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This information is current as of August 8, 2022.

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*J Immunol* 2003; 171:6251-6259; ;  
doi: 10.4049/jimmunol.171.11.6251  
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# Activity and Safety of CTLA-4 Blockade Combined with Vaccines in Cynomolgus Macaques

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The immune modulatory molecule CTLA-4 (CD152), through interactions with the B7 costimulatory molecules, has been shown to be a negative regulator of T cell activation in various murine model systems. Abs that block CTLA-4 function can enhance immune responses that mediate potent antitumor activity. However, CTLA-4 blockade can also exacerbate autoimmune disease. The safety and activity of anti-CTLA-4 Abs in primates has not been addressed. To that end, we generated human Abs against CTLA-4 using transgenic mice expressing human Ig genes. A high affinity Ab (10D1) that blocked the binding of CTLA-4 to the B7-1 and B7-2 ligands and had cross-reactivity with macaque CTLA-4 was chosen for further development. Administration of 10D1 to cynomolgus macaques significantly enhanced Ab responses to hepatitis surface Ag and a human melanoma cell vaccine. Anti-self Ab responses as measured by immunoassays using lysate from melanocyte-rich tissues were elicited in those animals receiving the melanoma cell vaccine and anti-CTLA-4 Ab. Remarkably, chronic administration of 10D1 did not result in measurable polyclonal T cell activation, significant alteration of the lymphocyte subsets, or induce clinically observable autoimmunity. Repeated dosing of the 10D1 did not elicit monkey anti-human Ab responses in the monkeys. These observations support the development of CTLA-4 blockade for human immunotherapy. *The Journal of Immunology*, 2003, 171: 6251–6259.

In vivo blockade of the CTLA-4, referred to as CTLA-4 blockade, represents a novel method for enhancing a patient's immune response to fight disease (1). In particular, the antitumor effect of CTLA-4 blockade has been well-documented in experimental syngeneic tumor transplant models (2–5). The efficacy of CTLA-4 blockade in these model systems appears to be related to the inherent immunogenicity of the tumor, as nonimmunogenic tumors (i.e., tumors in which irradiated cells do not confer protection to a subsequent challenge) are generally not sensitive to anti-CTLA-4 Ab treatment alone (6). However, with less responsive tumors a combination of anti-CTLA-4 Ab with additional treatments such as active immunization with tumor Ag vaccines (7, 8), surgery (9), or chemotherapy (10) constitutes effective therapy.

The mechanism by which antitumor responses are augmented as a result of treatment with anti-CTLA-4 mAbs is thought to be dependent on the inhibition of CTLA-4 engagement of the B7 costimulatory molecules expressed on APCs (1, 11). The B7 family of costimulatory molecules is critical in the induction of Ag-specific T cell activation through interactions with the constitutively expressed T cell molecule CD28 (12, 13). After a T cell becomes activated, CTLA-4 expression is induced on the surface of the cell. Due to a higher affinity, CTLA-4 efficiently competes with CD28 for binding to B7 molecules on the APC, leading to T

cell inactivation. This mechanism of T cell inactivation by CTLA-4 is important in regulation of peripheral T cell homeostasis, an activity that is most dramatically demonstrated in CTLA-4 knockout mice, which exhibit massive lymphoproliferation leading to death at 3–4 wk of age (14, 15). Abs that block CTLA-4/B7 interactions appear to reversibly prolong T cell activation by preventing negative signals provided by the B7/CTLA-4 interactions and in so doing lower the threshold for T cell activation to Ag.

Thus far, the majority of studies on CTLA-4 blockade have been performed in mice. Evidence for a similar function in humans comes from limited in vitro studies demonstrating augmentation of T cell responses by CTLA-4 blockade (16). Furthermore, epidemiological data on polymorphisms in the CTLA-4 gene suggest a correlation with certain autoimmune disorders (17, 18). Primate models and human clinical trials will be required to elucidate the full potential of CTLA-4 blockade in humans. To this end, human mAbs directed against human CTLA-4 were generated using transgenic mice carrying human rather than mouse Ig genes. These mice possess introduced human H and L chain transgenes that undergo class switching and somatic mutations to produce human IgG1 $\kappa$  and IgG3 $\kappa$  Abs (19). This study describes the in vitro and in vivo activity of a human mAb specific for CTLA-4 referred to as 10D1. We have demonstrated that administration of mAb 10D1 to cynomolgus monkeys is well-tolerated and enhances immune responses to vaccines.

## Materials and Methods

### Cell lines

BW-huCTLA-4/CD3 $\zeta$  is a murine T cell hybridoma that expresses human CTLA-4 constitutively. This line was constructed by expressing the extracellular and transmembrane domains of human CTLA-4 (aa 1–190) fused to murine CD3 $\zeta$  (aa 52–164). The CTLA-4/CD3 chimeric gene was then subcloned into the expression vector pBABE, which also contains a gene encoding for puromycin resistance, to create pBABE-huCTLA-4/CD3 $\zeta$  (20). pBABE huCTLA-4/CD3 $\zeta$  was transfected into the retroviral packaging line,  $\psi$ -2, and a pool of puromycin-resistant cells was selected. BW5147 cells (ATCC no. TIB-47; American Type Culture Collection, Manassas, VA) were cocultured with the retroviral producer cells and selected

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Received for publication May 9, 2003. Accepted for publication September 30, 2003.

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for resistance to puromycin. A clone expressing high levels of human CTLA-4 at the cell surface was selected (BW-huCTLA-4CD3 $\zeta$ -3#7).

L cells which constitutively express mouse CTLA-4 were generated by transfection using a gene composed of the extracellular and transmembrane domains of murine CTLA-4 (aa 1–190) fused to the intracellular domain of murine CD28 (aa 179–219). L cells were also generated that express rhesus CTLA-4. A genomic clone from the rhesus genomic library (BD Biosciences/Clontech Laboratories, Palo Alto, CA) was isolated by hybridization with a human cDNA clone for CTLA-4. An exon encoding the extracellular domain of rhesus CTLA-4 was sequenced and a fusion between the signal peptide of human CTLA-4 (aa 1–36), the extracellular domain of rhesus CTLA-4 (aa 37–162), the transmembrane domain of human CTLA-4 (aa 162–190), followed by the intracellular domain of CD28 (aa 179–219) was created. The sequence of rhesus macaque CTLA-4 has two amino acid differences from the human sequence (21).

The human melanoma cell line SK-MEL-3 (American Type Culture Collection) was transfected to produce human GM-CSF (Invivogen, San Diego, CA). A subclone stably expressing GM-CSF was used for the vaccine studies and termed SK-Mel-GM. GM-CSF production was monitored by ELISA (R&D Systems, Minneapolis, MN).

