AD_____

Award Number: DAMD17-97-1-7142

TITLE: The Effects of the MHC Class II Transactivator on the Growth and Metastasis of Breast Tumors

PRINCIPAL INVESTIGATOR: Brian K. Martin, Ph.D.
Dr. Jenny Ting

CONTRACTING ORGANIZATION: University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-1350

REPORT DATE: June 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2000	Final, (1 Jun 97 – 31 May 00)

4. TITLE AND SUBTITLE
The Effects of the MHC Class II Transactivator on the Growth and Metastasis of Breast Tumors

5. FUNDING NUMBERS
DAMD17-97-1-7142

6. AUTHOR(S)
Brian K. Martin, Ph.D.
Dr. Jenny Ting

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-1350

E-Mail: brian-martin@uiowa.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

I have finalized my research on the effects of the MHC class II transcriptional transactivator (CIITA) on the growth and metastasis of breast tumors. Mouse model systems have shown that CIITA expression, in and of itself, is at best not beneficial for tumor immunotherapy in the mouse model system. Surprisingly, even in the context of the immune costimulatory molecules CD80 (B7-1) and CD86 (B7-2), CIITA was unable to mount and immune response in tumor challenged mice. When the system was modified to examine the ability of CIITA to induce immunogenicity of the tumor, it was found that CIITA was actually detrimental to survival. This result is not surprising considering that dogma in immunology states that engagement of the MHC-TCR complex without additional costimulation can lead to T cell anergy. What is surprising is that the classical costimulatory molecules CD80 and CD88 are unable to provide this T cell costimulation in the context of CIITA. Additional experiments have also been undertaken to examine novel CIITA genes induced by CIITA. These studies provide a wealth of useful information regarding proposed human cancer therapy trials utilizing CIITA.

14. SUBJECT TERMS
CIITA; MHC class II; MHC class I, Immunotherapy

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

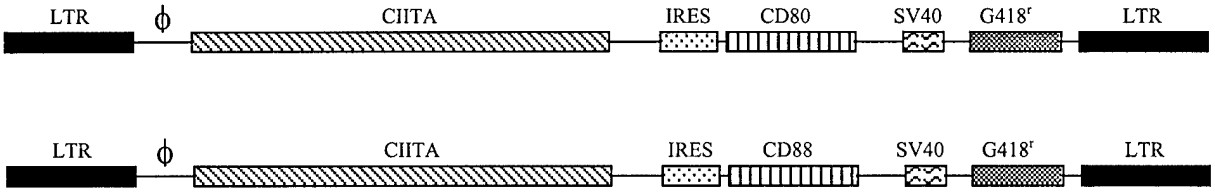
Introduction

MHC proteins are key regulators of the immune response. They present antigens to T lymphocytes and are key for the elicitation of T cell immunity. MHC class II proteins present peptides derived from extracellular sources to CD4+ T cells and in cases where there is costimulation, activate a helper response that can lead to an antibody response (TH2) or a cellular response (TH1). Previous work has shown that expression of α and β chains of MHC class II on a sarcoma cell line can lead to protective tumor immunity (1); however, other MHC class II pathway genes are not activated in this case. CIITA has been shown in many systems to induce several genes involved in the MHC class II antigen presentation pathway (2-5). In some instances, *de novo* expression of CIITA has lead to enhanced antigen presenting cell (APC) function (6-8). We and others have recently shown that, in addition to class II molecules, CIITA is able to induce MHC class I surface expression in cells deficient in expression of these molecules (9, 10). I hypothesize that *de novo* expression of CIITA in tumor cells will upregulate class II genes, and in the case of cells with low or no expression of MHC class I, class I genes as well. The expression of these molecules may induce an immune response against these cells, affecting growth, metastasis, and vaccine efficacy. Should this not induce a response, the coexpression of costimulatory molecules may be necessary for immune response induction. I hypothesize that CIITA expression has the potential to be a novel mechanism for induction of immunity to breast cancer.

