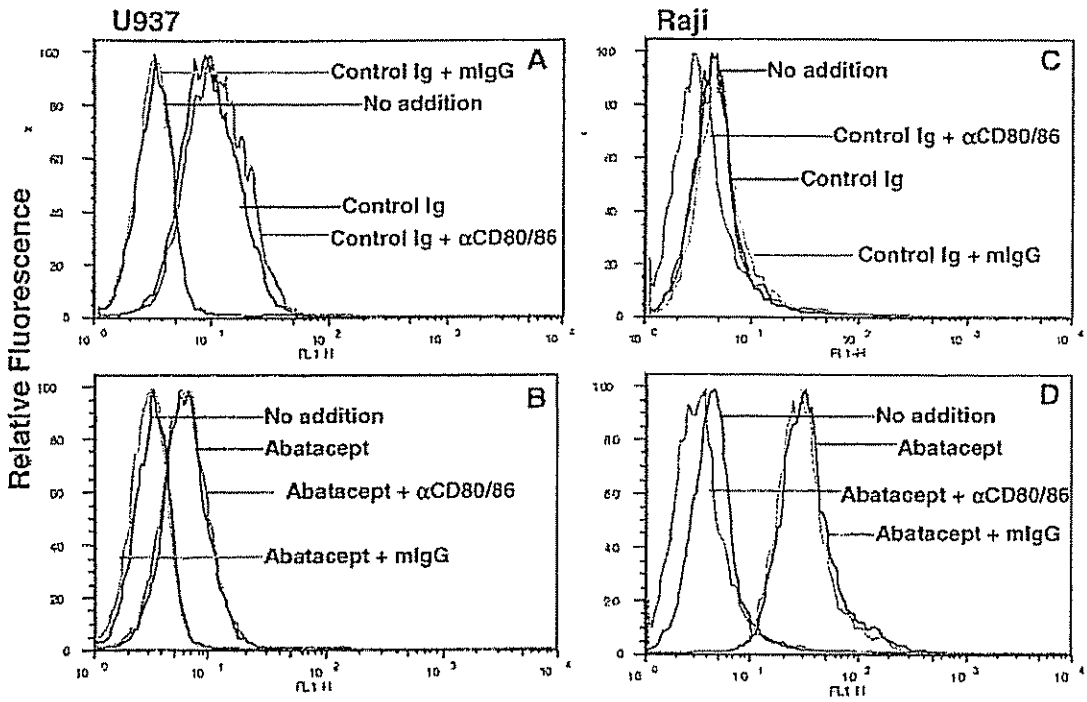

Figure 5. Abatacept binding to U937 and Raji cell lines as measured by flow cytometry. A. Background control binding to U937 cells. B. Abatacept binding to U937 cells. C. Background control binding to Raji cells. D. Abatacept binding to Raji cells.

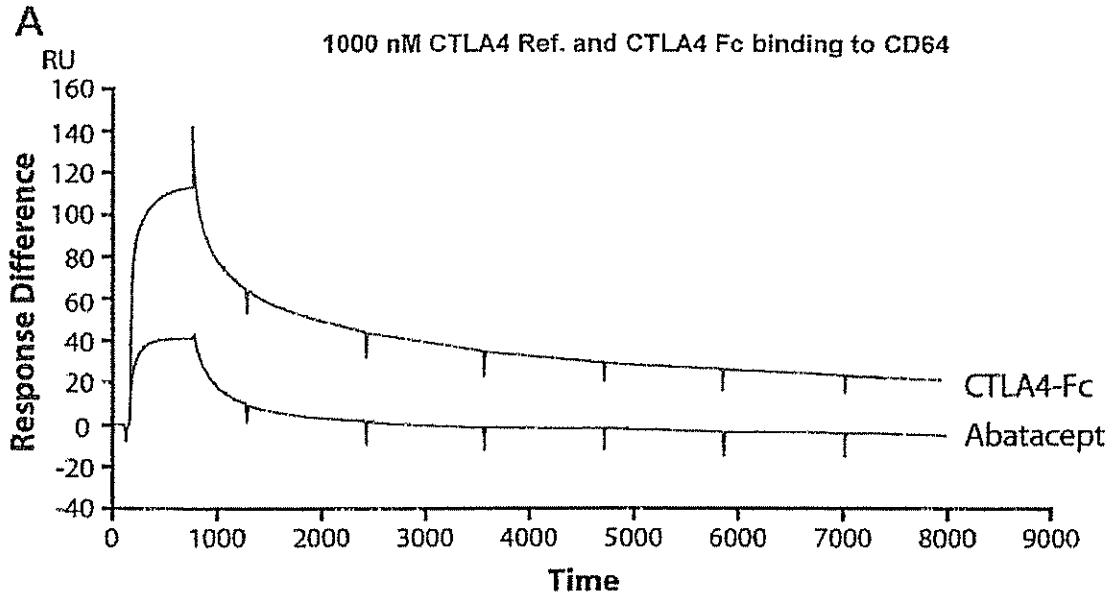

Figure 6 A. Sensorgram of abatacept and CTLA4-Fc binding to immobilized CD64 as measured by surface plasmon resonance (SPR).

