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13. ABSTRACT *(Maximum 200 words)*

I have continued my investigations into the effect of the MHC class II transactivator, CIITA, on the growth and immunogenicity in breast cancer. I have utilized three different model sytems; MT901, 4T1 and EMT6. In the MT901 cell line, CIITA expression does not change in vivo tumor growth properties. In the 4T1 model, expression of CIITA leads to direct killing of the tumor in vitro, suggesting that CIITA has a toxic effect in this cell line. We are actively investigating inducible CIITA expression systems to fully investigate this phenomenon. We have also investigated immunogenicity of CIITA expressing tumors. MT901 is immunogenic and cannot be used in these assays, therefore we have used the Line 1 lung carcinoma as a surrogate system (we have also started to examine EMT6 as an additional mammary tumor model). We have found that CIITA expression can result in more mice with tumor formation, suggesting a negative role for CIITA in the absence of costimulation. We have initiated preliminary investigations into novel genes induced by CIITA. The results of these experiments have important implications in proposed CIITA-human gene therapy trials.

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Bri K. Mac 6/29/99

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Introduction

MHC genes 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. In cases where there is costimulation, they may activate a helper response that can lead to a cellular response (TH1) or an antibody response (TH2). 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 led 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 the 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, induce 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 to obtain a protective effect. I hypothesize that CIITA expression has the potential to be a novel mechanism for induction of immunity to breast cancer.

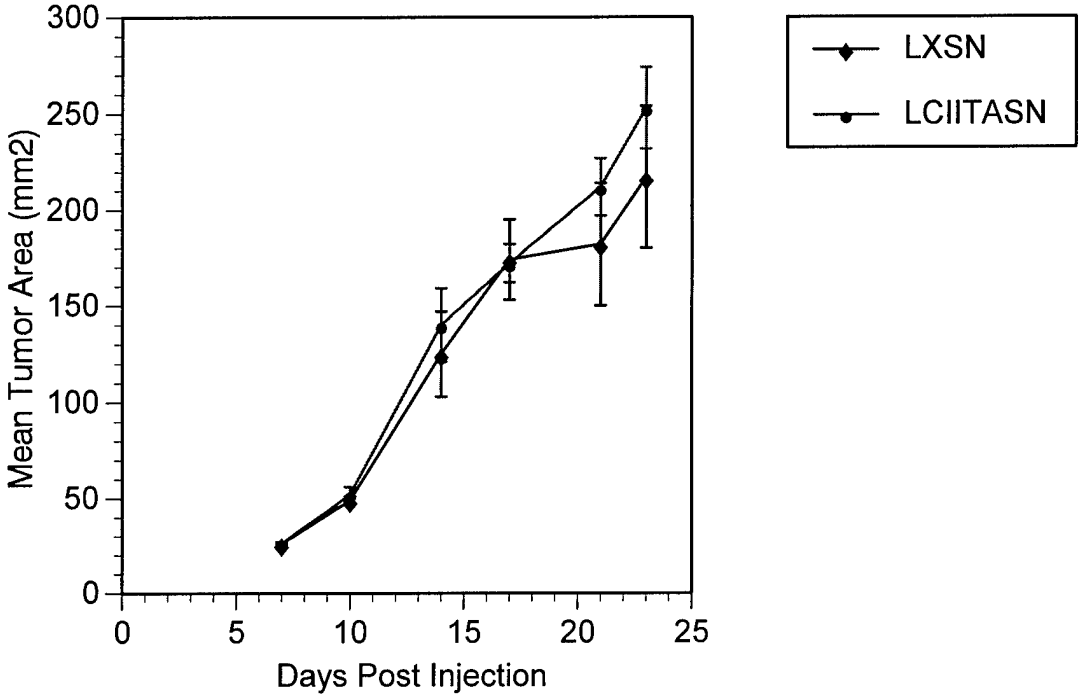
Body

Experimental Results

Tumor cell growth *in vivo*:

As demonstrated in last year's Annual Report, we had inoculated mice with the mouse BALB/c mammary tumor, MT901, expressing CIITA (this was an aim in the Year 2 Statement of Work, see Figure 1).

FIGURE 1



These data demonstrate that CIITA expression in the MT901 tumor model system does not change the in vivo growth characteristics of the tumor. Since I had already accomplished this Year 2 goal, we went on to investigate the growth of tumor cells after irradiation; ie., an immunogenicity experiment (and a Year 3 goal).

Primary tumor growth assays (ie., when mice are injected with live cells) are good experimental models; however this design does not closely mimic the clinical situation with human patients. Clinical protocols rely upon the resection of primary tumor, *ex vivo* modification and cellular irradiation followed by injection back into the patient. To more closely approximate this protocol, an approach that tests the immunogenicity of tumors has been employed. With this method, cells were modified, irradiated and injected into the mouse. This was followed by injection of unmodified cells and tumor growth was monitored. Unfortunately, the MT901 cell line is immunogeneic. That is, if unmodified wild-type cells are irradiated and injected into mice, all these mice will be protected from subsequent tumor challenge. These means that immunogenicity assays can not be conducted in this model system. We have subsequently used the Line 1 lung epithelial model system as a general system to examine immunogenicity, since this cell type has very low immunogenicity.

We have irradiated Line 1 cells and injected them intraperitoneally into syngeneic BALB/c mice. Two weeks later mice were challenged with unmodified cells and the growth of those tumor were compared to mice injected with wild-type cells. When mice were mock injected in the primary inoculation, no mice were tumor free at day 28. When irradiated vector control cells were injected, 53% of the mice were tumor free. Finally, when CIITA expressing cells were irradiated and injected, followed by challenge with unmodified cells, only 43% of the mice were tumor free at day 28. Table I demonstrates that more mice succumb to Line 1 tumor formation when injected with CIITA-expressing cell than do when injected with unmodified cells These data indicate that, at best, CIITA expression does not increase the immunogenicity of the tumor, and may allow tumors to form in more mice.

Table I-Changes in Immunogenicity Associated With CIITA Expression in Line 1 Tumors*

		Irradiated Cells Injected		
Experiment	Cells/mouse	None	Vector	CIITA
1	1000	0/5	3/5	2/5
2	1000	0/4	1/4	2/5
3	1000	0/4	3/4	3/5
4	5000	0/6	3/6	3/8
	Total Tumor-free at day 28	0%	53%	43%

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

These data demonstrate that expression of CIITA in epithelial tumors of the lung does not lead to increased immunogenicity in subsequent challenge. These data are not unexpected. As outlined in my research proposal, expression of MHC proteins in the absence of costimulatory molecules often results in either deletion of reactive T lymphocytes or anergy of those cells. This suggests that the proper course of action to pursue is to use costimulatory molecules in combination with CIITA, a Year 3 goal for my research.

The reason that these data are extremely important is that our lab has been approached several times to start clinical trials with CIITA tumor therapy. While we understand that these trials involve end stage cancer patients, we are loathe to initiate work when our data demonstrate that in some cases CIITA can actually allow increased tumor growth. For instance, in primary tumor growth assays, if CIITA expression is high the tumor actually grows faster than control tumors (see Figure 2).

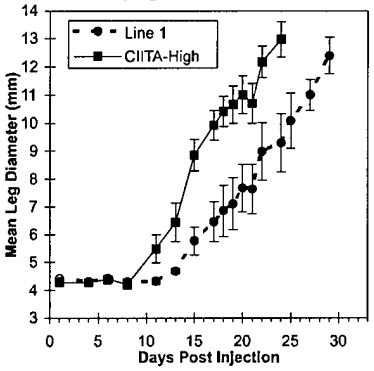


Figure 2

Inducible CIITA Systems and the 4T1 Tumor Line

We have had a major challenge with proposed experiments examining metastatic mammary tumor model systems. As explained in the Year 1 Annual Report, CIITA expression in the 4T1 metastatic model leads to cell death. This means that we have been unable to investigate changes of tumorigenicity and metastasis in this cell line. To attempt to address the qestion of how CIITA kills these cells, we have investigated inducible expression systems where CIITA expression is under the control of an inducible promoter. In our. initial attempts at examining this question, we placed the CIITA under the control of the metallothionine promoter. In cells other than 4T1, where we could monitor MHC class II expression to determine whether or not the promoter was off in the uninduced state, we found that CIITA was expressed at a level that allowed surface class expression. This is undoubtedly due to the fact that CIITA is present at very low levels in most MHC class II positive cells and even a small amount of leakiness in an inducible promoter system will lead to sufficient levels of CIITA to activate MHC class II expression.

