

PROGRESS REPORT Year 01

Title: Mechanisms of Immune System-mediated Tumor Regressions in Patients with Melanoma.

Principal Investigator: Antoni Ribas, M.D.

A. Lay Language Progress Report

The goal of the proposal funded by the MRF was to study the mechanisms of immune response and resistance of metastatic melanoma to antibodies blocking CTLA4. CTLA4 is a main negative regulator of the immune response, which may limit the ability of the immune system to get rid of melanoma in patients. Blocking CTLA4 with monoclonal antibodies may release this break of the immune system and result in cancer regressions. The cancer regressions induced by CTLA4 blocking antibodies in patients with metastatic melanoma are long lived, lasting years with very few tumor reoccurrences. However, these antibodies can also lead to important toxicities due to immune attack on normal tissues. Therefore, it is of critical importance to understand and develop markers for patients that may respond or would be resistant.

We proposed to study how the immune system is activated to fight melanoma by CTLA4 blocking antibodies. We have performed extensive studies in blood samples taken from patients receiving CTLA4 blocking antibodies. These studies followed the prevailing hypothesis on how CTLA4 blocking antibodies can activate the immune system arising from preclinical studies in the laboratory and in laboratory animals. The emerging results suggest that studying the number and function of immune cells specific for melanoma or infectious disease antigens does not allow to define which patients may or may not respond. Similarly, studying the number of immune suppressive cells (called T regulatory cells) in blood of patients has not provided clear evidence of how CTLA4 blocking antibodies lead to melanoma regression. These results have been presented at the annual meeting of the American Society of Clinical Oncology.

However, when we studied what is happening inside tumors, we have found that there are impressive changes in patients that go onto have their melanoma metastasis disappear. These tumors become massively infiltrated by killer cells from the immune system, called the CD8+ cytotoxic T cells. On the contrary, melanoma cancer lesions from patients that do not respond to the administration of CTLA4 blocking antibodies do not develop these immune cell infiltrates, and their tumors continue to be made up of melanoma cancer cells only. As proposed in the funded MRF grant, we have also studied if there is disappearance of immune suppressive cells, like the T regulatory cells or a special type of dendritic cell that expressed a protein called IDO. Our results suggest that these cells continue to be present both in the responding and non-responding tumors. These results are accepted for presentation at the annual meeting of the International Society of Biological Therapy for Cancer.

In conclusion, our ongoing studies have pointed us to expand our studies in tumor biopsies of patients receiving CTLA4 blocking antibodies to further study the mechanisms of response and resistance to this promising form of therapy for melanoma. In this regard, we are in the process of gaining approval from the UCLA Institutional Review Board (IRB) for a follow up clinical trial where all participating and consenting patients will be requested to donate tumor biopsies before and after receiving CTLA4 blocking antibodies.

B. Specific Aims (From the original submission)

Overview. In this grant we proposed to define the mechanisms of immune system-mediated tumor regressions after CTLA4 blockade in patients with melanoma. Ongoing clinical trials with a CTLA4 blocking monoclonal antibody (CP-675,206) provide clear evidence of immune sensitivity in a subset of patients, while the great majority of patient fail to respond. The nature of this selective activity is not well understood. We proposed to study the mechanisms of antitumor responses in patients with advanced melanoma receiving CP-675,206 within two aims:

B.1. Aim 1: Determine the Effects of Negative Regulatory Blockade on the Activation and Expansion of Melanoma-specific T Cells in Humans. In this aim we test the hypothesis that blockade of the CTLA4 negative signaling results in an increased number and activation of tumor antigen-specific T cells. Ongoing clinical trials using a CTLA4 blocking monoclonal antibody alone or after T cell priming with a dendritic cell (DC)-based vaccine provide samples to analyze the impact of these interventions using standardized modern immunological assays.

B.2. Aim 2: Role of Immune Suppressive Cells in Tumor Regression After Immunotherapy. Several immune cell subsets have a dominant immune suppressive role, and are critically involved in the maintenance of tolerance to self tissues and prevention of autoimmunity. However, they also dampen immune responses to cancer through CTLA4 signaling. In this aim we test the hypothesis that tumor responses after CTLA4 blockade are mediated by the inhibition of the suppressive function of immune suppressor cells.