Body

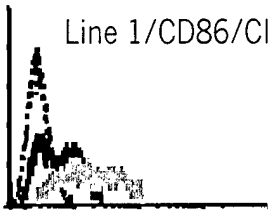
Year 3 Statement of work

As described in the original proposal, the first goal during year 3 was the procurement of genes for the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) and the generation of dual expression constructs. These constructs consisted of the gene for CIITA under the control of the LTR promoter plus the genes encoding either CD80 or CD86 whose expression was mediated by the Internal Ribosome Entry Sequence (IRES) (11). This sequence is from the encephalomyocarditis virus (ECMV) and allows the ribosome to enter in the middle of an RNA strand, creating a polycistronic eukaryotic message. A schematic of these constructs is presented below:



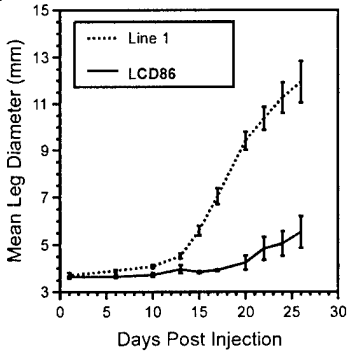
Unfortunately, these constructs presented two problems. First, because of the large size of CIITA (3.5 kb), the total size of the construct with CIITA, IRES, the C80 or CD86 genes and the genes for G418 resistance brought the total size of the inserts at or above the upper limit for efficient retroviral packaging (6.5 kb) (12). This meant that packaging of the inserts was very inefficient and resulted in low titer virus. Second, it was discovered that the IRES sequence used (from the pCITE-4a vector, Novagen) mediated only marginal translation (approximately 10-fold less than the same gene under control of the LTR promoter). As a result of these caveats, we were unable to construct effective combination retroviral vectors. For future experiments, it may be possible to create these combination vectors in adenoviral constructs, however these vectors usually mediate strong immune responses, rendering the results of such studies difficult to interpret.

Since we were unable to express the CIITA and costimualtory genes in a single vector, we decided to use two-plasmid transfection to overcome this obstacle. First cells were transduced with the CIITA-encoding retroviral construct (LXSN, G418 resistance). Next, cells were transduced with retrovirus encoding the genes for either CD80 or CD86 (LXSP, puromycin resistance). In this manner we were able to create cell lines that expressed both CIITA (and surface MHC class II) as well as the proteins for costimulatory molecules (CD80 or CD86). Shown below is a FACS profile of cells that express CIITA and CD86.



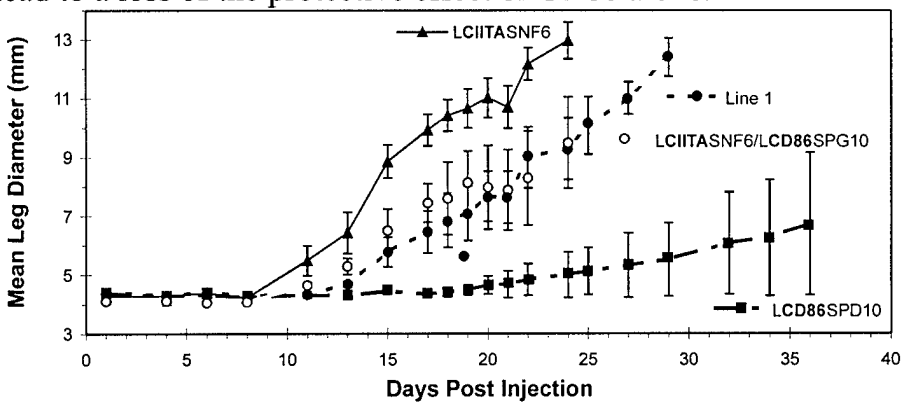
Cell surface expression of CD86 and MHC class II on cells transduced with retroviruses encoding the indicated proteins. Cells were transduced and selected in the appropriate antibiotic. Surface expression was measured by FACS analysis using antibodies for the indicated proteins.