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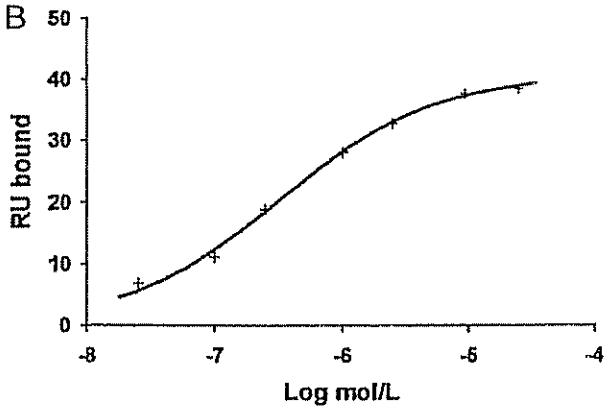

Figure 6. B. Abatacept binding to immobilized human CD64 as measured by SPR. Results are plotted using a 4-parameter curve-fit as described in Materials and Methods.

Table 1. Binding of abatacept and CTLA4-Fc fusion proteins as measured by surface plasmon resonance. Results are expressed in resonance units.

	CD16	CD32	CD64
Fc receptor density	1722	856	1563
Abatacept	–3	–2	40
CTLA4-Fc	219	55	113

toxicity associated with either CDC or ADCC mechanisms *in vitro*. In contrast, CTLA4-Fc, which differs from abatacept by containing a non-engineered Fc domain, was fully functional for both CDC and ADCC. These results indicate that directed mutations in the abatacept molecule appear to have yielded the intended function of diminishing cytotoxicity of CD80/CD86-expressing target cells.

To understand the mechanistic rationale for these results, *in vitro* binding studies using immobilized Fc receptors were conducted using SPR. The results demonstrate that abatacept bound only to the high-affinity Fc IgG receptor I (CD64). Although binding to additional allelic variants of the Fc receptors was not specifically investigated, there is a high degree of sequence homology in the extracellular domains of the 3 identified human CD32 genes and both CD16 alleles. As expected, CTLA4-Fc bound to all 3 immobilized Fc IgG receptors studied (CD16, CD32, and CD64). The binding of CTLA4-Fc to CD64 also occurred with higher affinity as compared to the binding observed for abatacept.

Cell-surface binding studies using Raji and U937 cell lines with flow cytometry analysis examined Fc binding in the context of cell-surface presentation of the Fc receptors. These studies confirmed that abatacept bound specifically via the Fc region to cells expressing CD64; however, in agreement with results from SPR, the observed binding for abatacept in cell-surface binding experiments was lower relative to CTLA4-Fc. Also in agreement with SPR, no specific binding of abatacept to CD32 was observed in the cell-surface binding experiments. These studies demonstrate that abatacept retains some level of Fc binding for the high-affinity Fc IgG1 receptor (CD64), but not the lower affinity Fc IgG receptors CD32 and CD16. Further, this binding is not functional *in vitro* for either CDC or ADCC-mediated activity. CD4-Ig fusion proteins that bind to CD64 but do not fix complement have been described[14]. The reduced binding of abatacept to CD64, undetectable binding to CD16 and CD32, and the lack of ADCC and complement fixation could be the result of steric hindrance or altered glycosylation, among other possibilities. For instance, the deletion of hinge cysteines makes conformational changes in the IgG portion of abatacept probable, and introduction of multiple serine residues makes altered glycosylation a possibility. For example, one of the 3 serines that replaced cysteines in the Fc region (serine 139) was found to be glycosylated. Together, these results support the concept that abatacept therapeutic activity is mediated through the binding of CD80/86 by the CTLA4 extracellular domain and not through activities mediated by the modified Fc domain.

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EXHIBIT 14

Journal of Clinical Immunology, Vol. 13, No. 4, 1993

Special Article

The Biology and Pathology of Fc Receptors

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INTRODUCTION

An interesting property of immunoglobulin molecules is their propensity to bind to cell surfaces. The selective cytophilia exhibited by antibodies turns out to be crucial to many of their physiological functions. Some classes of immunoglobulins bind directly to certain cells in the absence of antigen, such as the binding of IgE to mast cells, while other immunoglobulins bind to cell surfaces as immune complexes that form upon encounters with antigen. The cellular structures that specifically bind immunoglobulin constant regions were first designated Fc receptors by Paraskevas *et al.* in 1972 (1).