C. Progress in Year 01

C.1. Aim 1: Determine the Effects of Negative Regulatory Blockade on the Activation and Expansion of Melanoma-specific T Cells in Humans.

Administration of CTLA4 blocking monoclonal antibodies to patients with advanced melanoma are expected to induce tumor regressions by stimulating a cytotoxic T cell response. In this Aim we proposed to characterize melanoma-specific T cells in peripheral blood and in tumors of patients receiving CP-675,206.

C.1.A. Status of CP-675,206-based Clinical Trials that Provide Samples for Immune Monitoring Analysis.

We proposed to analyze the effects of administering the anti-CTLA4 antibody CP-675,206 to patients with advanced melanoma in terms of its ability to activate immune system cells. Key to this proposal was the enrollment of patients to clinical trials designed to adequately collect samples for laboratory analysis. Below is a description of the status of these clinical trials:

- 1. Performance Specifications of a Combined Tetramer/ICS Assay Compared to the Tetramer and ELISPOT Assays.** This clinical trial was a subaim in the proposal. It is the only clinical trial that did not involve the administration of CP-675,206. This is an investigator-initiated methodology study where healthy subjects and patients with melanoma with a defined population of cells specific for MART-1 (a melanoma antigen) or EBV or CMV (two infectious disease antigens) provided two blood draws (100 ml each) to allow us to optimize the performance of a new immune monitoring assay. This assay (a combination of MHC tetramer and intracellular cytokine staining) would ideally combine the benefits and robustness of the MHC tetramer assay with the functional information from assays that quantitate specific cytokine production upon antigen recognition. We have fully analyzed the samples from the 10 study subjects. To our surprise, assay performance based on the manufacture's instructions was unreliable. Begonya Comin-Anduix in our group has a close

relationship with scientists at Beckman Coulter in San Diego. Together, they figured out the problems with the assay and resolved them. We are preparing a manuscript on this experience in which we plan to acknowledge the support of the MRF.