#### Immunization of transgenic mice and development of hybridomas

Transgenic HuMAb mice, strain HC2/KCo7, having four distinct genetic modifications were used for immunizations (22). To generate Abs, mice were immunized with BW-CTLA4/CD3 $\zeta$  (5–10  $\times$  10<sup>6</sup>) injected i.p. Immune mice were given an i.v. boost with the extracellular domain of CTLA-4 4 days prior to harvesting spleens. The extracellular CTLA-4 fragment was prepared by proteolytic cleavage of the CTLA-4/Fc fusion protein (cat. no. 325-CT-200; R&D Systems) at a factor Xa protease cleavage site located after the C terminus of the CTLA-4 extracellular domain. Single cell suspensions of splenic lymphocytes from immunized animals were fused with the murine myeloma cell line P3X63Ag8.653 (American Type Culture Collection) in the presence of polyethylene glycol (23). Hybridomas were selected by the addition of hypoxanthine/aminopterin/thymidine 24 h after fusion. Hybridomas were first screened by a sandwich ELISA for human IgG $\kappa$  producers. Human IgG $\kappa$ -producing hybridomas were then selected based on reactivity with CTLA-4 by flow cytometry and ELISA.

#### Inhibition of CTLA-4/B7 binding

To demonstrate the ability of 10D1 to block the interaction of CTLA-4 to the ligands B7.1 and B7.2, competition assays were performed by flow cytometry and ELISA. Briefly, CTLA-4-expressing cells (BW-huCTLA-4/CD3 $\zeta$ ) were incubated with varying concentrations of human anti-CTLA-4 mAb 10D1 (F(ab')<sub>2</sub>) or murine anti-CTLA-4 (BNI-3; BD Biosciences/BD PharMingen, San Diego, CA) together with either recombinant human B7.1-Ig (0.1  $\mu$ g/ml) or B7.2-Ig (0.2  $\mu$ g/ml; R&D Systems). The cells were washed, fixed in 1% paraformaldehyde, and their fluorescence analyzed with a FACSCalibur instrument. For ELISA experiments the B7-Ig was labeled with biotin, and detected with a streptavidin-alkaline phosphatase probe. Percent inhibition was calculated from the formula: ((mean fluorescence intensity (MFI) no inhibitor) – (MFI with inhibitor))/MFI no inhibitor  $\times$  100%.

#### Immunohistochemistry

Immunohistochemistry evaluations were performed by Pathology Associates International (Frederick, MD). Human tissues were obtained from the National Disease Research Interchange (Philadelphia, PA), embedded in Tissue-Tek OCT medium, frozen on dry ice, and stored below –70°C. Tissues were sectioned at 5  $\mu$ m, fixed for 10 min in acetone before immunoperoxidase staining. Endogenous peroxidase activity was quenched through incubation with peroxidase solution for 5 min. FITC-labeled 10D1 was applied for 60 min at concentrations of 2.5 and 10  $\mu$ g/ml. Rabbit anti-fluorescein Ab was applied for 30 min at a dilution of 1/500 (PBS with 1% BSA and 1 mg/ml heat-aggregated IgG). Slides were reacted for 30 min with DAKO Envision kit peroxidase-labeled polymer (DAKO, Carpinteria, CA). Diaminobenzidine was applied for 8 min using the substrate-chromogen solution supplied in the DAKO Envision kit. Slides were counterstained with hematoxylin, dehydrated, and coverslipped for light microscopic evaluation.

#### Binding to mouse/human/macaque CTLA-4

Cells lines that constitutively express human, murine, and rhesus CTLA-4 were used to determine the species cross-reactivity of 10D1. Cells were incubated with 10  $\mu$ g/ml 10D1 or isotype control for 90 min at 4°C. After

washing, the cells were further incubated with goat anti-human IgG conjugated to FITC. The cells were fixed in 1% paraformaldehyde and their fluorescence was analyzed on a FACSCalibur instrument.

#### Experimental design for hepatitis B surface Ag (HBsAg)<sup>4</sup> vaccine study in cynomolgus macaques

Eight male and eight female cynomolgus monkeys (*Macaca fascicularis*), 3–4 years of age and 3.5–5.6 kg, were obtained from Scientific Research International (Sparks, NV). The animal husbandry, test article administrations, and sample collections were performed by Sierra Biomedical (Sparks, NV). mAb 10D1 or an isotype-matched control human IgG (anti-RSV humanized IgG1; Synagis, MedImmune, Gaithersburg, MD) was administered i.v. (10 mg/kg) on days 1 and 29. All of the animals were vaccinated (i.m.) with 10  $\mu$ g of HBsAg mixed with alum (Engerix-B; GlaxoSmithKline, Philadelphia, PA) on study days 2 and 30. Animals were monitored for general health twice daily and body weights were recorded weekly. At day 64, a macroscopic pathology examination was conducted and selected tissues were analyzed microscopically.

#### Ab responses to HBsAg

For the mouse studies, Ab responses were determined by ELISA using recombinant HBsAg protein (Seradyne, Indianapolis, IN). Briefly, wells were coated with HBsAg (0.5  $\mu$ g/ml), blocked, and then incubated with dilutions of plasma samples obtained before and after immunizations. Ab responses were measured with goat anti-murine IgG probe. The titer was determined as the highest dilution giving a positive value compared with preimmunization samples. For the macaque study, a commercial kit (Sanofi-Pasteur Diagnostics, Montreal, Quebec, Canada) was used for the quantitation of Abs to HBsAg. The HBsAg Ab concentration (mIU per milliliter) was determined from the standard curve according to the kit.

#### Surface marker expression and intracellular cytokine analysis

For determining surface marker expression, whole blood was divided into tubes (100–200  $\mu$ l/tube) and stained for surface markers for 15 min at room temperature (anti-CD3, CD4, CD8, CD69, HLA-DR, and CD25; BD Biosciences). The RBCs were lysed and the remaining leukocytes were fixed in 1% paraformaldehyde. For the analysis of intracellular cytokines, whole blood was incubated overnight (37°C, 5% CO<sub>2</sub>) in the presence of HBsAg (Aldevron, Fargo, ND) or medium only along with costimulatory Abs (anti-CD28, anti-CD49d; BD Biosciences) and brefeldin A. RBCs were lysed and the remaining leukocytes were permeabilized (Perm 2 Solution; BD Biosciences) before staining for intracellular cytokines and activation marker. A mixture of Abs with established reactivity to macaques was used (anti-CD3, anti-CD8, anti-CD69 with either anti-TNF- $\alpha$ , anti-IL-2, or anti-IFN- $\gamma$ ; BD Biosciences). The samples were analyzed on a (FACSCalibur) flow cytometer within 24 h of staining.