We next investigated a recently published inducible system based on a retroviral vector (11). The gene of interest is cloned into the retroviral vector under the control of a tetracycline regulated promoter. In the presence of tet, the operator is bound, resulting in a lack of expression. An additional control mechanism is that the gene of interest is cloned in the antisense orientation. If there is a low level of basal expression, the mRNA is expected to be bind to the sense message from the vector and be degraded by cellular RNAses. We obtained the original vector from Paulus et al. and in the last year we have created a vector that, by sequence, is perfect; however, in the uninduced state we have been seeing expression higher than in the induced state. We have attributed this to problems with early versions of the system that have not been subjected to quality control. Within the past few months, Stratagene Inc. has marketed an inducible vector system based upon this retroviral tet operon. We plan to purchase this system in order to continue our pursuit of the mechanism of CIITA induced killing of 4T1 mouse mammary tumor cells.

Since we have been unable to use the 4T1 system and the MT901 tumor model is immunogenic, we have begun investigations in the EMT6 mammary tumor, as proposed in the Statement of Work. Just this week (Jun. 28, 1999) we have transduced CIITA into this mouse model to begin these studies. The benefit of using this cell line is that it is poorly immunogenic so immunogenicity studies can be performed.

Gene Induction by CIITA

Finally, we have begun our pursuit of other genes induced by CIITA in tumor cells. We initially investigated this question by two different means. We ran 2-D SDS-PAGE gels with extracts from CIITA transfected and vector transfected cell lines, then the different banding patterns were compared between these cell types. We have sporadically observed CIITA specific spots on the gels, but we have not been able to obtain protein sequence from these bands. We have also attempted to use differential display to examine CIITA specific gene expression, but once again technical difficulties have prevented the acquisition of meaningful data. Finally, we have begun using a subtractive PCR technique pioneered by Dr. Janeil Shields in Dr. Channing Der's lab here in the Lineberger Comprehensive Cancer Center. Initial results suggest that we have

identified several CIITA induced genes and we are hopeful that additional analysis will provide us with novel gene sequences expressed in breast cancer cells.

Milestone questions to be answered in Year 2.

Does CIITA modification of tumor cells change the growth pattern *in vivo*? In the cancer models we have investigated (MT901 mammary tumor and the Line 1 lung carcinoma), CIITA expression does not change primary growth of mammary tumor cells. It does slightly retard primary tumor growth of Line 1 cells; however, in immunogenicity assays it has proven to be a negative factor.

Does CIITA induce antigen presentation and/or costimulatory genes molecules in breast tumors? We have reported that CIITA can induce antigen presentation in a sarcoma cell model (6). We have not seen any changes in the antigen presentation capacity as measured by cytotoxic T lymphocyte (CTL) assays from mice inoculated with CIITA expressing tumors. However, since CIITA mainly induced MHC class II expression, we would expect to see a CD4+ T lymphocyte response that may not involve CD+ CTL. Without transgenic T cells, this question will be difficult to address; however there is evidence that some cell types lack of a non-CIITA inducible gene (cathepsin S) that is required for an complete MHC class II processing and presentation pathway. What have therefore set out to clone murine cathepsin S by reverse transcription-DNA amplification. This should give us all known genes required in the MHC class II pathway required to reconstitute the pathway.

What is the mechanism of the CIITA-induce changes in tumor growth? We have not yet seen sufficient changes in CIITA transduced cells to address this question. We suspect that the changes seen in Line 1 tumor cells may be due to NK cells. Unfortunately, the NK deficient strain, Beige, is the H-2^b haplotype, while our cells are H-2^d, meaning that we can not directly test this hypothesis.

APPENDICES

Key Research Accomplishments:

- Established the growth properties in vivo of three different murine mammary tumor models (4T1, MT901, and EMT6).
- Determined that CIITA expression in 4T1 leads to induced cell death.
- Found that CIITA induces no change in MT901 growth and can be a negative factor in the Line 1 tumor model system. 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.
- Initiated experiments to examine CIITA mediated killing of 4T1 tumors cells using a inducible promoter system.
- Initiated experiments to examine other genes regulated by CIITA

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.

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 past two years I have applied for approximately 40 jobs. I have had four 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 am currently waiting as the second choice at the Medical School of Wisconsin. I have a verbal offer at the University of Tennessee at Memphis and am waiting to see what the final offer will be.

Copies of Manuscripts and Abstracts are attached.

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Combination Gene Therapy with CD86 and the MHC Class II Transactivator in the Control of Lung Tumor Growth[1]

Brian K. Martin,* John G. Frelinger,† and Jenny P.-Y. Ting[2]*

Early reports suggest that the costimulatory molecule CD86 (B7-2) has sporadic efficacy in tumor immunity, whereas changes in cancer immunity mediated by the MHC class II transactivator (CIITA) have not been extensively investigated. CIITA activates MHC class II expression in most cells; however, in the Line 1 lung carcinoma model system, CIITA activates MHC class I and well as class II. Here we show that CD86 is very effective in inducing a primary immune response against Line 1. Tumor cells expressing CD86 grew in only 50% of the mice injected with live cells, and those mice that developed tumors did so with significantly delayed kinetics. Furthermore, irradiated CD86-expressing Line 1 cells served as an effective tumor vaccine, demonstrating that CD86 is effective in inducing tumor immunity in the Line 1 system. These data suggest that if CIITA and CD86 cooperate, enhanced tumor immunity could be achieved. CIITA alone was mildly beneficial in slowing primary tumor growth but only when expressed at low levels. Clones expressing high levels of class II MHC grew as fast as or faster than parental tumor, and CIITA expression in a tumor vaccine assay lacked efficacy. When CIITA and CD86 were coexpressed, there was no cooperative immune protection from tumor growth. Cells that coexpress both genes also failed as a cancer vaccine, suggesting a negative role for CIITA in this lung carcinoma. These data suggest that human cancer vaccine trials utilizing CIITA gene therapy alone or in combination with CD86 should be approached with caution. *The Journal of Immunology,* **1999, 162: 6663–6670.**

Malignant cells utilize many different mechanisms to evade the immune recognition (reviewed in Ref. 1). A common defect in the recognition and killing of tumor cells by lymphocytes is the lack of a costimulatory signal. A central dogma in immunology states that when foreign (or mutated) peptides are recognized in the context of MHC class I or class II, lack of a costimulatory signal can lead to anergy or deletion of effector lymphocytes (reviewed in Refs. 2 and 3). The discovery of the costimulatory molecules CD80 (B7-1) (4, 5) and CD86 (B7-2) (6, 7) allowed the testing of the costimulatory hypothesis with regard to tumor immunity. It is well established that de novo CD80 expression in a wide variety of tumor model systems can lead to protective immunity (8). Interestingly, those models in which CD80 expression is ineffective often lack MHC class I expression (8). Less clear is the role of CD86 expression in tumor immunity. Some reports have suggested CD86 expression is not effective in generating tumor immunity (9–13); however, several tumor model systems do derive great benefit from CD86 expression (14–17). In the case of both CD80 and CD86, expression of these costimulatory molecules in the absence of MHC protein expression would be expected to be ineffective.

Another way in which cancerous cells evade immune recognition and destruction is via down-regulation of MHC class I through a variety of mechanisms (reviewed in Ref. 18). This is hypothesized to result in the lack of T lymphocyte surveillance of potential tumor Ags. One technique that has been suggested as an immunotherapeutic strategy for tumors (and is currently in clinical trials) is the introduction of genes encoding MHC class I molecules to restore the ability of the cells to present tumor-associated Ag(s). This was first proposed in the mid-1980s when it was discovered that the introduction of syngeneic MHC class I genes into some mouse cancer models led to tumor regression (19–21). Later, this observation was extended to MHC class II genes (reviewed in Ref. 22). One potential problem with these approaches is that although individual genes for the class I or class II molecules can be transfected into tumor cells, the full restoration of Ag processing and presentation requires other accessory proteins.

MHC class I Ag presentation is a complex process involving multiple steps (reviewed in Ref. 23). First, proteins in the cytosol are degraded by the proteasome complex. These peptides are then transported into the endoplasmic reticulum by the TAP system. In the endoplasmic reticulum, peptide associates with MHC class I-β_2-microglobulin, and this complex is shunted through the Golgi to the cell surface for presentation. In this process, the lack of β_2-microglobulin, proteasome proteins, and/or TAP can lead to the down-regulation of class I, even if there are sufficient heavy chain products being transcribed and translated within the cell (24, 25). In the MHC class II processing and presentation pathway, the required elements include the class II α- and β-chains, the invariant chain (Ii), and the DM molecules (26). There are at least two major difficulties with proposed cancer therapies for both the MHC class I and class II pathways. First, there are codominant alleles for a given MHC molecule. For instance, in humans there are HLA-A, B, and C class I proteins and the HLA-DR, -DQ, -DP class II molecules. If only a single molecule is introduced, the allele capable of recognizing tumor-specific peptide may not be present, resulting in a less than optimal immune response. Second, since for

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a given pathway there are several accessory proteins, the introduction of genes for the main complex chains (i.e., the heavy chain for class I and the α- and β-chains for class II) would not reconstitute the entire processing and presentation pathway. For instance, in the class II pathway, the lack of the DM heterodimer would lead to a failure to remove the Ii-derived peptide in the class II compartment, hence a failure to load foreign peptides. These caveats suggest that a global transcriptional transactivator of a given peptide processing and presentation pathway would be the most effective candidate in reconstituting the MHC class I and/or class II pathways.