The cytophilic nature of antibodies attracted the interest of investigators in the sixties, and since that time there has been an impressive growth in knowledge about its significance and about the nature of Fc receptors (FcR). During the seventies the cellular distribution of many Fc receptors was established. The first half of the eighties saw the development of anti-FcR monoclonal antibodies and the identification of cytokines and other elements that regulate the level of FcR expression. The second half of the eighties saw the cloning of several FcR and the establishment of some basic principles of FcR molecular structure. The FcR research of the early nineties has focused on FcR-associated proteins and their role in signal transduction. In addition, a major source of new information has come from the construction of chimeric FcR molecules that provide an elegant means to dissect the functional anatomy of discrete regions of FcR molecules. As genomic DNA sequence data begin to appear, it is providing insight into the organization of FcR genes and the mechanisms that regulate their expression. Information has developed at an explosive rate and has been the subject of several recent reviews (2–6).

During this period of expansion in basic knowledge about Fc receptors, reports in the clinical literature began to document alterations in FcR expression in human diseases. There is now an impressive number of pathologic conditions in humans and experimental animals in which alterations in FcR expression or function have been recognized. Because of the rapid growth in information about the biology and pathology of Fc receptors, it seemed worthwhile to review briefly some of the major developments. The goals of the present article are (a) to summarize some of the basic information about the families of FcR, (b) to consider some disease states in which FcR expression is known to be altered, (c) to review some examples of FcR participation in disease pathogenesis, and (d) to discuss briefly therapeutic aspects of FcR. Our search of the literature indicates that articles on FcR are being published at a rate of about one paper per day. To facilitate reader access to this large body of information, the References include a number of detailed review articles.

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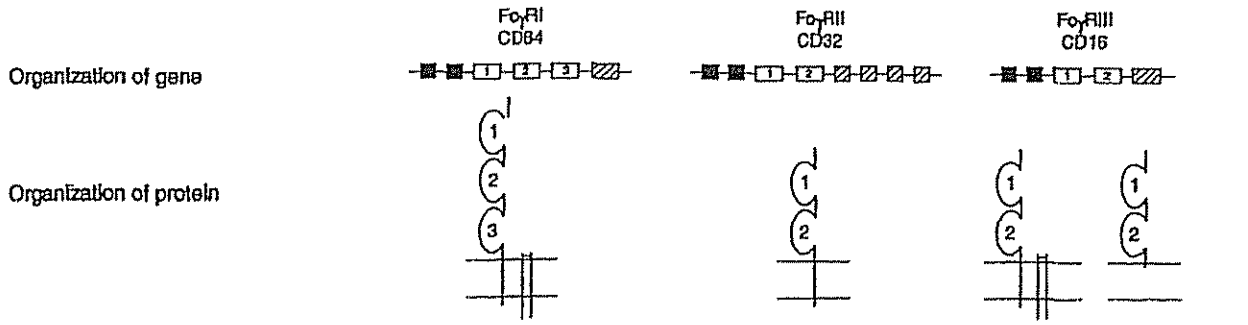


	FcγRI CD64	FcγRII CD32	FcγRIII CD16
Genes	3 (Ia,Ib,Ic)	3(IIa,IIb,IIc)	2(IIIa,IIIb)
Exons	6	8	5
Transcripts	4 (Ia,Ib1,Ib2,Ic)	6(IIa,IIa2,IIb1,IIb2,IIb3,IIc)	2(IIIa,IIIb)
Mw of protein	72 Kd	40 Kd	50-80 Kd
Non-IgG binding associated subunits	γ-γ	?	γ-γ, γ-γ-β, γ-ζ, ζ-ζ, ζ-η
IgG binding extracellular domain	2	2	2
Affinity for monomeric IgG	high	low	low
Specificity for IgG subclasses	IgG3>1>4>>2	3>1>>2*,4	1,3>>2,4
Cellular distribution	monocytes, macrophages granulocytes	monocytes, macrophages granulocytes, platelets Langerhans cells, B cells some T cells mesangial cells	NK cells, γδ T cells, monocytes, macrophages granulocytes
Representative monoclonal antibodies	32.1, 22, 10,1, 47	14.3, 2E1,KuFc79, 41.H16	3G8, Gran1, B73, Leu 11, a,b,c

Fig 1. FcγRI (CD64), FcγRII (CD32); and FcγRIII (CD16). (—▪—) Untranslated region; (—□—) extracellular domain (if numbered, an Ig-like domain); (—▨—) transmembrane and cytoplasmic domains. *FcγRIIa^{HR} allotype binds IgG2.

STRUCTURES, CELLULAR DISTRIBUTIONS, AND FUNCTIONS OF FcR

IgG Fc Receptors

The five major types of FcR are defined by their specificity for immunoglobulin isotypes. In humans the FcR that binds IgG consists of three related receptors: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). These receptors are encoded by eight genes located at q23–q24 on chromosome 1 (Fig. 1). Twelve transcripts have been characterized, some of which are generated by alternative splicing. Additional heterogeneity results from the occurrence of allelic forms of some receptors and the generation of soluble forms by proteolytic cleavage of surface receptors. The extracellular domains of IgG FcR exhibit a disulfide loop structure characteristic of immunoglobulin superfamily members.