We used the Line 1 lung carcinoma (13) as a model cancer system because it was found that in the MT901 breast cancer line described in previous project updates, transfection with CD80 or CD86 leads to tumor immunity (data not shown). The means that the combination of CIITA and these costimulatory molecules could not be examined in this cell line. We found that the CD80 molecule had no effect on Line 1 tumor growth in vivo, however, the CD86 protein lead to a marked decrease in the growth rate of tumors, as shown below.



High stable expression of CD86 on Line 1 cells leads to growth attenuation and decreased tumorigenicity. The Line 1 clone, LCD86SPD10, expressing high stable levels of CD86 was injected at 500 cells per mouse into the calf muscle of Balb/c mice. Mice were monitored individually for tumor growth. Each line represents the mean leg diameter of four to six mice per group. Error bars represent the SEM for each group. The dotted line represents unmodified Line1 growth, solid line is the CD86 group. Three of four mice injected with CD86 polyclonal cells did not grow tumors in this experiment.

As shown in my Year 2 research update, CIITA has a small effect on tumor cell growth in vivo, but only when expressed at low levels. The question then became; what is the effect of coexpression of CIITA and CD86? We engineered cells to express both proteins and found that, contrary to what we expected, CIITA expression in the context of CD86 lead to a loss of the protective effect of CD86 alone.



Polyclonal Line 1 cells coexpressing CD86 and CIITA have a growth phenotype intermediate to that of CIITA or CD86 expressed alone. The LCIITASN polyclonal population was transduced with CD86 expressing retrovirus and the polyclonal populations were injected into mice. Cells were injected at 500 cells per mouse into the calf muscle of Balb/c mice. Mice were monitored individually for tumor growth. Each line represents the mean leg diameter of four to six mice per group. Error bars represent the SEM for each group. Dotted line, filled circles is unmodified Line 1; dashed line, filled squares is the LCD86SP polyclone, line that is dashes and dots with filled triangles is the LCIITASN polyclone, solid line with open circles is the LCIITASN/LCD86SP polyclone.

These results demonstrate that CIITA and CD86 do not cooperate in tumor immunity with this model system. Further experiments utilizing the CD80 costimulatory molecule demonstrated that (a) CD80 alone had little effect on tumor growth in vivo and

(b) coexpression of CD80 and CIITA had little bearing on tumor growth rate (data not shown). These results are somewhat counterintuitive, given that dogma in immunology states that if a cell has both immunological signals provided by MHC and a costimulatory molecule, then they should be competent to mount an immune response. These results speak to the case for the special nature of tumor immunity induction. It appears that breaking immune privilege that tumor enjoy requires additional immune cell function.

Given that we saw little in the way of tumor specific immune induction in the case of CIITA, we did not pursue additional immune mechanisms. We have generated interesting data demonstrating that CIITA expression alone can have a negative role in immune induction. This may be due to natural killer (NK) cells (14). It is well recognized that NK cells survey the body for cells that have aberrant expression of MHC molecules. Cells that have down-modulated their MHC class I expression are subject to killing by NK cells. Many tumors have decreased MHC class I expression as a means to escape immune surveillance (15). These cells are subject to some level of NK control (not enough, however, to be completely killed and not grow to the point of being cancerous). When CIITA is expressed in these tumor cells, there is upregulation of MHC class I (16). This might suggest that these cells then lose their ability to be recognized by natural killer cells, leading to fast initial growth rates. This is supported by our data with the Line 1 tumor model.

To examine the role of CIITA and costimulation in a tumor immunogenicity model, we injected mice with irradiated Line 1 cells expressing CIITA, CD86 or both. Subsequently, mice were challenged with unmodified Line 1 cells and the number of mice that succumbed to tumor was reported. As shown below, more mice expressing CIITAdied as a result of their tumor than did control mice, again showing a potential negative role for CIITA. On the other hand, mice injected with cells expressing CD86 survived at a higher frequency than control mice. But when CIITA and CD86 were coexpressed, the number fell below that of CIITA alone.