#### Experimental design for melanoma vaccine study

Six male and six female cynomolgus monkeys (*M. fascicularis*), 7–8 years of age and 2.4–6.4 kg, were obtained from Primate Products (Miami, FL). The animal husbandry, test article administrations, and sample collections were performed by Huntingdon Life Sciences (East Millstone, NJ). Ten milligrams per kilogram of mAb 10D1 were administered i.v. on days 0, 28, 56, 84, and 140 to six animals. The SKmel-GM vaccine was administered to all animals on days 0, 28, 56, 84, and 140. To prepare the vaccine, SKmel-GM cells were grown to confluency and harvested. The cells were treated with mitomycin C, washed several times, and resuspended to 1  $\times$  10<sup>7</sup>/ml in saline. The cell preparation (0.5 ml) was injected s.c. into animals. Each vaccine preparation was tested for endotoxin (<2 EU/ml) and GM-CSF production after 48 h (2–8 ng/ml per 10<sup>6</sup> cells). Animals were monitored for general health twice daily and body weights were recorded weekly. Hematology, pharmacokinetic analysis, and functional assays were performed prior to study initiation and periodically throughout the study. A complete macroscopic and microscopic pathology examination was performed at day 167.

#### Ab responses to melanoma vaccine

**Flow cytometry.** SKmel-3 or other cell lines were washed and adjusted to 3  $\times$  10<sup>5</sup> cells/50  $\mu$ l in PBS containing 0.1% BSA, before incubation with the plasma samples (final dilution = 1/1000) at 4°C. After washing, the cells were incubated with a FITC-conjugated goat anti-human IgG

<sup>4</sup> Abbreviations used in this paper: HBsAg, hepatitis B surface Ag; MFI, mean fluorescence intensity; ADCC, Ab-dependent cellular cytotoxicity; T reg, T regulatory.

F(ab')<sub>2</sub>-specific reagent. The cell associated fluorescence was analyzed using a FACSCalibur, and the MFI of the samples was reported.

**ELISA.** Whole cell lysate was prepared from SKmel-3 cells or fresh frozen cynomolgus iris tissue by incubation of cells in Triton X-100 containing buffer. Solubilized proteins were separated from insoluble material by centrifugation. Plates were coated with lysates, and blocked with PBS containing 5% BSA. Plasma samples (1/200 dilution) were incubated on the plates. The bound monkey IgG was detected with alkaline phosphatase-labeled goat anti-human IgG Fc-specific probe. The assay was developed with a *p*-NPP substrate and the absorbance at 405 nm was determined.

#### Ab-dependent cellular cytotoxicity (ADCC)

Melanoma cell lines were used as targets for lysis by fresh human mononuclear cells purified from heparinized whole blood. Target cells were labeled with 100  $\mu$ Ci <sup>51</sup>Cr for 1 h prior to combining with mononuclear cells and monkey plasma specimens. After 4-h incubation at 37°C, supernatants were collected and analyzed for radioactivity. Cytotoxicity was calculated by the formula: % lysis = (experimental cpm – target leak cpm)/(detergent lysis cpm – target leak cpm)  $\times$  100%. Specific lysis = % lysis with sample – % lysis without sample. Assays were performed in triplicates.

#### Pharmacokinetics

Analysis of plasma concentration for mAb 10D1 was performed by ELISA. Briefly, microtiter wells were coated with CTLA-4-Ig (R&D Systems) and blocked with PBS containing 5% BSA. Dilutions of plasma samples were incubated on the plates, and the bound 10D1 was detected with alkaline phosphatase-labeled goat anti-human IgG (Fab') probe. The assay was developed with a *p*-NPP substrate and the absorbance at 405 nm was determined on a plate reader. Sample dilutions falling into the linear portion of a standard curve were used for determining the plasma concentrations.

#### Statistics

Statistical analysis of data was obtained using the Student *t* test and SIGMAPLOT software (SPSS Science, Chicago, IL).

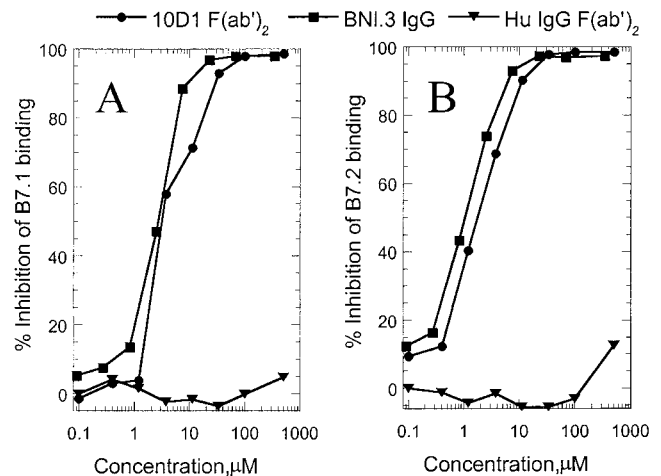
## Results

### Generation and characterization of human anti-CTLA-4 mAb 10D1

Fully human mAbs specific for human CTLA-4 were generated using transgenic mice expressing human Ig genes. Spleen cells from mice that developed human Ab titers against CTLA-4 were used to generate hybridomas using standard myeloma fusion technology originally developed by Kohler and Millstein (23). Approximately 70 hybridomas secreting human IgG mAbs were generated with specificity for CTLA-4. One hybridoma (10D1) was selected for further development based on binding specificity, affinity, and capacity to block ligand binding.

The predominant mechanism whereby Abs to CTLA-4 are thought to mediate their immune stimulatory activities is through blockade of the receptor-ligand interactions. Therefore, we characterized the ability of mAb 10D1 to inhibit binding between CTLA-4 and its costimulatory ligands, B7.1 and B7.2. Fig. 1 demonstrates blocking of ligand binding to CTLA-4-expressing cells by F(ab')<sub>2</sub> of 10D1 using a flow cytometry method. The murine anti-human CTLA-4 mAb (BNI-3) and 10D1 effectively blocked ligand binding with similar kinetics (IC<sub>50</sub> ~1–3  $\mu$ M). Similarly, mAb 10D1 was found to efficiently block B7 binding to CTLA-4 in ELISA-based competition assays with purified recombinant molecules (data not shown). In addition, 10D1 Ab inhibited the binding of soluble CTLA-4Ig to L cell transfectants expressing murine B7.2 (data not shown).

