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.	)	C.A. No. 06-500-SLR **PUBLIC VERSION**

DECLARATION OF DAVID A. OSTROV, PH.D. IN SUPPORT OF ZYMOGENETICS, INC.'S OPPOSITION TO BRISTOL-MYERS SQUIBB CO.'S MOTION FOR SUMMARY JUDGMENT OF NONINFRINGEMENT

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I, David A. Ostrov, declare:

1. I am an Assistant Professor in the Department of Pathology, Immunology and Laboratory Medicine at the University of Florida College of Medicine.

2. In 1995, I received a Ph.D. in Biological Structure from the University of Washington School of Medicine.

3. I solved the crystal structure of CTLA-4, a T cell surface molecule that is critical in the negative regulation of T cell responsiveness (Ostrov DA, Shi W, Schwartz JC, Almo SC, Nathenson SG. Structure of murine CTLA-4 and its role in modulating T cell responsiveness. Science. 2000 Oct 27; 290(5492):816-9.).

4. The structure revealed several surprising features, such as a novel mode of dimerization, which I, and the leaders in the field, believe has implications on T cell signaling in the immunological synapse (Schwartz JC, Zhang X, Nathenson SG, Almo SC. Structural mechanisms of costimulation. Nat Immunol. 2002 May; 3(5):427-34. Review.). Due to its dominant role in modulating T cell activity, CTLA-4 has received considerable attention as a target for therapeutic agents.

5. *In vivo*, evidence demonstrates that the CTLA-4 molecule exists as a homodimer that consist of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. *In vivo*, one can make a crystalline form of dimeric CTLA-4 in order to study the individual chains – but such studies do not address whether such a molecule need be a dimer for biological activity.

6. The biological activity of CTLA4 is to downregulate (attenuate) T cell activation. CTLA-4 is thought to play important roles in maintenance of T cell homeostasis by negatively regulating T cell signaling. There is evidence that ligation of CTLA-4 (CD152) with B7-1 or B7-2 (CD80 and CD86) inhibits T cell activation by several mechanisms, including by recruiting phosphatases such as PP2A and SHP-2 to the intracellular cytoplasmic tail of CTLA-4 (Thompson CB, Allison JP. The emerging role of CTLA-4 as an immune attenuator. Immunity. 1997 Oct; 7(4):445-50. Review.)

7. Resting naïve T cells do not typically express CTLA-4 on their cell surfaces.

However, 24-48 hours after cognate T cell receptor ligation and CD28 costimulation, a number of tyrosine kinases become activated and CTLA-4 is dramatically upregulated on cell surfaces. CTLA-4 on the cell surface is typically localized to the central region of the immunological synapse. Clustering of CTLA-4 in the immunological synapse results in the recruitment of phosphatases which counteract the positive (stimulatory) signaling effects of the protein kinases (thereby resulting in downregulation of T cell signaling). (Egen JG, Kuhns MS, Allison JP. CTLA-4: new insights into its biological function and use in tumor immunotherapy. Nat Immunol. 2002 Jul; 3(7):611-8. Review.).

8. The therapeutic mode of action for Orencia is to modulate the T-cell receptor CD28, by binding to the antigen presenting cell ligands CD80 (B7-1) and CD86 (B7-2) that interact with CD28. The end result is an inhibition of T-cell proliferation and cytokine release.

Non-Immunoglobulin Requiring Dimerization

9. It is my understanding that BMS argues that CTLA-4 is an immunoglobulin polypeptide. This argument is not consistent with the definition of immunoglobulin polypeptides in immunology textbooks and peer reviewed journals.

10. Immunoglobulin polypeptides are secreted by plasma cells and function as antibodies in the immune response by binding with specific antigens. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM. Immunoglobulins, or antibodies, are polypeptides expressed by B cells that: 1) serve as antigen receptors in the adaptive immune system, and 2) are encoded by gene segments that undergo somatic rearrangement during B cell development.

11. CTLA-4 is not considered to be an antigen receptor in the adaptive immune system (it forms non-antigen specific interactions with B7 molecules), and is not encoded by gene segments that undergo somatic rearrangement. That is to say, it is not an antibody.

12. The immunoglobulin 'superfamily' is one of the most prevalent families of protein domains in the animal kingdom. The functions of the various members of this superfamily are disparate and provide a notable example of selection acting on a modular

structure, the immunoglobulin domain fold, to form molecules with specialized functions in processes such as cell-cell adhesion and molecular recognition. Hemolin, an insect protein, DIM-1, in *C. elegans* (a roundworm,) and CTLA-4 are members of this large superfamily of proteins.

13. I understand that BMS believes CTLA-4 is "immunoglobulin-like" because it shares significant homology to the immunoglobulins. Such a statement is not scientifically correct. CTLA-4 does not share significant homology to the immunoglobulins.

14. The ability of CTLA-4 to function depends on a number of processes. Binding of CTLA-4 to B7 molecules is necessary but not sufficient for the normal biological activity of CTLA-4. Evidence demonstrates that dimers of CTLA-4 are required to engage in bivalent interactions within the central portion of the immunological synapse.

15. Although monomeric forms of CTLA-4 can be artificially generated, and have been shown to bind weakly to B7 ligands, these monomeric CTLA-4 molecules are not considered sufficient to account for the biological activities conferred by CTLA-4 *in vivo*.