One candidate master regulator for tumor immunotherapy is the MHC class II transactivator, CIITA.[3] CIITA was cloned by its ability to restore MHC class II expression in an in vitro mutagenized cell line and was subsequently demonstrated to be the defect in a subgroup of bare lymphocyte syndrome patients (27). This gene was analyzed and found to be a global regulator of the class II MHC genes. De novo expression of CIITA facilitates expression of all the classical MHC class II α- and β-chains (27), Ii, and the DM genes (28–31). Mice with a defective CIITA gene modified by homologous recombination have a phenotype similar to that of bare lymphocyte syndrome patients (32). Our recent work demonstrates that in one cell line, CIITA by itself is able to reconstitute class II processing and presentation (33), and others have also found that CIITA alone can reconstitute intact class II Ag presentation (34, 35). However, Mach et al. have shown that proper Ag presentation required an additional protease (cathepsin S) that is not induced by CIITA (36, 37). These studies demonstrate that the full reconstitution of the class II pathway via CIITA may be cell specific.

An additional reason that CIITA is an excellent candidate as a global inducer of an immune response to cancer is its ability to induce the expression of the heavy chain of MHC class I in addition to MHC class II (38, 39). We have found that CIITA can induce significant amounts of MHC class I in cells with low or no class I expression. Class I induction by CIITA provides an additional mechanism by which CIITA may initiate an antitumor immune response.

These studies suggest that CIITA is a good candidate for cancer immunotherapy; however, CIITA alone may not be ideal due to the lack of costimulation. The engagement of class I or class II MHC without an additional costimulatory signal may induce deletion of reactive T lymphocytes or induction of an anergic response (2). This indicates that CIITA alone could actually negatively impact the immune response to tumor cells. Indeed, we have found that CIITA expression in a sarcoma model, SaI, does not change tumor growth properties (33); however, CIITA does not modify the high levels of MHC class I in this cell line (38). Also, in that report, we did not examine the contribution of costimulatory molecules such as CD86. Another possible negative element in CIITA therapy is the lack of NK cell surveillance. NK cells have receptors that recognize cells with decreased MHC class I expression (40, 41). Tumors that have been CIITA modified and have induced class I expression may no longer be effective NK cell targets. To test the contribution of these events in CIITA therapy, both by itself and in the context of the costimulatory molecule CD86, we investigated the changes in tumor growth and immunogenicity in a lung carcinoma model that has not been examined previously.

Here we demonstrate that CD86 expression in Line 1 cells leads to a markedly reduced tumor growth rate and decreased tumor incidence. CIITA expression by itself was mildly effective in decreasing the tumor growth rate at a low level of expression, but actually increased tumor growth at higher levels. In contrast to expectations, coexpression of CIITA and CD86 had no additive beneficial effect and actually resulted in the loss of CD86 protection. These data suggest that great caution should be considered in the use of CIITA tumor therapy. The relevance of these findings to proposed human tumor therapy is discussed.

[3] Abbreviation used in this paper: CIITA, MHC class II transactivator.

Materials and Methods

Cell lines and culture conditions

Line 1 is a poorly immunogenic lung carcinoma and has been described previously (42). The cell line was cultured in DMEM-H (Life Sciences, Gaithersburg, MD) supplemented with 7% FBS (Life Sciences) and penicillin-streptomycin (Life Sciences).

Retroviral constructs and transduction

The derivation of the CIITA retroviral construct has been described previously (38). Murine CD86 was kindly provided by Peter Linsley, Bristol-Myers Squibb, Princeton, NJ. The CD86 gene was excised from the pcD-NAI/mB7-2 vector with *Xba*I and *Bam*HI, followed by filling in the overhanging ends with the Klenow fragment of DNA polymerase. It was cloned into the LXSP vector (kindly provided by John C. Olsen, University of North Carolina, Chapel Hill, NC) at the *Hpa*I site. Retroviral packaging and transduction of Line 1 cells was done as previously described (38). Briefly, plasmid DNA was transfected into the PA317 helper cell line via calcium phosphate precipitation, and the media were changed the following day. Forty-eight hours after transfection, the supernatant was collected, sterilized by filtration, and stored for later use at −70°C. Cells were transduced by adding 0.5 ml of virus supernatant to Line 1 cells with 8 μg/ml Polybrene (Sigma, St. Louis, MO) for 2 h. The media were changed, and the cells were allowed to grow for 48 h at which time they were split into selection media. Cells were selected in 400 μg/ml geneticin (Life Sciences) (in the case of LXSN-based clones) or 2.5 μg/ml puromycin (Sigma) (in the case of LXSP-based vectors). In cases where cells were transduced with both LXSN-based and LXSP-based vectors, they were first transduced with either LXSN or LCIITASN, then subsequently transduced with LXSP or LCD865P as indicated.

Flow cytometry

The Abs used for these studies were; mouse CD86 Ab (PharMingen, San Diego, CA) and class II Ab BP1072.2 (anti-I-Eb/I-Ab, reactive with haplotypes d, b, p, q, u, j) (provided by Dr. J. A. Frelinger). Secondary Abs used were goat anti-mouse IgG-FITC conjugate (PharMingen) and goat anti-rat IgG FITC (Sigma).

For flow cytometry, cells in mid-log growth phase were harvested and washed twice with 1× PBS containing 0.1% sodium azide. The cells were resuspended at 1×10^7 cells/ml, and 100 μl were used for each sample. The cells were incubated for 30 min with diluted primary Ab (20 μl total volume per sample). The cells were washed three times with 1× PBS-sodium azide and then incubated for 20 min in diluted secondary Ab (20 μl total volume per sample) followed by three washes with 1× PBS-sodium azide. These cells were either analyzed immediately or stored in 2% paraformaldehyde for <1 wk before analysis.

Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, CA) and analyzed using Cyclops software (Cytomation, Fort Collins, CO). Data are presented in table form as the fold induction of the secondary Ab vs the Ab in question. For instance, if the mean channel fluorescence of secondary Ab was 4.0 and the mean channel fluorescence of CD86 Ab was 24.0, then the fold induction is 6.0×.

Tumor studies

BALB/c mice were either purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in University of North Carolina facilities from breeders purchase from Jackson. In all cases, cells in mid-log growth were harvested, washed three times in PBS, and then resuspended at the appropriate concentration. For primary tumor growth assays, mice were injected with the indicated tumor dose (500–1000 cells in 50 μl) in the calf muscle of the hind limb. The mice were individually monitored for tumor growth. In each experiment, there were four to six mice per group. Graphs indicate the mean tumor size ± SE. Each experiment was repeated two to four times, and a representative experiment is shown.

For tumor challenge studies, 2.5×10^6 parental or modified Line 1 cells were irradiated at 10,000 rads. These cells were injected into BALB/c mice i.p. in a volume of 100 μl. One group of mice in each experiment was not injected and served as the control group. One week later, the mice were

Table I. *Expression of CD86 and MHC class II in transduced Line 1 cells*

Name	Used in	Mean Channel Fluorescence vs Control[a]	
		MHC class II	CD86
A. Line 1	Figs. 1–6	1.0	1.2
B. LCD86SP poly	Figs. 1, 5	1.0	12.0
C. LCD86SPD10	Fig. 2	1.0	44.0
D. LCIITASN poly	Figs. 3, 5	7.7	1.1
E. LCIITASNA5	Fig. 4	7.7	1.2
F. LCIITASND12	Fig. 4	6.0	1.1
G. LCIITASNH8	Fig. 4	12.0	0.7
H. LCIITASNF7	Fig. 4	17.1	1.1
I. LCIITASNF6	Fig. 4	18.4	1.1
J. LCIITASNF6/LCD86SPG10	Fig. 5	6.9	46.2
K. LXSN/LXSP	Fig. 6, Table II	1.1	1.4
L. LXSN/LCD86SP	Fig. 6, Table II	1.0	12.0
M. LCIITASN/LXSP	Fig. 6, Table II	2.0	1.3
N. LCIITASN/LCD86SP	Fig. 6, Table II	2.2	11.9

[a] Data are expressed as the fold induction: i.e., the mean channel fluorescence of the specific Ab divided by the mean channel fluorescence of the secondary Ab control. Boldface type indicates the gene coded for by that construct.

injected with wild-type Line 1 cells at the indicated dose. The mice were individually monitored for tumor growth. In each experiment, there were four to eight mice per group.

