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TITLE: Novel MHC Class II Breast Cancer Vaccine Using RNA Interference (RNAi) to Down Regulate Invariant Chain (Ii)

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| 14. ABSTRACT<br><br>Our goal is to induce a strong CD4+ T cell response against tumor antigens by preferentially presenting endogenous tumor antigens via class II major histocompatibility complex molecules (MHC II). MHC II can present endogenous tumor antigens if expressed in the absence of Invariant chain (Ii). We have up-regulated MHCII and down regulated Ii without affecting MHC II expression in tumor cells. Using the key transcription factor class II trans-activator (CIITA) we have coordinately up-regulated all class II MHC molecules (DR, DP, DQ) and associated molecules such as the Invariant chain in a Human mammary carcinoma (MCF10). We have successfully down regulated the invariant chain in MCF10 cells, up regulated for MHC II, using retroviral vectors that express siRNAs as hairpin loops. Immuno-fluorescence shows no down regulation of MHC II molecules on the cell surface after Ii was down regulated. We will test the ability of our vaccine to present tumor antigen by observing whether these cells can stimulate HER2/neu restricted CD4+ or CD8+ T cells. These tumor cells could be used as a vaccine stimulating both CD4+ and CD8+ T cells in close proximity inducing a powerful long-term immune response against tumor sharing common tumor antigen with the vaccine. |             |                                  |                            |                                                          |                                           |
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## Introduction:

The immune system is able to mount a response to tumors. The adaptive arm of the Immune system is adept at differentiating between normal (non-malignant cells) and cancerous cells due to subtle differences in the cells repertoire of peptides(1). Unfortunately many patients die even while immune cells are mounting a response to tumor. Therefore, many groups are applying various strategies to amplify any tumor specific immune response. Activation of tumor specific CD4+ T cells (helper T cells) has shown to improve tumor specific immune responses and CD8+ T cell (cytotoxic) memory (2). Key players in the adaptive immune response known as professional antigen presenting cells (APC) take up peptides and cross present them to CD4 T cells(3). These peptides are bound to the peptide binding groove of class II Major Histocompatibility Molecules (MHC II) which traffic to the cell surface and bind to specific T cell receptors which then, in the proper context, become activated. These activated T cells clonally expand and secrete cytokines that influence trafficking and activation of other cellular components of the immune system. In an attempt to facilitate the presentation of a greater repertoire of tumor antigens Suzanne Ostrand-Rosenberg *et. al.* have developed a method of modifying the tumor itself to present a broader repertoire of its own endogenous antigens via the MHCII system(4-16). By modifying these cells to express MHC II but not the class II accessory molecule called the invariant chain (Ii), the MHC II antigen processing and presentation is modified to better present endogenously produced antigens. The repertoire of antigens presented in the absence of Ii is different than those presented with Ii. In normal circumstances Ii is always present when MHC II is expressed. We propose that the antigens presented in the absence of Ii are unique antigens previously not “seen” by the host immune system and therefore they may activate a set of T cells that have not been previously tolerized. Tipping the balance in favor of a robust immune response to tumor antigens, to which a patient has not been tolerized, may lead to better immunotherapy for patients with existing tumors and help prevent metastasis.

Since, co-expression of MHC II and Ii favors presentation of exogenously-derived epitopes, we (8-10, 13, 14) and others (17-19) have proposed that in the absence of Ii, newly synthesized MHC II molecules may bind and present peptides derived from endogenously synthesized proteins. We have exploited this non-traditional pathway to generate cancer vaccines that consist of tumor cells transduced with syngeneic MHC II  $\alpha$  and  $\beta$  chain and costimulatory molecule genes. These vaccines mediate tumor regression in experimental mouse models (4, 5, 7, 8, 10, 11, 20, 21) and activate tumor-specific human CD4+ T cells in vitro (15) (22). The activated CD4+ T cells in both mouse and human systems are specific for antigens encoded by the vaccine cells, suggesting that the transfected MHC II molecules are fully functional in the absence of Ii. To date, the vaccines have been produced by transducing MHC class II negative tumor cells with individual HLA-DR  $\alpha$  and  $\beta$  genes to generate tumor cells that express a single HLA-DR allele (4, 7, 8, 10, 11, 15, 20). Since tumor cells are likely to contain multiple tumor antigen epitopes, and HLA-DQ, and -DP alleles may present additional epitopes, we have hypothesized that tumor cell expression of the CIITA in conjunction with down-regulation of Ii may also generate efficacious vaccines. To test this hypothesis, we have designed small interfering RNAs (siRNA) expressing vectors specific for down

regulation of Ii and transduced them into CIITA and costimulatory molecule expressing tumor cells. The CIITA/siRNA transduced cells efficiently activate CD4+ and CD8+ T cells to tumor antigen epitopes, demonstrating that this strategy may be effective for vaccine development. In addition, the presence of properly conformed and functional HLA-DR heterodimers in the Ii down-regulated tumor cells demonstrates that functionally correct MHC II molecules can be produced in the absence of Ii.

We have shown that CD4+ T cells are activated by MHC II+ tumors as shown by interferon gamma (IFN- $\gamma$ ) secretion together with magnetic bead cell depletions of subpopulations of cells within PBMC as shown by analysis of the T cell receptor repertior of the expanded CD4+ T cell population activated by cells with or without Ii. It has also been shown that a different set of antigens are presented in the absence of Ii and that these Ii- cells better activate CD4+ T cells.

These studies will provide useful information on the role of tumor cells as antigen presenting cells that activate or anergize host T lymphocytes in breast cancer patients, and may provide a powerful tool for activation of the immune system against primary tumor and metastatic disease.

## **Body:**

### **Statement of Work (SOW)**

**Technical Objective 1: Develop siRNA for Ii and test its ability to down-regulate Ii in human and mouse breast cancer cells that constitutively express MHC II or are induced by IFN $\gamma$  to express MHC II.**

**Task 1:** Months 1-2: Use flow cytometry to ascertain that the human mammary carcinoma cell line SUM159 and the mouse mammary carcinoma 4T1 express MHC class II when induced with IFN $\gamma$ .

Accomplished. 4T1 data unpublished; SUM159 published (15) We have also modified 4T1 to express the class II transactivator (CIITA) as a second method for upregulating MHCII and Ii (Unpublished Data).

**Task 2:** Months 2-4: Design 21bp DNA oligos complimentary to 15 different sites on the human invariant chain (Ii) sense and antisense strands. Anneal the strands together and make siRNA in vitro with T7 RNA polymerase.

Accomplished. Described in 2005 progress report and Thompson et al 2005, appendix II (22).

**Task 3:** Months 3-8: Test individual siRNAs for effective down-regulation of Ii in breast cancer cell lines described in Task 1.

Accomplished. Using our small hairpin RNAs we can successfully down-regulate Ii by over 95%. Described in 2005 progress report and Thompson et al 2005, appendix II. (22)

**Task 4:** Months 3-8: Clone human CD80 cDNA into pLPCX retroviral vector (Clontech).

Accomplished (22)

**Task 5:** Months 6-10: Clone human U6 RNA Polymerase III promoter with a multi-cloning site downstream into retroviral vector pLPCX/CD80 from Task 4.

Accomplished. (unpublished). Due to promoter interference from the 3'LTR of the retroviral vector we found that the U6 promoter was inoperable in this vector. We opted to use a commercially available retroviral vector pSIREN Retro-Q siRNA expression vector with the U6 promoter, which has the promoter function of the 3'LTR eliminated (Clontech, Carlsbad CA) (22).

**Task 6:** Months 8-12: Clone DNA oligonucleotide coding for the sense-loop-antisense of successful siRNAs downstream of U6 promoter in pLPCX/CD80/U6 from Task 5. Clone DNA oligonucleotide coding for control siRNA (Lamin A/C) described by Paul et al (Paul, 2002 #23).

Accomplished. Using our small hairpin RNA for three different siRNA sequences (32, 48 and 53) we can successfully down-regulate Ii by over 95%. When expressed by the stable retroviral vector we can stably down regulate Ii indefinitely (22).

**Task 7:** Months 10-14: Transfect packaging cell line (293T Human Embryonic Kidney cells) with pLPCX/CD80/Ii<sup>-</sup> retroviral vector from Task 6. Harvest virus and titer on NIH3T3 mouse fibroblasts.

Accomplished using our pSIREN Retro-Q/hIi32 (22).

**Task 8:** Months 13-16: Transduce human SUM159 and mouse 4T1 mammary carcinoma cells with retrovirus encoding CD80 and siRNA for Ii and select transductants by drug selection. Limit dilution clone if necessary.

Accomplished using MCF10CA1 (malignant epithelial mammary carcinoma) (22). The MCF10CA1 cell line may be more indicative of a breast cancer than SUM159. MCF10CA1 cells are derived from the non-malignant MCF10A cell line giving a good negative control for immune response against malignant versus non-malignant phenotypes. Also MCF10CA1 is IFN-gamma inducible and can be up-regulated by CIITA expression for HLA-DR7 a common allele in the Caucasian population making it easy to match patient/donor peripheral blood mononuclear cells (PBMC).

A separate siRNA was designed to down regulate mouse Ii. The human siRNA could not be used because the sequence homology of the mouse and human Ii was not similar enough. The mouse siRNA were designed targeting the same region of the mouse Ii as showed good targets for human siRNA. The same small hairpin RNA expression vector used for our human siRNA was used to clone mouse siRNA for Ii. This vector has been

shown to have promoter activity in both human and mouse (personal communications with Clontech, Calsbad, CA). Mouse cell lines 4TI (mammary sarcoma) and SaI (acites) were transduced with the siRNA expression vector. Mouse siRNA 54 down-regulated Ii greater than 95% as shown in figures 1 and 2, Appendix I. SaI tumor was used to confirm the functionality of the mouse Ii siRNA in another cell line.

**Task 9:** Months 16-24: Induce cells generated in Task 8 with  $IFN\gamma$ . Use flow cytometry to verify MHC II and CD80 expression and functionality of the siRNA to down regulate Ii.

Accomplished (22). Cells were transduced to express CIITA giving stable class II expression.

**Technical Objective 2: As a model system in which to test “proof of principle” we will test the siRNA approach in vivo in mice with metastatic mammary carcinoma.**

**Task 10:** Months 20-24: Inoculate naïve BALB/c mice with genetically modified 4T1 cells and follow for survival and quantify number of metastatic cells in the lungs, liver, bone marrow, and brain using the clonogenic assay.

**Task 11:** Months 24-30: Inoculate BALB/c mice with wild type 4T1 tumor cells and allow primary tumors to metastasize. Surgically remove primary tumors and treat mice with irradiated retrovirally transduced  $CD80^+ Ii^-$  mouse 4T1 cells that have been in vitro treated with  $IFN\gamma$ . Follow mice for survival and quantify the numbers of metastatic cells in distant organs per task 10.

In progress: Because of the difference in sequence between human and mouse Ii we had to develop a separate siRNA for mouse Ii. We have successfully developed siRNA specific for mouse Ii, which has been tested in SaI/CIITA and 4TI/CIITA (figures 1-2, appendix I). The pSIREN Retro-Q/mouse Ii siRNA54.2 down-regulates Ii protein expression by over 95%. We are in the process of transducing 4TI/CIITA/siRNA54 cells with CD80. These cells will be used in tasks 10 and 11.

**Technical Objective 3: Test  $MHC II^+ CD80^+ Ii^-$  human breast cancer cells for their ability to activate breast-cancer-specific  $CD4^+ T$  cells from PBMC of tumor-bearing individuals.**

**Task 12:** Months 18-22: Using primary tumor for which autologous PBMC are available (provided by our collaborator, Dr. A. Stopeck), identify primary breast cancer cells, which are  $IFN\gamma$  inducible or constitutively express MHC II.

**Task 12 modified:** Because of the advantages of MCF10CA1 (Listed in the response to Task 8) we will be working with this line and using partially HLA matched PBMC from healthy donors to study T cell responses to vaccine.

HLA-matched patient PBMCs will be used in future experiments.

**Task 13:** Months 22-25: Transduce the primary human breast cells identified in Task 12 with the CD80<sup>+</sup> siRNA retrovirus. Induce cells with IFN $\gamma$  and use flow cytometry to ascertain expression of MHC II and CD80, and lack of Ii expression.

The MCF10CA1 cell line has been transduced with CD80, CIITA, and Ii siRNA expression vector and shown by flow cytometry and western analysis to express MHC II, and CD80 but not the Ii (appendix II) (22).

**Task 14:** Months 25-32: Perform antigen presentation assays using autologous PBMCs as responding lymphocytes and retrovirally-modified autologous breast cancer cells from task 13 as antigen presenting cells. Quantify T cell activation by measuring IL-2 production by ELISA.

PBMC are primed with either Tumor antigen HER2 p98, p776 or with tumor vaccine cells (MHCII+, CD80+, Ii-) then expanded on IL-2 or IL-15 for one week then boosted with tumor vaccine or control tumor lines.

We have successfully boosted PBMC, that were primed with a known breast cancer tumor antigen HER2 (p98 and p776), with tumor vaccine (22) (appendix II). We have successfully primed and boosted partially matched PBMC with tumor vaccine (figure 3, appendix I).

PBMC primed and boosted with Ii negative tumor vaccines consistently activated a greater CD4 T cell response when compared with Ii positive tumor vaccines, as shown by increased IFN-gamma secretion (Figure 4, appendix I). We have also shown that the responding cells in the activated PBMC are CD4+ T cells, as shown by CD4 depletions (Figure 4, appendix I). The vaccine cells are only MHC class II matched to the PBMC. Tumor vaccines require MHC II to activate PBMC (Figure 4, appendix I).

PBMC primed with Ii- tumors (MCF10/DR7/CD80) give significantly greater IFN-gamma secretion when boosted with Ii- tumors than with Ii+ tumors (Figure 5a and b, appendix I). PBMC primed with Ii+ tumors and boosted with Ii+ or Ii- tumors have no significant change in IFN-gamma secretion (Figure 5a,b, appendix I). Further analysis of PBMC primed with Ii positive tumor showed that these PBMC when boosted with Ii negative tumor gave significantly increased activation in many experiments (Figure 6 appendix I). This data supports other studies that show that in the absence of Ii different antigens are presented than in the presence of Ii (18). This also supports our theory that in the absence of Ii novel tumor antigens are presented for which T cells have not previously been selected against or tolerized. This data also suggest that Ii negative tumors many if not all the antigens presented in the presence of Ii but present other antigens not presented by Ii positive cells. Therefore, a greater array of antigens are presented in the absence of Ii some of which may activate a greater immune response than those presented normally in the presence of Ii.

To further support the theory that different antigens are presented in the absence of Ii PBMC were primed and boosted with Ii positive or negative tumor vaccines. Boosted PBMC were labeled with antibodies specific to particular V beta T cell receptors (TcR-V $\beta$ ). Cells were gated on CD4<sup>+</sup> cells and analyzed for their particular TcR-V $\beta$ . PBMC primed and boosted with Ii negative tumor (MCF/DR7/CD80) expanded/activated a greater percentage of TcR than those activated with Ii positive tumor (MCF/DR7/CD80/Ii). Some T cells were activated to a greater extent by Ii positive tumor but for the most part most T cells of a given TcR were activated to a greater extent without Ii (Figures 7a and b, appendix I). Figure 7a shows the percentage of a given T cell receptor out of CD4<sup>+</sup> T cells and figure 7b shows the percent difference in activation of a given TcR. This data suggest either the Ii negative tumors are presenting a broader range of antigens activating more variants of any given TcR Vbeta family or the antigens are inducing a more prolific expansion of those Tcells.

**Task 15:** *Identify the responding T cells in Task 14 by blocking MHC class I or II and/or by depleting CD4 or CD8 T cells before setting up the antigen presentation assays. Ascertain the type of CD4 response (Th1 or Th2) by assessing IL-4 and IFN $\gamma$  production by ELISA and in situ cytokine capture.*

We have published that CD4 T cells are activated by our tumor vaccine as shown by IFN-gamma ELISA (22) (appendix II). We have shown that tumor vaccine cells induce a Th1 response as shown by high IFN-gamma and low IL-4 production as ascertained by ELISA (data not shown).

### **Key Research Accomplishments:**

- Development of siRNA that down regulates the mouse Invariant chain by over 95%.
- Creation of mouse mammary sarcoma cell line 4TI which expresses CIITA inducing expression of MHC II as well as Ii. These cells were further transduced with a stable small hairpin RNA expression vector which down regulates Ii by over 95% giving a mouse tumor vaccine, which is MHC II positive and Ii negative.
- Creation of a human Invariant chain vector and transduction of MCF10/DR7/CD80/Ii vector to aid in studying the effects of Ii on antigen processing and presentation and how that effects efficacy of tumor vaccines.
- Priming and boosting of donor PBMC with tumor vaccine alone, giving very high IFN-gamma secretion, indicative of high T cell activation.
- Identified, through depletion of T cell subpopulations, the responding CD4<sup>+</sup> T cells to MHC II matched vaccines.
- Showed that Ii negative tumors vaccines induce a more prolific activation of CD4<sup>+</sup> T cells than Ii positive tumor vaccines.
- Showed that Ii negative tumor vaccines activate a broader range of T cells to a greater extent than Ii positive tumor vaccines.