FcγRI (CD64). It has recently been reviewed (3) that three genes for FcγRI (CD64) encode four distinct transcription products (Fig. 1). The only protein characterized to date is the gp72 high-affinity (10e8–10e9 M/L) receptor for monomeric IgG that is expressed on monocytes, macrophages, and some neutrophils (Fig. 1). Recently, it has been found that this receptor can associate with the γ-subunit (see below), which can be important in the transmembrane signaling process.

FcγRII (CD32). FcγRII (CD32) is a gp40 with a wide cellular distribution that is summarized in Fig. 1. Three genes have been reported to encode FcγRII (CD32). Molecular cloning of transcripts predicts the existence of six distinct FcγRII proteins, some of which are the products of alternative splicing (Fig. 1). The complexity of the FcR system is even greater because of the occurrence of allotypes (reviewed in 7). The FcγRIIa has two allelic

forms that differ in two amino acids. One allotype (HR) binds IgG2 poorly, while the other (LR) binds IgG2 well. The affinity of CD32 for soluble monomeric IgG is negligible, but IgG complexes are avidly bound. The finding that serine esterases enhance the binding of immune complexes by CD32 may be relevant to the broad cellular distribution of CD32 on cells involved in acute and chronic inflammatory responses (7).

FcγRIII (CD16). FcγRIII is encoded by two highly homologous genes with a broad cellular distribution (Fig. 1). FcγRIIIa is a membrane-spanning form with a 25-amino acid cytoplasmic tail, and FcγRIIIb is a truncated form of the receptor that is anchored to the cell surface via a phosphatidylinositol–glycan bridge. FcγRIIIb is expressed at very high levels on neutrophils (>10ε5 receptors/cell) and is a major source of the soluble form of FcγR found in human plasma. Like CD32, CD16 exhibits a low binding affinity for soluble, monomeric IgG but avidly binds IgG immune complexes. A crucial detail in the cell surface expression of FcγRIIIa is the requirement for pairing with an associated dimeric protein (9). Interestingly, the dimeric partner can be a homodimer or heterodimer composed of the γ-subunit of FcεRI and/or the ζ-subunit of the TCR/CD3 complex. This arrangement effects a molecular linkage between IgG recognition mediated by CD16 and signal transduction, a process in which the γ- and ζ-subunits are known to participate. The physical association of FcR molecules with components of cellular activation cascades has been reported from several laboratories and is an issue being actively investigated.

The murine FcγR system is similar but somewhat simpler. To date, only one gene has been described for each of FcγRI, FcγRII, and FcγRIII. As in the human, FcγRII has alternative splice products with different cellular distributions, but a PIG-anchored form of FcγRIII has not been found.

STRUCTURE–FUNCTION RELATIONSHIPS

The molecular anatomy of human FcγR exhibits two interesting and important features. First, the extracellular segments of the different FcγR have very similar structures that consist of repeating units of immunoglobulin-like domains. Despite clear-cut differences in binding affinity and Ig subclass specificity, the same region of the second extracellular domain of the different receptors appears to be crucial for Ig binding. Second, the cytoplasmic segments of the different FcγR exhibit major diversity in structure and in the usage of associated chains. This heterogeneity is found not only among the three families of FcγR, but also within members of a single family, and could represent the structural basis for the multiplicity of functions that have been reported to be mediated through FcγR on different cell populations.

There is increasing evidence that, in addition to IgG, FcγR also bind non-Ig ligands. For example, transfected FcγRII has been shown to bind IgE immune complexes (10) and oxidized low-density lipoproteins (11). FcγRI binds the acute-phase protein CRP with a high affinity (12). It is likely that other non-Ig ligands will be found. Conversely, besides conventional FcR, there are other functionally important IgG-binding molecules such as soluble and cell-bound forms of rheumatoid factors and C1q, lectins, and soluble CD4 (13). Some neonatal vertebrates express an IgG-binding receptor on gut epithelial cells. In rats this IgG receptor has been shown to resemble closely class I MHC molecules, having a 55-kD α-chain which is associated with β2-microglobulin (14). This IgG transport receptor is important for the uptake of maternal IgG in milk to provide immunological competence for the newborn. As discussed later, a number of viruses and bacteria also express IgG-binding molecules.

A broad array of functions has been proven or proposed for IgG Fc receptors. Until recently our understanding of the functions of IgG FcRs has not developed as rapidly as our understanding of their molecular structures. However, a greatly expanded molecular knowledge base has catalyzed research efforts directed at understanding FcR functions and the field is now entering a phase of accelerating growth.

On myeloid cells FcγR participate in activation events that trigger effector functions. These include ADCC, lysosomal enzyme release, phagocytosis, endocytosis, antigen presentation, cytokine production, and superoxide generation. On NK cells FcR are participants in ADCC and in the regulation of lymphokine release. On B cells FcγR function to modulate various activities. For example, FcγR cross-linked to sIg downregulates B-cell activation (15), while ligation of B-cell FcγR results in tyrosine phosphorylation of multiple target molecules including the FcγR (16, 17). Soluble forms of FcγR released from murine T cells have been shown to bind to sIg on B cells and modulate cellular proliferation and Ig secretion (18). On certain T cells

FcγR are involved in ADCC (19). On platelets FcγR are involved in the aggregation response and in mediator release (20).