All mouse experiments were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. University of North Carolina animal facilities are accredited by the American Association for the Accreditation of Laboratory Animal Care.

Results

CD86 expression increases immunity against the Line 1 carcinoma

Line 1 is a poorly immunogenic spontaneous lung tumor derived from BALB/c mice (38). CD80 has been shown to cause a decrease in primary tumor growth in this system by enhancement of NK cell killing and not increased CTL activity (43). However, the influence of CD86 in Line 1 immunity has not been investigated. We began our studies by investigating the effect of CD86 on the primary tumor growth in syngeneic BALB/c mice. Line 1 cells were transduced with recombinant retrovirus encoding the gene for murine CD86. This virus also contained the gene for puromycin resistance. Cells were selected in puromycin, and the polyclonal population was analyzed for CD86 expression by flow cytometry.

As shown in Table I, row A, Line 1 cells are negative for MHC class II and CD86. However, after transduction with CD86 retrovirus, surface CD86 expression was increased 12-fold in the polyclonal population (Table I, row B). Parental cells and CD86-transduced cells exhibited no difference in growth rates in vitro (data not shown). This polyclonal population of cells was injected into immunocompetent BALB/c mice in the calf muscle. Mice injected with unmodified Line 1 cells grow tumors progressively and require sacrifice after 19 to 26 days. CD86-modified polyclonal Line 1 tumors grew significantly slower than control tumors, delaying tumor growth by ~10 days (Fig. 1). The mean leg diameter of Line 1 control mice at day 15 was similar to the mean leg diameter of CD86-expressing tumors at day 25. These data show that CD86 is beneficial in the Line 1 tumor model, and a test of a cloned population of CD86 expressing tumor was warranted because the CD86 polyclonal cells did have a low but significant number of puromycin-resistant cells that had undetectable surface expression of CD86. When using a polyclonal population, it is possible that the CD86-negative cells eventually outgrew the CD86-expressing

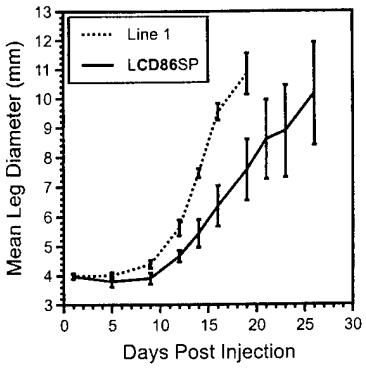

FIGURE 1. Polyclonal Line 1 cells transduced with CD86 show a decreased tumor growth rate. Line 1 cells were transduced with the vector LCD86SP and selected for puromycin resistance. This polyclonal cell population was injected at 500 cells/mouse into the calf muscle of BALB/c mice. Mice were monitored 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. ·····, Unmodified Line 1 growth; ——, mice injected with polyclonal CD86 Line 1 cells.

cells, because any immune response elicited by these cells was insufficient to overcome the growth of these negative cells. To investigate this possibility, the LCD86SPD10 clone was isolated by limiting dilution and tested by flow cytometry (Table I, row C). This clone expressed high, stable levels of CD86 and maintained stable expression over time in culture (data not shown). This clone was injected into BALB/c mice, and these mice were monitored for tumor growth. As shown in Fig. 2, LCD86SPD10 grew with greatly delayed kinetics. At the day of sacrifice for the control mice, the difference in the tumor size of the mice in the two groups was highly significant ($p < 0.005$). In fact, of eight mice in two experiments, four did not develop tumors, demonstrating the great beneficial effect of CD86 expression in the Line 1 model system.

CIITA expression in the Line 1 carcinoma can be beneficial or detrimental depending on expression level

Line 1 expresses low to undetectable levels of MHC class I and is MHC class II negative (38). However, IFN-γ can effectively induce MHC class I but not MHC class II in these tumor cells (data

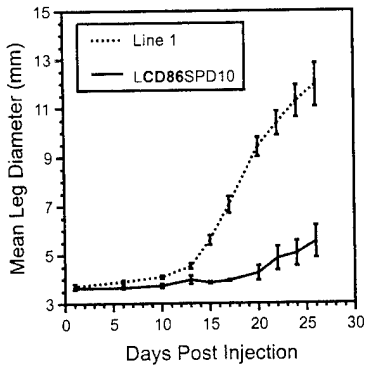

FIGURE 2. High stable expression of CD86 on Line 1 cells leads to growth attenuation and decreased tumorigenicity. The Line 1 clone, LCD86SPD10, expressing high stable levels of CD86, was injected at 500 cells/mouse into the calf muscle of BALB/c mice. Mice were monitored individually for tumor growth. Each line represents the mean leg diameter of four to six mice per group. Error bars represent the SEM for each group. ·····, Unmodified Line 1 growth; ——, CD86 group. Three of four mice injected with CD86 polyclonal cells did not grow tumors in this experiment.

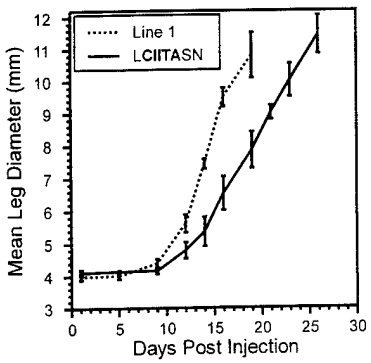

FIGURE 3. Polyclonal Line 1 cells transduced with CIITA show a decreased tumor growth rate. Line 1 cells were transduced with the vector LCIITASN and selected for geneticin resistance. This polyclonal cell population was injected at 500 cells/mouse into the calf muscle of BALB/c mice. Mice were monitored 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. ·····, Unmodified Line 1 growth; ——, CIITA group.

not shown) (44). We have previously shown that CIITA-transduced Line 1 cells up-regulate expression of both MHC class I and class II, whereas in a sarcoma model only MHC class II is induced (38). This suggests that Line 1 cells modified to express CIITA may have potential changes in tumorigenicity and immunogenicity that could be mediated by MHC class I, MHC class II, or both. The use of CIITA in combination with CD86 was also worthy of analysis because cooperative interaction between these molecules in tumor immunity induction has not been determined.

To study the effectiveness of CIITA/CD86 therapy, we first determined the effect, if any, that CIITA alone has in the Line 1 model. Cells were transduced with CIITA coding retrovirus, selected for G418 resistance, and analyzed for MHC class II expression (see Table I, row D). CIITA effectively induced class II expression, with >60% of the polyclonal population expressing MHC class II Ags. As shown in Fig. 3, polyclonal CIITA expression in Line 1 led to a small but significant decrease in the overall tumor growth rate of this tumor line. High stable expression of CIITA could lead to enhanced Ag presentation, hence increased tumor immunity. However, it was also possible that high expression of class I MHC leads to loss of surveillance by NK cells, hence hastening tumor growth. Also, high expression in the absence of costimulation may, in fact, lead to anergy, exacerbating tumor growth.

To test these possibilities, several different CIITA-transduced clones that expressed a range of MHC class II proteins were examined. MHC induction in clones tested for growth in mice ranged from 6- to 18-fold enhancement of class II expression. Based on their surface class II phenotype, these clones could be grouped into two classes; lower expressers (clones LCIITASNA5 and LCIITASND12, Table I, rows E and F, respectively); and higher expressers (clones LCIITASNH8, LCIITASNF7, and LCIITASNF6; Table I, rows G, H, and I, respectively). These clones were injected into mice, and the rate of tumor growth was monitored individually. The results of these experiments are presented in Fig. 4.