## Reportable Outcomes:

### Publications:

- **James A. Thompson**, Samudra K. Dissanayake, Keith L. Knutson, Mary N. Disis, and Suzanne Ostrand-Rosenberg. Tumor Cells Transduced With the MHC Class II Transactivator Activate Tumor-Specific CD4<sup>+</sup> T cells Whether or Not They are Silenced for Invariant Chain. Cancer Res. 2006 66: 1147-1154
- Dissanayake SK, **Thompson JA**, Bosch JJ, Clements VK, Chen PW, Ksander BR, Ostrand-Rosenberg S. Activation of tumor-specific CD4+ T lymphocytes by major histocompatibility complex class II tumor cell vaccines: a novel cell-based immunotherapy. Cancer Res. 2004 64:1867-1874.

### Invited Oral Presentations:

- **Thompson, J. A.**, Dissanayake S. K., Ostrand-Rosenberg S., Novel MHC class II breast cancer vaccine using RNA interference (RNAi) to down regulate invariant chain. Department of Defense Breast Cancer Research Program Meeting: Era of Hope, Philadelphia, PA, USA; 2005
- **Thompson, J. A.** & Ostrand-Rosenberg S., Novel MHC class II breast cancer vaccine using RNA Interference (RNAi) to down regulate invariant chain: An MHC II+ li- tumor vaccine approach. University of Maryland Graduate Student Association of Biological Sciences (GABS) Symposium, Baltimore, MD, USA; 2004

### Poster presentations:

- **Thompson, J. A.**, Dissanayake S. K., Ostrand-Rosenberg S., Tumor Cells Transduced with the MHC Class II Transactivator and Silenced for Invariant Chain Activate Tumor-Specific CD4+ T Lymphocytes and are Potential Cancer Vaccines. Cancer Research Institute (CRI) Cancer Vaccines, New York, NY, USA; 2005
- **Thompson, J. A.**, Dissanayake S. K., Ostrand-Rosenberg S., Tumor cells can stably express MHC II in the absence of the Invariant chain (li): RNAi down regulation of li does not effect surface levels of HLA-DR7. Tumor Vaccine and Cell Therapy (TVACT), Anehiem, CA, USA; 2005 & Basic Aspects of Tumor Immunology, Keystone, CO, USA; 2005
- **Thompson, J. A.**, Dissanayake S. K., Ostrand-Rosenberg S., Novel MHC class II breast cancer vaccine using RNA interference (RNAi) to down regulate invariant chain. Department of Defense Breast Cancer Research Program Meeting: Era of Hope, Philadelphia, PA, USA; 2005

## Conclusions:

We have hypothesized that MHC II positive Ii negative tumor cells could present a greater array of tumor antigens and possibly novel tumor antigens to CD4<sup>+</sup> T cells. We have shown that not only do MHC II positive tumors activate T cells but they have the potential to activate as good or better a CD4<sup>+</sup> T cell response than those attained by using large quantities of a model tumor antigen. Also MHC II<sup>+</sup>, Ii<sup>-</sup> tumors activate a greater response than MHC II<sup>+</sup>, Ii<sup>+</sup> tumors, supporting our theory that Ii<sup>-</sup> tumors express more and possibly novel tumor antigens. Further we have shown that T cells primed with MHC II<sup>+</sup>, Ii<sup>-</sup> tumors respond better when boosted with Ii<sup>-</sup> cells as opposed to Ii<sup>+</sup> cells. This further suggests that T cells specific to antigens presented in the absence of Ii are not activated in the presence of Ii because these antigens are either not presented or are presented to a lesser extent in the presence of Ii. When we further examine the T cell receptor repertoire we find that Ii negative tumor expands T cells identified by most V beta T cell receptor families more so than Ii positive tumors. Very few T cells are expanded more in the presence of Ii and of those that are the percentage of these cells out of total CD4<sup>+</sup> T cells is very low when compared to those activated better in the absence of Ii. We therefore conclude that MHC II<sup>+</sup>, Ii<sup>-</sup> tumor vaccines can activate CD4 helper T cells and that these Ii negative cells activate a broader repertoire of T cells. Ii negative tumor vaccines also induce a more prolific CD4<sup>+</sup> T cell response than Ii positive tumor vaccines. Therefore any tumor vaccine aimed at activating CD4<sup>+</sup> T cells should be down regulated for Ii.

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## Appendix I (Figures)

**Figure 1**

Transductants labeled for mouse Invariant chain: li-siRNA down regulates the mouse li more than 95% in two mouse cell lines

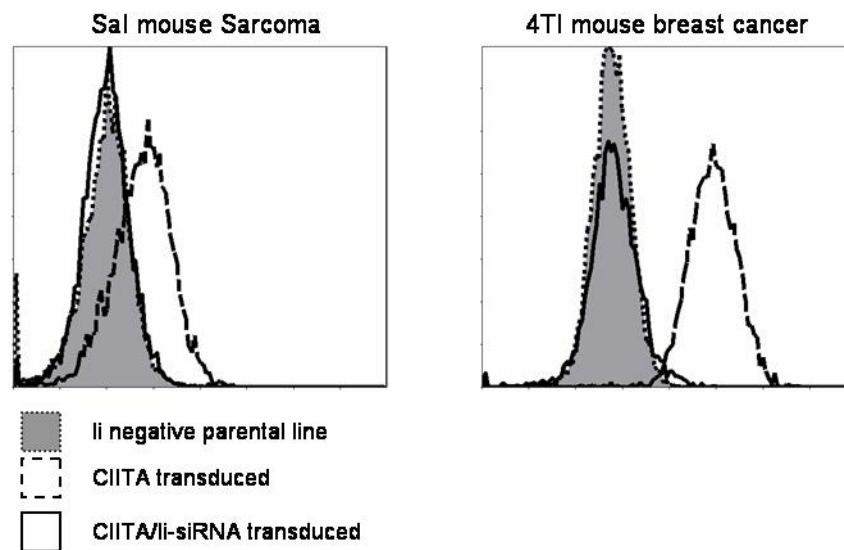


**Figure 2**

Mouse Ii siRNA down regulates Ii by over 95% as shown by Western analysis

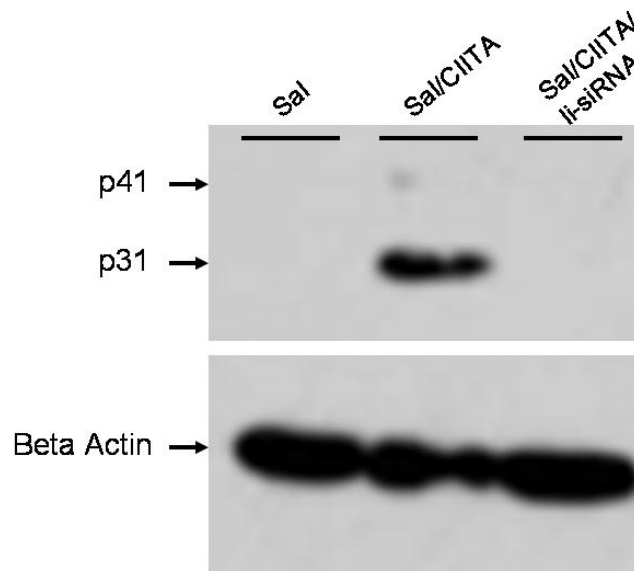


**Figure 3**

### II- vaccine cells prime and boost MHC II matched PBMC

MHC II matched CD4 depleted PBMC Primed with MCF.DR7.CD80 and boosted MCF variants

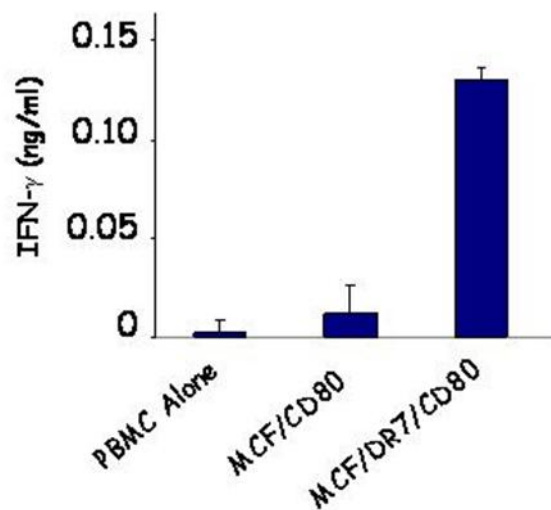


Figure 4

CD4 T cells are activated in PBMC when primed and boosted with MHC II matched tumor vaccine

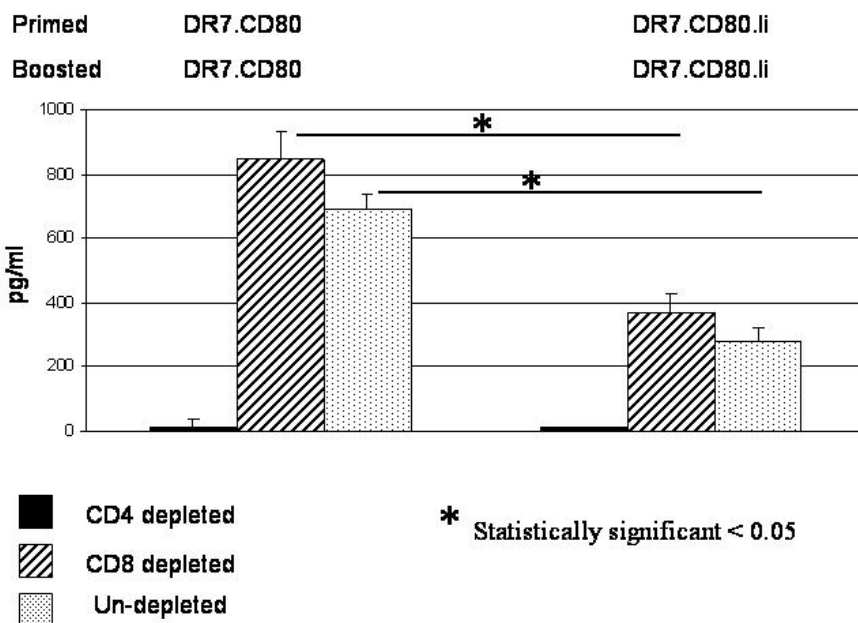


Figure 5a

PBMC primed and boosted with MHC II matched Ii positive or negative tumor vaccine

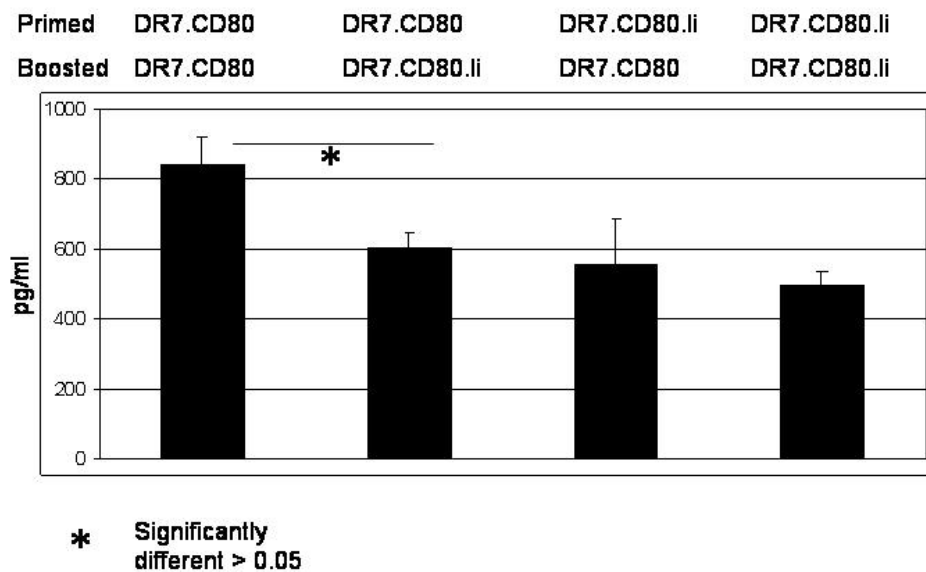


Figure 5b

Ii- vaccine cells present different antigens than Ii+ cells

MHC II matched CD4 depleted PBMC Primed with MCF.DR7.CD80 and boosted MCF variants

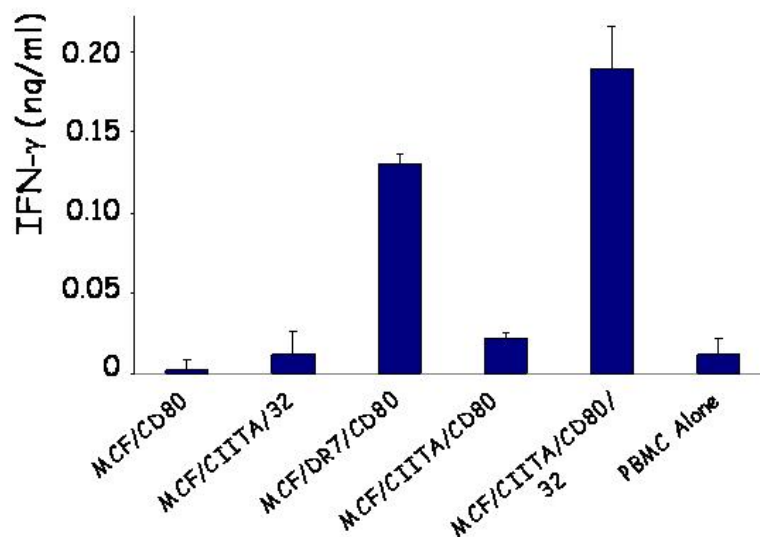


Figure 6

PBMC primed with li positive tumor vaccine and boosted with li negative tumor vaccine give increased activation

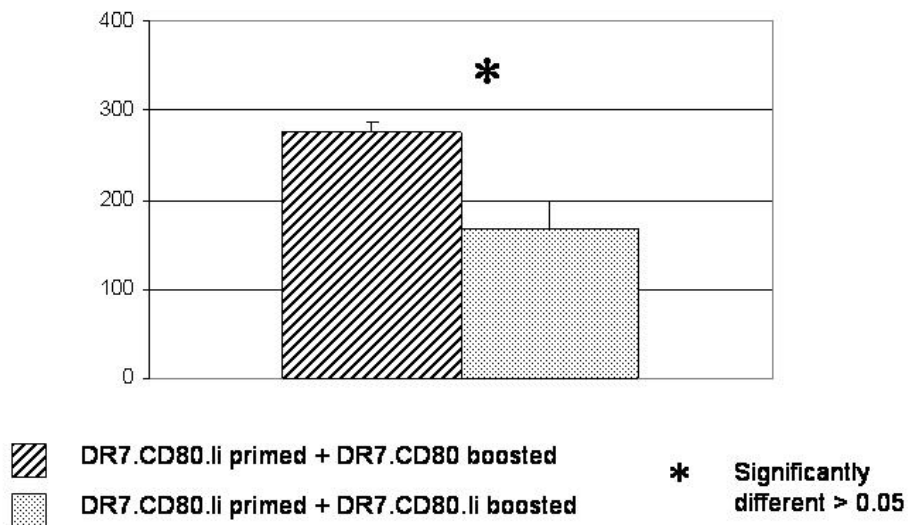


Figure 7a

li negative tumor vaccine activates different T cells as shown by V-beta T cell receptor repertoire difference when priming and boosting with or without li

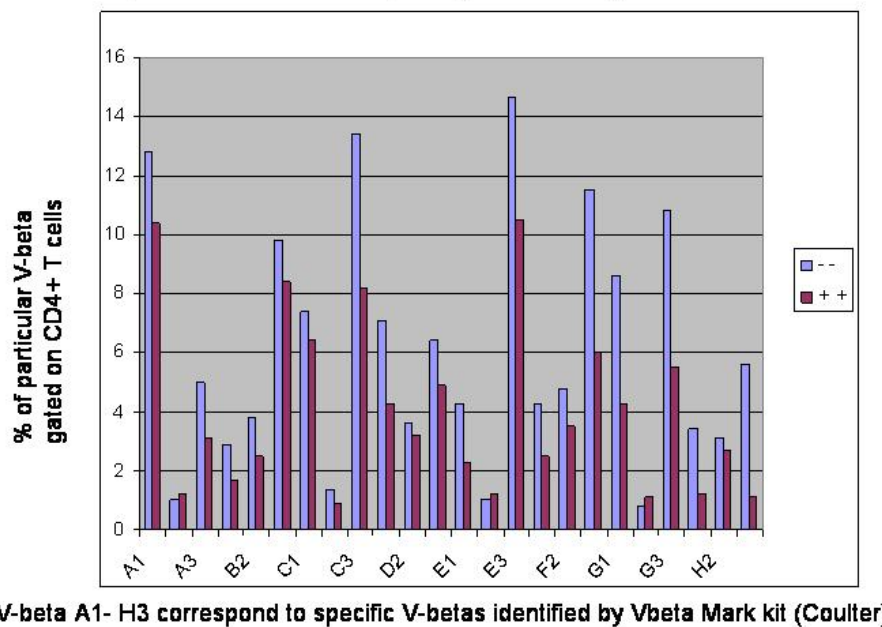
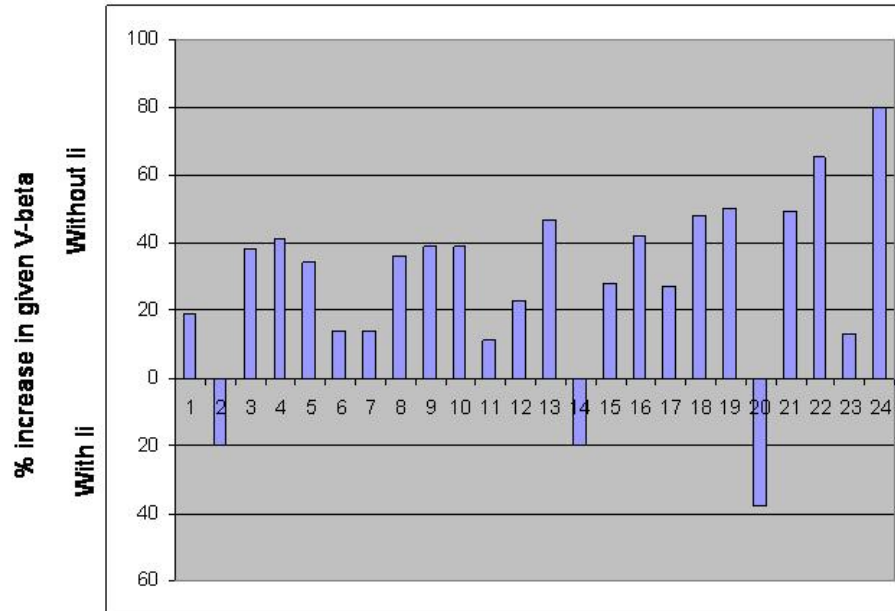