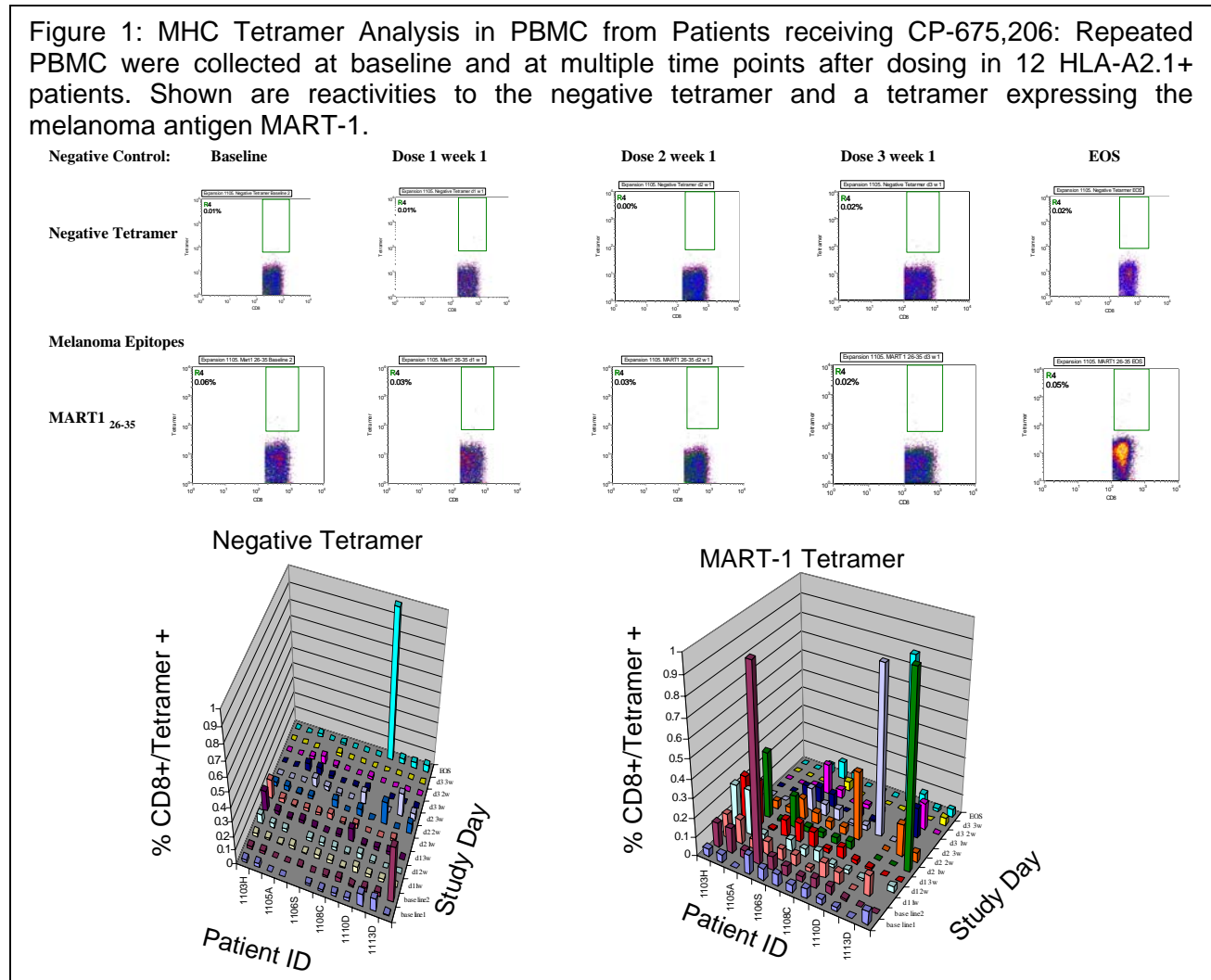
2. **1002 Expansion: Administration of CP-675,206 to HLA-A2.1 Patients with Serial PBMC Collection.** This clinical trial had completed accrual at the time of grant submission, and immunological monitoring was ongoing. We have now performed most of the immunological monitoring, which includes analysis of tumor antigen and infectious disease-specific T cell responses in peripheral blood after administration of the anti-CTLA4 antibody, evaluation of T cell activation and migration markers, quantitation of T regulatory (Treg) cells and preliminary analysis of intratumoral changes. The data generated thus far has been presented in two abstracts, one at the 2006 meeting of the American Society of Clinical Oncology (ASCO) and a presentation at the upcoming International Society of Biological Therapy for Cancer (iSBTc) (1-3). We are working on generating a manuscript with these data, in which we will acknowledge the support of the MRF.
3. **0003: Combination Immunotherapy with MART-1₂₇₋₃₅ Peptide Pulsed DC (MART-1₂₇₋₃₅/DC) and CP-675,206.** This investigator-initiated clinical trial is ongoing. Six additional subjects have been accrued since the grant submission. They have been accrued to two cohorts: i) receiving CP-675,206 at 10 mg/kg monthly, and ii) receiving CP-675,206 at 10 mg/kg every 3 months (90 days). Baseline and follow-up PBMC samples have been cryopreserved from the 7 patients accrued thus far. An amendment to the original protocol allows the collection of a pre- and post-dosing leukapheresis, which is providing sufficient cells for the more detailed analysis of antigen-specific T cells and T regulatory cells in patients accrued to the next two cohorts (as proposed in the MRF grant).
4. **Biopsies Protocol in Patients Receiving CP-675,206.** This is a new investigator-initiated clinical trial where we propose to collect baseline and follow up biopsies and leukapheresis products from 21 patients receiving CP-675,206 at the dose used in the ongoing registrational trials, 10 mg/kg every 3 months (90 days). This clinical trial was not directly proposed in the grant submission, but is a result of our findings to date that point us to closely study the intratumoral events as opposed to the events in peripheral blood. The clinical trial is undergoing regulatory review at UCLA with UCLA IRB number # 06-06-093 and title "A Phase 2, Open Label, Single Arm Clinical Trial to Study the Mechanism of Action of CP-675,206 in Patients with In-Transit and Metastatic Melanoma Amenable to Repeated Outpatient Tumor Biopsies."