16. As noted above, CTLA-4 is considered to function as a dimer for its biological activity *in vivo* for several reasons. First, CTLA-4 can be purified as a dimer from cells and tissues. Second, there is evidence of higher avidity interactions between dimeric forms of CTLA-4 and B7 ligands compared to monomeric forms of CTLA-4. Third, CTLA-4 has a propensity to dimerize in solution. Fourth, structures of CTLA-4 solved by x-ray crystallography show that it is in the dimeric form bound and unbound to B7 ligands. Thus there is overwhelming evidence that CTLA-4 exists as a homodimer *in vivo*, and it is the dimeric form that is biologically active. My opinion is based on these findings which have been reported in high caliber peer reviewed journals, in addition to my personal experience working with CTLA-4 and solving its structure by x-ray crystallography (reported in Science).

17. I not aware of any studies proving that a monomeric chain of native CTLA-4 exists. And if such a form did exist, it would be highly unlikely to be biologically active as CTLA-4 is an obligate dimer.

Dimerizing Protein

18. I have personally experimented on CTLA-4Ig (Orencia). During the course of experiments designed to obtain a crystalline form of Orencia®, I determined that the C_H2 and C_H3 domains of Orencia interacted to form a dimer.

19. It is my understanding that BMS believes that the Ig portion of Orencia does not "drive" the dimerization of the extracellular domain of CTLA-4 found in Orencia. CTLA-4Ig dimerizes in solution. Forms of CTLA-4 without the Ig portion do not, as shown in BMS' studies. Linsley, P.S. et al., (1995), J. Biol. Chem. 270:15417-15424.

20. The C_H2C_H3 domains of IgG, which are found in Orencia, are considered elements that dimerize, and can therefore be called a "dimerizing protein". A large body of evidence demonstrates the propensity of C_H2C_H3 domains of IgG to dimerize, including by x-ray crystallography (Deisenhofer, J. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-A resolution. Biochemistry, v20 pp. 2361-2370, 1981).

Soluble Monomer

21. I have reviewed the journal articles regarding the reported "soluble form" of CTLA-4. In each instance, the reports appear to be claiming that a "spice variant" or truncated form of the native CTLA-4 exists in the serum of humans. These reports are insufficient scientific evidence that a functional soluble form of truncated CTLA-4 exists in humans. All the studies failed to present any data indicating such a form of CTLA-4 functions as a monomer. Nor are there any reports that I am aware of presenting data that native CTLA-4 on the T cell surface can be biologically active as a monomer.

22. The Magistrelli study (Magistrelli et al., Eur. J. Immuno. (1999) 29:3596-3602) reports expressing a truncated form of CTLA-4 in COS cells. The authors appear to detect an immunoreactive protein in the serum of humans. In this study, no evidence presented that shows the protein expressed in the COS cells is the same protein detected in the human sera. That is to say, the authors did not characterize the human sera sufficiently to determine if the detected protein there was the same as that detected from the COS cells.

Furthermore, the molecular mass (or size) of the protein transcribed from the reported splice variant in COS cells is inconsistent with the expected size. In this study, the authors did not examine whether or not the so-called "soluble form" was a dimer or a monomer. Thus, this study fails to conclusively determine whether or not a soluble form of CTLA-4 exists in human sera, and if it did, the study fails to examine if such a form is a dimer or a monomer. Interestingly, however, the splice variant clone reported in this study contains a cysteine residue that may facilitate disulfide bonding, although such disulfide bonds were not observed in x-ray crystallographic studies.

23. The Oaks study (Oaks et al., Cellular Immunol. (2000) 201:144-153) is a similar study examining a splice variant of CTLA-4. This clone was expressed in insect cells. As with the Magistrelli study, the actual cells that might be producing such a truncated form of CTLA-4, the T cells, were not examined. The immunoreactive protein detected in human sera does not appear to be the same as the insect cell-produced protein. Furthermore, no studies were done to indicate whether or not the insect cell-produced protein, or the protein detected in the human serum, was a dimer or monomer. Thus, as in the Magistrelli study, there is no conclusive scientific evidence is presented that the protein detected in the serum is the same as that produced in the lab, or that such proteins exist as monomers.

24. The Saverino study (Saverino et al., Clin. Immun. (2007) 123:190-198) is consistent with the Magistrelli and Oaks studies in not providing conclusive evidence showing monomeric CTLA-4 to be active in human sera. In this case, the immunoreactive protein detected in the human sera appears to be inconsistent with the molecular mass expected for the reported monomeric splice variant of CTLA-4. The ELISA studies reported do not utilize optimal methods of using samples to establish a standard curve (for use in computing concentration.) Finally, it is noteworthy that while the authors also failed to present data on whether or not the splice variant or protein in the human serum existed as a dimer or a monomer, it is represented in figure 4b as a dimeric, soluble form.

I declare under penalty of perjury under the laws of the United States that all

statements made herein of my own knowledge are true and correct, and that all statements made on information and belief are believed to be true.

Dated: August 16, 2008

David A. Ostrov, Ph.D.

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CERTIFICATE OF SERVICE

I, Philip A. Rovner, hereby certify that on August 25, 2008, the within document was filed with the Clerk of the Court using CM/ECF which will send notification of such filing(s) to the following; that the document was served on the following counsel as indicated; and that the document is available for viewing and downloading from CM/ECF.

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