Figure 7b

# T cells expand better when activated with li negative tumor vaccine



# Tumor Cells Transduced with the MHC Class II Transactivator and CD80 Activate Tumor-Specific CD4<sup>+</sup> T Cells Whether or Not They Are Silenced for Invariant Chain

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## Abstract

The specificity and potency of the immune system make immunotherapy a potential strategy for the treatment of cancer. To exploit this potential, we have developed cell-based cancer vaccines consisting of tumor cells expressing syngeneic MHC class II and costimulatory molecules. The vaccines mediate tumor regression in mice and activate human CD4<sup>+</sup> T cells *in vitro*. Previous vaccines were generated by transducing MHC II negative tumor cells with a single *HLA-DR* allele. Because expression of multiple MHC II alleles would facilitate presentation of a broader repertoire of tumor antigens, we have now transduced tumor cells with the MHC class II transactivator (*CIITA*), a regulatory gene that coordinately increases expression of all MHC II alleles. Previous studies in mice indicated that coexpression of the MHC II accessory molecule invariant chain (Ii) inhibited presentation of endogenously synthesized tumor antigens and reduced vaccine efficacy. To determine if Ii expression affects presentation of MHC class II-restricted endogenously synthesized tumor antigens in human tumor cells, HLA-DR-MCF10 breast cancer cells were transduced with the *CIITA*, CD80 costimulatory molecule gene, and with or without small interfering RNAs (siRNA) specific for Ii. Ii expression is silenced >95% in *CIITA*/CD80/siRNA transductants; down-regulation of Ii does not affect HLA-DR expression or stability; and Ii<sup>+</sup> and Ii<sup>-</sup> transductants activate human CD4<sup>+</sup> T cells to DRB1\*0701-restricted HER-2/*neu* epitopes. Therefore, tumor cells transduced with the *CIITA*, CD80, and with or without Ii siRNA present endogenously synthesized tumor antigens and are potential vaccines for activating tumor-specific CD4<sup>+</sup> T cells. (Cancer Res 2006; 66(2): 1147-54)

## Introduction

Immunotherapy is a potential approach for the treatment and/or prevention of cancer because of its specificity, sensitivity, potency, and long-term memory. T lymphocytes, the cellular arm of the immune response, are particularly promising because they have the capability of localizing to tumor sites and directly killing tumor cells. Because of these characteristics, vaccines and/or immunotherapy may facilitate the destruction of existing disseminated metastatic tumor cells and protect individuals against the recurrence of primary tumors and/or the outgrowth of latent metastatic cells (1, 2).

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T cells that are cytotoxic for tumor cells are typically CD8<sup>+</sup> T lymphocytes, and optimal activation of these cells usually requires coactivation of CD4<sup>+</sup> T helper lymphocytes (3, 4). CD4<sup>+</sup> T lymphocytes are also required for generating CD8<sup>+</sup> T memory cells (5–7). Because of these critical roles for CD4<sup>+</sup> T cells, we are developing cancer vaccines that specifically target the activation of CD4<sup>+</sup> T cells while concurrently activating cytotoxic CD8<sup>+</sup> T lymphocytes.

CD4<sup>+</sup> T lymphocytes are activated to peptide antigen that is presented by MHC class II molecules. Because MHC II molecule expression is usually limited to professional antigen-presenting cells (APC), immunity to most pathogens requires that professional APCs acquire antigen from exogenous sources. To facilitate the presentation of endocytosed antigen, professional APCs contain the MHC class II-associated accessory molecule, invariant chain (Ii). Ii hinders the presentation of endogenously synthesized peptides and favors the presentation of antigen acquired by endocytosis. It mediates this effect by binding to newly synthesized MHC class II molecules in the endoplasmic reticulum and preventing them from acquiring peptides of endogenously synthesized molecules. The Ii chain also contains trafficking signals, which guide newly synthesized MHC II molecules to the endocytic pathway where Ii protein is degraded and peptides derived from endocytosed proteins bind (reviewed by refs. 8, 9). In professional APCs, MHC class II and Ii molecules are coordinately regulated at the transcriptional level by the MHC class II transcriptional activator (*CIITA*), a master regulatory gene that controls expression of all MHC II alleles (10–13). This coordinate regulation assures that professional APCs efficiently present antigenic peptides acquired from extracellular sources.

Ii has been considered essential for MHC II function. Its requirement is supported by the finding that Ii knockout mice have dysfunctional or very low levels of MHC class II molecules, and CD4<sup>+</sup> T cell activation is minimal (14–18). In contrast, some Ii-negative nonprofessional APCs when transfected or transduced with MHC class II genes present antigen and activate CD4<sup>+</sup> T cells (16, 19–22), suggesting that MHC class II molecules can be fully functional in the absence of Ii.

Based on the assumption that coexpression of Ii blocks endogenous antigen presentation, we have produced cancer vaccines by transducing MHC II tumor cells with syngeneic MHC II and costimulatory molecule genes. The vaccines mediate tumor regression in mice and activate tumor-specific CD4<sup>+</sup> T cells (20, 23–26). Activated CD4<sup>+</sup> T cells in both mouse and human systems are specific for antigens encoded by the vaccine cells. These vaccines have been produced by transducing MHC class II tumor cells with a single *HLA-DR* allele. Because expression of multiple

MHC II alleles may facilitate presentation of a broader repertoire of tumor antigens, we have now transduced tumor cells with the CIITA. To determine if Ii coexpression affects T-cell activation to endogenous antigen, we have introduced small interfering RNAs (siRNA) specific for Ii into human tumor cells transduced with the CIITA and CD80 costimulatory molecule genes. The transductants efficiently activate human CD4<sup>+</sup> T cells to HER-2/*neu* tumor antigen epitopes, suggesting that this strategy may be useful for vaccine design. Properly conformed and functional MHC II heterodimers are present in transductants with or without Ii siRNA, indicating that Ii is not essential for the HLA-DR function. Surprisingly, transductants with or without the Ii siRNA are equally efficient at activating CD4<sup>+</sup> T cells, indicating that in this system, Ii does not impair endogenous antigen presentation.

## Materials and Methods

**Cells.** SUM159PT, Jurkat, Sweig, 293T, and peripheral blood mononuclear cells (PBMC) were handled as described (20). The human breast cancer line MCF10CA1 (hereafter called MCF10) and its nonmalignant counterpart MCF10A were cultured in MCF10 medium (DMEM/Hams F12, 1:1; 5% heat-inactivated FCS; 0.029 mol/L Na bicarbonate; 10 mmol/L HEPES; ref. 27) or MCF10A medium [MCF10 medium supplemented with 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin (all from Sigma, St. Louis, MO), and 20 ng/mL epidermal growth factor (Invitrogen, Carlsbad, CA)]. MCF10 transductants were supplemented with puromycin (0.3 µg/mL; Clontech, Palo Alto, CA) or hygromycin (150 µg/mL; Calbiochem, San Diego, CA). The OMM2.3 human ocular melanoma line (28) was grown in RPMI with 10% heat-inactivated FCS and  $5 \times 10^{-5}$  mol/L β-mercaptoethanol. All cell lines and procedures were approved by the institutional review boards of the participating institutions.

**siRNA.** Complementary sequences in the coding region of the human Ii gene (Genbank accession no. NID NM\_004355) were identified using the Ambion siRNA target finder search engine (Ambion, Inc., Austin, TX). Sequences with no homology to other known human mRNAs were chosen at random from the 3' to 5' end of the Ii mRNA. siRNAs were produced by *in vitro* transcription with T7 RNA polymerase (29) using the Ambion Silencer siRNA Construction kit and were made homologous to sequences 4, 8, 16, 24, and 50 (double adenine regions found by the Ambion target finder search engine). siRNAs were transfected into 293T/CIITA cells (20) using 40 ng siRNA. siRNAs producing 4- to 50-fold decreases in Ii expression were identified, and oligonucleotide siRNA expression cassettes were prepared by MWG Biotech (High Point, NC) and inserted into the pSIREN Retro-Q vector (Clontech) according to the manufacturer's directions (Clontech). The forward and reverse primers were annealed and ligated to the linearized pSIREN-RetroQ vector with *Bam*HI and *Eco*RI "sticky ends."

**Retroviral constructs, transductions, and drug selection.** The human CIITA gene was cloned from pcDNA1-amp/tagCIITA (30) into Litmus28 (New England Biolabs, Beverly, MA) using *Xba*I and *Eco*RI and then cloned into a modified pLNCX retroviral vector, pLNCX2(AvrII) (neo resistance; ref. 20) using *Bgl*II and *Avr*II.

The HLA-DRB1\*0701 cDNA (in RSV.5 vector; ref. 31) contained two point mutations: a guanine instead of an adenine at base 13 and an adenine instead of a thymine at base 191. These errors were corrected using Splicing by Overlapping Extensions (SOEing; ref. 32) using the following four primers in successive SOEing reactions: primer 1, AGTACCCGGGATGGTGTG-TCTGAAGCTCCCTG; primer 2, AGCGCACGAACCTCTCTGTTATAGAA; primer 3, TTCTATAACCAGGAGGAGTTCGTGCGCT; primer 4, TAGTGCG-GCCGCTCAGCTCAGGAATCCTGTTG. Reaction 1: 10 pmol/L RSV.5/DRB1\*0701 template and 0.5 µmol/L of primers 1 and 2; cycle at 95°C for 2 minutes, then 30 cycles of 95°C for 30 seconds, 60.2°C for 30 seconds, 72°C for 1 minute, then 72°C for 10 minutes. Reaction 2: 10 pmol/L RSV.5/DRB1\*0701 template and 0.5 µmol/L primers 1 and 2; same as PCR1, but annealing temperature was 62.3°C. Reaction 3: 10 pmol/L of product from reactions 1 and 2 were mixed with primers 1 and 4 and incubated at 95°C for

2 minutes followed by five rounds of 95°C for 30 seconds followed by 60.2°C for 30 seconds followed by 72°C for 1 minute. Then five rounds more of the same reaction with annealing temperature at 62.3°C followed by 23 rounds of the same reaction at 64.8°C followed by 72°C for 10 minutes. All reactions used 2 units of PFU turbo polymerase (Invitrogen) according to the manufacturer's specifications. These primers added the *Xma*I and *Not*I restriction sites on the 5' and 3' ends of the cDNA, respectively. The corrected sequence was confirmed by sequencing of both strands.

Using the same restriction sites as for cloning of pLNCX2/HLA-DR1, the HLA-DRB1\*0701 or HLA-DRB1\*0401 genes were cloned into the downstream site of the pIRES/DRA0101 vector containing the HLA-DRA\*0101 gene in the upstream site. The DRA0101-IRES-DRB1 section was excised from the resulting vector and cloned into the retroviral vector pLNCX2(AvrII). The pLHCX/CD80 retroviral construct, retrovirus production, and transductions were previously described (20).

Transduced cells were selected as follows: CD80 transductants (150 µg/mL hygromycin); DR4, DR7, and CIITA transductants (300 µg/mL G418); siRNA transductants (0.3 µg/mL puromycin). If 2 to 3 weeks of drug selection did not yield homogeneous populations of transgene-expressing cells, the transductants were sorted by magnetic bead selection (Miltenyi, Auburn, CA) according to the manufacturer's directions.

**Peptides, antibodies, reagents, and immunofluorescence.** HER-2/*neu* peptide 98-114 (RLRIVRGTLQFEDNYAL) and peptide 776-790 (GVGSPYVSRLLGICL; refs. 33, 34) were synthesized at the University of Maryland Biopolymer Laboratory. Monoclonal antibodies (mAb: HLA-DR-FITC and CD80-PE), streptavidin-PE, FITC-isotype, and PE-isotype controls were from BD PharMingen (San Diego, CA). Biotinylated HLA-DR1 mAb (BIH0126) was from One Lambda, Inc. (Canoga Park, CA); HLA-DQ-PE and HLA-DP-FITC were from Chemicon (Temecula, CA); rat anti-mouse IgG-FITC was from ICN (Costa Mesa, CA); *c-neu* (Ab-2) was from Oncogene (Cambridge MA); CD4-FITC, CD8-FITC, and anti-human IgG-FITC were from Miltenyi Biotec; and human IgG-FITC was from Cappel (West Chester, PA). Culture supernatants of hybridomas W6/32 (pan HLA-A,B,C), L243 (pan anti-HLA-DR), PIN1.1 (anti-Ii), and 28.14.8 (anti-H-2D<sup>b</sup>L<sup>d</sup>) were prepared, and tumor cells and PBMCs were stained for cell surface markers (MHC class I, class II, CD80, CD4, CD8, and immunoglobulin) or fixed and stained for Ii as described (20).

**Western blots.** Ii Western blots were done as described (20). Blots for MHC II were done as for Ii with the following modifications: cell lysates were loaded onto SDS-PAGE gels using nonreducing loading dye [0.2% SDS, 20% glycerol, 1.25 mol/L Tris (pH 6.8), and 0.4 mg/mL bromophenol blue]. Half of each sample was boiled for 5 minutes immediately before loading. Blocking buffer was 2% bovine serum in TBST. Membranes were incubated with undiluted supernatant from hybridoma L243, and the last wash was done for 1 hour.

**T-cell priming.** PBMCs from healthy donors ( $2 \times 10^7/4$  mL/well) were cultured in PBMC medium [Iscove's modified Dulbecco's medium, 10% FCS, 1% penicillin, 1% streptomycin (BioSource, Rockville, MD), 2 mmol/L Glutamax (Bethesda Research Laboratories/Life Sciences, Grand Island, NY)] with 2 µg/mL of HER2 p98 or p776 in six-well tissue culture plates at 37°C and 5% CO<sub>2</sub> for 5 days. Nonadherent cells were harvested, washed twice with PBMC medium, and replated in 24-well plates with 20 units/mL of recombinant human interleukin 2 (IL-2; R&D Systems, Minneapolis, MN) at  $1 \times 10^6/2$  mL/well. HER2-activated nonadherent cells were harvested 7 days later; live cells were isolated using Histopaque-1077, cultured 1 to 5 days without exogenous IL-2, and used the following day. For some experiments, after incubation with IL-2, nonadherent PBMCs were cultured at  $1 \times 10^7/4$  mL/well with  $8 \times 10^6$ , 50 Gy-irradiated SUM/DR7/CD80 cells for 5 days, washed and cultured as above with IL-2 for 7 days, and washed and rested for 1 day before use.

Alternatively, PBMCs were obtained from HER-2/*neu*-immunized patients with stage III or IV breast, ovarian, or non-small cell lung cancer participating in a University of Washington Food and Drug Administration-approved phase I trial (35). Patients were immunized intradermally once a month for 6 months to the same regional draining lymph node site with three different peptides derived from HER-2/*neu* and admixed with 100 µg granulocyte macrophage colony-stimulating factor. PBMCs were

collected 1 month after the last immunization and cryopreserved. For *ex vivo* boost, PBMCs were thawed at 37°C, washed twice, and resuspended at  $3 \times 10^6$ /mL in X-VIVO media [10% human AB serum, 2 mmol/L L-glutamine, 20 mmol/L HEPES buffer, and 10 mmol/L acetylcysteine solution (USP)]. The cells were stimulated with 10 mg/mL of HER-2/*neu* peptides (p98, p776, or p98+p776) and incubated at 37°C in 5% CO<sub>2</sub> for 12 days. On days 4/5 and 8, 10 units/mL of recombinant human IL-2 (Chiron Corp., Emeryville, CA) and 10 ng/mL of recombinant human IL-12 (R&D System) were added. On day 12, the cells were harvested, washed, counted, tested by flow cytometry and enzyme-linked immunospot (ELISPOT), and resuspended at  $1 \times 10^6$ /mL in fresh media containing  $1 \times 10^5$ /mL of anti-CD3/CD28-coated beads to a final concentration of 1 or 10 bead(s) per T cell. Between days 14 and 23, the cell concentration was evaluated every 2 to 3 days, and the cells were diluted to 0.5 to  $1 \times 10^6$ /mL with fresh media as needed. On days 15, 18, 20, and 22, IL-2 was added to a final concentration of 30 units/mL, and on day 25, the expanded cells were harvested, washed, counted, and evaluated by flow cytometry and ELISPOT (36).