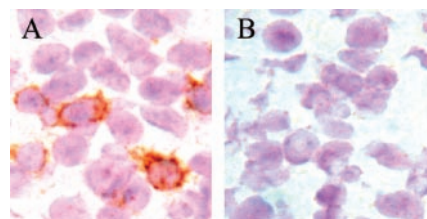
Cross-reactivity studies of mAb 10D1 to human and monkey tissues and cell lines expressing human, monkey, and murine CTLA-4 were performed to determine specificity and establish appropriate animal models for further studies. By means of immunohistochemistry, the reactivity of mAb 10D1 on frozen sections from human tissues corresponding to all of the major organs and tissues was assessed. In most tissues, rare to occasional immuno-



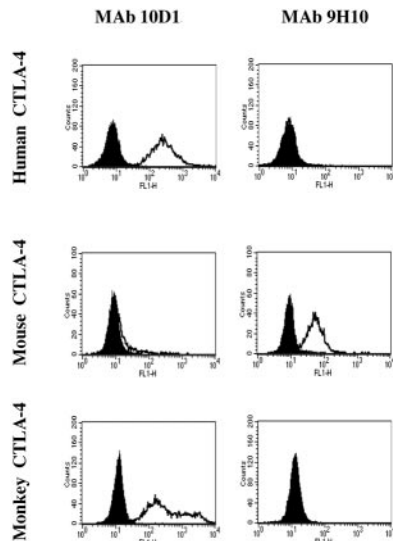
**FIGURE 1.** Ligand blocking by mAb 10D1. Inhibition of recombinant B7.1 (A) and B7.2 (B) binding to CTLA-4-expressing T cells. B7-Ig fusion proteins were incubated with huCTLA4 transfectants in the presence of varying concentrations of 10D1 F(ab')<sub>2</sub> or isotype control F(ab')<sub>2</sub>. The mouse anti-human CTLA-4 mAb BNI-3 was also included as a positive control. B7-Ig binding was detected using goat anti-human IgG (Fc-specific)-PE probe. The cell-associated fluorescence was determined by flow cytometry, and percent inhibition of binding was determined as described in *Materials and Methods*. Representative data from one of two experiments are shown.

reactive cells were present and identified as lymphocytes based on morphology and location. Tonsil was consistently the most reactive tissue, presumably due to inflammation that may have been present at the time of surgery (Fig. 2). The mAb 10D1 specifically stained discrete, round granules at the membrane and cytoplasm immediately below the membrane. The results were consistent with the expected pattern of CTLA-4 expression and no unanticipated cross-reactivity was observed. The immunohistochemistry analysis also revealed a similar reactivity pattern in selected tissue sections from rhesus and cynomolgus macaques (data not shown). In addition, specific binding to human and rhesus CTLA-4 was demonstrated by flow cytometry with cell lines engineered to express CTLA-4 cloned from these species (Fig. 3). Cells expressing murine CTLA-4 did not bind mAb 10D1. These studies suggest that macaques represent an appropriate species for evaluation of mAb 10D1.

Lastly, the affinity of mAb 10D1 was determined using surface plasmon resonance technology on a BIAcore instrument. When CTLA-4Ig was bound to the chip (RU 1400), the rate constants for



**FIGURE 2.** Reactivity of 10D1 with lymphocytes in human tonsil tissue. Immunohistochemistry was performed on frozen sections of human tonsil with FITC-conjugated 10D1 (A, 10  $\mu$ g/ml), or FITC-conjugated isotype control (B, 10  $\mu$ g/ml). Ab reactivity was detected with secondary rabbit-anti-FITC and developed using the DAKO Envision kit. Slides were counterstained with hematoxylin, dehydrated, and coverslipped for light microscopic evaluation. Magnification,  $\times$ 100.



**FIGURE 3.** Specificity of mAb 10D1 for human and macaque CTLA-4. Transfected cell lines expressing human, macaque (rhesus), and murine CTLA-4 were incubated with 10  $\mu\text{g}/\text{ml}$  10D1, 9H10 (hamster anti-mouse CTLA-4), or isotype control mAbs. Bound Abs were detected with FITC-labeled anti-human or anti-hamster secondary Abs. The cell-associated fluorescence was determined by flow cytometry. Filled histograms represent control mAb, open histograms represent 10D1 or 9H10 binding.

Ab association and dissociation were determined to be  $K_a = 2.59(\pm 0.06) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  and  $K_d = 6.70(\pm 0.22) \times 10^{-4} \text{ s}^{-1}$ . This results in an apparent avidity ( $K_D$ ) of  $2.59(\pm 0.13) \times 10^{-9} \text{ M}^{-1}$ . Similar data were obtained ( $K_D$  of  $2.99(\pm 0.09) \times 10^{-9} \text{ M}^{-1}$ ) when lower amounts of CTLA-4Ig were bound to the chip (RU 366). These results show that 10D1 displays a high affinity and specificity for its target.

#### CTLA-4 blockade enhances humoral responses to HBsAg vaccine

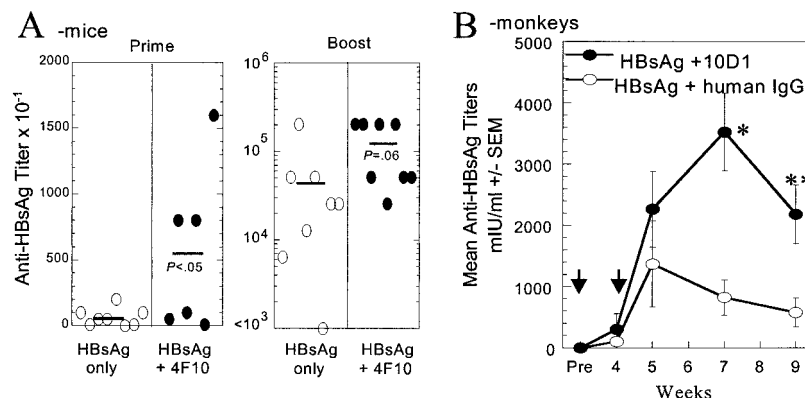
Studies regarding CTLA-4 blockade in mice have focused on cellular responses and have not reported on humoral immune responses. However, as Ab titers are an important and readily measured immune correlate for vaccines, we performed a preliminary

study in mice to examine the effect of CTLA-4 blockade on Ab responses to HBsAg. Administration of the hamster anti-murine CTLA-4 (75  $\mu\text{g}/\text{dose}$ ,  $\approx 3 \text{ mg}/\text{kg}$ ) at both the prime and the boost immunizations enhanced the Ab titers to HBsAg compared to the control group (Fig. 4A). After the boost, 50% of the animals treated with anti-CTLA-4 mAb reached high anti-HBsAg titers ( $>10^5$ ), compared to only one of eight in the control group, although this difference did not quite reach statistical significance. We did not observe a difference in the IgG isotype responses to HBsAg, which generated a predominantly Th2 profile with or without anti-CTLA-4 mAb.