The extraordinary scope of functions mediated by FcγR is finding molecular counterparts in the multiplicity of molecular species of FcγR (2, 3). For example, human CD32 is encoded by three genes, and from one of these, FcγRIIB, two alternatively spliced transcripts are generated that encode distinct isoforms of CD32. A B-cell expressed isoform, β1, contains an extra exon in the cytoplasmic tail which disrupts the amino acid sequence required for endocytosis (21). The macrophage-expressed β2 isoform of CD32 contains the intact endocytic motif that links this isoform to endocytosis, phagocytosis, and antigen presentation.

As listed in Fig. 1, the major biological functions that are mediated by the various IgG FcR include IgG transport (22), regulation of IgG synthesis (18), regulation of B-cell activation (15), phagocytosis (23), clearance of immune complexes (24), and antibody-dependent cell-mediated cytotoxicity (25).

The expression of IgG FcR on such a diversity of highly specialized cells and the existence of receptor isoforms account for many of the different functions that are mediated by IgG FcR. The considerable heterogeneity within the three classes of human IgG FcR is further complicated by the occurrence of polymorphisms (7). Allelic forms of the GPI-linked FcγRIIIb have been implicated in transfusion reactions and alloimmune neutropenias. Furthermore, the phagocytic activities markedly differ between allelic forms of FcγRIIIb on human neutrophils (26). The binding of human IgG2 by allelic variants of CD32 on human moncytes differs considerably and could influence the outcome of infections in which host resistance depends on IgG2 antibody responses (7).

IgE Fc RECEPTORS

The two best-described IgE-binding structures are FcεRI and FcεRII (CD23) (Fig. 2).

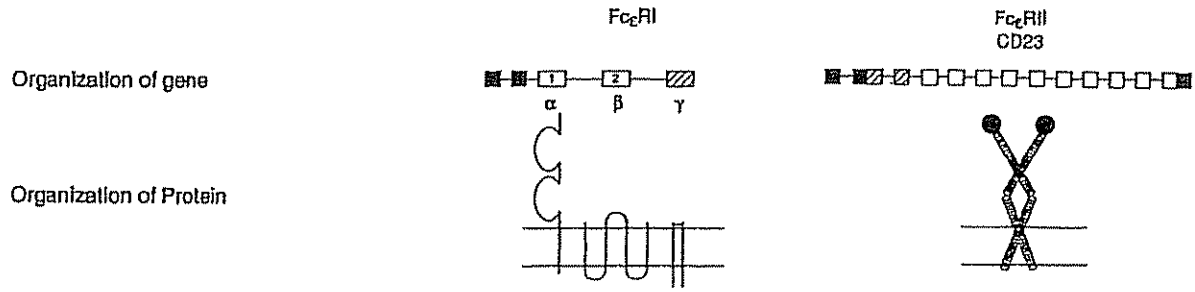
FcεRI is a polypeptide complex composed of three chains. The IgE-binding α-chain is closely related to CD16 in its genomic organization and sequence. The β- and γ-chains of FcεRI are important in signal generation through the receptor. CD16 also can associate with the β- and γ-chains. A detailed review of the structure, genetics, and signaling through FcεRI was published by Metzger and Kinet (27). The cellular distribution of the receptor is quite restricted to mast cells and basophils, and recently it has been found on Langerhans cells in human skin (28).

FcεRII (CD23) is a member of the lectin family of proteins. This class II membrane glycoprotein (COOH– is extracellular and NH2– is intracellular) contains a C-lectin domain connected to an alpha-helical coiled coil, all of which are extracellular. Similar stalked structures occur in the asialoglycoprotein receptor and in CD72. Near the extracellular COOH– terminus of human CD23, there is an RGD element which is a potential site for interaction with integrin molecules. In humans CD23 occurs in two isoforms, α and β, which differ in their responsiveness to IL-4 and in the terminal five amino acids of their cytoplasmic tails (29). The pentapeptide cytoplasmic termini of the CD23 isoforms are encoded by alternative exons under the control of different promoters and expression of the isoforms is cell lineage dependent. CD23a is B cell restricted, while CD23β has a broader cellular distribution. The tail piece of CD23a contains a tyrosine that is important for endocytosis on B cells. The CD23β form has an Asn–Pro site which is critical for phagocytosis (32).

CD23 binds IgE with a low affinity, but it also binds carbohydrate in a Ca^{2+}-dependent manner (30, 31). It has recently been shown that CD21, which is a complement receptor (CR2), is a ligand of CD23 (33). CD21 is a receptor for C3d and for the Epstein–Barr virus. It is very interesting that an EBV promoter (EBNA) regulates CD23 expression in EBV-infected cells. The functional significance of the interactions between CD23 and CD21, both of which are elements in B-cell activation pathways, remains unclear.