**Antigen presentation assays.** Antigen presentation assays and T-cell depletions were done as described (20) using Miltenyi human CD4 and CD8 beads with the following modifications: stimulator cells were used at  $2.5 \times 10^4$  per well. MCF10-derived and MCF10A stimulator cells were not irradiated; all other stimulators were 50 Gy irradiated. Antibody blocking experiments included 20 µg/mL L243 (anti-HLA-DR), W6-32 (anti-class I MHC), or 28.14.8. For exogenous HER2 peptide presentation, assays were as for endogenous antigen presentation, except soluble HER2 peptide p98 or p776 was included at 2 µg/mL.

**HLA-DR nomenclature and genotypes.** Normal donor PBMCs are A24, A29, B44, B35, DR7, DR11, and DRβ3.4. SUM159PT cells are A2, A24, B5, B15, DR4, and DR13. OMM2.3 cells are A11, A29, B7, and B52. MCF10 and MCF10A cells are A33, B55, B22, DR7, and DR4. HLA genotypes were determined by PCR typing and are referred to by their short-hand form (e.g., HLA-DR7 is DRB1\*0701).

**Statistical analyses.** Means, SDs, and statistical significance as measured by Student's *t* test were calculated using Excel v2002.

## Results

**siRNA down-regulates Ii.** The human breast cancer epithelial cell line MCF10 (27), which does not constitutively express MHC class II or Ii molecules, was transduced with a retrovirus encoding the human *CIITA* gene (Fig. 1A) and selected by magnetic bead sorting for MHC class II expression. CIITA-transduced MCF10 (MCF10/CIITA) cells are MHC class II (HLA-

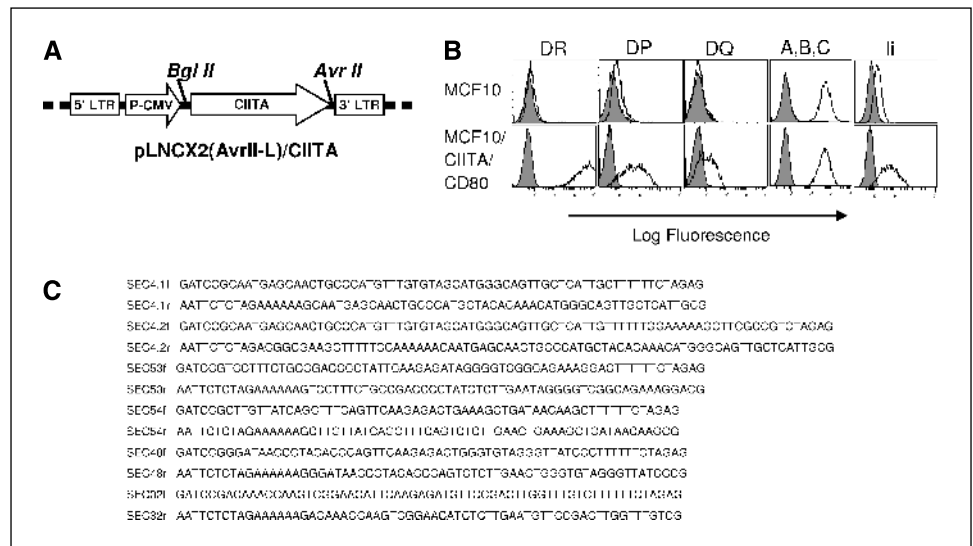
DR, DP, and DQ) and Ii positive as shown by immunofluorescence (Fig. 1B).

Retroviruses expressing siRNAs for Ii were constructed to down-regulate Ii in MCF10/CIITA cells. Four sequences in the human *Ii* gene, starting with AA and having low GC content, were selected (sequences 4, 38, 16, and 50). Double-stranded RNA molecules of these sequences were prepared and were transiently transfected into 293T cells that had previously been transduced with the CIITA retrovirus. Sequences 4 and 50 but not 38 or 16 down-regulated Ii by 4- to 50-fold (data not shown). To obtain stable transductants, sequence 4 was cloned into the pSIREN vector with two different termination signals giving vectors 4.1 and 4.2, with 4.2 containing additional sequence following the six thymidines. Sequence 50 was not used because it contained a four-thymidine repeat that is a stop of transcription for the U6 polymerase III promoter. Additional sequences starting with AA and having low GC content were selected adjacent to sequence 50 and were inserted into pSIREN (sequences 48, 53, and 54). An additional sequence 32 was also randomly selected and inserted into pSIREN. Sequences were inserted using the forward and reverse primers shown in Fig. 1C. Retroviruses containing these six siRNAs were prepared and used to transduce MCF10, MCF10/CIITA, and CD80-expressing cells (MCF10/siRNA, MCF10/CIITA/siRNA, MCF10/CIITA/CD80/siRNA cells). Transduced cells were analyzed by flow cytometry 3 days after transduction. Lines containing siRNAs 32, 53, and 48 showed a marked down-regulation of Ii but not complete loss, whereas cells containing siRNAs 4.1, 4.2, and 54 had minimal down-regulation of Ii (data not shown).

Western analyses for Ii (mAb PIN1.1) were done 3 days after transduction to confirm that Ii expression was down-regulated. The predominant form of Ii in MCF10/CIITA cells is p35, with a smaller amount of p33 (Fig. 2A). The p35 isoform, which is translated via an alternative translation initiation site, is normally the less abundant isoform of Ii (37). Others have noted an increase in p35 in tumors (38). The p35 and p33 isoforms are both down-regulated >95% in the siRNAs 53, 48, or 32 transductants, and there is a slight down-regulation in siRNA 4.1. siRNAs 4.2 and 54 do not affect Ii expression. Interestingly, at 3 days after transduction, all of the down-regulated cell lines contain a 23-kDa band that corresponds to an Ii degradation product. To ascertain if p23

**Figure 1.** CIITA-transduced breast cancer cells express HLA-DR, HLA-DP, HLA-DQ, HLA-A, HLA-B, HLA-C, and invariant chain. A, CIITA retroviral vector. B, parental MCF10 and transduced MCF10/CIITA/CD80 cells were stained for HLA-DR (mAb L243), HLA-DQ (mAb CBL118P), HLA-DP (mAb CBL100F), HLA-A,B,C (mAb W6/32), or Ii (PIN1.1) and analyzed by flow cytometry. Unfilled peaks, specific antibody-stained cells; filled peaks, isotype control stained cells.

C, forward (f) and reverse (r) oligonucleotides used to construct siRNA cassettes homologous to human Ii mRNA.



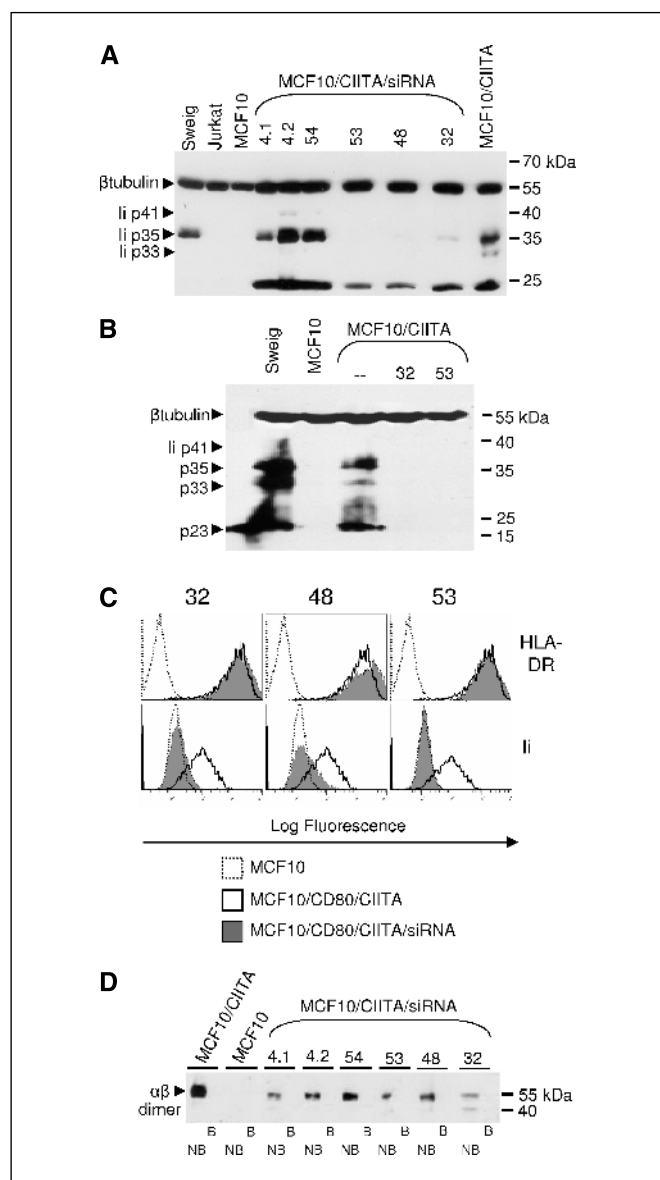


**Figure 2.** Ii siRNAs silence Ii expression in CIITA-transduced cells without altering HLA-DR expression. MCF10 cells were transduced with retroviruses containing the CIITA and siRNAs for Ii. **A** and **B**, Western blots of MCF10, transduced MCF10, and control Swei and Jurkat cells probed for Ii with the PIN1.1 mAb, 3 days after transduction (**A**) or 3 weeks after transduction (**B**). **C**, parental and transduced MCF10 cells stained for HLA-DR (mAb L243) or Ii (mAb PIN1.1). **D**, Western blots of parental and transduced MCF10 cells probed for HLA-DR (mAb L243) after 3 weeks of drug selection. Samples were either boiled (**B**) or not boiled (**NB**) before loading on the gel. 4.1, 4.2, 32, 48, 53, 54 identify independent Ii siRNAs.

persists, MCF10/CIITA/siRNA 32 and 53 were cultured in selective media for 3 weeks and subsequently tested by Western analysis. Neither full-length Ii nor the Ii degradation product is present 3 weeks after selection (Fig. 2B). Similar results were obtained with MCF10/CD80/CIITA/siRNA cells (data not shown).

To determine if siRNA-mediated down-regulation of Ii affected cell surface expression of MHC class II molecules, the transductants were analyzed by immunofluorescence 3 weeks after siRNA transduction. HLA-DR expression is the same in siRNA and non-siRNA transductants (Fig. 2C, top). Ii is absent in MCF10/CD80/CIITA/siRNA 53 and reduced >95% in lines 32 and 48

(bottom). Therefore, down-regulation of Ii by siRNA eliminates Ii expression without affecting MHC class II expression.

**MCF10/CIITA/CD80 cells down-regulated for Ii contain stable MHC class II heterodimers.** Formation of stable  $\alpha\beta$  heterodimers that dissociate upon boiling is a hallmark of correctly conformed MHC class II molecules (39). To determine if the siRNA transductants have properly conformed MHC II molecules, lysates of >3-week drug-selected cells were either boiled or not boiled before Western blotting with the L243 mAb, which is specific for MHC II  $\alpha\beta$  dimers. Nonboiled samples contain bands migrating at ~55 kDa that correspond to stable MHC class II  $\alpha\beta$  heterodimers (Fig. 2D). Therefore, tumor cells transduced with the CIITA and Ii siRNA express properly conformed MHC class II molecules in the absence of Ii.

**MCF10/CIITA/CD80/siRNA cells present endogenous HER-2/neu peptides and activate CD4<sup>+</sup> T cells.** To assess if MHC class II is functional in the absence of Ii, MCF10/CD80/CIITA/siRNA 32 cells, which are down-regulated for Ii >99%, were used as APCs for the activation of PBMCs to tumor-encoded epitopes. HER-2/neu, a growth factor receptor that is overexpressed by many tumors, was used as the tumor antigen because (a) MCF10 cells constitutively overexpress HER-2/neu (Fig. 3A); (b) HER-2/neu contains two HLA-DR7-restricted peptides (p98 and p776; refs. 33, 34); and (c) MCF10/CD80/CIITA cells express DR7. To control for HLA-DR specificity, ocular melanoma OMM2.3 and breast cancer SUM159PT were transduced with CD80 and DR7 or DR4 retroviruses (Fig. 3B). OMM2.3 and SUM159PT cells express HER-2/neu and do not express Ii, and the respective transductants express CD80 and DR4 or DR7 (Fig. 3C).

To determine if the CIITA/siRNA cells present endogenously synthesized tumor peptides, HER-2/neu peptide p98 and/or p776-activated T cells were cocultured with transduced tumor cells, and IFN- $\gamma$  production was measured. Soluble peptide p98 or p776 was added to some wells to determine if the transductants preferentially present peptides from exogenous sources. MCF10/CIITA/CD80/siRNA 32 and MCF10/CIITA/CD80 present peptides p98 and p776 from endogenously synthesized HER-2/neu (Fig. 4A and B). Peptide p776 is presented equally well by MCF10/CIITA/CD80/siRNA 32 and MCF10/CIITA/CD80, whereas MCF10/CIITA/CD80 cells are slightly better presenters of p98 ( $P < 0.05$ ). Both MCF10 cell lines present exogenously pulsed HER-2/neu peptides. Exogenous peptide presentation is not significantly better than presentation of endogenous peptide and is not affected by Ii expression. HER-2/neu presentation is DR7 restricted because cells lacking DR7 (MCF10/CD80, OMM2.3/DR4/CD80, and SUM159/CD80) do not induce significant IFN- $\gamma$  release. T-cell activation is limited to MHC-restricted antigen, and there is no activation to allogeneic MHC antigens because neither MCF10/CD80 nor SUM159/CD80 cells induce significant IFN- $\gamma$  release. Therefore, down-regulation of Ii by siRNA slightly diminishes presentation of peptide p98 but does not significantly affect presentation of p776, suggesting that presentation of some epitopes may be Ii dependent, whereas presentation of others is independent of Ii. In either case, removal of Ii does not render MHC class II molecules unable to present antigen, indicating that MHC class II antigens are functional in the absence of Ii.

To rule out that PBMCs were responding to secreted HER-2/neu that is subsequently endocytosed, PBMCs were mixed with supernatants from MCF10/CIITA/CD80/siRNA 32 cells. PBMC were pulsed with p98 as a positive control. Peptide-pulsed PBMC

produced IFN- $\gamma$ ; however, supernatant-pulsed cells did not (data not shown). Therefore, MCF10/CIITA/CD80/siRNA 32 cells are presenting endogenously synthesized molecules.

Peptides p98 and p776 also activate CD8<sup>+</sup> T cells, suggesting that they may contain nested MHC class I epitopes (33, 34). The HER-2/*neu*-activated T cells share DR7 with the transduced MCF10 cells, and DR7 and A24 with SUM159PT/DR7/CD80 cells. No MHC class I alleles are common between p98- and p776-activated PBMCs and transduced MCF10 cells. Because peptides p98 and p776 are presented by both DR4 and DR7, there is the potential for the activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells by SUM159PT/DR7/CD80 but not by MCF10/CIITA/CD80/siRNA 32 cells. To identify which T cells are activated, PBMCs were primed with p98 and p776 and subsequently incubated with vaccine cells in the presence of antibodies to MHC class I and/or MHC class II. Antibodies to MHC class II block antigen presentation by MCF10/CIITA/CD80/siRNA 32 and MCF/CIITA/CD80, whereas antibodies to both MHC I and II block antigen presentation by SUM159/DR7/CD80 cells (Fig. 4C). To confirm the activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, PBMCs were primed with p776 and depleted for CD4<sup>+</sup> or CD8<sup>+</sup> T cells, before activation by MCF10 or SUM159PT transductants. Depletion of CD4<sup>+</sup> T cells completely eliminates T-cell activation by both MCF10/CIITA/CD80/32 and SUM/DR7/CD80 cells. Depletion of CD8<sup>+</sup> T cells significantly reduces T-cell activation by SUM159PT transductants and has a smaller effect on MCF10/CIITA/CD80/32-induced T-cell activation (Fig. 4D and E). This latter effect is probably nonspecific because MCF10/CIITA/CD80/32 cells do not share MHC class I alleles with PBMCs. Similar results were obtained with p98-primed PBMCs (data not shown).