C.1.B. Study of the Effects of CTLA4 Blockade on the Number and Functional Status of Tumor Antigen-specific T Cells in Peripheral Blood.

We are using our standardized conditions for tetramer and ELISPOT assays to quantitate circulating antigen-specific T cells (4), which allows us to adequately assess which change in the assay results represents a positive or negative immune response after administration of CP-675,206 to patients with melanoma. The threshold of changes in circulating antigen-specific T cells from baseline with a 99% significance is defined as the Reference Change Value (RCV).

The most complete analysis to date has been performed in samples obtained from the 1002 Expansion study. In this study, twelve HLA-A2.1+ patients with stage IIIc or IV melanoma and baseline circulating MART-1-specific T cells above the low limit of detection by tetramer assay (LLD, 0.03% of CD8+ T cells) received CP-675,206 at 10 mg/kg monthly. Two 40 ml blood samples were collected at baseline, and one at 1 and 2 weeks after each dose for 4 cycles. Tetramer and ELISPOT assays were run for reactivity to two negative control epitopes (AFP and the Immunonics Negative control), two positive control infectious disease epitopes (CMV

and EBV), and three melanoma antigens (MART-1, gp100, tyrosinase). Results were interpreted according to previously-defined RCV for immune response {Comin-Anduix, 2006 #3761}. The primary endpoint was immune response for MART-1 by tetramer assay, for which the RCV is 80% (expressed as percent change from baseline).



Clinical results of this study included the development of toxicity (grade 3 diarrhea in 2 patients, grade 2 hypophysitis in 1 patient, and grade 3 hepatitis in 2 patients). This study also demonstrated the anti-tumor activity of CP-675,206 in advanced melanoma: complete response in 1 patient (15+ months), partial response in 2 patients (9+, 8 months), and stable disease in 3 patients (11, 8+ and 4 months); the rest had disease progression at 4 months or less.

A mean of 2 baseline and 7 follow up (range 5-9) time points were tested by tetramer and ELISPOT assays. Figure 1 provides an example of the data generated when testing with a negative control tetramer and the melanoma antigen MART-1. Similar data has been generated for the 7 epitopes described above for the 12 patients and the multiple follow up time points for both tetramer and ELISPOT assays. As a summary of the full set of data, 4 patients had an increase in MART-1-specific T cells beyond the RCV on 1 or more occasions, but no patient had a consistent pattern of change over time. Indeed, there was no consistent pattern of change in

any patient in circulating T cells specific for MART-1, gp100, tyrosinase, EBV, or CMV by tetramer or by ELISPOT assay.

From this experience, we concluded that CP-675,206 administered monthly induces immune-related phenomena and tumor responses in patients with melanoma without a demonstrable expansion of circulating melanoma antigen-specific T cells. This data was presented at the 2006 meeting of ASCO (1).

In addition, we have also studied the surface expression of the T cell activation marker HLA-DR and the T cell memory marker CD45RO on both CD4 and CD8 T cells in peripheral blood samples obtained before and after CP-675,206 dosing. Ten patients had at least one baseline and one postdosing aliquots of PBMC for this analysis, for a total of 81 analyzed samples. Results were converted to percentage change from baseline as described {Maker, 2005 #3768}, and were then assigned a score from 0 to -4 or +4 for statistical analysis. There was a significant increase in the expression of these two surface markers comparing pre- and post-dosing samples using when analyzed by paired t-test ($p = <0.0001$ for CD4-HLA-DR, $p = 0.327$ for CD8-HLA-DR, $p = 0.019$ for CD4-CD45RO, $p = <0.0001$ for CD8-CD45RO). These results confirm prior data from others using both ipilimumab {Maker, 2005 #3768} and CP-675,206 {Reuben, 2006 #3860}.