A third IgE-binding molecule merits brief comment. Murine macrophages, eosinophils, dendritic cells, and some epithelial cells express a 32-kD protein designated Mac-2, which binds carbohydrates and IgE in an isotype-specific manner (34). A human counterpart of Mac-2 exists, but it has not been thoroughly investigated.

A number of important physiological functions are mediated by IgE receptors. It is widely appreciated that the degranulation response of mast cells and basophils is mediated by FcεRI. More recently it has been shown that FcεRI also mediates the release of interleukins 4, 5, and 6 from mast cells (35). Based on studies in immature mast cells, cytokine release mediated by FcεRI is established



	FcεRI	FcεRII CD23
Number of genes	1	1
Number of exons	5	11
Transcripts	1	2 (a,b)
MW of protein	55 Kd	49 Kd
non-IgE binding associated subunits	γ-γ-β	--
Affinity for IgE	high	low
IgE binding domain	second Ig like domain	lectin domain
binding site on IgE	Cε2 and Cε3 domains	Non-carbohydrate portion of the Cε3
Cellular distribution	Mast cell, basophils Langerhans cells	B cells, some T cells monocytes, platelets eosinophils

Fig 2. FcεRI and FcεRII (CD23). (—▪—) Untranslated region; (—□—) extracellular domain (if numberd, an Ig-like domain); (—▨—) transmembrane and cytoplasmic domains.

in mast cells prior to the developmental stage at which granule formation begins to occur.

Multiple functions have been linked to FcεRII (CD23) (reviewed in Refs. 6, 31, and 36). The majority of investigations has been carried out with B cells, where it has been shown that CD23 can influence the process of B-cell activation, can modulate Ig production in B cells, and can participate in endocytosis, in IgE-dependent antigen processing and presentation, and in homotypic cellular adhesion. It has been found that cross-linking of sIg and CD23 on B lymphocytes generates an activation signal. A role for CD23 in signal transduction has been raised by the finding of Sugie *et al.* (37) that CD23 is physically associated intracellularly with p59 (*fyn*), a *src* family tyrosine kinase that also associates with the CD3/TCR complex on T lymphocytes. CD23 on T cells and soluble forms of CD23 have been shown to regulate the synthesis of IgE.

While the majority of published studies have focused on B-cell CD23, it has been clearly established that T cells in humans and rodents also synthesize CD23 (36). In a sequence of studies using IgE-producing murine hybridoma cells, CD23+/CD8+ T cells have been shown to down-regulate IgE synthesis selectively at the transcriptional level by repressing the activity of the Ig heavy-chain intronic enhancer (38). Soluble CD23 has been shown to function with IL-1 as a differentiation factor in the development of T-lymphoid cells and myeloid cells (reviewed in Ref. 6). It has also been reported that soluble CD23 can function as an inhibitor of macrophage migration (6) and that CD23 on eosinophils can direct an IgE-ADCC effect to parasite targets (39). CD23 is also expressed on

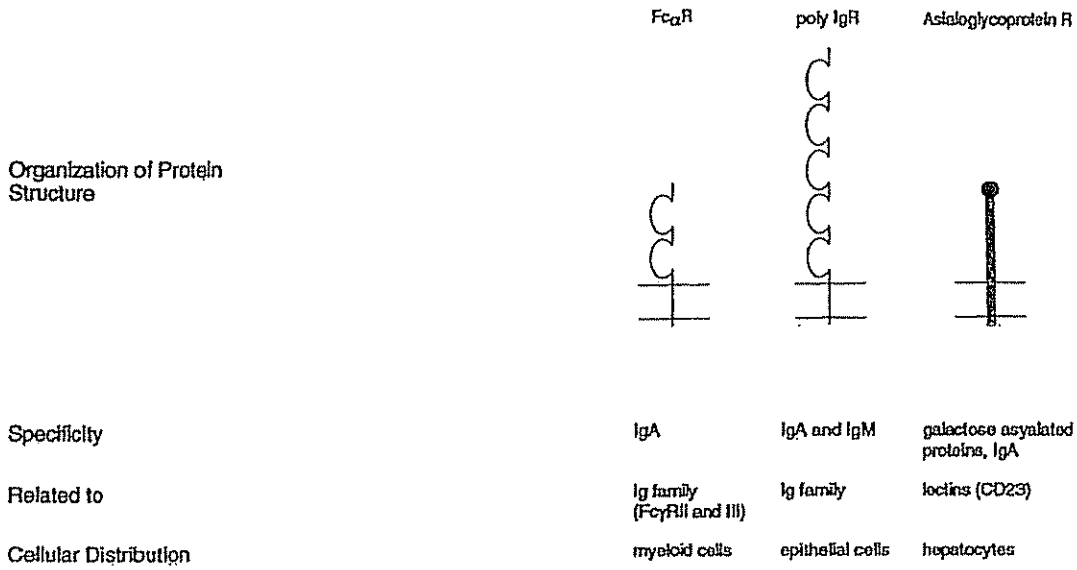


Fig 3. FcαR, poly-Ig receptor, and asialoglycoprotein receptor.

platelets, macrophages, and dendritic cells in germinal centers, but the functional significance remains to be established.