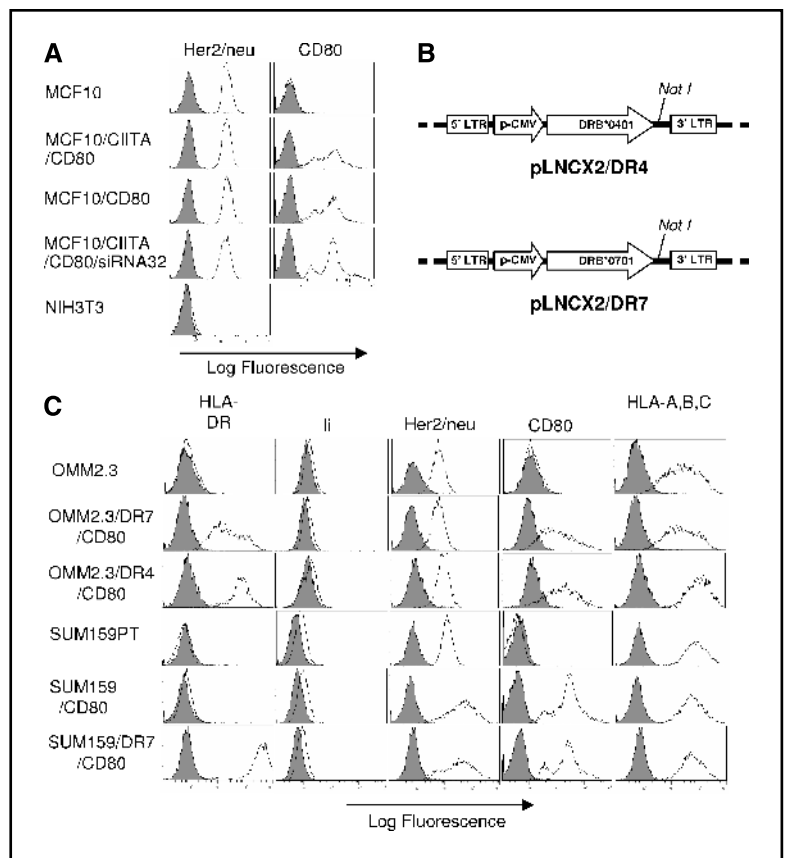
MCF10/CIITA/32 cells were also included in this experiment (Fig. 4D) to determine if coexpression of CD80 enhances boosting of HER-2/*neu*-specific CD4<sup>+</sup> T cells. In agreement with earlier findings (20), CD80-expressing transductants are better stimulators. Therefore, the transductants activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells if they share common alleles with the responding PBMCs, and coexpression of CD80 enhances activation.

**Nonmalignant cells do not activate T cells.** A potential problem with cell-based vaccines is that they will activate T cells against nonmalignant cells due to cross-reactivity with normal self-antigens. To determine if the MHC II vaccines induce reactivity against nonmalignant cells, PBMCs were activated with HER-2/*neu* p776 peptide and tested on MCF10 cells and their nonmalignant counterpart, MCF10A cells. As measured by flow cytometry, MCF10A cells express HER-2/*neu*, although at slightly lower levels than MCF10 cells (Fig. 5A compare with Fig. 3A). Unlike MCF10 cells, MCF10A cells do not express MHC II molecules; however, they are inducible for MHC II if incubated for 48 hours with 1,000 units/mL of rIFN $\gamma$ . As seen in Fig. 5B, neither untreated nor IFN $\gamma$ -treated MCF10A cells activate T cells. Therefore, tumor-specific T cells are activated by MHC II<sup>+</sup>CD80<sup>+</sup> tumor cell-based vaccines and not by nonmalignant cells of the same tissue origin.

## Discussion

A goal of tumor immunotherapy is to activate T lymphocytes to tumor-encoded antigens. Although some tumor peptides have been identified, many are unknown, and it is unclear how diverse an immune response is needed to eradicate tumor cells *in vivo*. The

**Figure 3.** MCF10, OMM2.3, and SUM159PT cells overexpress HER-2/*neu* and transduced HLA genes and do not express li. A, parental and transduced MCF10 and control NIH3T3 cells labeled with antibodies to HER-2/*neu* or CD80 and analyzed by flow cytometry. B, HLA-DR4 and HLA-DR7 retroviral constructs. C, parental and transduced OMM2.3 and SUM159 cells stained with antibodies to HLA-DR (mAb L243), li (mAb PIN1.1), HER-2/*neu*, CD80, or HLA-A,B,C and analyzed by flow cytometry. Unfilled peaks, specific antibody-stained cells; filled peaks, isotype control stained cells.



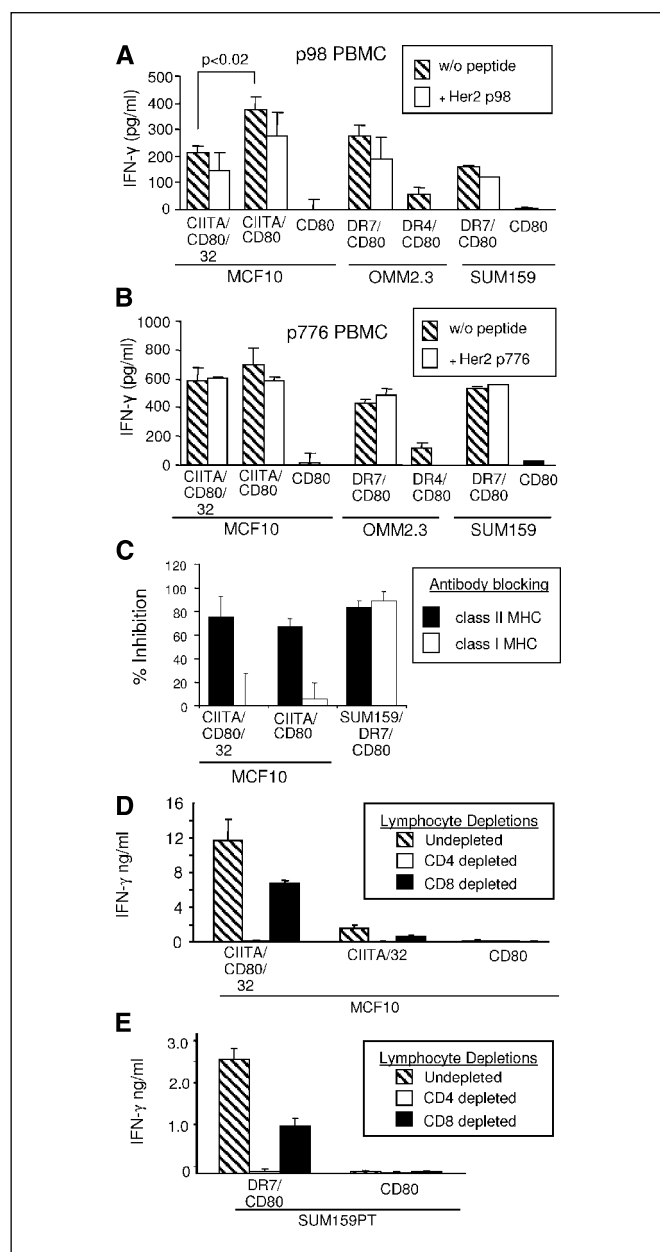


**Figure 4.** CIITA- and CD80-transduced MCF10 cells down-regulated for Ii by RNAi present endogenously synthesized HER-2/*neu* epitopes and activate CD4<sup>+</sup> T cells. HLA-DR7-restricted peptide 98-specific T cells (A) or peptide 776-specific T cells (B) were cocultured with MCF10, OMM2.3, or SUM159PT parental cells or transductants, and T-cell activation was quantified by measuring IFN-γ release. Exogenous peptide was added to some wells. C, p98- and p776-primed T cells were cocultured with transduced MCF10 or SUM159PT cells in the presence of antibodies to HLA-DR (mAb L243) or HLA-A,B,C (mAb W6/32). HLA-DR7-restricted, p776-primed PBMCs were depleted for CD4<sup>+</sup> or CD8<sup>+</sup> T cells before incubation with MCF10 (D) or SUM159PT (E) transductants as in (A) and (B).

vaccines (transductants) described here circumvent the identification of tumor antigens and potentially present a diversity of immunogenic peptides. The vaccines were designed to preferentially present endogenously synthesized MHC II-restricted epitopes (1). In previous studies, we made vaccines by transducing MHC class II alleles into Ii-negative tumor cells (20, 23, 24, 40, 41). We now show that immunogenic cells can also be made by transducing tumor cells with the CIITA with or without Ii.

Our vaccine design was based on the hypothesis that coexpression of Ii would inhibit the presentation of endogenously synthesized tumor peptides, a hypothesis supported by our own earlier work and extensive work of others in nontumor systems (8, 9). Recent mass spectroscopy studies (42) provide direct biochemical support for this hypothesis and also provide an explanation for the lack of inhibition of Ii for HER-2/*neu* peptides p98 and p776. These investigators showed that the MHC II molecules of MHC II<sup>+</sup>Ii<sup>-</sup> cells contain peptides presented by MHC II<sup>+</sup>Ii<sup>+</sup> cells plus additional novel peptides, which are not presented by MHC II<sup>+</sup>Ii<sup>+</sup> cells (42). Because p98 and p776 were originally identified as epitopes presented by MHC II<sup>+</sup>Ii<sup>+</sup> professional APCs (33, 34), they are most likely in the category of epitopes that are presented by both Ii<sup>+</sup> and Ii<sup>-</sup> APCs. However, the findings of ref. (42) make it likely that the MHC II<sup>+</sup>Ii<sup>-</sup> transductants also present novel tumor antigen epitopes that are not presented by professional APCs. In this fashion, Ii RNAi vaccines may activate a more diverse repertoire of tumor-specific CD4<sup>+</sup> T cells than professional APCs and may activate T cells that have not previously been tolerized by the tumor.

Clinical studies also support an inhibitory role for Ii. Chamuleau et al. have shown that acute myelogenous leukemia (AML) patients in complete remission whose HLA-DR<sup>+</sup> myeloid leukemic blasts have low levels of the MHC class II-associated Ii peptide (CLIP), a degradation product of Ii, have a significantly better clinical prognosis than patients whose blasts are DR<sup>+</sup>CLIP<sup>+</sup> (43). Similar to AML blasts of progressor patients, DM-deficient mice also have DR<sup>+</sup>CLIP<sup>+</sup> APCs, which are inefficient presenters of endogenously synthesized molecules (44). Preferential expression of the Ii p35 isoform is also associated with increased malignancy in chronic lymphocytic leukemia, and this effect has been attributed to reduced presentation of endogenously synthesized

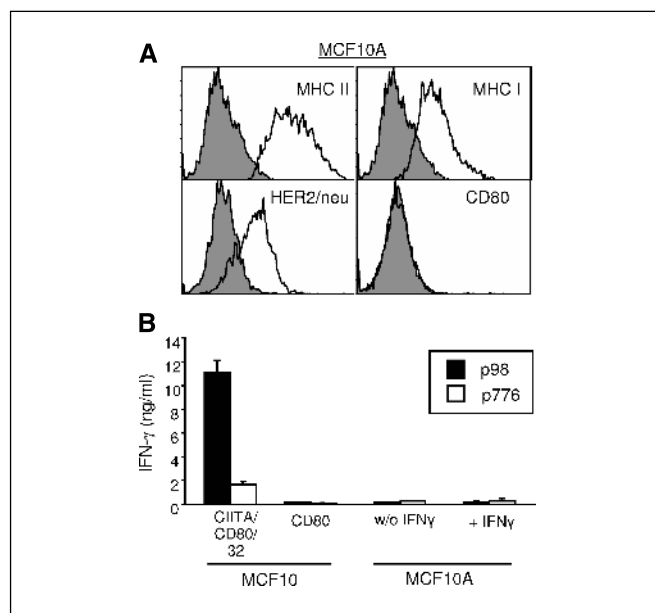


**Figure 5.** Nonmalignant breast cells do not activate T cells. A, untreated MCF10A cells were stained for CD80 or HER-2/*neu*, and IFN-γ-treated (1,000 units for 48 hours) MCF10A cells were stained for HLA-DR or HLA-A,B,C and analyzed by flow cytometry. Unfilled peaks, specific antibody-stained cells; filled peaks, isotype control stained cells. B, PBMCs were pulsed with p776 or p98 and incubated with MCF10, MCF10A, or IFN-γ-treated MCF10A cells, and the supernatants were analyzed for IFN-γ as per Fig. 4.

tumor antigens (38). Expression of CLIP is also associated with polarization towards a type 2 CD4 (Th2) response (45, 46), which may favor tumor progression (47).

Although early studies suggested that Ii expression was essential for MHC class II function (14, 15, 17), there are now many reports showing that MHC II alleles are properly conformed and functional in the absence of Ii (19, 20, 25, 48, 49). The present report extends this conclusion and shows that peptide affinity for MHC II is not affected by Ii, because peptide binding to surface MHC II molecules is similar for Ii<sup>+</sup> and Ii<sup>-</sup> cells. Therefore, although Ii may be required during development for expression of some mouse MHC II alleles, most MHC II alleles are stable and functional in the absence of Ii.

We envision that the vaccine strategy described here will be used to generate MHC II allele-specific vaccines from established cell lines. We propose a "cocktail" approach in which a patient will be treated with a mixture of multiple cell lines expressing MHC class I and II molecules matched to their genotype. Following HLA typing, a patient's "semicustomized cocktail" would be prepared from stocks of frozen transduced cells. This approach depends on the existence of shared tumor antigens and eliminates the need for autologous tumor cells, making it feasible to treat most patients. Although retroviruses could be used to induce MHC II and CD80 molecules, alternative techniques that are less controversial would be preferable.

This vaccine strategy has the potential to activate T cells to self-antigens that are also expressed on nonmalignant cells. Autoimmunity has not been observed in the three mouse tumor systems studied *in vivo*,<sup>4</sup> and the absence of reactivity with the nonmalignant breast line MCF10A suggests that autoimmunity against normal cells will also not be a problem in patients. In addition, a DR4<sup>+</sup>DR7<sup>+</sup> patient with advanced metastatic ocular melanoma has been treated with irradiated OMM2.3/CD80/DR4 and OMM2.3/CD80/DR7 vaccines, and no autoimmune or other complications were noted.<sup>5</sup>

Vaccines consisting of tumor cells transduced with the CIITA and Ii siRNAs have several potential advantages over previous vaccines in which MHC II nonexpressing tumor cells were transduced with individual MHC II alleles. Vaccines made by transducing single *HLA-DR* alleles are limited to presenting tumor antigen epitopes restricted by the transduced allele(s). In contrast, CIITA-transduced

vaccine cells express multiple *HLA-DR* alleles, as well as *DP* and *DQ* alleles, and hence have the potential to present a much broader repertoire of tumor antigen epitopes. The CIITA/siRNA vaccines also differ from the previous MHC II vaccines in that expression of the CIITA up-regulates accessory molecules, such as HLA-DM. Although our previous studies have not shown that HLA-DM expression facilitates vaccine efficacy (50), studies by others have shown that HLA-DM expression stabilizes MHC II in the absence of Ii, aids MHC II traffic, and helps edit the MHC II peptide repertoire (42).

The CIITA/Ii siRNA strategy also expands the choice of tumor cells which could be used to generate vaccines to include tumors that constitutively express MHC II or are inducible for MHC II by treatment with IFN $\gamma$ . Tumor cells that constitutively coexpress MHC II and costimulatory molecules, such as some leukemias (43), are particularly attractive targets for Ii siRNA therapy because creating a vaccine would only require down-regulating Ii via RNA interference (RNAi). This approach is supported by studies in which Ii was down-regulated in MHC II-positive, Ii-positive mouse tumor cells by antisense RNA (51–55). Mice immunized with the Ii antisense down-regulated tumor cells were protected against later challenge with wild-type tumor. Because siRNA is more effective in down-regulating Ii than antisense RNA, Ii siRNA vaccines may have more therapeutic efficacy than Ii antisense vaccines. Therefore, tumor cells expressing the CIITA and costimulatory molecules may be useful reagents, and concomitant down-regulation of Ii via RNAi may further improve vaccine efficacy and protect and/or treat tumor recurrence and/or metastatic disease.

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# Activation of Tumor-specific CD4<sup>+</sup> T Lymphocytes by Major Histocompatibility Complex Class II Tumor Cell Vaccines: A Novel Cell-based Immunotherapy

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## ABSTRACT

Mouse tumor cells transfected with syngeneic MHC class II and costimulatory molecule genes are therapeutic vaccines in mice, provided they do not coexpress the class II-associated invariant chain (Ii). We demonstrated previously that the vaccine cells present tumor peptides via the endogenous antigen presentation pathway to activate CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Because of their efficacy in mice, we are translating this vaccine strategy for clinical use. To obtain MHC class II<sup>+</sup>CD80<sup>+</sup>Ii<sup>-</sup> human tumor cells, we developed retroviruses encoding HLA-DR and CD80. The HLA-DR virus encodes the DR $\alpha$  and DR $\beta$ 0101 chains using an internal ribosomal entry site to coordinate expression. SUM159PT mammary carcinoma and Mel 202 ocular melanoma cells transduced with the retroviruses DRB1/CD80 express high levels of DRB0101 and CD80 on the cell surface in the absence of Ii. Irradiated SUM159PT/DR1/CD80 vaccines stimulate proliferation of non-HLA-DRB0101 peripheral blood mononuclear cells and present an exogenous DR1-restricted tetanus toxoid (TT) peptide, indicating that the transduced DRB0101 is functional. SUM159PT/DR1/CD80 vaccines were further transduced with a retrovirus encoding the TT fragment C gene, as a model tumor antigen. These cells stimulate IFN- $\gamma$  release from TT-primed human DRB0101 peripheral blood mononuclear cells, demonstrating their ability to present “endogenous” tumor antigen. Depletion and antibody blocking experiments confirm that MHC class II-restricted, endogenously synthesized epitopes are presented to CD4<sup>+</sup> T cells. Therefore, the MHC class II vaccines are efficient antigen-presenting cells that activate tumor-specific MHC class II-restricted, CD4<sup>+</sup> T lymphocytes, and they are a novel and potential immunotherapeutic for metastatic cancers.

## INTRODUCTION

A key goal of cancer vaccine development is to generate therapeutic reagents that provide protection against development and outgrowth of metastatic tumor cells. Because metastatic disease for many tumors appears at varied intervals after diagnosis of primary tumor, the most effective vaccines will provide long-term immune memory. We (1, 2) and others (3–5) have focused on the critical role of CD4<sup>+</sup> T cells in cancer vaccines, because these cells, in conjunction with CD8<sup>+</sup> T lymphocytes, are likely to provide maximal antitumor immunity with long-term immunological memory.