C.1.C. Effects of CTLA4 Blockade on the Number and Functional Status of Tumor Antigen-specific T Cells Inside Tumors.

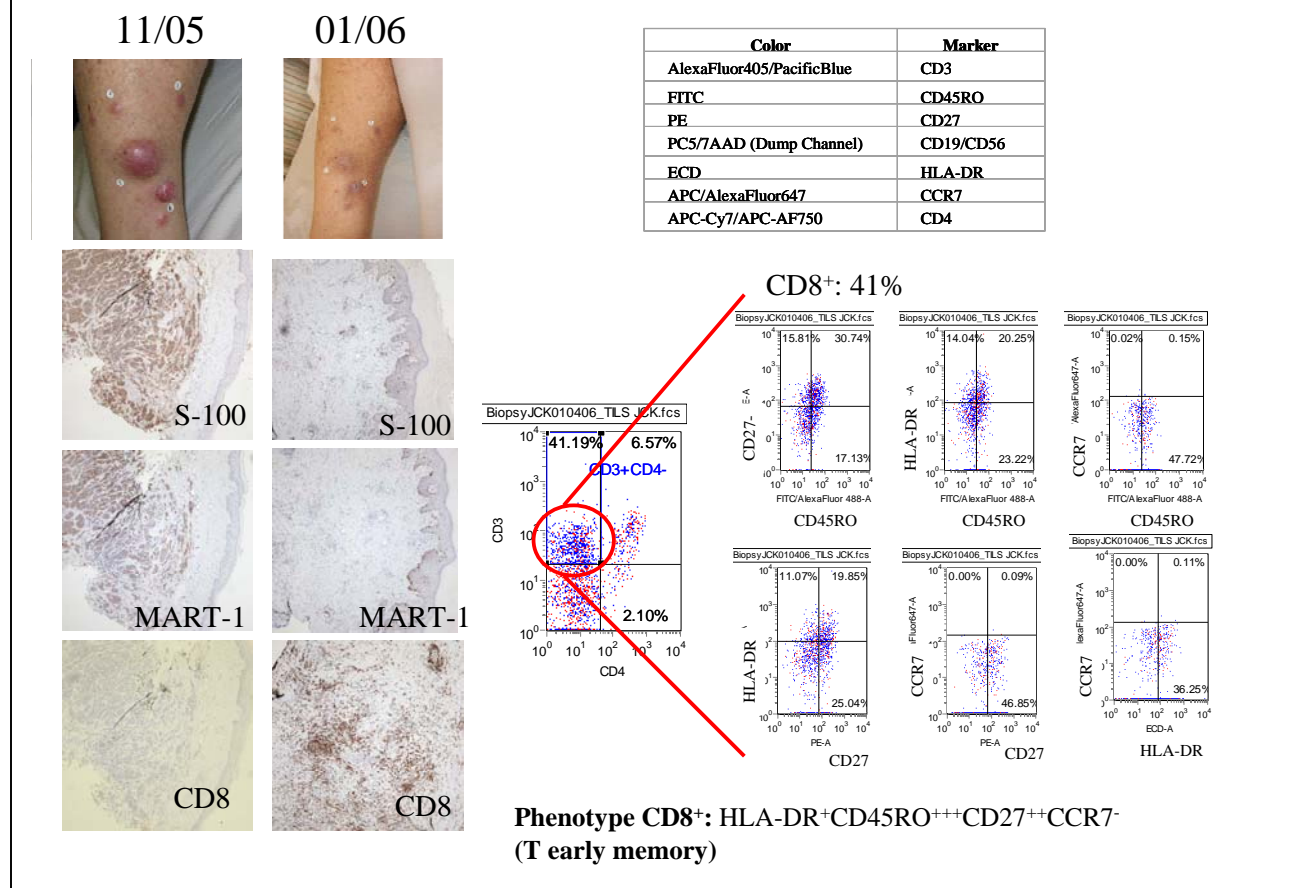
Since our extensive analysis of samples obtained from peripheral blood failed to reveal a clear impact of CTLA4 blockade on circulating melanoma antigen-specific T cells, as proposed in the funded grant, we turned our attention to analyzing intratumoral changes.