Table I-Changes in Immunogenicity Associated With CD86 and/or CIITA Polyclonal Expression in Line 1 Tumors*

	Irradiated Cells Injected				
	None	Vector	CD86	CIITA	CIITA/CD86
Total Tumor-free at day 28	0%	53%[1]	70%[1,2]	43%	38%[2]

*Number of mice tumor free at day 28 versus total injected

[1]$P < 0.05$, vector comparing Vector with CD86

[2]$P < 0.05$, CIITA/CD86 compared with CD86

These data again suggest that CIITA and CD86 do not cooperate; indeed, CIITA had a dominant negative role with respect to the positive effect of CD86.

We were unable to examine the role of CIITA in metastasis. As previously shown in a research update, the 4T1 mouse mammary tumor metastasis model (17) undergoes cell death when transfected with CIITA, therefore could not examine this

process. We were unable to generate a CIITA inducible expression system for the 4T1 cell line. As described in previous research updates, expression of CIITA in this cell line leads to apparent cell death. We have tried to develop two different CIITA inducible model systems for this cell type. The apparent failure appears to stem from the large size of CIITA (over 3500 bp). The inducible vectors are derived from retroviral vectors and the size of CIITA in conjunction with other control element appears to cause some problems with function. We are, however, still convinced that CIITA does induce apoptotic death in some cell types.

What are the conclusions of our studies on the role of CIITA and costimulatory molecules in cancer? These data show that CIITA is ineffective in several different tumor model systems, in and of itself. At times, CIITA represents a negative factor. We have found little evidence for cooperation with costimulatory molecules in the control of tumor growth. However, the combination of CIITA and CD86 may be effective in other tumor models; the experiences with CD86 in several models suggest that the effects of this coexpression may be different in other systems tested. However, these results do bring into question the prudence of beginning human CIITA vaccine trials without being able to ascertain whether CIITA could lead to the induction of tolerance to the tumor being treating in proposed CIITA human tumor vaccine trials. Caution must be exercised when proceeding with these studies. It is possible that other costimulatory molecules may be more efficacious for induction of tumor immunity. These might include CD40 (18) and ICAM-1 (19).

Finally, we have examined genes induced by CIITA by representation differential analysis and by differential display. To our surprise, we did not find a large number of genes induced by CIITA. In fact, the majority of genes were classical MHC class II gene products. We did find discordant regulation of the DNα and DOβ genes. These data suggest that the CIITA protein very specifically regulates the MHC class II genes (and in some cells MHC class I genes).

Milestone Questions answered in Year 3

What is the effect of coexpression of CIITA with costimulatory molecules on breast tumor growth, metastasis and vaccine efficacy?

We have found little effect of CIITA on tumor cell growth when expressed in combination with the CD80 and CD86 costimulatory molecules. These data suggest that in the tumor model systems thus far examined, there is very little efficacy of CIITA, either with or without costimulation in the induction of tumor immunity. These results do not preclude the use of other potential costimulatory molecules in tumor immunity induction.

What is the effect of costimulatory molecules on tumor growth, metastasis and vaccine efficacy?

As described, CD86 induced tumor immunity in the Line 1 model system. This was in the context of both primary tumor growth and in a vaccine model. Due to the problems with CIITA induced expression, we did not examine the role of costimulatory molecules in metastasis.

What is the immune mechanism for the effects observed with CIITA in the mouse mammary cancer model systems?

Since we did not see any positive effect of CIITA expression on tumor growth, we did not rigorously pursue this question. It is postulated that the negative effect of CIITA on tumor growth may be due to natural killer cells.

Key Research Accomplishments:

- Created retroviral constructs for the CD80 and CD86 genes (with puromycin selection marker).
- Found that CD86 expression in and of itself can induce both primary and secondary tumor immunity in the Line 1 model system.
- Found that CIITA in combination with CD86 does not induce additional tumor immunity. In fact, CIITA in either primary or secondary assays appears to induce a dominant negative effect on tumor immunity. This finding is very important since we have been approached on several occasions to start human clinical trials with CIITA in end-stage cancer patients. Although we understand that their prognosis is grim, our data suggests that at best CIITA expression will have no effect and at worse may be a negative factor.
- Found that CIITA does not induce many genes that are unknown outside the MHC class II pathway.