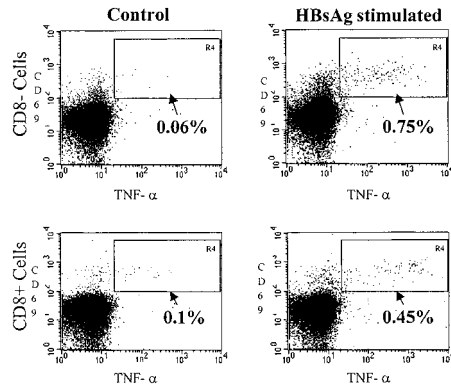
A similar design was used for examining the efficacy of 10D1 in cynomolgus monkeys. Groups of four animals received two i.m. immunizations with a HBsAg vaccine on days 1 and 29. One day prior to the vaccine (days 0, 28), animals were administered an i.v. bolus of mAb 10D1 or an isotype control at dose of 10 mg/kg. Plasma samples were analyzed for Ab responses to HBsAg (Fig. 4B). Combination of the vaccine with 10D1 significantly enhanced the humoral responses over the control group ( $p < 0.05$ ) at 7 and 9 wk.

T cell activation was also investigated in vaccinated animals. Analysis of Ag-specific T cell responses was attempted using recombinant HBsAg protein to stimulate intracellular cytokine responses with fresh samples of whole blood. One animal (of four) in the 10D1 group demonstrated a consistent, Ag-specific stimulation of TNF- $\alpha$  and IL-2 in both CD8 $^-$  and CD8 T cells (at weeks 7 and 9). Fig. 5 illustrates the Ag-specific TNF- $\alpha$  response at week 7. IFN- $\gamma$  was not detected, however, this may have been due to the relatively weak staining with anti-IFN- $\gamma$  Ab used in these studies. Using whole protein to prime in vitro T cell responses limits the sensitivity of these assays, therefore, we may be underestimating the Ag-specific T cell responses.

There were no significant changes (relative to controls or pre-treatment samples) observed in total populations of activated CD4 or CD8 T cells examined for expression of activation markers (CD69, HLA-DR, CD25) by flow cytometry (data not shown). Furthermore, there was no evidence of 10D1-related lymphocytic inflammation/lesions in the histology review of tissue sections from intestines or colon. The results from this study document that 10D1 enhanced immune responses to a vaccine in primates in the absence of observed side effects.



**FIGURE 4.** CTLA-4 blockade enhances humoral responses to immunization with HBsAg. **A**, Anti-HBsAg titers were determined in mice following a primary immunization (prime) and booster (week 6) of recombinant HBsAg (1  $\mu\text{g}/\text{mouse}$ ) given i.m. Mice were administered either hamster anti-mouse CTLA-4 mAb 4F10 or isotype control (75  $\mu\text{g}/\text{mouse}/\text{dose}$  i.p.) on days 0, 1, and the day of the booster immunization. **B**, Groups of four cynomolgus monkeys (two male, two female) were immunized with HBsAg vaccine after i.v. administration of 10 mg/kg 10D1 or an isotype control human Ab (24 h prior to each vaccine injection). Plasma samples obtained at the indicated times were analyzed for the level of anti-HBsAg Abs by commercially available kits. Statistical significance was calculated by the Student  $t$  test; \*,  $p < 0.05$ , \*\*,  $p < 0.01$ . Anti-HBsAg responses were confirmed in duplicate assays and by a contract laboratory.



**FIGURE 5.** Ag-specific T cell activation measured by intracellular cytokine response. Fresh whole blood drawn at week 7 from an animal treated with 10D1 and HBsAg was incubated overnight with or without recombinant HBsAg, and with brefeldin A and costimulatory Abs CD28 and CD49d. Cells were stained for surface markers, and then stained for TNF- $\alpha$  after permeabilization. Staining for intracellular cytokines analyzed on a flow cytometer within 24 h of staining. Representative from two samples (the other at 9 wk) from the same animal.

*Potential of immune responses to a melanoma vaccine in cynomolgus monkeys with mAb 10D1*

Based on the encouraging results from the initial study with HBsAg vaccine, a second experiment was initiated to examine the efficacy and safety of mAb 10D1 in combination with a cancer vaccine. The vaccine used in this study was a human melanoma cell line (SKmel-3) transfected to express GM-CSF (SKmel-3-GM). Previous studies in murine models have demonstrated synergistic effects of CTLA-4 blockade combined with a similar vaccine (7, 8, 24). In addition, a whole cell vaccine would allow for investigation of autoimmune reactions to a variety of normal tissues, despite the fact that the cellular vaccine was of human origin. A protocol was designed for support of potential clinical applications, and to investigate chronic dosing of mAb 10D1.

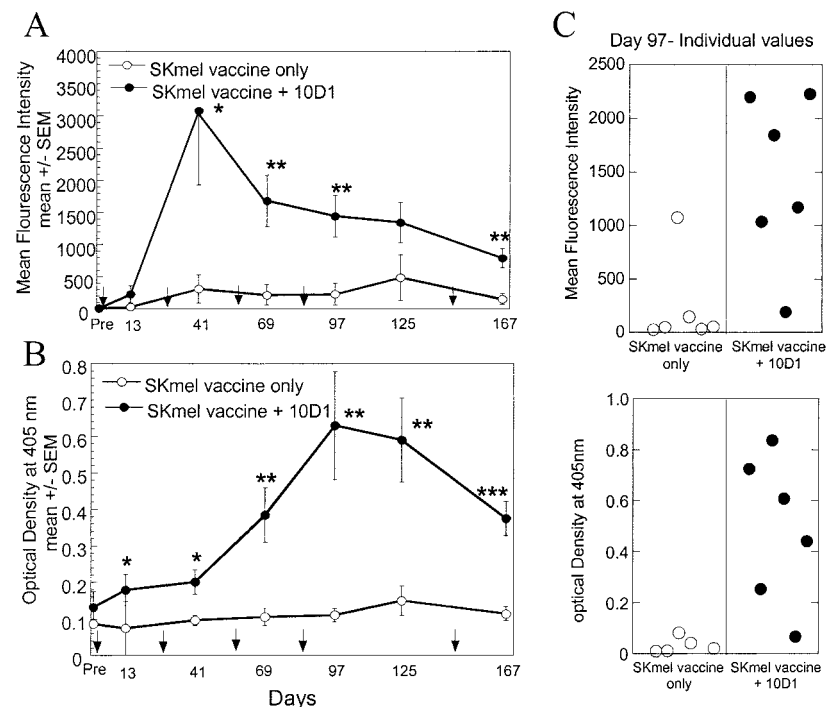
Groups of six cynomolgus monkeys were dosed monthly (except for the fourth month) with s.c. injections of  $5 \times 10^6$  SKmel-3-GM cells for a total of five doses. The SKmel-3-GM cells were pretreated with mitomycin C, and produced  $\sim 2\text{--}8$  ng of GM-CSF/ $10^6$  cells/48 h. One group of monkeys was given an i.v. bolus of mAb 10D1 (10 mg/kg) on the same days as the vaccine. Samples were drawn for immune response analysis 2 wk following each immunization.