We have accumulated a series of pre- and post-dosing biopsies in patients with both regressing and progressing tumors after administration of CP-675,206. Figure 2 shows an example of a responding patient. Immunohistochemistry (IHC) analysis of a pre-dosing lesion revealed strong staining with the melanoma markers S-100 and MART-1, and lack of intratumoral infiltration by CD8+ cytotoxic T lymphocytes (CTL). On the contrary, a biopsy taken 2 months after the first dose of CP-675,206 stained negative for the two melanoma markers. This post-dosing lesion was heavily infiltrated by CD8+ CTL.

Figure 2 also demonstrates our ability to perform multicolor FACS staining of tumor infiltrating lymphocytes (TIL) obtained from small biopsy samples processed in the laboratory. As proposed in the funded grant, TIL are collected from tumor biopsies that are mechanically and enzymatically-digested and plated overnight in tissue culture-treated flasks. The next morning, non-adherent cells are collected, which are enriched for TIL. All flow cytometry testing is done with a sample of 10^5 TIL (without *ex vivo* expansion) using multicolor flow cytometry analysis with an optimized combination of fluorochrome-labeled antibodies. In the example provided in Figure 2, a simultaneous 7-color analysis allowed us to characterize the CD8+ CTL infiltrating the regressing tumor as having a T early memory phenotype.

Our studies to date analyzing the collected tumor biopsies from before and after dosing patients with CP-675,206 demonstrate that 4 out of 4 analyzed responding lesions had 2-3+ diffuse intratumoral infiltrates of CD8+ cells. On the contrary, none of the 3 non-regressing lesions analyzed had patchy CD8+ intratumoral infiltrates. Two patients with regressing lesions had a concomitant CD4+ infiltrate with a frequency similar to CD8, and 2 had increased CD8+ cells without an increase in CD4+ cells. These studies are part of an oral presentation at the upcoming International Society of Biological Therapy for Cancer (iSBTc) (2).

Figure 2: Analysis of Tumor Infiltrating Lymphocytes (TIL) in Regressing Melanomas. Administration of the CTLA4 blocking antibody CP-675,206 results in objective responses in a subset of patients, like the one depicted here. These responses are mediated by massive tumor infiltration with CD8+ CTL (IHC images, brown is positive IHC staining). We have refined our immune monitoring assays to a level where we can take a small outpatient biopsy and define the specificity and phenotype of TIL using multicolor flow cytometry techniques. In this case, we could define that the TIL infiltrating the regressing lesion were CD8+ T cells with an early memory phenotype with 7 color staining of a single sample.



C.2. Aim 2: Role of Immune Suppressive Cells in Tumor Regression After Immunotherapy.

Alternative hypothesis to explain melanoma tumor responses to CTLA4 blocking monoclonal antibodies are the possibility of: i) depleting T regulatory cells (Treg), a professional immune suppressor cell subset that constitutively expresses CLTA-4, or ii) the blockade of negative signaling provided by CTLA4 expressed on Treg that results in the induction of tolerizing DC (5, 6). These tolerizing DC can be recognized by their expression of indolamine 2,3 dioxygenase (IDO), a rate-limiting step in the catabolism of the essential amino acid tryptophan. Aim 2 of the MRF grant proposed to test these hypothesis using samples collected from patients receiving CP-675,206.

C.2.A. Impact of CP-675,206 on Circulating Treg.

Circulating Treg can be detected by the expression of forkhead box P3 (FoxP3), a transcription factor that controls Treg differentiation (7). We have explored the possibility that CP-675,206

would deplete Treg by analyzing available samples from the 1002 Expansion study described above. Samples have been analyzed by quantitative reverse transcription real time PCR (qRT-PCR) and by intracellular staining with a specific antibody to FoxP3 on cells with concomitant high surface expression of CD4 and CD25.