Reportable Outcomes.

Manuscripts-

Martin, B. K., J. G. Frelinger and J. P.-Y. Ting. 1999. Combination gene therapy with CD86 and the MHC class II transactivator in the control of lung tumor growth. J Immunol. 162:6663-70.

Martin, B. K. and J. P.-Y. Ting. 1999. Expression of complement protein C5a in a murine mammary carcinoma leads to tumor regression. In revision.

Martin, B. K., K. P. McKinnon and J. P.-Y. Ting. 1999. A rapid, quantitative, high-throughput cell migration assay: application for examination of functional C5a. Submitted.

Abstracts-

Brian K. Martin, Gene H. MacDonald, Robert E. Johnston, and Jenny P.-Y. Ting. 1999. Novel Cancer Therapy Utilizing Tumor-Specific Dendritic Cell Immune Responses. Keystone Meeting: Immunogenetics of Human Disease-MHC/TCR and Peptide. January 1999. (Recipient of Keystone Travel Award)

Martin, B. K., J. G. Frelinger and J. P.-Y. Ting. 1998. MHC Class II Transactivator (CIITA) is Ineffective in the Stimulation of Primary Line 1 Tumor Immunity and Does Not Cooperate with B7-2. Experimental Biology. March 1998.

Patents Applied For-

The use of pre-existing immunity in the prevention and treatment of cancer. Gene H. MacDonald, Brian K. Martin, Robert E. Johnston, and Jenny P.-Y. Ting.

Employment Opportunities

Over the last two years on the DOD training grant I applied for approximately 50 jobs. I have had six interviews. The University of Nebraska Medical School in Omaha was

interested in me, but I felt that the position was not appropriate for my qualifications. I was the second choice at the Medical School of Wisconsin. I had interviews at the University of Connecticut Health Sciences Center and Moffitt Cancer Center in Tampa, Florida. I had a job offer at the University of Tennessee at Memphis that I eventually turned down. I accepted an assistant professor position in the Department of Microbiology at the University of Iowa and started June 4, 2000. I am very happy with the university and my position.

Conclusions

I have conducted a concise examination of the potential role of the MHC class II transactivator, CIITA, as a tumor immunotherapy. It was initially hypothesized by various groups (including ourselves) that expression of CIITA leads to increased immunogenicity of the tumor. This modified tumor could then be used as an immunogen to induce antitumor immunity. The alternative hypothesis was that, in the absence of costimulatory molecules, CIITA expression would induce an anergic response, leading to faster tumor growth. Our results favor the second hypothesis. CIITA expression, by itself, has not been found to induce tumor immunity and in some instances has been a negative factor for tumor growth. The second major part of this research involved the coexpression of CIITA and the costimulatory molecules CD80 and CD86. Once again, we found little evidence for cooperation between theses proteins in tumor immunity induction. In fact, CIITA was found to be a negative factor with regards to CD86 mediated immunity. Our results strongly suggest that proposed human CIITA cancer therapy trials should be approached with caution. Until the stimulatory capacity of the modified tumor can be accessed, these experiment are not recommended.

Finally, I have been very happy with the training opportunities that the US Army DOD postdoctoral training grant has provided. The funds that I received through this award allowed me to travel to several excellent meetings in addition to purchasing supplies for my experiments. This training also allowed me to interview for several good academic research positions and ultimately lead to my appointment as an assistant professor at the University of Iowa.

References

1. Ostrand-Rosenberg, S. 1994. Tumor immunotherapy: the tumor cell as an antigen-presenting cell. *Curr.Opin.Immunol 6:722.*
2. Chin, K.-C., C. Mao, C. Skinner, J. L. Riley, K. L. Wright, C. S. Moreno, G. R. Stark, J. M. Boss, and J. P.-Y. Ting. 1994. Molecular analysis of G1B and G3A IFN gamma mutants reveals that defects in CIITA or RFX result in defective class II MHC and Ii gene induction. *Immunity 1:687.*
3. Chang, C. H., J. D. Fontes, M. Peterlin, and R. A. Flavell. 1994. Class II transactivator (CIITA) is sufficient for the inducible expression of major histocompatibility complex class II genes. *J Exp Med. 180:1367.*
4. Chang, C. H., and R. A. Flavell. 1995. Class II transactivator regulates the expression of multiple genes involved in antigen presentation. *J. Exp. Med. 181:765.*
5. Steimle, V., C. A. Siegrist, A. Mottet, B. Lisowska-Grospierre, and B. Mach. 1994. Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science 265:106.*