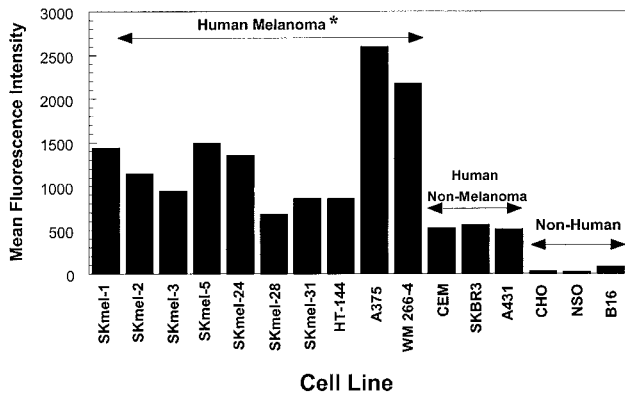
Treatment with 10D1 induced a dramatic enhancement of humoral responses to the vaccine as observed by both flow cytometry and ELISA (Fig. 6). Ab responses to cell surface Ags were noted after the first vaccine dose, peaked after the second dose, and remained elevated throughout the study. Interestingly, Ab responses to total cellular Ags (ELISA with whole cell lysate) peaked later in the course of the study (day 97). In the 10D1 group, five of six animals developed a strong Ab response to the vaccine (Fig. 6C). In contrast, only one of six animals in the vaccine alone group had a good response at the dilution used in these assays.

Further characterization of the Ab responses in the 10D1-treated animals was performed using pooled plasma samples from the time point of peak response to cell surface Ags (day 41). As expected, a significant part of the Ab response was directed against human Ags shared on various cell types, yet a stronger reactivity was observed with the human melanoma cell lines compared with the nonmelanoma lines ( $p < 0.05$ ) (Fig. 7). Although these cell lines did not control for variability in polymorphic proteins including HLA molecules, five of nine melanoma cell lines did not share HLA A or B subtypes with the immunizing cell suggesting some of the response was elicited against melanoma-specific Ags (Fig. 7). Interestingly, there was also a greater reactivity with the mouse melanoma cell line (B16 F1) compared to a mouse myeloma or Chinese hamster ovary cells.

As a correlate of biological activity, we investigated whether the Ab responses elicited against the melanoma vaccine could mediate ADCC against melanoma targets. Pooled serum from the vaccinated animals was used in combination with human mononuclear cells to mediate the lysis of three different melanoma cell lines in

**FIGURE 6.** CTLA-4 blockade with mAb 10D1 enhances Ab response to a melanoma vaccine in monkeys. Groups of six cynomolgus monkeys were given s.c. injections of  $5 \times 10^6$  SKmel-GM cells alone (arrows) or in combination with a bolus i.v. administration of mAb 10D1 (10 mg/kg). Analysis of Ab responses to SKmel-3 cells by flow cytometry (A) or ELISA (B) was determined from plasma samples taken 2 wk after each immunization. The values represent the mean  $\pm$  SE from plasma samples, 6/group, tested at a 1/1000 dilution for flow cytometry or 1/200 for ELISA. Statistically significant differences by the Student *t* test are noted; \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.005$ . C, The individual animal responses by flow cytometry or ELISA (pretreatment values subtracted) at day 97. All samples were analyzed in a minimum of two assays with consistent results.



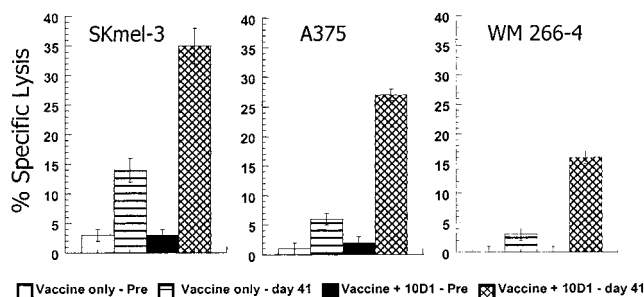


**FIGURE 7.** Ab responses to melanoma and nonmelanoma cell lines in animals treated with melanoma vaccine and mAb 10D1. Pooled plasma from six cynomolgus monkeys given SKmel-3-GM-CSF vaccine and mAb 10D1 (day 41) was tested for reactivity to a variety of melanoma and nonmelanoma cell lines by flow cytometry (1/1000 dilution). \*,  $p < 0.05$  human melanoma cell binding vs human nonmelanoma cells. Representative of two experiments.

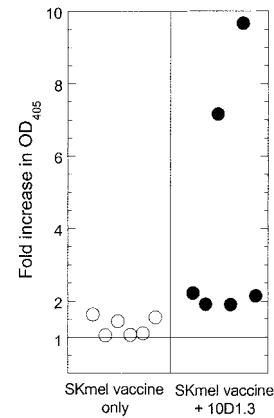
chromium-release assays. For all three cell lines, greater ADCC activity was observed with samples from animals receiving the combination of vaccine and anti-CTLA-4 mAb (Fig. 8).

To determine whether the Abs generated to the vaccine might cross-react with monkey melanocyte Ags, ELISA were performed with lysates from cynomolgus iris tissue, which is rich in melanocytes. Only the 10D1-treated animals had significant reactivity to the iris lysate, with two animals having particularly strong responses (Fig. 9). The samples were also tested on lysates from tissues not expected to have significant expression of melanocyte-specific Ags. The iris-reactive samples did not react with lysate prepared from stomach tissue, although reactivity was observed with lysates from brain and kidney (data not shown), indicating that a portion of the anti-self response was not melanocyte-specific. Collectively, these data suggest that the combination of the vaccine and 10D1 resulted in Ab responses to both nonmelanoma and to shared melanoma Ags. Furthermore, monkeys (in particular two of six animals) treated with anti-CTLA-4 mAb developed significantly greater levels of self-reacting Abs.

In accordance with the previous study, no significant effects were observed on total T cell populations or on T cells expressing the activation markers CD25, CD69, or HLA-DR (Table I). Ag-



**FIGURE 8.** CTLA-4 blockade enhances ADCC activity in response to the melanoma vaccine. Animals were immunized as described in Fig. 6. Pooled plasma samples from vaccinated animals with or without 10D1 mAb were taken prior to immunization (Pre) or 2 wk after the second immunization (day 41). The samples were tested for ADCC activity on three melanoma cell lines at a 1/500 dilution as described under *Materials and Methods*. The data represent the mean  $\pm$  SD of triplicates from a representative experiment.