The tumors could be grouped into three categories based on their in vivo growth rates: those that grew with delayed kinetics relative to parental tumors (Fig. 4*A*); those that grew at approximately the same rate as parental tumors (Fig. 4*B*); and the one tumor that grew faster than parental Line 1 (Fig. 4*C*). The two tumors demonstrating the slowest growth also had the lowest expression of MHC class II (see Table I, rows E and F). All tumors that grew as fast as parental tumor had expression higher than that of the LCIITASN polyclonal cells. The clone that demonstrated faster growth than that of the parental clone (Fig. 4*C*) had the

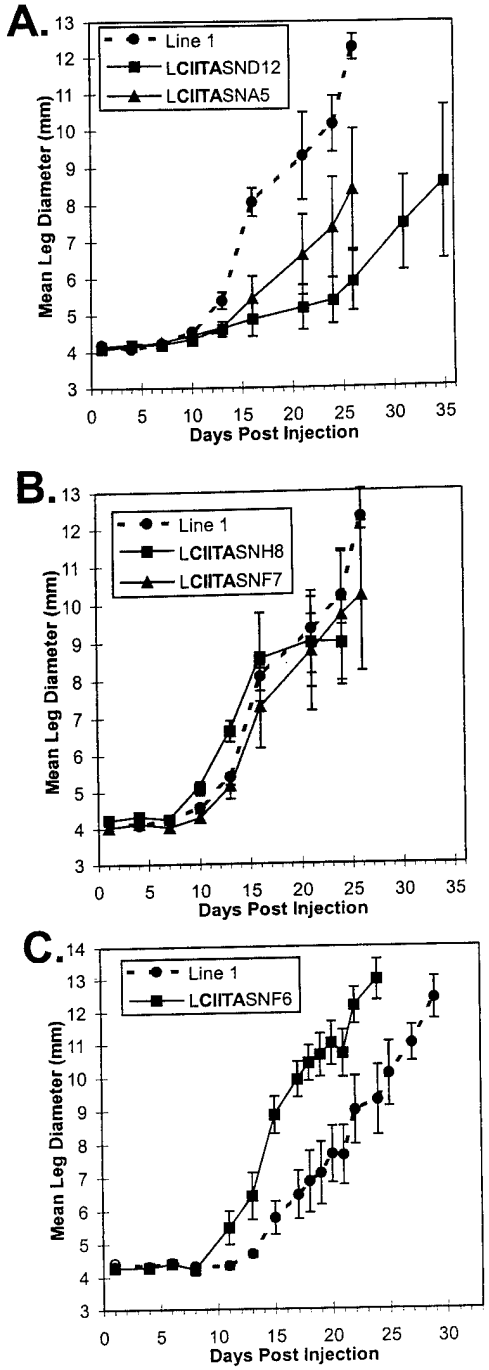

FIGURE 4. Line 1 clones expressing low levels of CIITA have attenuated growth whereas clones expressing high levels of CIITA have unchanged growth or grow faster than mice injected with control cells. Line 1 clones expressing varying levels of CIITA (as assessed by surface class II expression; see Table I) were injected into mice at 500 cells/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. ●, Unmodified Line 1. *A*, results for clones LCIITASNA5 (▲) and LCIITASND12 (■). *B*, results for clones LCIITASNF7 (▲) and LCIITASNH8 (■). *C*, results for clone LCIITASNF6 (■).

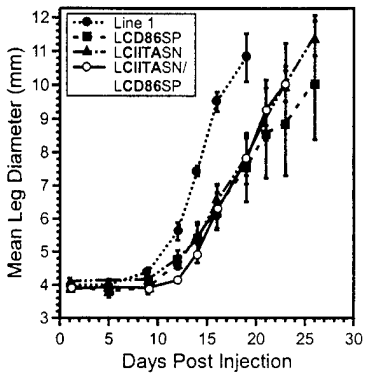

FIGURE 5. Polyclonal Line 1 cells coexpressing CD86 and CIITA have a growth phenotype intermediate to that of CIITA or CD86 expressed alone. The LCIITASN polyclonal population was transduced with CD86-expressing retrovirus, and the polyclonal populations were injected into mice. Cells were injected at 500 cells/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. ●, Unmodified Line 1; ■, LCD86SP polyclone; ▲, LCIITASN polyclone; ○, LCIITASN/LCD86SP polyclone.

highest expression level of MHC class II (see Table, 1 row I). In the experiment shown in Fig. 4*B*, one tumor cell clone (LCIITASNH8) had a significantly faster initial growth rate that leveled out in time. These data suggest that high levels of class II and/or class I has a deleterious effect on the primary growth of the Line 1 tumor.

Lack of cooperation between CIITA and CD86 in the induction of tumor immunity

The results with CD86 suggest that if CIITA and CD86 cooperate in the induction of an antitumor response, then coexpression of both proteins on the surface of cancer cells may increase the immunity against the tumor. If they did not cooperate, then no difference in overall growth should be observed. To test this hypothesis, the Line 1 polyclonal population expressing CIITA was additionally transduced with the CD86 retroviral construct (puromycin resistant). Double-resistant cells were isolated as a polyclonal population and examined by flow cytometry as shown in Table I, row N. These polyclonal cells had lower levels of MHC class II expression than did the singly selected pools, perhaps demonstrating the loss of expression during the second selection. These polyclonal CIITA/CD86-expressing Line 1 cells were injected into mice and compared with the both the CIITA and CD86 singly transduced cells. As shown in Fig. 5, the doubly transduced pool did not have significantly changed growth kinetics from that of either the CIITA or CD86 single populations. These data suggest that CIITA and CD86 do not cooperate to protect animals from primary tumor growth.

If our hypothesis that high expression of MHC class I and/or class II in the Line 1 lung carcinoma has a deleterious effect in tumor immunity is correct, then the expression of CIITA in the context of high stable expression of CD86 should lead to an increased tumor growth rate and greater tumor incidence. To this end, we transfected the fast growing CIITA clone (LCIITSNF6) (see Table I, row I, and Fig. 4*C*) with CD86 and examined clones for expression of CD86 equal to that of the CD86 clone shown in Fig. 2. As shown in Table I, row J, this double clone had levels of expression of MHC class II lower than that of LCIITASNF6, again perhaps reflecting loss of expression during the second transduction and selection; however, CD86 expression was equal to that of the LCD86SPD10 clone. This clone (LCIITASNF6/LCD86SPG10) was injected into mice, and the growth rate was compared with that of parental Line 1 and both single clones. As shown in Fig. 6, this clone did not exhibit the slower growth rate of the CD86-transduced LCD86SPD10 clone, but it did grow more slowly than the CIITA-transduced clone LCIITASNF6. Additionally, all mice injected with the CIITA/CD86 clone grew tumors progressively, as opposed to the 50% of mice injected with the CD86 tumor that remained tumor free (Fig. 2). We interpret these data to mean that high expression of CIITA is actually deleterious and reversed the beneficial effects obtained from high stable expression of CD86 in the Line 1 tumor model system.

Vaccination with CD86-expressing tumor induces immunity whereas CIITA expression is deleterious

All the data presented up to this point are primary tumor growth assays in which the cells are modified and injected into mice. Clinical protocols rely on the resection of primary tumor, ex vivo modification, and cellular irradiation followed by injection back into the patient. To more closely approximate this approach, cells were modified, irradiated, and injected into the mouse. This was followed by injection of unmodified cells, and tumor growth was monitored. Since Line 1 is a poorly immunogenic tumor, we could test the ability of CIITA- and/or CD86-modified tumors to stimulate an immune response to subsequent challenge with control tumor. For this experiment, we chose an injection and challenge scheme that would give ~50% tumor incidence in the group injected with vector control. This was necessary because if CIITA and/or CD86 were beneficial, fewer mice would grow tumors, but if CIITA and/or CD86 were detrimental, then more mice would develop a tumor burden.

As shown in Table II, no mice were tumor free at day 28 without injection of irradiated cells. However, 53% of the mice injected with irradiated vector control cells had no measurable tumors at day 28. On the other hand, the CIITA group had slightly fewer tumor-free individuals than did vector control (compare 53% with

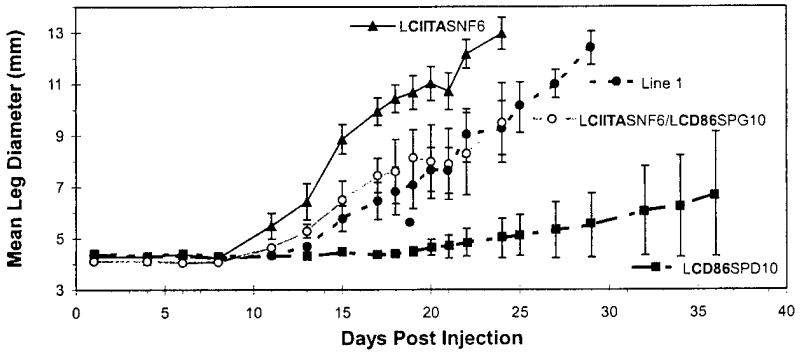

FIGURE 6. Primary tumor growth assays of clonal tumor cells lines expressing CIITA and CD86. Clonal cell populations described in Table I were injected at 500 cells/mouse into the calf muscle of BALB/c mice. Mice were monitored individually for tumor growth. Each line represents the mean leg diameter of four to six mice per group. Error bars represent the SEM for each group. ●, Unmodified Line 1; ■, LCD86SPD10; ▲, LCIITASNF6; ○, LCIITASNF6/LCD86SPG10.