6. Armstrong, T. D., V. K. Clements, B. K. Martin, J. P. Y. Ting, and S. Ostrand-Rosenberg. 1997. Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor- specific immunity. *Proc.Natl.Acad.Sci.U.S.A. 94:6886.*
7. Hershberg, R. M., D. H. Cho, A. Youakim, M. B. Bradley, J. S. Lee, P. E. Framson, and G. T. Nepom. 1998. Highly polarized HLA class II antigen processing and presentation by human intestinal epithelial cells. *Journal of Clinical Investigation 102:792.*
8. Sartoris, S., M. T. Valle, A. L. Barbaro, G. Tosi, T. Cestari, A. D'Agostino, A. M. Megiovanni, F. Manca, and R. S. Accolla. 1998. HLA class II expression in uninducible hepatocarcinoma cells after transfection of AIR-1 gene product CIITA: acquisition of antigen processing and presentation capacity. *Journal of Immunology 161:814.*
9. Martin, B. K., K.-C. Chin, C. A. Skinner, J. C. Olsen, A. Dey, K. Ozato, and J. P.-Y. Ting. 1997. Induction of MHC class I expression by the MHC class II transactivator (CIITA). *Immunity 6:591.*
10. Gobin, S. J. P., A. Peijnenburg, V. Keijsers, and P. J. van den Elsen. 1997. Site alpha is crucial for two routes of IFN-gamma-induced MHC class I transactivation: The ISRE-mediated route and a novel pathway involving CIITA. *Immunity 6:601.*
11. Martinez-Salas, E. 1999. Internal ribosome entry site biology and its use in expression vectors. *Curr Opin Biotechnol 10:458.*
12. Miller, A. D., D. G. Miller, J. V. Garcia, and C. M. Lynch. 1993. Use of retroviral vectors for gene transfer and expression. *Methods Enzymol. 217:581-99:581.*
13. McAdam, A. J., B. A. Pulaski, S. S. Harkins, E. K. Hutter, E. M. Lord, and J. G. Frelinger. 1995. Synergistic effects of co-expression of the TH1 cytokines IL-2 and IFN-gamma on generation of murine tumor-reactive cytotoxic cells. *Int.J.Cancer 61:628.*
14. Lanier, L. L. 2000. The origin and functions of natural killer cells. *Clin Immunol 95:S14.*
15. Cohen, E. P., and T. S. Kim. 1994. Neoplastic cells that express low levels of MHC class I determinants escape host immunity. *Semin.Cancer Biol. 5:419.*
16. Martin, B. K., K. C. Chin, J. C. Olsen, C. A. Skinner, A. Dey, K. Ozato, and J. P. Ting. 1997. Induction of MHC class I expression by the MHC class II transactivator CIITA. *Immunity. 6:591.*
17. Pulaski, B. A., D. S. Terman, S. Khan, E. Muller, and S. Ostrand-Rosenberg. 2000. Cooperativity of Staphylococcal aureus enterotoxin B superantigen, major histocompatibility complex class II, and CD80 for immunotherapy of advanced spontaneous metastases in a clinically relevant postoperative mouse breast cancer model. *Cancer Res 60:2710.*
18. Toes, R. E. M., S. P. Schoenberger, E. I. H. van der Voort, R. Offringa, and C. J. M. Melief. 1998. CD40-CD40Ligand interactions and their role in cytotoxic T lymphocyte priming and anti-tumor immunity. *Semin Immunol 10:443.*
19. Schlom, J., and J. W. Hodge. 1999. The diversity of T-cell co-stimulation in the induction of antitumor immunity. *Immunol Rev 170:73.*