**FIGURE 9.** CTLA-4 blockade with mAb 10D1 generated cross-reactive Ab responses with monkey tissues rich in melanocytes. Lysates prepared from fresh iris tissue were used as a source of melanocyte Ags for ELISA. Reactivity of individual plasma samples (day 97) from animals given SKmel-GM vaccine alone (○) or in combination with 10D1 (●) were analyzed by ELISA at a 1/200 dilution. To normalize for individual background variability, values are expressed as the fold-increase in OD<sub>405</sub> relative to pretreatment values. Pretreatment (background) OD<sub>405</sub> values ranged from 0.051 to 0.152.

specific T cells were difficult to analyze in the absence of known Ags. We attempted to use autologous dendritic cells primed with SKmel-3 cells, yet were unable to observe significant Ag-specific responses by a number of different methods. Parenthetically, one animal (10D1 group) gave a strong, Ag-specific proliferative response (both CD8<sup>-</sup> and CD8<sup>+</sup>), which correlated with injection site reactions in that animal.

Chronic dosing was well-tolerated and the animals did not mount measurable Ab responses to 10D1 during the course of the study. The mean plasma concentration for mAb 10D1 in the treated animals peaked between 175 and 315  $\mu\text{g/ml}$  on the day postinfusion, and remained above 20  $\mu\text{g/ml}$  during the six-month study (Fig. 10). Clinical chemistry, cage-side observations, and complete histology analysis did not reveal any significant alterations related to the Ab or vaccine administration. Therefore, chronic CTLA-4 blockade with mAb 10D1 was achievable in a safe and effective manner.

## Discussion

Studies in mouse models have greatly advanced our understanding of CTLA-4 function and its potential as a target for immunotherapy (reviewed in Ref. 1). However, in the absence of good surrogate in vitro assays to study the effect of an antagonist Ab on human cells, studies in a more closely related species were needed to help translate the murine studies to human clinical trials. We developed a high affinity human mAb reactive with human and macaque CTLA-4 that blocks ligand binding. Administration of mAb 10D1 in combination with a cellular vaccine and a recombinant viral Ag vaccine to monkeys resulted in a substantial increase in Ag-specific Abs relative to animals receiving the vaccines alone. These data provide the first clear evidence that CTLA-4 blockade augments immune responses to vaccines in primates, suggesting that cynomolgus monkeys are an appropriate model for investigating the safety and efficacy of CTLA-4 blockade.

In this report, we demonstrate that Ab-mediated inhibition of CTLA-4 increased Ag-induced Abs in both mice and primates. Our findings are in contrast to the study reported by Horspool et al. (25), which showed CTLA-4 blockade suppressed humoral responses to a DNA vaccine. The reason for this discrepancy is

Table I. The effect of anti-CTLA-4 mAb 10D1 on T lymphocyte populations in monkeys<sup>a</sup>

T Cell Marker	Group (n = 6)	Pre	Day 1	Day 29	Day 57	Day 141
CD3 <sup>+</sup> /CD4 <sup>+</sup> /CD25 <sup>+</sup>	Vaccine only	3.8 ± 2.3 <sup>b</sup>	6.4 ± 3.5	6.2 ± 4.5	2.3 ± 2.9	4.0 ± 2.6
	Vaccine with 10D1	9.1 ± 0.8	9.2 ± 1.1	10.3 ± 1.5	8.3 ± 2.1	7.7 ± 2.6
CD3 <sup>+</sup> /CD8 <sup>+</sup> /CD25 <sup>+</sup>	Vaccine only	8.9 ± 2.5	10.6 ± 2.8	10.7 ± 3.3	6.2 ± 1.7	5.5 ± 2.2
	Vaccine with 10D1	6.7 ± 1.3	8.4 ± 1.3	8.5 ± 2.0	6.6 ± 1.0	6.0 ± 2.2
CD3 <sup>+</sup> /CD4 <sup>+</sup> /CD69 <sup>+</sup>	Vaccine only	7.3 ± 4.6	9.2 ± 3.8	11.9 ± 7.2	4.7 ± 1.3	12.2 ± 11.6
	Vaccine with 10D1	6.0 ± 3.4	7.0 ± 2.3	7.4 ± 3.7	4.4 ± 3.8	11.4 ± 4.2
CD3 <sup>+</sup> /CD8 <sup>+</sup> /CD69 <sup>+</sup>	Vaccine only	12.9 ± 8.4	15.9 ± 10.8	13.9 ± 6.6	11.5 ± 7.7	19.5 ± 10.2
	Vaccine with 10D1	13.3 ± 5.2	13.8 ± 5.2	12.7 ± 6.8	11.4 ± 6.5	18.2 ± 5.8
CD3 <sup>+</sup> /CD4 <sup>+</sup> /HLA-DR <sup>+</sup>	Vaccine only	1.1 ± 1.8	0.5 ± 0.5	1.1 ± 0.6	0.6 ± 0.4	0.5 ± 0.6
	Vaccine with 10D1	0.9 ± 0.3	0.3 ± 0.3	0.4 ± 0.6	0.3 ± 0.5	0.4 ± 0.6
CD3 <sup>+</sup> /CD8 <sup>+</sup> /HLA-DR <sup>+</sup>	Vaccine only	1.8 ± 1.0	1.2 ± 0.7	1.2 ± 0.9	1.5 ± 1.0	0.6 ± 0.6
	Vaccine with 10D1	1.3 ± 1.0	0.6 ± 0.7	0.9 ± 0.7	0.9 ± 0.5	0.7 ± 0.5

<sup>a</sup> Flow cytometric analysis of surface expression of T cell markers.

<sup>b</sup> Values represent the percent positive cells (based on total lymphocyte gate) expressed as the mean percent ± SD.

unclear, but most likely is related to differences in the vaccine models used. In agreement with our findings, Zheng and Monestier (26) have recently reported that CTLA-4 blockade augments autoantibody production in a mouse model of mercury-induced autoimmunity. The mechanism by which CTLA-4 blockade may enhance Ab responses may involve enhancing T cell help for germinal center B cells. Walker et al. (27) have recently demonstrated that CTLA-4 expression down-regulates germinal center B cell proliferation. In their mouse model, CTLA-4 blocking Abs combined with immunization significantly enhanced the size of germinal centers.