To better activate tumor-specific CD4<sup>+</sup> T cells, we have designed cell-based vaccines that facilitate the presentation of MHC class II-restricted tumor peptides to responding CD4<sup>+</sup> T cells (2). We have reasoned that tumor cells present a variety of MHC-restricted peptides that are potential tumor antigens, and that if they constitutively express MHC class I molecules and are transduced with syngeneic MHC

class II and costimulatory molecules, they could function as antigen-presenting cells (APCs) for MHC class I- and class II-restricted tumor peptides. This approach is appealing for several reasons: (a) identification of specific tumor antigen epitopes is not required; (b) multiple class I- and class II-restricted epitopes will be presented concurrently; and (c) CD4<sup>+</sup> T cells may be activated to novel MHC class II-restricted tumor epitopes not presented by professional APCs.

To test our approach, cell-based vaccines were generated from three independent mouse tumors that constitutively express MHC class I molecules and do not express MHC class II molecules (mouse Sal sarcoma, B16 melanoma, and 4T1 mammary carcinoma). The mouse tumor cells were transfected with syngeneic MHC class II  $\alpha$ - and  $\beta$ -chain genes and with costimulatory molecule (CD80) genes. This vaccine approach was adapted for two reasons:

(a) In conventional immunity, activation of CD4<sup>+</sup> T cells requires the uptake of soluble antigen by professional APCs and the cross-presentation of the processed antigen to specific CD4<sup>+</sup> T cells. If antigen is limiting, as it may be when tumor burden is low, available antigen may not be sufficient for the activation of tumor-specific CD4<sup>+</sup> T cells. Our vaccine design bypasses the requirement for professional APCs and soluble tumor antigen because the genetically modified tumor cell vaccines function as the APC.

(b) Because each vaccine cell expresses both MHC class I and class II molecules and their associated tumor peptides, a given vaccine cell could be an APC for both MHC class I- and class II-restricted tumor antigen epitopes and concurrently activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. If CD4<sup>+</sup> and CD8<sup>+</sup> T cells are simultaneously activated by the same APC and are in close proximity to each other, then the transfer of “help” from the activated CD4<sup>+</sup> to the CD8<sup>+</sup> T cell should be highly efficient (2, 6), thereby maximizing the therapeutic effect. CD4<sup>+</sup> T cell “help” could be provided to CD8<sup>+</sup> T cells via the classical mechanism of soluble cytokine production or by the alternative mechanism of up-regulation of CD40 on the vaccine cells (APCs; Ref. 7). Regardless of the mechanism of help, the activated CD4<sup>+</sup> T cells do not need to directly interact with wild-type tumor cells or with professional APCs, because their only role is to provide help to CD8<sup>+</sup> T cells.

Extensive studies using a variety of mouse tumor models have shown that immunization/immunotherapy with the MHC class II plus CD80-modified vaccines induces a potent antitumor immunity against wild-type tumor that confers prophylactic protection (1), delays or eliminates growth of primary solid tumors (8), reduces both experimental (9) and spontaneous metastasis, and extends survival (10, 11). Immunization studies using genetically marked vaccine cells have demonstrated that the vaccine cells themselves are the relevant APCs *in vivo* (12–14), and that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are required for the optimal antitumor effect (8, 11). Therefore, by circumventing the traditional cross-presentation pathway for activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, these genetically modified cancer vaccines induce a potent tumor-specific immunity against wild-type tumor cells.

The efficacy of the vaccines depends on their ability to present endogenously synthesized, MHC class II-restricted tumor antigen epitopes to activate CD4<sup>+</sup> T cells. Presentation of endogenous antigen

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is favored when levels of the class II-associated accessory molecule, invariant chain (Ii), are limiting (reviewed in Ref. 15). Because MHC class II and Ii are coordinately regulated and Ii expression blocks vaccine efficacy (14, 16, 17), we have used tumor cells that do not constitutively express MHC class II (or Ii) as the “base” line for the vaccines.

Because of its therapeutic efficacy in mice, we are translating this vaccine approach for the treatment of human cancers. Our experimental design is to express syngeneic MHC class II and costimulatory molecules in established human tumor cell lines that constitutively express MHC class I molecules and do not constitutively express MHC class II or Ii. To achieve this goal, we are using retroviral transduction to express HLA-DR and CD80 molecules in two human tumor lines, an ocular melanoma (Mel 202) and a mammary carcinoma (SUM159PT). The resulting HLA-DRB0101-transduced cells stably express high plasma membrane levels of functional HLA-DRB0101, as measured by immunofluorescence, activation of allogeneic peripheral blood mononuclear cells (PBMCs), and presentation of a DR1-restricted peptide. To ascertain that the transductants activate CD4<sup>+</sup> T lymphocytes to endogenously synthesized antigens, we have shown that tetanus toxoid (TT) fragment C-transduced vaccine cells activate TT-specific HLA-DRB0101-restricted CD4<sup>+</sup> T cells. Therefore, human tumor cells genetically modified by gene transfer to express syngeneic MHC class II and costimulatory molecules express functional HLA-DR molecules and may serve as useful therapeutics for activating tumor-specific CD4<sup>+</sup> T lymphocytes of cancer patients.

## MATERIALS AND METHODS

**Construction of Retroviral Vectors.** For the pLNCX2/DR1 construct, DRA cDNA in the RSV.5 vector (18) was PCR amplified including 5' *NheI* and 3' *XhoI* restriction sites: DRA 5' primer, TGTCGCTAGCATGGCCATA-AGTGGAGT; and DRA 3' primer, ACTGCTCGAGTTACAGAGGCCCTGCGTT. The PCR product was cloned into the pCR2.1-TA vector (Invitrogen, Carlsbad, CA), excised with *NheI* and *EcoRI*, and inserted into the multiple cloning site (MCS)-A of *NheI*- and *EcoRI*-digested pIRES plasmid (Clontech, Palo Alto, CA). DRB0101 in the RSV.5 vector (18) was PCR amplified including 5' *XmaI* and 3' *NotI* sites and subcloned into the 5' *XmaI* and 3' *NotI* sites of the MCS-B of the pIRES vector: DRB0101 5' primer, AG-TACCCGGGATGGTGTGTCTGAAGCTC; and DRB01013' primer, TAG-TGCGGCCGCTCAGCTCAGGAATCCTGTG. PCR conditions for both DRA and DRB0101 amplifications were: denature at 94°C for 2 min, denature at 94°C for 1 min, anneal at 60.9°C or 62.9°C (DRA and DRB0101, respectively) for 1 min, extend at 72°C for 3 min (High Fidelity Taq; Roche, Basel, Switzerland); repeat the last three steps 30 times and extend at 72°C for 7 min. The resulting construct is pIRES/DR1 (Fig. 1A).

The pLNCX2 retroviral vector (Clontech) was modified to include a linker containing an *AvrII* site in the MCS. To make the linker, equimolar amounts of the oligonucleotides (5'-GATCTCGAGCTCCTAGGAATTGTTGGCCGAGGC-3' and 3'-AGCTCGAGGATCCTTAACAAACCGGCTCCGCCGG5'-) were mixed, heated at 95°C for 5 min, and then incubated at 22°C for 1 h. The resulting linker was ligated to *BglII*- and *NotI*-digested pLNCX2. The resulting construct is pLNCX2/*AvrII*.

The DRA-IRES-DRB0101 fragment of the pIRES/DR1 was digested with *NheI* and *NotI* and gel purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA) and then ligated to *AvrII*- and *NotI*-digested pLNCX2/*AvrII*. The final MHC class II construct is pLNCX2/DR1 (Fig. 1A).

For the pLHCX/CD80(HPH) construct, pLHCX (hygromycin resistance; Clontech) was modified to include a 5' *BamHI* site and a 3' *HindIII* site by inserting an oligonucleotide linker between the *HindIII* and *ClaI* sites of the MCS. The original *HindIII* in the vector was deleted by insertion of the linker. *XhoI*, *HpaI*, *AvrII*, and *NotI* restriction sites were included in the linker for future cloning purposes. The linker sequence was: L1, 5'-AGCTGCTCGAGT-TAACGGATCCTAGGAAGCTTGC GGCCGCAT-3'; and L2, 5'-CGAT-GCGGCCGCAAGCTTCCTAGGATCCGTTAACTCGAGC-3'.

Human CD80 was excised from the pREP10/B7.1 vector with *BamHI* and

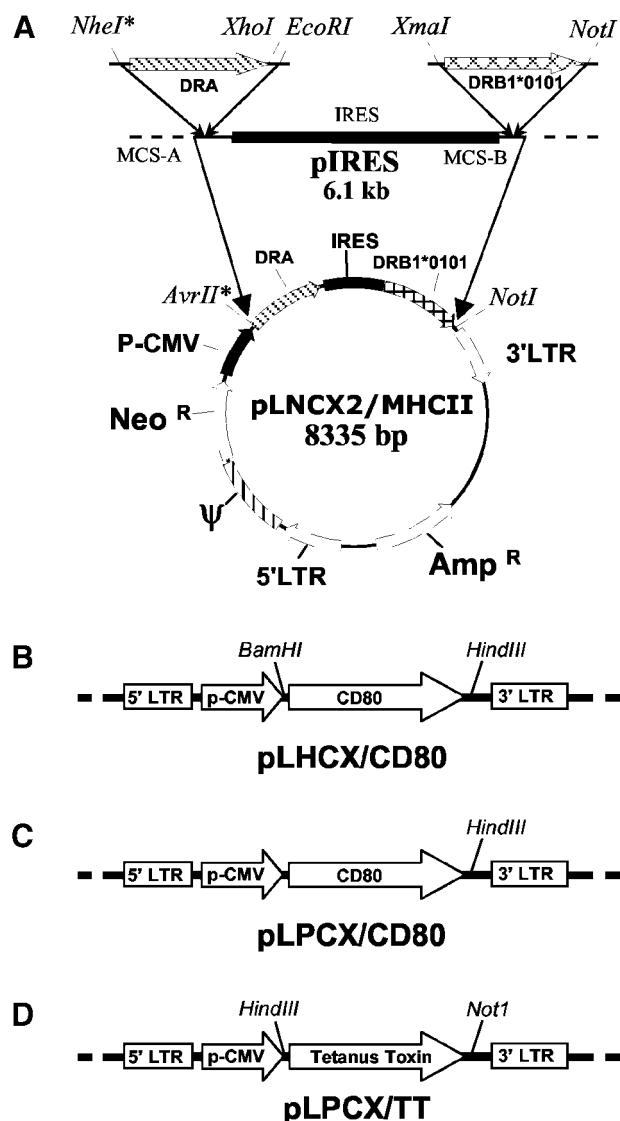


Fig. 1. Retroviral constructs made and used in these studies. A, the pLNCX2/MHC II construct contains the DRA and DRB0101 cDNAs flanking an IRES and under the control of the cytomegalovirus (CMV) promoter and contains the *G418* resistance gene. B and C, the pLHCX/CD80 and pLPCX/CD80 constructs encode the human *CD80* gene and contain the hygromycin or puromycin resistance genes, respectively. D, the pLPCX/TT construct encodes the TT fragment C gene and contains the puromycin resistance gene. LTR, long terminal repeat.

*HindIII* and inserted into the modified pLHCX vector using the *BamHI* and *HindIII* sites (Fig. 1B).

For the pLPCX/CD80 (Puro) construct, the *CD80* gene was excised from pREP10/B7.1 by digestion with *BglII* and *HindIII* and ligated into pLPCX digested with *BamHI* and *HindIII*. The *BamHI* and *BglII* sites were deleted during this process (Fig. 1C).

For the pLPCX/TT construct, TT fragment C DNA was PCR amplified from pCR Blunt (19) to include an ATG start codon and *HindIII* site at the 5' end and a *BamHI* site at the 3' end: 5' primer sequence, CCGCCGAGCT-TGCCACCATGAAAAACCTTGATTGTT; and 3' primer sequence, CTGT-TCGGATCCTTAGTCGTTGGTCCAA. PCR conditions were: denature at 94°C for 5 min, denature at 94°C for 1 min, anneal at 55°C for 1 min, extend at 72°C for 1 min (*Taq*DNA polymerase; Invitrogen); repeat the last three steps 35 times and extend at 72°C for 10 min. The resulting PCR product was inserted into the TA cloning vector, pGEM-T-Easy (Invitrogen). The modified TT fragment C gene was then excised with *HindIII* and *BamHI* and inserted into the mammalian expression vector pCDNA3.1/Zeo(+) (Invitrogen). A *HindIII*-*NotI* fragment containing the TT fragment C gene was then excised from pCDNA3.1/Zeo(+) and subcloned into the *HindIII*-*NotI* site of the MCS of pLPCX(Puro) to produce the pLPCX/TT vector.

Table 1 Tumor cell vaccines (transductants) used in these studies

| Cell line        | HLA-DRB0101 | CD80 | TT <sup>a</sup> | Drug selection                                           |
|------------------|-------------|------|-----------------|----------------------------------------------------------|
| SUM/DR1          | +           |      |                 | G418 <sup>b</sup>                                        |
| SUM/CD80         |             | +    |                 | HPH <sup>c</sup>                                         |
| SUM/TT           |             |      | +               | Puro <sup>d</sup>                                        |
| SUM/DR1/CD80     | +           | +    |                 | G418 <sup>b</sup> + Puro <sup>d</sup>                    |
| SUM/DR1/CD80/TT  | +           | +    | +               | G418 <sup>b</sup> + HPH <sup>c</sup> + Puro <sup>d</sup> |
| SUM/DR1/TT       | +           |      | +               | G418 <sup>b</sup> + Puro <sup>d</sup>                    |
| SUM/CD80/TT      |             | +    | +               | HPH <sup>c</sup> + Puro <sup>d</sup>                     |
| Mel 202/DR1      | +           |      |                 | G418 <sup>b</sup>                                        |
| Mel 202/CD80     |             | +    |                 | HPH <sup>c</sup>                                         |
| Mel 202/DR1/CD80 | +           | +    |                 | G418 <sup>b</sup> + Puro <sup>d</sup>                    |

<sup>a</sup> Tetanus toxin fragment C.<sup>b</sup> 600 µg/ml.<sup>c</sup> 200 µg/ml.<sup>d</sup> 0.2 µg/ml.<sup>e</sup> 75 µg/ml.

**Cells.** Media for all cell lines contained 1% gentamicin, 1% penicillin/streptomycin (all from BioSource, Rockville, MD), and 2 mM Glutamax (BRL/Life Sciences, Grand Island, NY). All cells and T-cell activation assays were cultured at 37°C in 5% CO<sub>2</sub>. SUM159PT was obtained from the Michigan Breast Cell/Tissue Bank<sup>3</sup> and was maintained in Ham's F-12 medium with 10% heat-inactivated FCS (Hyclone, Logan, UT), 1 µg/ml hydrocortisone, and 5 µg/ml insulin (both from Sigma, St. Louis, MO). Mel 202 (20) was grown in RPMI 1640 (BioSource, Rockville, MD) with 10% FCS, 0.01 M HEPES (Invitrogen, Grand Island, NY), and 5 × 10<sup>-5</sup> M β-mercaptoethanol (J. T. Baker, Inc., Phillipsburg, NJ). Transductants were grown in the same medium as their parental cells, supplemented with G418 (Sigma), puromycin (Clontech, Palo Alto, CA), or hygromycin (Calbiochem, San Diego, CA; see Table 1 for dosages), depending on their transgenes. Sweig and Jurkat cells were obtained from the American Type Culture Collection and were maintained in Iscove's modified Dulbecco's medium (BioSource) supplemented with 10% fetal clone I (FBP; Hyclone). EBV B cells were grown in RPMI 1640 with 10% FCS and 0.01 M β-mercaptoethanol. Peripheral blood mononuclear cells (PBMCs) were grown in Iscove's modified Dulbecco's medium with 5% human AB serum (Gemini Bio-Products, Woodland, CA). All cell lines and procedures with human materials were approved by the Institutional Review Boards of the participating institutions.

**Retrovirus Production.** 293T cells (obtained from the Harvard Gene Therapy Institute) were plated in a 6-cm dish at 9 × 10<sup>5</sup> cells/4 ml of 293T medium [DMEM (BioSource, Rockville, MD), 1% gentamicin, 1% penicillin/streptomycin, 1% Glutamax, and 10% heat-inactivated FCS] and cultured at 37°C. Twenty h later, the growth medium was replaced with 4 ml of 37°C Iscove's modified Dulbecco's medium containing 25 mM HEPES (BioSource), 1% Glutamax, and 10% heat-inactivated FCS. Three h later, the 293T cells were transfected with pLNCX2/DR0101, pLHCX/CD80, pLPCX/CD80, or pLPCX/TT plasmids (8 µg) plus pMD.MLV gag.pol (6 µg) and pMD.G (2 µg) using CaPO<sub>4</sub> (21). Twelve to 16 h after transfection, medium was replaced with 293T growth medium containing 10 mM HEPES. Virus was collected 48 h later and either used immediately or stored at -80°C.