Table II. *Changes in immunogenicity associated with CD86 and/or CIITA polyclonal expression in Line 1 tumors*[a]

Exp.	Cells/Mouse	Irradiated Cells Injected				
		None	Vector	CD86	CIITA	CIITA/CD86
1	1000	0/5	3/5	4/5	2/5	2/5
2	1000	0/4	1/4	3/5	2/5	3/5
3	1000	0/4	3/4	3/4	3/5	1/5
4	5000	0/6	3/6	4/6	3/8	3/9
Total tumor free at day 28		0%	53%[b]	70%[b,c]	43%	38%[c]

[a] Number of mice tumor free at day 28 vs total injected.
[b] $p < 0.05$, vector compared with CD86.
[c] $p < 0.05$, CIITA/CD86 compared with CD86.

43%); however, this difference was not significantly different. Mice injected with irradiated CD86 tumors had significantly more tumor free mice than did vector control (70% compared with 53%, respectively). Only 38% of mice injected with CIITA/CD86 coexpressing tumors were tumor free at day 28. The significant decrease in the protection afforded by CD86 indicates that CIITA expression in the Line 1 system abrogates CD86-induced immunity, resulting in an increased number of mice with tumor burden. These data demonstrate a positive role for CD86 in the induction of Line 1 immunity, while CIITA appears to have a deleterious role in tumor immunity in this model system.

Discussion

CD86 is important for the induction and maintenance of an immune response. Engagement of MHC class I and/or class II molecules without an additional costimulatory signal can lead to the induction of anergy. Mice with a genetic disruption of the CD80 costimulatory molecule retained much of their costimulatory capacity (6). It was later discovered that CD86 could provide a compensatory signal in these CD80 knockout mice, underscoring the importance of the CD86 protein (6, 7). MHC proteins are expected to be critical for the ability of CD86 to induce lasting protective immunity. CIITA is a master regulatory of the MHC class II processing and presentation pathways that has been also shown to induce MHC class I in some cell lines (38, 39). The concomitant expression of class I and class II with CD86 is a likely protocol for tumor gene therapy.

In this report, we show that introduction of the gene coding for the mouse CD86 costimulatory molecule into the murine lung carcinoma, Line 1, results in a markedly decreased tumor growth rate. CD86 expression in a tumor vaccine model also exhibits some efficacy. These results are interesting since expression of CD86 without MHC molecule expression would be expected to be ineffective. However, Line 1 does express very low levels of class I, the levels of which are inducible with cytokines such as IFN-γ (38, 44). It may be possible that when cells are injected into mice, cytokine expression in the local environment leads to up-regulation of MHC class I and cooperative interaction with CD86 on engineered cells, leading to tumor rejection. These findings lend additional support for the emerging theory that CD86 can be an important costimulatory molecule to be considered for tumor immunotherapy.

Early reports suggest that CD86 expression was ineffective in primary tumor therapy model systems. When direct comparisons were made between CD80 and CD86, CD80 was found to be the most effective (9–12). Furthermore, CD86 was totally ineffective in other tumor systems (13). To some degree, the ineffectiveness of CD86 therapy was believed to be due to the propensity of CD80 to stimulate a Th1 T cell response, while CD86 was thought to induce a Th2 phenotype (45). Although CD86 was shown to be effective in the reduction of primary tumor growth in the CMT93 tumor, this expression actually led to a loss of immunogenic potential (46). These observations have undoubtedly deterred some researchers from pursuing the use of CD86 as a potential immunotherapeutic in their tumor systems. Increasingly, however, reports are showing that CD86 has marked effectiveness in several model systems. CD86 has been shown to induce T cell proliferation in a MLR and can effectively generate CTL (47). This suggests that CD86 can induce a CTL response against cancerous cells expressing CD86. It is also possible that a Th2-mediated Ab response can be protective in tumor immunity, since Abs have been shown to be efficacious in some cancers (48, 49). Several other tumor model systems have tested the effectiveness of CD86 therapy. CD86 expression in a vaccinia delivery system leads to protective tumor immunity (14). Other reports demonstrate the tumor model specificity of the CD86-mediated immunity (15, 16). In one study, expression of CD86 was clearly superior to that of CD80 in adenocarcinoma and melanoma systems (17). These reports and the data presented here show that the potential for immune recognition via the CD86 molecule varies according to the system being used. In human tumor systems it is possible that a means for testing the potential of tumor immunity via CD86 (such as in vitro T cell stimulation) can be used to determine in advance whether a given costimulatory molecule will be advantageous in human immunotherapy.

CIITA expression in the Line 1 system leads to increased survival in primary tumor challenge when the expression levels of CIITA are low (as measured by surface class II expression) (Figs. 3 and 4*A*). However, when expression levels of CIITA are high, the cells lose their slow growth phenotype (Fig. 4*B*), and in certain situations, the cells can even grow faster than unmodified tumor (Fig. 4*C*). Furthermore, when CIITA-modified cells are used in a tumor vaccine setting, there is no protection from subsequent control tumor challenge (see Table II). Indeed, even more mice succumb to their tumors than when injected with vector control tumor. Mice injected with CIITA expressing tumor (either polyclonal or various clones) did not have increased CTL activity against Line 1 as measured using T cells obtained from tumor-infiltrating lymphocytes or from splenic lymphocytes (data not shown). These data indicate a negative role for CIITA expression in the absence of costimulatory molecules, perhaps through tolerance induction.

There are several possible explanations for the ineffectiveness of CIITA immunotherapy. In instances where we have used clones, it is possible that simple clonal variation may play a factor; however, the data using polyclonal pools agree with the findings with clones, suggesting that clonal variation contributes minimally. There may also be nonimmune factors at work, such as the ability or inability to vascularize, but no other data in the literature indicate that CIITA and/or CD86 affects these processes. Finally, the changes in growth of the transductants may reflect differences in host immunity to those cells. This is the hypothesis that will be discussed in detail.

Part of the central tenet of MHC class I and class II presentation is that efficient induction of a T cell response must involve at least two signals. If there is MHC-peptide recognition in the absence of costimulation, an anergic response or the deletion of those reactive T cells may result (2, 3). In the situation where CIITA-expressing cells are presenting tumor Ag to T lymphocytes, the lack of a costimulatory signal on the tumor cell may lead to one of these events. We have also demonstrated that the coexpression of CIITA and CD86 does not lead to enhanced tumor immunity as measured by either primary tumor growth or tumor vaccination. Indeed, in

the case of the fast growing LCIITASNF6 clone, coexpression of CD86 leads to an intermediate phenotype of growth faster than that of cells expressing B7-2 alone. In tumor challenge assays, coexpression of CIITA and B7-2 abrogated the protective effect of CD86 alone. These data show that CIITA is ineffective in this model system and in some cases represents a negative factor. However, the combination of CIITA and CD86 may be effective in other tumors. These results bring into question the prudence of beginning human CIITA tumor vaccine trials without being able to ascertain whether CIITA could lead to the induction of tolerance to the tumor that is being treated in proposed CIITA human trials.

The second possibility for failure of CIITA therapies involves NK cells. NK cells are lymphocytes that survey cells for those that have aberrant expression of MHC class I. Much progress has recently been made on the identification of NK cell receptors that are responsible for this surveillance (reviewed in Refs. 40 and 41). Line 1 has very low to nondetectable expression of MHC class I, making it a potential NK cell target (43). As we have shown previously, CIITA expression in the Line 1 system leads to up-regulation of transcription and surface expression of MHC class I (38). The Line 1 clones that have the highest MHC class II expression also have the highest MHC class I expression (data not shown). This suggests that cells with high CIITA-mediated MHC class I expression may not be susceptible to NK cell killing. This would be expected to lead to faster initial growth as is seen with the LCIITASNF6 clone. We believe that the most likely reason CIITA expression abrogates CD86 protection is the lack of NK cell surveillance in the coexpressing CIITA/CD86 transfectant. This hypothesis would be best tested in NK-deficient mice. However, the beige strain is not on the H-2^d background; therefore, experiments conducted in nude mice are the only viable option by which to elucidate the mechanism of this effect.