IgA Fc RECEPTORS

Recently, an IgA receptor was cloned from human myeloid cells (40). The structures of its two extracellular Ig-like domains and transmembrane segment indicate that this IgA FcR is related to the families of FcγR and to FcεRI. Using northern blotting and specific monoclonal antibodies, this IgA receptor has been detected so far only on myeloid cells (41). Since T- and B-lymphoid cells have been shown to bind IgA, these cells may display other forms of IgA receptors. The first Ig-binding receptor that was cloned was designated the poly-Ig receptor (42) (Fig. 3). This receptor is a member of the Ig superfamily as reflected by an extracellular portion which has five Ig-like domains. The poly-Ig receptor binds polymeric forms of IgM and IgA and mediates their transport through epithelial cells. The extracellular portion of the receptor is cleaved from the surface of epithelial cells and forms the secretory component of IgA and IgM.

Binding of IgA by hepatocytes is mediated by the asialoglycoprotein receptor (43) (Fig. 3). This lectin, which binds asialated proteins, specifically binds IgA. The poly-Ig receptor and the asialoglycoprotein receptor are important molecules in the enterohepatic transport of IgA, the former responsible for movement of IgA into intestinal secretions and the latter responsible for hepatic uptake of IgA. The asialoglycoprotein receptor, both in its lectin domain and in the extracellular stalk of the lectin domain, is structurally related to FcεRII. Other molecules which bind IgA include the enzyme β-galactosyltransferase, which has been shown to be expressed on the surface of lymphoid cells (43).

The physiological functions of FcαR on myeloid cells include a regulatory role in the activation of granulocyte/macrophage effector functions including superoxide generation (41) (Fig. 3). On T cells FcαR have been shown to influence IgA responses in B cells (44). Cross-linking of the FcαR on B cells has been shown to inhibit polyclonal B-cell antibody responses. On NK cells and monocyte/macrophages, FcαR have been shown to mediate IgA-specific ADCC reactions (45).

IgM Fc RECEPTORS

The IgM FcR on human peripheral blood T cells attracted considerable attention when the classic studies of Moretta and colleagues (46) were reported almost 20 years ago. Insights into the biology and pathology of IgM FcR on lymphocytes have been slow to develop and this remains an important area for further investigation.

Lineages of hematopoietic cells that exhibit IgM binding include B cells, T cells, NK cells, macrophages, and granulocytes. Little is known about the

molecular structure(s) of these operationally defined IgM FcR. A 60-kD protein has been precipitated from human B cells by IgM (47). Several reports indicated that the early T-cell differentiation antigen CD7 binds IgM (48). Experiments using CD7-transfected cells clearly established that CD7 by itself was not sufficient to render an IgM-binding capacity (49).

An immunoregulatory role for IgM FcR on murine CD4+/Th2 cells was suggested when the FcμR on T cells were shown to bind to the sIgM on B cells and promote cytoconjugate formation (50). Likewise, cross-linking the IgM FcR on CD4+ T cells modulated their production of IL-4 (unpublished observations). When murine CD4+ T cells are activated through their CD3/TCR complex, FcμR are induced on the T cells. Interestingly, there is preferential induction on the Th2 subset of CD4+ T cells (51), a finding with some resonance to the original report of Moretta *et al.* (46) with human T cells. There is a report that the IgM FcR on macrophages can mediate ADCC reactions (52).

As mentioned above, IgM is bound by the poly-Ig receptor present on certain epithelial cells. This receptor selectively binds polymeric IgM and IgA, mediates their transepithelial passage, and, in a final proteolytic step, donates secretory component to the released antibody molecule. The poly-Ig receptor is a member of the immunoglobulin gene superfamily and is distinct from the Ig-M receptors present on lymphoid and myeloid lineage cells.

THE PATHOLOGY OF Fc RECEPTORS

Changes in Lymphocyte FcR Expression in Pathologic States

The number of diseases in which FcR expression on lymphoid cells is changed continues to increase. Considering that FcR expression on lymphoid cells is altered by cellular activation, immunoglobulin levels, and changes in the patterns and amounts of circulating cytokines, it is inevitable that this list will continue to grow. Whether particular patterns of FcR alteration will have diagnostic or prognostic utility, or will lead to a better understanding of the pathogenesis of the disease or its complications, remains to be determined.

The first studies that identified alterations in FcR expression in disease were in mice with plasmacytomas (reviewed in Ref. 53). Large numbers of peripheral T lymphocytes were found to express FcR whose specificity matched the heavy chain class of the monoclonal Ig secreted by the tumor. This was observed for all five heavy-chain classes in the mouse and has been seen in humans with IgG myeloma (54). While changes in lymphocyte IgA FcR levels have been reported in patients with selected IgA deficiency and in IgA nephropathy (55), with the exception of Ig-secreting tumors, most studies of FcR alteration in disease have focused on lymphocyte CD23. Some parasitic diseases in experimental animals are accompanied by changes in B-lymphocyte CD23 expression (unpublished observations). The mechanisms involved are still undetermined, but certain parasitic diseases are accompanied by elevated IgE, polyclonal lymphocyte activation, biased expansion of lymphoid cell subsets, and changes in cytokine levels. Each of these events is known to influence CD23 expression. It is interesting that the protozoan parasite *Leishmania donovani* induces a selected loss of CD23 expression on murine B lymphocytes *in vitro* and *in vivo* by a mechanism that requires direct contact of the promastigote with the B lymphocyte and results in accelerated shedding of CD23 (56). The multiplicity and complexity of lymphocyte FcR regulatory pathways could make it difficult to distinguish cause from effect in any analysis of FcR changes in disease.