**Retroviral Transduction.** Tumor cells were plated in 6-well plates at 1.2-3 × 10<sup>5</sup> cells/3 ml growth medium/well. Approximately 16 h after plating, when cells were in log phase, growth medium was replaced with 500 µl of viral supernatant mixed with 500 µl of 293T medium containing 4 µg/ml polybrene (Sigma) and 10 mM Hepes. Cells were incubated for 5-6 hrs at 37°C, washed twice with excess PBS and maintained in growth medium for 2 days before adding G418, puromycin, and/or hygromycin.

**Peptides, Antibodies, Reagents, and Immunofluorescence.** TT p2 peptide TT<sub>830-844</sub> (QYIKANSKFIGITEL; Ref. 22) was synthesized at the University of Maryland Biopolymer Laboratory. Formaldehyde-inactivated TT was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY).

Monoclonal antibodies [mAbs; HLA-DR-FITC, CD80-phycoerythrin (PE), and anti-TT], streptavidin-PE, FITC-isotype, and PE-isotype controls were purchased from BD PharMingen (San Diego, CA). Biotinylated HLA-DR0101 was purchased from One Lambda, Inc. (Canoga Park, CA). Rat anti-mouse IgG-FITC was purchased from ICN (Costa Mesa, CA), and CD4-FITC, CD8-FITC, and anti-human IgG-FITC were purchased from Miltenyi Biotech (Au-

burn, CA). Human IgG-FITC was purchased from Cappel (West Chester, PA). Culture supernatants of hybridomas W6/32 (pan HLA-A,B,C), L243 (pan anti-HLA-DR), 28.14.8 (anti-H-2L<sup>d</sup>, D<sup>b</sup>), and PIN1 (anti-Ii) were purified on protein A or protein G affinity columns as described previously (1). Tumor cells and PBMCs were stained for cell surface markers (MHC class I, class II, CD80, CD4, CD8, and immunoglobulin) or fixed and stained for internal markers (Ii, TT) by direct or indirect immunofluorescence as described previously (1, 16). PBMCs were collected by venipuncture from HLA-typed healthy donors and isolated using Histopaque 1077 separation medium as described previously (20). For some experiments, PBMCs were provided by Dr. D. Mann (University of Maryland Baltimore). PBMCs were stored at 1 × 10<sup>7</sup> cells/ml at -80°C until used.

**Western Blots.** Western blot analyses were performed as described (14) using 10% SDS-PAGE. Blots were incubated with PIN1.1 mAb (0.003 µg/ml) followed by sheep anti-mouse HRP at a 1:10,000 dilution (Amersham).

**Allogeneic T-Cell Activation.** Responder PBMCs (1 × 10<sup>5</sup>/well) were cultured in triplicate with 5 × 10<sup>3</sup> or 1 × 10<sup>4</sup> irradiated (CS-137 irradiator; Kewaunee Scientific, Statesville, NC) stimulator SUM159PT (50 Gy) or 5 × 10<sup>5</sup> allogeneic PBMCs (40 Gy) per well in 200 µl/well of culture medium (RPMI, 10% FCS, 1% penicillin/streptomycin, 2 mM Glutamax, and 0.01 M β-mercaptoethanol) in flat-bottomed 96-well microtiter plates (Corning, Inc., Corning, NY). Cells were incubated at 37°C in 5% CO<sub>2</sub> for 6 days and pulsed with [<sup>3</sup>H]thymidine (2 µCi/well) during the final 18 h, after which the cells were harvested onto glass fiber filter mats using a Packard Micromate 196 cell harvester (Downers Grove, IL). Filter mats were sealed into plastic bags with 5 ml of betaplate scintillation fluid (Perkin-Elmer, Gaithersburg, MD) and counted using a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer). Samples were run in triplicate.

Stimulation Index (SI)

$$= \frac{(\text{cpm of transduced experimental tumor cells} + \text{allogeneic PBMC}) - (\text{cpm of transduced tumor cells alone})}{\text{cpm of allogeneic PBMCs alone}}$$

Responder PBMCs do not express DRB0101.

**TT-boosted PBMCs.** DRB0101 PBMCs (2 × 10<sup>7</sup> cells/4 ml/well) were cultured with 1 µg/ml of exogenous TT (Accurate, Westbury, NY) in 6-well tissue culture plates (Corning). After 5 days of culture, nonadherent cells were harvested, washed twice with Iscove's modified Dulbecco's medium, and replated in culture medium with 20 units/ml of recombinant human interleukin 2 (R&D Systems, Minneapolis, MN) at 1 × 10<sup>6</sup> cells/2 ml/well in 24-well plates (Corning). Remaining nonadherent cells were harvested 7 days later, and live cells were isolated using Histopaque-1077 separation medium. TT-activated, nonadherent cells were maintained in culture medium without exogenous interleukin 2 overnight and used the following day.

**Antigen Presentation Assays for Endogenous TT and Exogenous TT Peptides.** Irradiated (50 Gy) stimulator cells (1 × 10<sup>4</sup> or 2.5 × 10<sup>4</sup> cells/well) were cocultured in triplicate with adherent cell-depleted, TT-primed DRB0101 responder PBMCs (5 × 10<sup>4</sup> cells/well) in 200 µl/well in flat-bottomed 96-well microtiter plates (Corning). After 2 days of culture, supernatants were collected and assayed by ELISA for IFN-γ according to the manufacturer's instructions (Endogen, Woburn, MA). For the antibody blocking experiments, 1 × 10<sup>4</sup> stimulator cells were incubated with 10 µg/ml or 12.5 pg/ml of L243 (anti-HLA-DR) or 28.14.8 (isotype-matched irrelevant mAb) in 100 µl/well for 45 min before the addition of responder PBMCs. Values are the averages of triplicate points with their SDs.

For exogenous TT peptide p2 presentation, assays were as for endogenous antigen presentation, except soluble TT peptide p2 (22, 23) was added at the beginning of the 2-day culture period, and antigen-presenting cells not transduced with TT were used.

**CD4, CD8, and CD19 Cell Depletions.** Adherent cell-depleted, TT-primed PBMCs were depleted for CD4<sup>+</sup>, CD8<sup>+</sup>, or CD19<sup>+</sup> cells using magnetic beads, LD columns, and the QuadroMACS separation system according to the manufacturer's instructions (Miltenyi Biotech). Purity of depleted fractions was confirmed by flow cytometry.

**HLA-DR Nomenclature.** The PBMCs used in these studies were HLA typed by PCR; hence, they are known to be HLA-DRB0101. The HLA-DR gene used in these studies was sequenced and identified as HLA-DRB0101 and

<sup>3</sup> Internet address: www.cancer.med.umich.edu/breast\_cell/umbnkbdb.htm.

is abbreviated as "DR1" in the names of the transductants. The TT p2 peptide has been identified as a DR1-restricted epitope; however, its DR1 subtype is not known.

## RESULTS

**Construction of Retroviruses Encoding HLA-DR $\alpha$  Plus HLA-DR $\beta$ , CD80, and TT Fragment C.** To generate human tumor cells expressing high levels of MHC class II molecules, retroviruses encoding HLA-DR $\alpha$  plus HLA-DR $\beta$  genes have been generated. The HLA-DRB0101 allele was selected because it is one of the more common alleles in the Caucasian population and is a frequently used restriction element (24, 25). A novel bicistronic retroviral vector that drives coordinate expression of approximately equimolar amounts of HLA-DR $\alpha$  and HLA-DR $\beta$  was developed using the pLNCX2(neo) retroviral backbone. DR $\alpha$  and DR $\beta$ 0101 cDNAs (18) were cloned upstream and downstream, respectively, of the internal ribosomal entry site (IRES) of the vector pIRES. The DRA-pIRES-DRB segment was then excised from the pIRES vector and ligated into the pLNCX plasmid to yield the pLNCX/DR $\beta$ 1 plasmid (Fig. 1A). This construct will produce a single-chain mRNA driven by the cytomegalovirus promoter in which DR $\alpha$  is translated by a CAP-dependent mechanism and DR $\beta$  is translated via the IRES in a CAP-independent manner.

Because of the critical role of costimulatory molecules in the activation of naive T cells (26), we have also generated retroviral plasmids encoding human CD80 (hCD80). The *hCD80* gene was excised from the pREP10/B7.1 plasmid and ligated into the retroviral vector pLHCX(HPH) or pLPCX(Puro) to form the pLHCX/CD80 (Fig. 1B) or pLPCX/CD80 (Fig. 1C) plasmids, respectively.

To monitor presentation of endogenously synthesized antigen, a retroviral plasmid encoding the TT fragment C was generated. The TT fragment C gene was excised from the pCR Blunt plasmid, an ATG start codon was inserted at its 5' end, and the resulting construct was ligated into the pLPCX(Puro) vector to form the pLPCX/TT retroviral plasmid (Fig. 1D). All retroviral plasmids were packaged in 293T cells, and supernatants containing infectious retroviruses were harvested and used to transduce target tumor cells.

**Transduced Human Tumor Cells Express Cell Surface HLA-DRB0101 and CD80 and Internal TT.** The human ocular melanoma cell line Mel 202 and the mammary carcinoma cell line SUM159PT were transduced with different combinations of the pLNCX2/DR1, pLHCX/CD80, pLPCX/CD80, and pLPCX/TT retroviruses. The resulting transductants are shown in Table 1. SUM159PT and Mel 202 tumors were chosen because they do not constitutively express MHC class II molecules and hence should not express Ii, which we have shown previously inhibits presentation of MHC class II-restricted endogenous antigens (14, 17). To assess the magnitude and stability of transgene expression, transductants were tested by immunofluorescence and flow cytometry 1 week after being placed on drug selection (see Table 1 for drug selection conditions for each transductant line) and intermittently for 6 months thereafter. As shown in Fig. 2, Mel 202 and SUM159PT transductants express high levels of cell surface HLA-DR (L243 mAb), CD80 (CD80-PE mAb), and internal TT (polyclonal anti-TT ab), as measured at 6 months after transduction. HLA-DR-expressing Mel 202 and SUM159PT cells were also biotinylated, and the cell extracts were immunoprecipitated with anti-HLA-DR mAbs to assure proper structural conformation of cell surface-expressed, transduced class II molecules. Both lines displayed high levels of SDS-stable MHC class II  $\alpha\beta$  dimers, indicating proper conformation and peptide binding.<sup>4</sup> The parental lines and transduc-

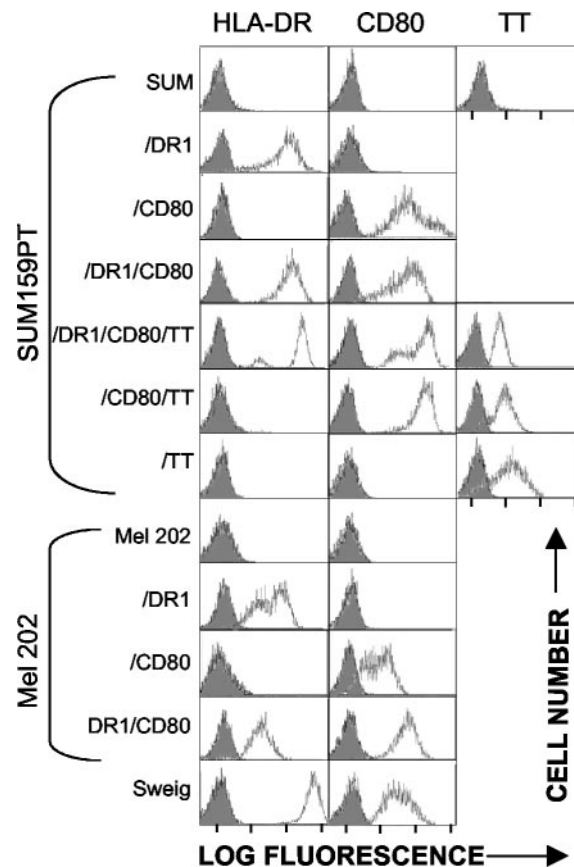


Fig. 2. SUM159PT and Mel 202 transductants express MHC class II HLA-DR and CD80 on the cell surface and TT internally. Live transductants were stained by direct immunofluorescence for plasma membrane HLA-DR (L243-FITC) or CD80 (CD80-PE). Fixed cells were stained by indirect immunofluorescence for internal TT (TT mAb plus fluorescent conjugate). Gray peaks denote staining with fluorescent conjugate alone or isotype control; white peaks represent staining with directly coupled primary antibody or primary antibody plus fluorescent conjugate. These data are from one of three to five independent experiments.

tants were also stained for MHC class I molecules (W6/32 mAb). All lines showed strong class I expression, with transductants displaying levels roughly comparable with their parental lines (data not shown).

To ascertain that the MHC class II expression is allele specific, SUM/DR1 and SUM/DR1/CD80 cells were stained for cell surface expression of HLA-DR1 using the HLA-DR1-specific mAb. As shown in Fig. 3, pLNCX2/DR1-transduced SUM cells express high levels of DR1 and only stain at background levels with an irrelevant HLA-DR2-specific mAb. Therefore, SUM/DR1/CD80, SUM/DR1, SUM/CD80, SUM/DR1/CD80/TT, Mel 202/DR1, Mel 202/CD80, and Mel 202/DR1/CD80 transductants express high levels of the transduced *HLA-DR*, *CD80*, and/or *TT* genes as measured by antibody reactivity and immunofluorescence.

**SUM159PT and Mel 202 Cells Do Not Express Invariant Chain.** Because coexpression of Ii inhibits endogenous antigen presentation by MHC class II vaccine cells (14, 17), SUM159PT and Mel 202 cells were tested to ascertain that they do not express Ii. Cells were permeabilized, stained with the Ii-specific mAb PIN-1, and analyzed by flow cytometry. As shown in Fig. 4A, neither tumor line contains Ii, whereas the human B cell line, Sweig, which constitutively expresses Ii, is strongly positive. To further confirm the absence of Ii, detergent extracts of SUM159PT, Mel 202, Ii-positive Sweig, and Ii-negative Jurkat cells were electrophoresed by SDS-PAGE and analyzed by Western blotting for Ii expression. As shown in Fig. 4B, neither SUM159PT, Mel 202, Mel 202/DR1/CD80, nor SUM/DR1/

<sup>4</sup> V. Clements, unpublished results.

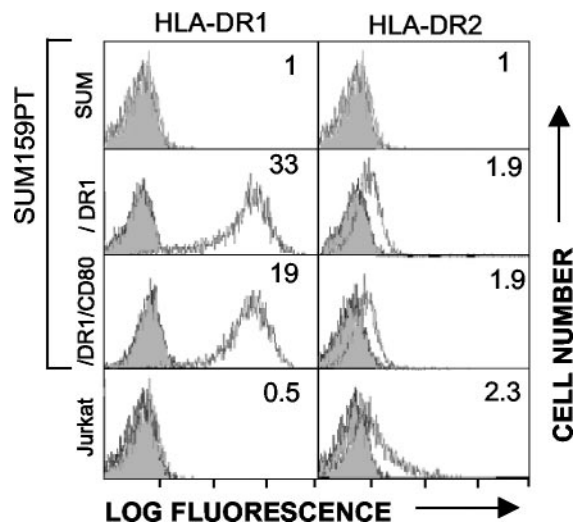


Fig. 3. SUM/DR1 and SUM/DR1/CD80 transductants express HLA-DR1 at the cell surface. Live cells were stained by indirect immunofluorescence for plasma membrane HLA-DR1 (mAb HLA-DR1 biotin) or with an irrelevant Ab (HLA-DR2-biotin) plus an avidin-PE conjugate. Jurkat is a DR1<sup>-</sup> cell line. Gray peaks denote staining with fluorescent conjugate without primary antibody; white peaks represent staining with primary antibody plus fluorescent conjugate. Numbers in the upper right-hand corner of each profile are the mean channel fluorescence for the antibody stained peak. These data are from one of two to five independent experiments.

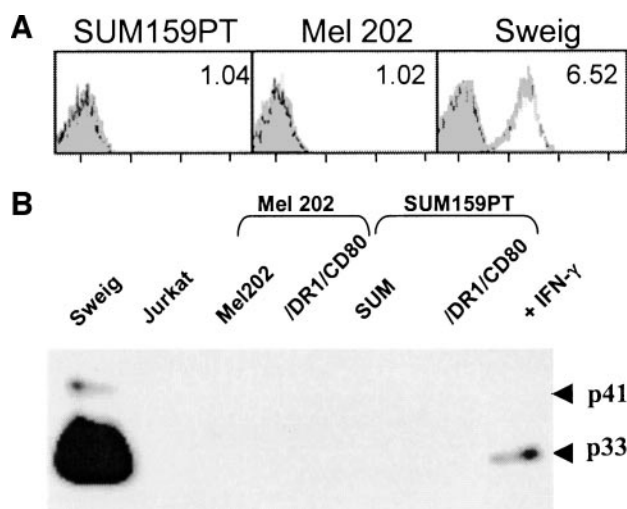


Fig. 4. SUM159PT and Mel 202 cells do not express Ii chain. **A**, fixed cells were stained by indirect immunofluorescence for Ii (mAb PIN1.1; white peaks) or with fluorescent conjugate alone (gray peaks). These data are representative of three independent experiments. **B**, uninduced or IFN- $\gamma$ -treated (+ IFN $\gamma$ ) cells were detergent lysed, electrophoresed on 10% SDS-PAGE gels under reducing conditions, and transferred to nitrocellulose. Blots were stained for Ii with the mAb PIN1.1. Sweig and Jurkat cells are Ii<sup>+</sup> and Ii<sup>-</sup> cell lines, respectively. These data are from one of two to three independent experiments.

