

Combination Gene Therapy with CD86 and the MHC Class II Transactivator in the Control of Lung Tumor Growth¹

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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.

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 pcDNA1/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 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$ geneticin (Life Sciences) (in the case of LXSN-based clones) or 2.5 $\mu\text{g}/\text{ml}$ 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 LCITASN, 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 $1 \times \text{PBS}$ 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 $1 \times \text{PBS}$ -sodium azide and then incubated for 20 min in diluted secondary Ab (20 μl total volume per sample) followed by three washes with $1 \times \text{PBS}$ -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 $6.0 \times$.

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 \pm 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

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

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. LCIIITASN poly	Figs. 3, 5	7.7	1.1
E. LCIIITASNA5	Fig. 4	7.7	1.2
F. LCIIITASND12	Fig. 4	6.0	1.1
G. LCIIITASNH8	Fig. 4	12.0	0.7
H. LCIIITASNF7	Fig. 4	17.1	1.1
I. LCIIITASNF6	Fig. 4	18.4	1.1
J. LCIIITASNF6/LCD86SPG10	Fig. 5	6.9	46.2
K. LXS/N/LXSP	Fig. 6, Table II	1.1	1.4
L. LXS/N/LCD86SP	Fig. 6, Table II	1.0	12.0
M. LCIIITASN/LXSP	Fig. 6, Table II	2.0	1.3
N. LCIIITASN/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

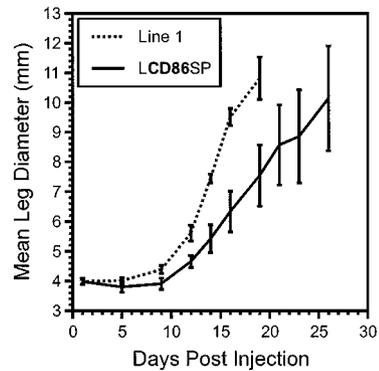


FIGURE 1. Polyclonal Line 1 cells transduced with CD86 show a decreased tumor growth rate. Line 1 cells were transduced with the vector LCD86SP and selected for puromycin resistance. This polyclonal cell population was injected at 500 cells/mouse into the calf muscle of BALB/c mice. Mice were monitored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

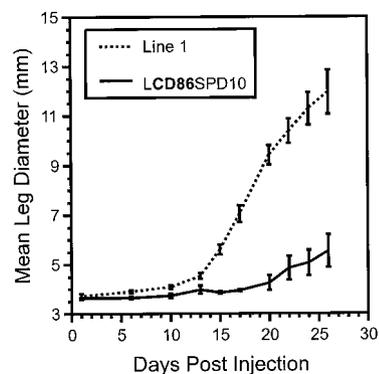


FIGURE 2. High stable expression of CD86 on Line 1 cells leads to growth attenuation and decreased tumorigenicity. The Line 1 clone, LCD86SPD10, expressing high stable levels of CD86, was injected at 500 cells/mouse into the calf muscle of BALB/c mice. Mice were monitored individually for tumor growth. 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.

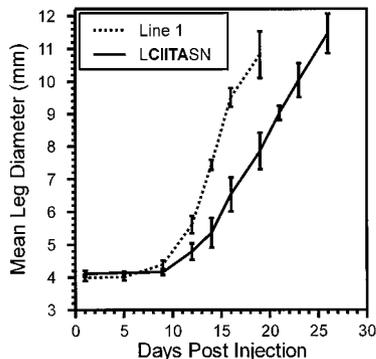


FIGURE 3. Polyclonal Line 1 cells transduced with CIITA show a decreased tumor growth rate. Line 1 cells were transduced with the vector LCIITASN and selected for geneticin resistance. This polyclonal cell population was injected at 500 cells/mouse into the calf muscle of BALB/c mice. Mice were monitored 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. \cdots , 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. 4A); those that grew at approximately the same rate as parental tumors (Fig. 4B); and the one tumor that grew faster than parental Line 1 (Fig. 4C). The two tumors demonstrating the slowest growth also had the lowest ex-