A final reason that CIITA expression alone may be ineffective in this model system is the incomplete reconstitution of the class II processing and presentation pathway. An early report by Siegrist et al. (36) demonstrated that a human melanoma cell line engineered to express CIITA had induced surface expression of MHC class II but was unable to properly process and/or present exogenous Ag. Surface loading of MHC class II with free peptide was possible and led to the induction of an immune response. This was the first suggestion that CIITA alone did not reproduce the entire class II pathway. A subsequent report showed that the molecule missing in this cell line was the serine protease, cathepsin S (37). The gene for this molecule was not inducible by CIITA in this cell line. We and others have shown that in several other cell systems CIITA expression alone can reconstitute class II processing and presentation (33–35). CIITA expression induces only the heavy chain, not accessory proteins (38, 39). This indicates that CIITA enhancement of class I presentation is limited to cells in which there are sufficient accessory proteins to support an increased level of heavy chain product. These reports suggest once again that the ability of CIITA to fully reconstitute class I Ag presentation may be cell type dependent.

One of the earliest papers dealing with CIITA and Ag presentation suggested the benefit of CIITA in immunotherapy, either with or without costimulation (36). Our results with the SaI sarcoma model system (33) and now with the Line 1 system suggest that by itself, CIITA expression may have little benefit. In a worst case scenario, CIITA may be a negative factor in vaccination strategies. Even combination gene therapy with CD86 and CIITA lacked efficacy in our model system. This report indicates that the use of CIITA in clinical protocols without additional costimulation or without first defining the costimulatory capacity of the tumor being treated should be approached with extreme caution. On the other hand, CIITA therapy with other costimulatory molecules such as CD40 ligand, CD80, and ICAM-1 should be tested to assess what contribution they might have for CIITA tumor immunotherapy.

Acknowledgments

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413 THE CANCER PROCESS AS A TYPE OF IMMUNOCOMPLEX HYPERSENSIBILITY INVOLVING C3b, NATURAL KILLER CYTOTOXICITY AND ANTIBODY-DEPENDENT CELL CYTOTOXICITY TY: PROPOSALS FOR TUMOUR IMMUNOTHERAPY AND VACCINE

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ABSTRACT - I have previously assumed that stem tumour cells are 'para-embryonal cells' (PECs) poor or missing in major histocompatibility complex (MHC) antigens and rich in heat shock proteins (HSPs). PECs might induce adjoining differentiated hyperplastic cells to also express tumoral phenotype and properties, thus transforming them into 'differentiated para-embryonal cells' (DPECs), MHC-endowed. In such a way, PECs, MHC-lacking, would be automatically surrounded by DPECs, MHC-endowed: this tumour organization was experimentally found by Cordon-Cardo et al in a variety of cancers. Now, I suggest that such a tumour histology might preferentially induce an anti-DPEC T cell immune response which, sparing PECs, might release increasing amounts of DPEC antigens in the peritumour site. DPEC antigens might increase synthesis of specific antibodies and subsequent immunocomplex formation at the peritumour site. Here, abundant immunocomplexes might react through their Fc pieces with CD16 receptors of ADCC-endowed immune cells. These cells would thus be stimulated to secrete their lytic factors before and without their coming into contact with target tumour cells. On the other hand, abundant immunocomplexes at the peritumour site might massively activate the complement system, thus generating large amounts of C3b. C3b might react with CD11b receptors of natural killer (NK) cells, stimulating them to also secrete their lytic factors in an ectopic way at the peritumour site, thus impairing NK cytotoxicity. In such a way, in the absence of ADCC and NK cytotoxicity, a tumour cell enhancement might easily occur. In the light of these ideas, a strategy for antitumour immunotherapy is then proposed, aimed at avoiding interference phenomena between humoral factors and NK cytotoxicity or ADCC, and at gradually removing tumour histologic organization that impairs and upsets the immune response. Moreover, a strategy for antitumour vaccine is proposed, in which only association between pre-sensitized B cells and ADCC-endowed cells might give simultaneously specificity and immunological memory in the immunesurveillance against newly arising MHC-lacking HSP-rich stem tumour cells (PECs).

414 **Novel Cancer Therapy Utilizing Tumor-Specific Dendritic Cell Immune Responses**

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Dendritic cells have been the focus of intense interest in recent years given their ability to mediate tumor rejection. A difficulty with this strategy is the time consuming *ex vivo* manipulations that are required to generate effector cells. We have used two approaches to specifically target an immune response via dendritic cells *in vivo*. First, we used a virus that specifically infects dendritic cells at the inoculation site. Injection of mice with this vector coding a gene for a model tumor antigen renders the host immune to subsequent tumor challenge. Viral injection leads to both antibody and CTL responses against the model tumor antigen. Of the mice that survive tumor challenge, 50% are also immune to wild type challenge, suggesting epitope spreading. Our second approach has been to modify tumor cells to express the complement component C5a, a chemotactic factor for granulocytes, macrophages and dendritic cells. We show that tumor cells expressing C5a induce dendritic cell chemotaxis. Mice injected with C5a tumors grow these tumors for a short time, then spontaneously reject the tumor. Survivors are immune to subsequent wild type challenge. These studies suggest that dendritic cell immunotherapy can be accomplished without *ex vivo* modification. Supported by U.S. Army Breast Cancer Grant #DAMD-17-97-1-7142 to BKM and NIH-AI-29564 and NCI-48185 to JPYT.

415 Tolerance induction by way of gene therapy.

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Efficient methods for gene transfer into hematopoietic cells may enable the expression of antigens involved in autoimmune processes for the induction of tolerance. We have developed a retroviral construct which displays antigens at the N-terminus of an IgG heavy chain, and expressed this in bone marrow progenitor cells and in peripheral B cells for tolerance induction in mice. We also used transgenic mice that express a foreign antigen constitutively in B cells. While resting B cells had no effect on an ongoing immune response *in vivo*, LPS-stimulated B-cell blasts from the transgenic mice induced tolerance in already primed BALB/c mice. Since LPS and CD40L upregulate B7.2 expression, the tolerogenicity of activated B cells can not be explained by the lack of costimulatory ligands. Interestingly, even activated transgenic B cells failed to present the endogenous produced protein to a specific T cell clone, althought we know that they secrete nanomolar amounts of fusion protein. Nonetheless, these cells were competent to present the exogenous protein to a specific T cell clone; therefore, there is no general defect in APC capacity of these transgenic B cells.

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416 **Experimental autoimmune encephalomyelitis induced in B6.C-H-2^{bm12} mice by oligodendrocyte glycoprotein: Effect of MHC class II mutation on immunodominant epitope selection and fine epitope specificity of encephalitogenic T cells**

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The bm12 mutation in the MHC class II I-A^b molecule can profoundly influence experimetal autoimmune diseases. We have studied the effect of this mutation on experimental autoimmune encephalomyelitis (EAE), induced in H-2^b mice by myelin oligodendrocyte glycoprotein (MOG). MOG 35-55 peptide (pMOG 35-55), representing the immunodominant encephalitogenic region for H-2^b mice, is also a strong encephalitogen for H-2^{bm12} mice. Although the differences in fine epitope specificity and TCR V gene usage between encephalitogenic T cells from H-2^b and H-2^{bm12} mice were subtle, H-2^b and H-2^{bm12} antigen presenting failed to effectively cross-present pMOG 35-55 non-syngeneically to I-A^b/ and I-A^{bm12}/pMOG 35-55-specific T cells. In contrast to pMOG 35-55-induced EAE, the incidence and clinical severity of the disease induced by recombinant human MOG (a.a. 1-121) (rhMOG) in H-2^{bm12} mice were considerably reduced, as compared to those in H-2^b mice. The primary response to rhMOG was associated in H-2^b mice with a dominant response to pMOG 35-55, while in H-2^{bm12} mice a co-dominant response to pMOG 35-55 and pMOG 94-116 was observed. pMOG 94-116 is a cryptic epitope in H-2^b mice, as specific T-cells selected from mice immunized with pMOG 94-116 did not react to rhMOG in contrast to I-A^{bm12}/pMOG 94-116-specific T-cells. pMOG 94-116 was not encephalitogenic for H-2^b or H-2^{bm12} mice, and the reduction in clinical incidence and severity of rhMOG-induced EAE in H-2^{bm12} mice may be related to regulation by pMOG 94-116-reactive T-cells in these mice.

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ROLE OF RECOMBINANT INTERFERON-ALPHA IN AN EFFECTIVE MURINE EL-4 TUMOR IMMUNOTHERAPY. T. Steele AND M. Shoop. Mercer University School of Medicine, Macon, Georgia, 31207.