Table 1 provides the results of these studies. We found no evidence that samples obtained after administration of CP-675,206 to patients with melanoma had any effect on the levels of circulating Treg determined by FoxP3 expression at the mRNA and protein levels. In addition, we found little correlation between the results of FoxP3 determination by qRT-PCR and intracellular staining.

Table 1: FoxP3 analysis by intracellular cytokine staining and qRT-PCR

	1103		1105A		1110		1108		1111	
	ICS	Q-RTPCR	ICS	Q-RTPCR	ICS	Q-RTPCR	ICS	Q-RTPCR	ICS	Q-RTPCR
Mean Baseline	0	0	0	0	0	0	0	0	0	0
d1 1w	3	1	1	1	-	-1	-	-	4	-1
d1 2w	1	1	-	-1	4	-2	-1	-1	1	-2
d1 3w	-2	-1	-	-2	-2	-2	1	-2	-1	-2
d2 1w	-	-	-	-1.00	-	-2	-	-2	-1	-
d2 2w	-	-	3	1.00	-	1	-	-2	-1	-1
d2 3w	-	-	3	-1.00	-	-	1	-	1	-1
d3w1	-	-	3	2.00	-	-	-	-	-	-
d3w2	-	-	-1	-	-2	-	2	-2	-	-
EOS	-	-	0	2.0	-	-	-	-2	1	1

In conclusion, our results argue against the ability of anti-CTLA4 blocking antibodies to deplete circulating Treg. These studies are part of a poster presentation at the upcoming International Society of Biological Therapy for Cancer (iSBTc) (3).

C.2.B. Impact of CP-675,206 on Intratumoral Treg.

To further examine the contribution of Treg on the antitumor activity of CTLA4 blocking antibodies, we examined their presence in tumor biopsies from patients with responding and non-responding tumors by IHC for FoxP3. As proposed in the application, IHC staining is being analyzed with the help of Dr. Alistair Cochran, an experienced melanoma pathologist.

Similar analysis 3 cases with paired samples demonstrated increased numbers of Foxp3+ cells in post-antibody biopsies in 2 cases (1+ pre- vs. 2+ post-). There was a difference in frequency of Foxp3+ cells between regressing (2+) and non-regressing tumors (1+) in an individual with coexisting regressing and progressing lesions. One patient with progressive melanoma had many Foxp3+ cells (2-3+). In another non-responding lesion, < 10% of TIL stained positive for Foxp3. Foxp3 intracellular flow cytometry provided similar results.

In conclusion, our emerging results strongly argue against a negative effect of CP-675,206 on Treg. In fact, some of the results suggest that FoxP3 positive cells may increase after administering this CTLA4 antagonistic antibody. These studies are part of an oral presentation at the upcoming International Society of Biological Therapy for Cancer (iSBTc) (2).

C.2.C. Impact of CP-675,206 on Intratumoral IDO Positive Cells.

We have analyzed the possibility of CP-675,206 blocking the negative signaling provided by CTLA4 positive Treg onto immune suppressive DC through the induction of IDO. There is no

evidence that IDO positive cells may circulate through peripheral blood, since they have been described as being residents in peripheral tissues at sites of immune privilege (8). Therefore, we focused our attention to the analysis of tumor samples.

IDO IHC analysis has been performed in 3 regressing lesions for which we had paired pre-dosing samples. Pathological analysis showed that the intensity of IDO staining increased in 1 patient, decreased in 1 patient and did not change in 1 patient (all ranging 1-2+). In a patient with simultaneously regressing and non-regressing lesions, the intensity of IDO staining was higher (2-3+) in the regressing compared to non-regressing lesion. Biopsy from 1 patient with progressive melanoma had substantial IDO staining (2+) while another had weaker staining (1+).

In conclusion, our emerging results strongly argues against a negative effect of CP-675,206 on IDO expressing DC. These studies are part of an oral presentation at the upcoming International Society of Biological Therapy for Cancer (iSBTc) (2).