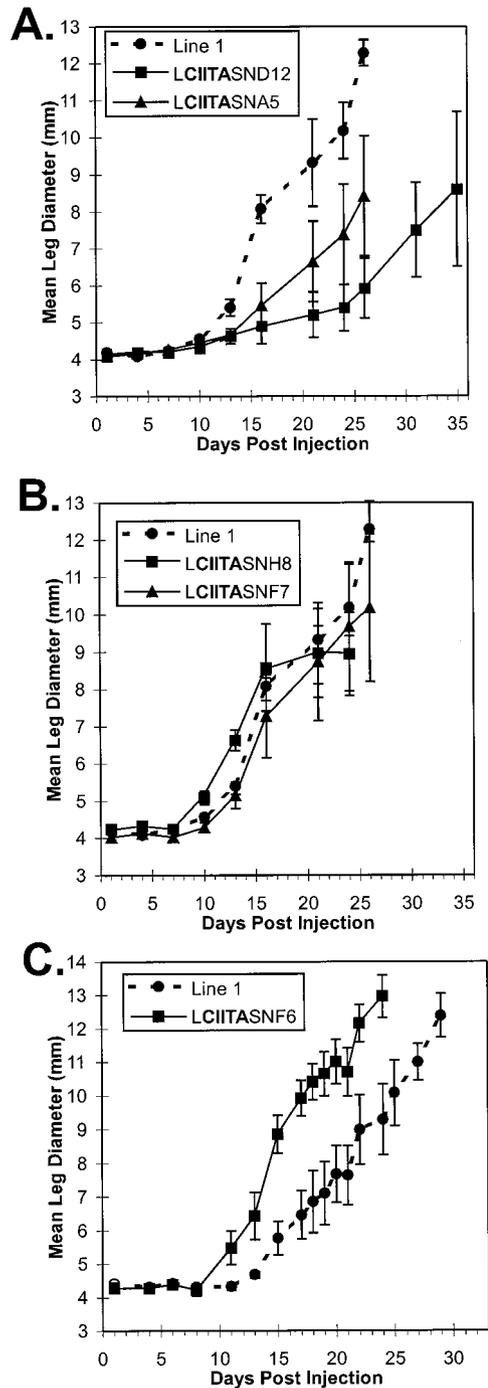


FIGURE 4. Line 1 clones expressing low levels of CIITA have attenuated growth whereas clones expressing high levels of CIITA have unchanged growth or grow faster than mice injected with control cells. Line 1 clones expressing varying levels of CIITA (as assessed by surface class II expression; see Table 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. \bullet , Unmodified Line 1. A, results for clones LCIITASNA5 (\blacktriangle) and LCIITASND12 (\blacksquare). B, results for clones LCIITASNF7 (\blacktriangle) and LCIITASNH8 (\blacksquare). C, results for clone LCIITASNF6 (\blacksquare).

pression 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. 4C) had the

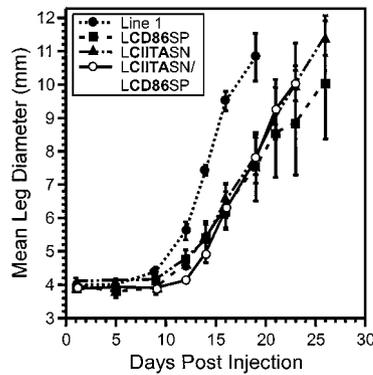


FIGURE 5. Polyclonal Line 1 cells coexpressing CD86 and CIITA have a growth phenotype intermediate to that of CIITA or CD86 expressed alone. The LCIITASN polyclonal population was transduced with CD86-expressing retrovirus, and the polyclonal populations were injected into mice. Cells were injected at 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 D). In the experiment shown in Fig. 4B, 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 transduced the fast growing CIITA clone (LCIITSNF6) (see Table I, row I, and Fig. 4C) 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 LCIITASN6, 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 (LCIITASN6/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 LCIITASN6. 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

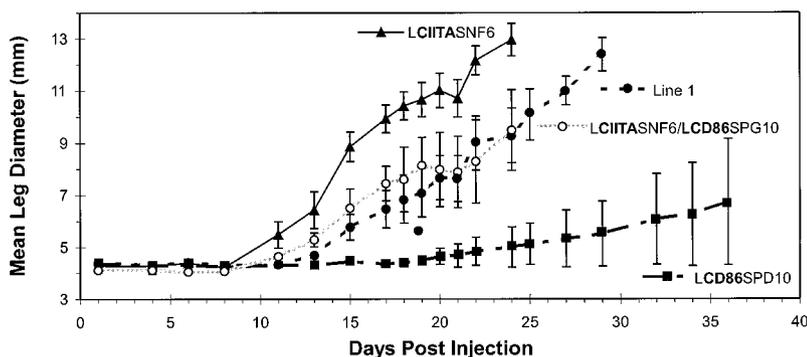


FIGURE 6. Primary tumor growth assays of clonal tumor cell lines expressing CIITA and CD86. Clonal cell populations described in Table I were injected at 500 cells/mouse into the calf muscle of BALB/c mice. Mice were monitored individually for tumor growth. Each line represents the mean leg diameter of four to six mice per group. Error bars represent the SEM for each group. ●, Unmodified Line 1; ■, LCD86SPD10; ▲, LCIITASN6; ○, LCIITASN6/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 co-expressing 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 complementary 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 4A). However, when expression levels of CIITA are high, the cells lose their slow growth phenotype (Fig. 4B), and in certain situations, the cells can even grow faster than unmodified tumor (Fig. 4C). 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 LCITASN6 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 LCITASN6 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.

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