Reovirus type 3 is a potent inducer of alpha/beta interferons in mice. We have investigated whether murine recombinant interferon-alpha (IFN-alpha) could replace reovirus in a murine tumor immunotherapy. The therapy is administered as follows. EL-4 tumor-bearing B6D2F1 mice are treated at day 4 (following tumor injection) with 9 mg/kg 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) followed at day 6 with 1 x 109 plaque-forming units of reovirus type 3. 65-80% of the therapy mice are cured, whereas 0% and 20% of tumor-bearing mice survive that were treated with reovirus or BCNU alone, respectively. Administration of IFN-alpha could replace reovirus in the therapy when doses as small as 500 units were used. 10,000 units/ml produced survival levels equivalent to reovirus. Although IFN-alpha treatment could produce high levels of natural killer cell activity, cyclosporine could abrogate the effects of the therapy without affecting natural killer cell activity, signifying that natural killer cells did not play a significant role in eliminating tumor in our system. IFN-alpha was found to inhibit the growth of EL-4 tumor in cell culture, suggesting that inhibition of tumor growth may be one mechanism whereby IFN-alpha may aid in the eradication of tumor. Currently we are quantifying the amount of IFN-alpha that is produced in response to virus. In addition, we are investigating whether anti-IFN antibodies can abolish the effect of the reovirus therapy.

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RECOMBINANT SV40 T-AG IMMUNIZATION PROVIDES TUMOR IMMUNITY IN AN EXPERIMENTAL PULMONARY METASTASIS MODEL. A.M.Watts, M.H.Shearer, R.C. Kennedy
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We have examined the ability of recombinant simian virus 40 (SV40) large tumor antigen (T-ag) to prevent the establishment of experimental pulmonary tumor foci and subsequent metastasis. The experimental murine pulmonary metastasis model used in this study employs computer-assisted video image analysis to allow for accurate measure of tumor foci establishment and growth in the lungs at various days post-challenge. Survival data for various doses of SV40-transformed tumorigenic cells and metastasis to distal organs have also been characterized in this model and serve as additional end points in determining levels of protection. Baculovirus-derived recombinant SV40 T-ag was injected into BALB/c mice prior to challenge with an intravenous injection of syngeneic SV40-transformed tumorigenic cells. Following challenge, lung tumor foci enumeration and survival data for the groups of mice were obtained. No detectable sign of lung tumor foci development was observed in animals immunized with recombinant SV40 T-ag. In addition, immunized animals survived greater than 90 days post-challenge. Control mice developed extensive lung tumor foci and succumbed to lethal tumor within 4 weeks post-challenge. Antibodies specific for SV40 T-ag were detected in the serum of immunized mice by ELISA. These data indicate that immunization with the recombinant SV40 T-ag induces complete protection from pulmonary tumor metastasis.

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Subtractive cloning of a novel GAS gene family member associated with the lymphoid lineage and B lymphomagenesis C.-X. Wang, B.C. Fisk, Y.-K. Chow, J. Braun. Dept. of Pathology, UCLA School of Medicine, Los Angeles 90095-1732

We have previously established a murine model of B cell lymphomagenesis, using premalignant cell lines (DACs) which are tumorigenic only in immunodeficient mice, and malignant progressor daughter lines (MVs) which are tumorigenic in wildtype mice. RDA (Representational Difference Analysis) was employed to identify the differentially expressed genes in DACs versus MVs. 853 genes were screened by multi-array expression analysis and DNA sequencing, revealing 106 differentially expressed genes in four functional gene families. Surprisingly, most differences involved expression loss in the MV cells. One novel gene identified in this screen was GAS-2.24, which was highly expressed in DAC but not MV lines. GAS-2.24 is a new member of the GAS (growth-arrest-specific) gene family, the first associated with the lymphoid lineage. Members of this gene family encode a putative surface glycoprotein with four transmembrane domains. GAS-2.24 is expressed in normal B cell development, and is selectively lost in fully malignant B cell tumors and cell lines. GAS genes have been previously limited to the neuronal, epithelial, and mesenchymal cell lineages, and play roles in growth-regulatory signaling and intercellular junction formation. The normal developmental pattern of GAS-2.24 expression, and gene transfer of wild-type and dominant-negative GAS-2.24 on host-tumor interaction will be presented. Supported by NIH CA12800,the Lymphoma Research Foundation, and Jonsson Comprehensive Cancer Center.

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THE MHC CLASS II TRANSACTIVATOR (CIITA) REDUCES TUMORIGENICITY AND IMMUNOGENICITY IN A MURINE LUNG CARCINOMA MODEL. B. K. Martin[1], J. G. Frelinger[2], and J. P.-Y. Ting[1]. [1]Lineberger Comp. Cancer Ctr., Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295. [2]Dept. of Micro. and Immunol., Univ. of Rochester Sch. of Med., Rochester, NY, 14642.

Our previous work has shown that in the Line1 murine lung carcinoma model, transduction of the MHC class II transactivator (CIITA) results the induction of MHC class I as well as class II expression. These studies suggested that tumors expressing both MHC class I and class II induced by CIITA may be less tumorigenic and more immunogeneic. In the current studies, we demonstrate that mice harboring CIITA expressing Line1 tumors have a modest increase in mean survival time as compared to mice with control tumors. Similarly, B7-2 expressing Line1 tumors have a delayed growth pattern. However, tumors expressing both CIITA and B7-2 show no cooperative decrease in tumorigenicity. The costimulatory molecule B7-1 had little effect on tumor growth, either alone or in combination with CIITA. Preliminary studies on the immunogenicity of CIITA expressing Line1 tumors show that CIITA expressing tumors induce a protective immune response that results in a low number of mice surviving wildtype challenge relative to mice injected with control tumor. Further, tumors expressing both CIITA and B7-2 or B7-1 show enhanced immunogenicity such that fewer than half of the mice grow tumors. These studies show that CIITA may have an impact in human tumor immunotherapy. These studies were supported by NIH grants AI29564 and NCI48185 to J.P.-Y.T. and US Army Breast Cancer Fellowship DAMD17-97-1-7142 to B.K.M.

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gp96 Engineered for Secretion of Tumor Peptides and for Vaccination against Cancer. K. Yamazaki, J. Spielman, G. Spruill, and E.R. Podack. Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Fl 33101

Mouse gp96 can induce specific immunity to the tumor from which it is isolated and may have great practical importance for vaccination and immunotherapy against cancer. In this study, we developed a generic vector expressing human gp96, by deleting the endoplasmic reticulum retention signal, KDEL, and adding the CH2 and CH3 domain of murine IgG1 in order to facilitate detection by ELISA and purification by affinity chromatography. After transfection, gp96-Ig was detected in the culture supernatant of NIH3T3, EL4, E.G7 (EL4 transfected with ovalbumin), LLC, P815, MC57, B16F10 and SCLC (small cell lung cancer) cell lines by ELISA. SDS PAGE of the purified product reveals 3-4 closely spaced 120kD bands. E.G7-gp96-Ig was rejected in C57BL/6 mice, while E.G7 developed tumor after subcutaneous injection. After two vaccinations of live E.G7-gp96-Ig, rechallenged E.G7 was rejected. However, gp96 purified from NIH3T3-gp96-Ig-Ova could not protect E.G7 rechallenge. These results suggest that gp96 secreted from E.G7-gp96-Ig holds tumor peptides of E.G7 and can induce tumor immunity against E.G7. These results also suggest that ovalbumin peptides are not as potent in inducing immunity as the mixed peptides produced by ovalbumin transfected EL4.

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VACCINATION OF PMEL17, A SELF MELANOMA-ANTIGEN REJECTS THE MELANOMA IN PMEL17 DEFICIENT MICE. Anjaiah Srirangam, Godwin Q.Wolisi, Willem Overwijk, Nicholas P. Restifo, Vinay S. Dass and Byoung S. Kwon. Indiana Univ. Sch. of Med., Indianapolis, IN, 46202, and National Cancer Inst., Bethesda, MD, 20892

Pmel 17, self melanoma-antigen, is a member of melanosomal proteins shown to be a potential candidate for the development of tumor vaccine for the treatment of melanoma. Pmel 17 vaccination, however, showed less than satisfactory results in treating murine and human melanoma..We have hypothesized that the high affinity CTLs, that are capable of rejecting melanoma have been deleted in the normal mice. To test this hypothesis, we have generated Pmel 17 KO mice and studied the immune response to Pmel 17 vaccination. Mice in each group (Pmel 17-/-, Pmel 17+/- and Pmel 17+/+) were immunized either with rVV-β-gal or rVV-mpmel17. After three weeks of immunization, all mice were challenged with B16 melanoma cells subcutaneously. Only Pmel 17 KO mice, which were vaccinated with rVV-mPmel 17, rejected B16 melanoma completely. Melanoma was developed in the mice of all other groups. These results demonstrate that the immune responses that are properly directed to Pmel 17 can irradicate melanoma. The National Institutes of Health and American Heart Association supported this study.