D. List of Publications Acknowledging Funding from the MRF

1. **Antoni Ribas**. Update on immunotherapy for melanoma. *J Natl Compr Canc Netw*, 4: 687-694, 2006.
2. **Antoni Ribas**, Douglas C. Hanson, Dennis A. Noe, Robert Millham, Deborah J. Guyot, Steven H. Bernstein, Paul C. Canniff, Amarnath Sharma, Jesus Gomez-Navarro. CP-675,206, a CTLA4 Blocking Monoclonal Antibody in Clinical Development for Patients with Cancer. (submitted).
3. Begonya Comin-Anduix, Yohan Lee, Jason Jalil, Alain Algazi, Pilar de la Rocha, Imran Ahmad, Luis H. Camacho, Viviana Bozon, Cecile Bulanghari, Elisabeth Seja, Arturo Villanueva, Bradley Straatsma, Alistair Cochran, Antonio Gualberto, James S. Economou, John A. Glaspy, Jesus Gomez-Navarro, **Antoni Ribas**. Changes in the Surface Expression of Activation Markers, but not Changes in Antigen-specific T Cells or in FoxP3 mRNA, are Related to Melanoma Tumor Responses After CTLA4 Blockade. (in preparation).
4. **Antoni Ribas**, Begonya Comin-Anduix, Timothy R. Donahue, Pilar de la Rocha, Jason Jalil, John A. Glaspy, James S. Economou, Jesus Gomez-Navarro, Alistair Cochran. Intratumoral Changes in Immune Cell Infiltrates, Foxp3 and Indoleamine 2,3-dioxygenase (IDO) in Patients Receiving CTLA4 Blocking Antibodies. (in preparation).

E. References

1. Ribas, A., B. Comin-Anduix, V. A. Bozon, L. H. Camacho, C. Bulanhangi, J. Jalil, E. Seja, A. Gualberto, J. S. Economou, J. A. Glaspy, and J. Gomez-Navarro. 2006. Antigen-specific T cell responses in patients with melanoma treated with the CTLA4 blocking mAb ticilimumab. *Proc Am Soc Clin Oncol (Abstract) Abstract*.
2. Ribas, A., T. R. Donahue, B. Comin-Anduix, P. de la Rocha, J. A. Glaspy, J. S. Economou, J. Gomez-Navarro, and C. A.J. 2006. Changes in Intratumoral Immune Cell Infiltrates, Foxp3 and Indoleamine 2, 3-dioxygenase (IDO) Expression with the CTLA4 Blocking mAb CP-675,206. *Proc Int Soc Biol Ther Cancer (Abstract)*.
3. Comin-Anduix, B., Y. Lee, J. Jalil, P. de la Rocha, E. Seja, V. A. Bozon, L. H. Camacho, J. S. Economou, J. A. Glaspy, J. Gomez-Navarro, and A. Ribas. 2006. Immunological Assays to Differentiate Responders from Non-Responders after CTLA4 Blockade with CP-675,206. *Proc Int Soc Biol Ther Cancer (Abstract)*.
4. Comin-Anduix, B., A. Gualberto, J. A. Glaspy, E. Seja, M. Ontiveros, D. L. Reardon, R. Renteria, B. Englahner, J. S. Economou, J. Gomez-Navarro, and A. Ribas. 2006. Definition of an immunologic response using the major histocompatibility complex tetramer and enzyme-linked immunospot assays. *Clin Cancer Res* 12:107.

5. Grohmann, U., C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M. L. Belladonna, R. Bianchi, M. C. Fioretti, and P. Puccetti. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat Immunol* 3:1097.
6. Munn, D. H., M. D. Sharma, D. Hou, B. Baban, J. R. Lee, S. J. Antonia, J. L. Messina, P. Chandler, P. A. Koni, and A. L. Mellor. 2004. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J Clin Invest* 114:280.
7. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057.
8. Munn, D. H., M. D. Sharma, J. R. Lee, K. G. Jhaver, T. S. Johnson, D. B. Keskin, B. Marshall, P. Chandler, S. J. Antonia, R. Burgess, C. L. Slingluff, Jr., and A. L. Mellor. 2002. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science* 297:1867.