In the absence of macaque melanoma cell lines, we used a human cell vaccine in this study. The xenogeneic cells clearly elicited Ab responses that were not specific to the melanoma vaccine. However, by comparison of several human tumor cell lines with varying HLA molecules we observed a consistently greater reactivity to melanoma-derived cell lines, indicating that at least some of the anti-vaccine Abs may have specificity for melanoma Ags. Furthermore, two of the six animals treated in combination with anti-CTLA-4 developed significant anti-self Abs that reacted

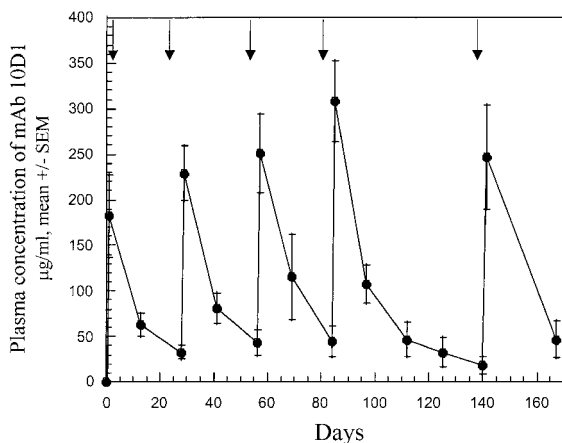
strongly with lysate derived from melanoma-rich tissue. The biological consequences of the enhanced immune responses induced with anti-CTLA-4 treatment are unclear. Abs can have significant antitumor activity, and to support the biological significance of the elicited immune response, we demonstrated enhanced ADCC activity against melanoma cell lines in the animals treated with anti-CTLA-4.

In addition to Ab responses, we measured Ag-specific T cell activity in 2 of 10 monkeys that were treated with mAb 10D1, compared to 0 of 10 animals given only the vaccines. However, as these studies were unable to use MHC-matched peptides for inducing T cell activation, we have possibly underestimated the number of animals with Ag-specific T cells.

The potent effect of anti-CTLA-4 Abs on immune responses raises the concern of inducing unrestricted anti-self reactions that lead to autoimmune disease. In fact, transgenic mice deficient in CTLA-4 succumb to a lymphoproliferative disease at 3–4 weeks of age (14, 15). Moreover, combining CTLA-4 blockade with experimental autoimmune diabetes and encephalomyelitis exacerbates the disease in those models (28–32). However, studies in most mouse models indicate that Abs to CTLA-4 given to adult animals enhance responses specifically to the Ags used for vaccination, without generating nonspecific autoimmunity (1). More recently, the expression of CTLA-4 on regulatory T cells (T reg) that contribute to tolerance of self-Ags has raised additional concerns that CTLA-4 blockade can induce undesired autoimmunity (33, 34).

Our experiments in nonhuman primates have shown that chronic administration (6 mo) of mAb 10D1 did not result in treatment-related pathology upon complete macroscopic and microscopic evaluation, with the exception of slight irritation at the vaccine injection site of two animals. Furthermore, there were no changes in hematology, coagulation, or clinical chemistry at any time interval related to administration of the Ab. The absence of side effects is noteworthy despite the ability of CTLA-4 blockade to establish potential autoreactive anti-self responses in these animals. Importantly, the monkeys did not develop any detectable Ab response to the 10D1 mAb and high levels of active circulating Ab were maintained for duration of the study.

During the course of the study, no significant changes were observed in the lymphocyte subsets that were evaluated. However, phenotyping of T cells using the standard activation markers CD25, CD69, and HLA-DR cannot address the effects of CTLA-4



**FIGURE 10.** Pharmacokinetic profile of mAb 10D1 during chronic dosing. Plasma concentration of 10D1 was analyzed using an ELISA with recombinant CTLA-4-Ig. mAb 10D1 was detected by a goat anti-human IgG (Fab')-specific alkaline phosphatase probe, and plasma concentrations were determined from a standard curve. Arrows indicate infusion time points. Values represent the mean of six animals ± SEM.



blockade on small numbers of Ag-specific lymphocytes. Nevertheless, these results are noteworthy in that CTLA-4 blockade does not significantly impact large numbers of T cells in a model where "normal" complement of naive and memory T cells are present. This is distinguished from the murine system where the majority of in vivo models describe the effects of CTLA-4 blockade on naive T cells. Studies of the role of CTLA-4 in memory cell responses suggest that the effects of CTLA-4 blockade may be more potent in secondary responses than in naive cells (35).

CTLA-4 is constitutively expressed at the surface of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells in mouse and human (33, 34, 36). Yet, the role of CTLA-4 in the function of these cells is controversial. Anti-CTLA-4 Abs administered in high doses can moderate the protective function of CD25<sup>+</sup>CD4<sup>+</sup> T reg cells, leading to gastritis and intestinal inflammation in murine models (33, 34). However, Suttmuller et al. (37) have demonstrated that removal of this subset is synergistic with CTLA-4 blockade in a murine model of tumor immunotherapy, suggesting that anti-CTLA-4 treatment does not impact the T reg population. In vitro studies in the human system have suggested that anti-CTLA-4 Abs have no effect on the suppressive function of these cells (38, 39). Although we have not directly assessed the CD4<sup>+</sup>CD25<sup>+</sup>CTLA-4<sup>+</sup> subset in primates, we have demonstrated that there is no alteration in the overall numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells after chronic exposure to anti-CTLA-4 Ab. The lack of pathology associated with anti-CTLA-4 treatment suggests that interfering with T reg cells by CTLA-4 blockade is not sufficient to induce clinically significant autoimmune reactions in healthy primates.

The safe toxicology profile of anti-CTLA-4 treatment in monkeys has permitted the initiation of clinical studies in cancer patients with mAb 10D1 (referred to as MDX-010). These clinical trials used a single dose of anti-CTLA-4 at 3 mg/kg in patients with hormone refractory prostate cancer and metastatic melanoma. Ab treatment with a single dose was tolerated well and demonstrated clear signs of immunological activity and antitumor effects, including significant clinical regressions. In addition, several of the patients in the melanoma trial had received prior immunotherapy including various vaccines. From immunohistochemical analysis of post-treatment tumor biopsies from these patients, it appears that CTLA-4 blockade enhanced tumor-directed immune responses in patients that received cellular vaccines (40). These promising initial results have prompted several studies using MDX-010 as a single agent and as a single agent in combination with chemotherapy as well as combinations with vaccines. In a preliminary report of 14 patients receiving anti-CTLA-4 together with melanoma peptides, several tumor responses (two complete responses and one partial response) have been observed (41). Interestingly, in these responder patients as well as some patients that did not meet the criteria for a frank clinical response, significant autoimmune reactions were manifested in specific organs, including skin, gut, liver, and adrenal gland. This suggests that CTLA-4 blockade activates anti-self responses; in contrast, while anti-self responses were also noted in the nonhuman primate studies, there was no evidence of these or any other autoimmune pathology. These initial studies in primates and in humans suggest that the negative regulatory function of CTLA-4 is an important target in the manipulation of the immune system for effective antitumor therapy.

## Acknowledgments

We gratefully acknowledge the continuous involvement of Dr. J. Allison from the inception of this project. We also acknowledge Dr. Jennifer Rojko for her expert analysis of the immunohistochemistry studies. We thank Mark Karlok, Scott Beard, Jill Elliot, and Dr. Weijun Lin at Nexstar Phar-

maceuticals (Boulder, CO) for generating and supplying cell lines expressing CTLA-4. We thank Dr. David King for input on BIAcore analysis.

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