Abnormalities in FcR as Pathogenetic Elements

In paroxysmal nocturnal hemoglobinuria (PNH) a defect in phosphatidylinositol anchoring results in an absence of GPI-anchored CD16 on neutrophils and impaired phagocytosis of immune complexes (57). The subject of FcγR genetic polymorphisms and their clinical significance has recently been reviewed (7). An allotypic polymorphism of the GPI-linked form of CD16 designated NA1/NA2 is the cause of certain transfusion reactions and allo- or autoimmune neutropenias (26). It has been found that neutrophils from individuals of allotype NA2 exhibit decreased phagocytic ability compared to individuals of allotype NA1. An allotypic polymorphism designated HR/LR involves CD32 and it has been proposed that the HR allele, which does not bind IgG2, predisposes to certain bacterial infections (7). It has recently been found that individual homozygous for HR and NA2, who also have a defect of C6 or C8, are at high risk for meningococcal disease. Thus, the presence of certain FcγR phenotypes have diagnostic and clinical relevance

and should be considered in the evaluation of patients with recurrent bacterial infections (Fjien *et al.*, submitted for publication).

Mice with the autosomal dominant mutation designated as "tight skin" develop a dense fibrosis of the skin and have autoimmune antibodies including antibodies directed to the GPI-linked form of CD16 (58). This is of particular interest because antibodies specific for the GPI-linked form of CD16 have been shown to trigger the release of mediators from neutrophils and to generate superoxide. Conceivably the anti-FcR antibodies contribute to tissue injury by promoting the release of mediators from granulocytes. Autoantibodies directed to human FcγR have also been found in patients with progressive systemic sclerosis (59). An additional example of FcR as a pathogenetic element is the recent finding that the presence of an FcγRI variant gene that lacks a portion of the cytoplasmic tail correlates with the expression of the diabetic trait in *NOD* mice.

FcR Utilization by Pathogens

Since some isoforms of FcγR facilitate cellular uptake of immune complexes, it is conceivable that certain complexes, once internalized, might be detrimental to the cell. This has been shown for the arborvirus of Dengue fever, where viral-specific IgG antibodies enhance viral uptake into host macrophages, a permissive site for viral replication. This mechanism needs to be considered in the development of vaccination strategies for obligate intracellular pathogens, including other viruses such as HIV.

It is well-known that bacteria such as streptococcus and staphylococcus express Fc-specific Ig-binding molecules which are structurally unrelated to host Fc receptors (60). The significance of these microbial FcR in the pathogenesis of infection remains unclear. Certain FcR derived from bacteria are valuable immunochemical laboratory reagents. During infection with *Herpes zoster,* FcR encoded by the viral gene gE is expressed, but their functional significance remains unclear. Viral-associated FcR is a topic that has recently been reviewed (61).

Therapeutic Implications of Fc Receptors

The therapeutic use of monoclonal antibodies can be frustrated by FcR binding and accelerated removal of the antibody by cells of the RES. Injury to those cells can ensue when the monclonal antibody is a conjugate with a toxic substance. Some target-cell effects of monclonal antibodies are influenced dramatically by FcR. Soluble anti-CD3 antibodies suppress T-cell responses, while the FcR-bound form of the same antibody cross-links the CD3 molecules and induces T cells to proliferate and produce cytokines.

Many murine anti-human antibodies are humanized by genetic engineering techniques in order to minimize immune responses during long-term therapy (reviewed in Ref. 62). The use of the IgG4 constant region, the least cytophilic subclass of IgG, and mutations at the hinge region, a site crucial to the binding to all forms of FcγR, can significantly minimize cytophilia of these IgGs.

Therapeutic antibodies directed against FcR and conjugated with another antibody to create bispecific reagents that direct effector cells to tumor cell targets have shown promise in experimental systems (63). Bispecific antibodies in which one component is directed against Fc receptors and the other against a virus, a cytokine, or some other substance can be used to accelerate removal via FcR+ cells of the RES.

PROSPECTUS

The molecular and genetic foundations of the field of Fc receptors is rapidly being established. This new information is already impacting in the clinic, where recombinant FcR molecules are successfully modulating GVH reactions and B-cell responses. As even newer knowledge is generated, it is likely that it will influence the practice of clinical immunology in the upcoming decade.

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EXHIBIT 15

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EXHIBIT 16

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EXHIBIT 17

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