CD80 cells contain detectable Ii, although Ii expression is inducible in SUM159PT cells by a 48-h treatment with IFN- $\gamma$ . Therefore, SUM159PT and Mel 202 tumor cells do not constitutively express Ii; therefore, Ii will not be present in the transduced vaccine cells to inhibit binding and presentation of endogenously synthesized peptides.

**HLA-DRB0101 Transductants Stimulate HLA-DR Allogeneic PBMCs.** Coculture of cells expressing functional HLA-DR molecules with allogeneic CD4<sup>+</sup> T lymphocytes results in T-cell proliferation (27). Therefore, to determine whether the HLA-DRB0101 molecules expressed by the transduced tumor cell vaccines are functional, we cocultured the various transductants with allogeneic PBMCs. Responder non-HLA-DRB0101 PBMCs were mixed with various

numbers of irradiated transductants, and proliferation was assessed by measuring the SI at the end of 6 days of culture. Irradiated allogeneic PBMCs were used as a positive control. As shown in Fig. 5A, SUM/DR1/CD80 cells induce high SI, whereas SUM, SUM/CD80, or SUM/DR1 transductants produce only background levels. Therefore, the cell-based vaccines activate allogeneic PBMCs, provided they coexpress DRB0101 and CD80.

**Transduced Tumor Cells Present an HLA-DR1-restricted TT Peptide.** TT peptide p2 is an HLA-DR1-restricted epitope (22). If the HLA-DRB0101 molecules of the transductants are properly conformed and functional, when pulsed with the TT p2 peptide, the transductants should activate TT-specific HLA-DRB0101 lymphocytes. Because the TT-specific CD4<sup>+</sup> T-cell precursor frequency in peripheral blood of the DRB0101 donor was low (data not shown), the HLA-DRB0101 PBMCs were boosted *in vitro* with TT to expand the number of TT-reactive T cells. TT-booster PBMCs were incubated at various ratios with tumor cell transductants pulsed with various quantities of TT p2 peptide to determine whether the transductants present this HLA-DR1-restricted epitope. T-cell activation was assessed by measuring IFN- $\gamma$  release. As shown in Fig. 5B, SUM/DR1/CD80 tumor cells activate the TT-specific T cells as or more efficiently than EBV-transformed HLA-DR1 B cells (DR1-EBV B cells), whereas HLA-DR1-negative parental SUM cells do not activate. Therefore, SUM/DR1/CD80 tumor cells are effective APCs for an HLA-DR1-restricted epitope, further demonstrating that the transduced MHC class II molecules are functional.

**HLA-DR1/CD80 Tumor Cell Transductants Present Endogenous TT and Activate TT-specific T Lymphocytes.** We have generated the DR1/CD80 transductants to use as cancer vaccines to immunize patients and activate their T lymphocytes to tumor-encoded

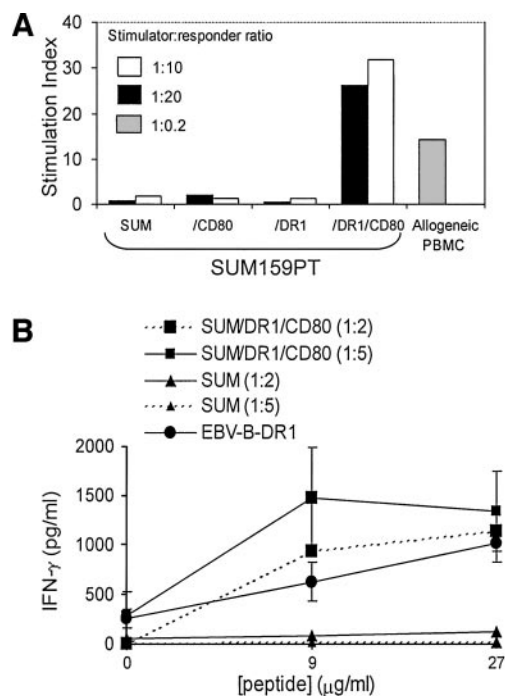


Fig. 5. SUM/DR1/CD80 cells induce proliferation of allogeneic T cells and present a DR1-restricted peptide to DRB0101 PBMC. **A**, irradiated SUM transductants expressing HLA-DRB0101 and/or CD80 or allogeneic PBMCs were cocultured with non-DRB0101 PBMCs at various ratios of APCs to responder lymphocytes. Proliferation was assessed by measuring the SI after 6 days of culture. These data are from one of three independent experiments. **B**, SUM, SUM/DR1/CD80, or DRB0101-expressing EBV B cells were pulsed with the DR1-restricted TT peptide, p2, and cocultured with TT-primed DRB0101 PBMCs at various ratios of APCs to PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN- $\gamma$ . These data are from one of three independent experiments. Bars, SD.

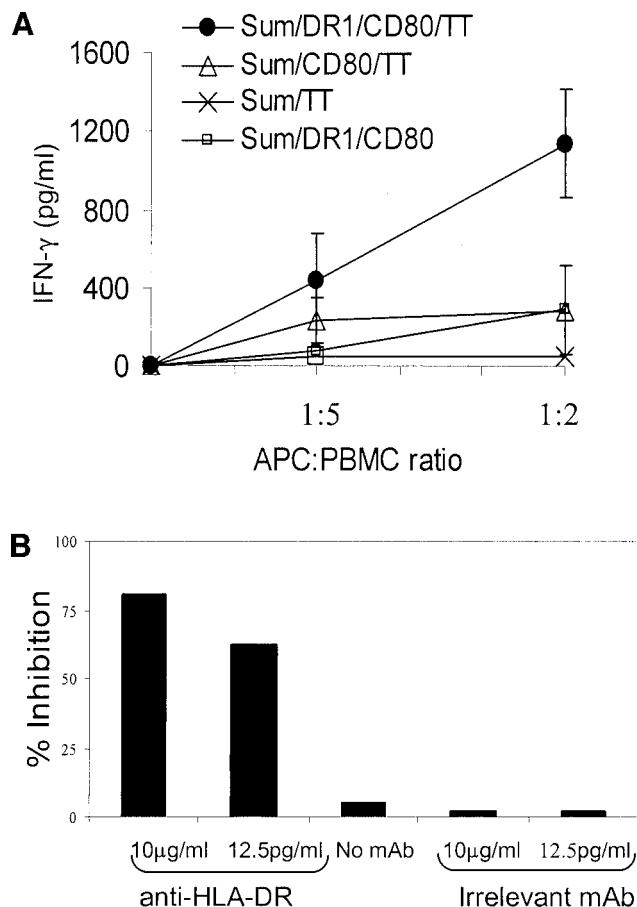


Fig. 6. SUM/DR1/CD80/TT cells activate HLA-DR-restricted DRB0101 PBMCs to tumor-encoded TT. A, irradiated SUM transductants were cocultured with TT-primed DRB0101 PBMCs at various ratios of APCs to responder PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN- $\gamma$ . These data are from one of three independent experiments; bars, SD. B, HLA-DR-specific (L243) or irrelevant (28-14-8) mAb was added to culture wells containing irradiated SUM/DR1/CD80/TT cells before addition of TT-primed responder DRB0101 PBMCs at a ratio of 1:2 APCs to responder cells. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN- $\gamma$ . These data are from one of two independent experiments.

tumor peptides. To achieve this goal, the transductants must not only express functional HLA-DR molecules, but the DR molecules must also bind and present endogenously synthesized tumor peptides. To determine whether the transductants have this capability, we tested SUM/DR1/CD80/TT cells as APCs for endogenously encoded TT. Because the TT construct does not contain a signal sequence, TT protein will reside in the cytoplasm and serve as a “model” tumor antigen for a cytoplasmically localized tumor antigen.

Adherent cell-depleted HLA-DRB0101 PBMCs were boosted *in vitro* with TT as per the experiment of Fig. 5B and cocultured at various ratios with irradiated transduced SUM cells. Activation was assessed by measuring IFN- $\gamma$  release. As shown in Fig. 6A, SUM/DR1/CD80/TT tumor cells activate a potent T-cell response, whereas SUM transductants without DRB0101 (SUM/CD80/TT), without TT (SUM/DR1/CD80), or without DRB0101 and CD80 (SUM/TT) do not activate. Because SUM/TT and SUM/CD80/TT cells do not activate, TT is not being released into the culture medium and being presented by other APCs in the PBMC population. Therefore, tumor cells transduced with *HLA-DRB0101*, *CD80*, and *TT* genes are effective APCs for endogenously encoded molecules.

To further analyze whether the presentation of endogenous TT is DR1 restricted, anti-HLA-DR mAb (L243) was added at various concentrations at the beginning of the assay. As shown in Fig. 6B, in

the presence of the highest dose of antibody, T-cell activation is inhibited >80%, whereas an irrelevant isotype-matched mouse H-2L<sup>d</sup>-specific mAb does not inhibit.

#### DR1/CD80/TT Tumor Cells Activate CD4<sup>+</sup> T Lymphocytes.

To identify the PBMCs that are specifically activated by the vaccine cells, adherent cell-depleted, TT-primed DRB0101 PBMCs were depleted for CD4<sup>+</sup> or CD8<sup>+</sup> T cells or for B cells and then used as responding cells in antigen presentation assays with SUM/DR1/CD80/TT transductants. T and B cells were depleted by magnetic bead separation. To ascertain the efficiency of the depletions, PBMCs before and after depletion were tested by flow cytometry for the percentage of CD4<sup>+</sup>, CD8<sup>+</sup>, and immunoglobulin<sup>+</sup> (B) cells. As shown in Fig. 7A, antibody depletion eliminated 98–99% of the target lymphocytes. The relatively high percentage of CD4<sup>+</sup> T cells and low percentage of CD8<sup>+</sup> T cells in the undepleted, TT-boosted population probably reflects the preferential activation of CD4<sup>+</sup> T cells during the *in vitro* boosting process.

After T- and B-cell depletion, the resulting PBMCs were cocultured with irradiated vaccine cells and endogenous TT presentation assessed by ELISA. As shown in Fig. 7B, CD4-depleted PBMCs stimulated with SUM159/DR1/CD80/TT vaccine cells are not activated, as measured by IFN- $\gamma$  release. In contrast, CD8-depletion did not affect IFN- $\gamma$  release. Likewise, depletion of CD19<sup>+</sup> cells did not affect IFN- $\gamma$  release, demonstrating that cross-priming by B cells is not occurring. Stimulation of undepleted PBMCs with SUM159/DR1/CD80 APCs also did not cause IFN- $\gamma$  release, demonstrating that

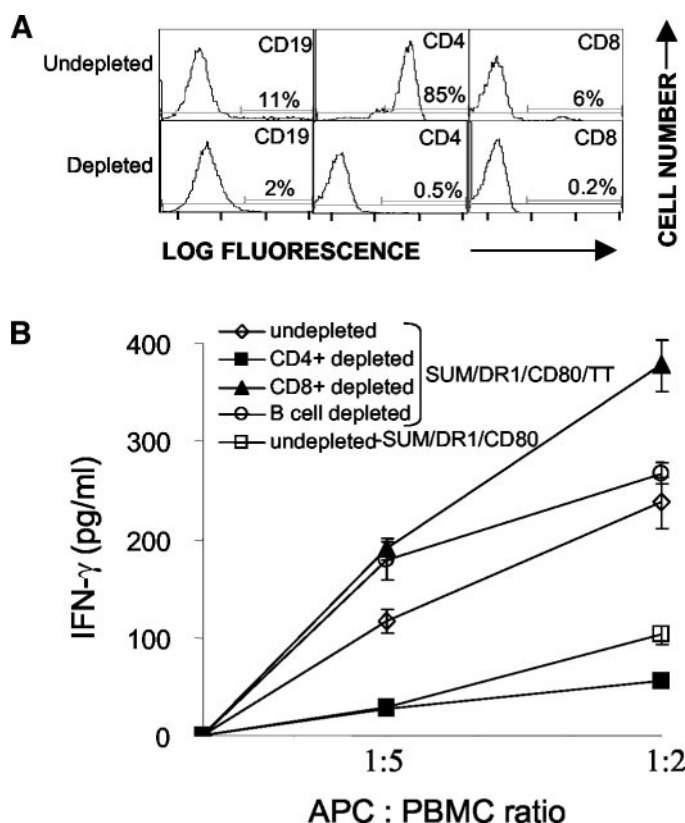


Fig. 7. SUM/DR1/CD80/TT cells activate CD4<sup>+</sup> T lymphocytes to tumor-encoded antigen. A, DRB0101 PBMCs were primed *in vitro* with TT, and separate aliquots were depleted for CD4<sup>+</sup>, CD8<sup>+</sup>, or CD19<sup>+</sup> cells. The resulting cells were stained by direct immunofluorescence for these populations. Values in the lower right-hand corners of each profile represent the percentage of the indicated cells. B, irradiated SUM/DR1/CD80/TT or SUM/DR1/CD80/transductants were cocultured with CD4, CD8, or CD19-depleted, or not depleted, TT-primed DRB0101 PBMCs at various ratios of APCs to responder PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN- $\gamma$ . These data are from one of two to four independent experiments; bars, SD.

PBMC activation is TT specific. Therefore, CD4<sup>+</sup> PBMCs are activated by the vaccine cells, and the activation is mediated by direct presentation of endogenously synthesized TT by the genetically modified tumor cell transductants.

## DISCUSSION

Recent animal studies and some clinical trials have indicated that the use of genetically engineered tumor cells as vaccines may have therapeutic efficacy for the treatment of cancer (28–30). Parallel studies have recognized the critical role played by CD4<sup>+</sup> T cells in orchestrating the host immune response against cancer and have developed methods to activate CD4<sup>+</sup> T cells (2–5, 31–33). Because CD4<sup>+</sup> T cells play a central role in enhancing antitumor immunity, our laboratory has focused on facilitating the activation of these cells. We have hypothesized that tumor cells that constitutively express MHC class I molecules do not contain Ii and are genetically modified to express syngeneic MHC class II molecules, and costimulatory molecules will function as APCs for endogenously synthesized MHC class I- and class II-restricted tumor antigen epitopes. If used as immunogens in tumor-bearing individuals, such cells will serve as “vaccines” to activate tumor-specific CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes that will facilitate regression of wild-type tumor (2, 6). Because the efficacy of these vaccines against wild-type primary tumors and experimental and spontaneous metastatic disease has been demonstrated in multiple mouse models (8, 10, 11, 34, 35), the goal of this study was to translate this strategy for clinical use.

Activation of tumor-specific CD4<sup>+</sup> T cells by the cell-based vaccines is based on the supposition that the MHC class II molecules of the vaccine cells bind peptides synthesized within the tumor cells and directly present these peptides to CD4<sup>+</sup> T lymphocytes. This mode of presentation is different from that of professional APCs that typically bind peptides derived from endocytosed, exogenously synthesized antigens (36). This fundamental difference is attributable to the absence of the MHC class II-associated accessory molecule, Ii, in the vaccine cells. If APCs express Ii, Ii binds to newly synthesized MHC class II molecules, thereby preventing the binding of endogenously derived peptides and favoring the binding of exogenously synthesized peptides (37). However, in the absence of Ii, MHC class II molecules bind peptides derived from endogenously synthesized antigens (14). Because the MHC class II and Ii genes are coordinately regulated and coordinately induced by IFN- $\gamma$  (38), professional APCs and tumor cells that constitutively express MHC class II genes and/or are induced by IFN- $\gamma$  are unlikely to be APCs for endogenously synthesized tumor antigens. Studies with Ii<sup>+</sup> and Ii<sup>−</sup> MHC class II<sup>+</sup> tumor cells support this concept and demonstrate that the most efficacious vaccines are MHC class II<sup>+</sup>Ii<sup>−</sup> (14, 16, 17).

Early studies suggested that expression of MHC class II molecules without coexpression of Ii produces reduced levels of class II molecules that are improperly conformed and unable to function as antigen presentation elements (39–41). More recent studies have demonstrated that the Ii dependency of MHC class II molecules is allele specific (42, 43), and that many MHC class II alleles do not require Ii expression for stability or antigen presentation function (44). The studies reported here demonstrating efficient antigen presentation by MHC class II<sup>+</sup>Ii<sup>−</sup> tumor cell vaccines add HLA-DR0101 to the list of MHC class II alleles whose expression and function are independent of Ii coexpression.

In addition to the absence of Ii for maximal vaccine efficacy, the studies reported here demonstrate that optimal vaccine activity requires coexpression of CD80 for delivery of a costimulatory signal. This observation agrees with extensive mouse and human studies showing the requirement for costimulation for optimal T-cell activa-

tion (reviewed in Ref. 26), as well as many studies that showed that CD80 expression facilitates tumor rejection (45–47).

Several lines of evidence support the hypothesis that the MHC class II tumor cell-based vaccines activate CD4<sup>+</sup> T cells by direct antigen presentation of endogenously encoded tumor antigens, rather than by cross-priming or indirect presentation via host-derived APCs, as suggested by other investigators for other cell-based vaccines and/or tumor cells (48–50):

(a) If tumor-encoded antigens were presented by host-derived APCs such as B cells or other APCs in the PBMCs, then SUM/DR1/TT, SUM/TT, and SUM/CD80/TT cell lines should be just as effective APCs as are SUM/DR1/CD80/TT. However, only SUM/DR1/CD80/TT vaccine cells activate PBMCs.

(b) If professional APCs, rather than the tumor cell vaccines, are the relevant APCs, then removal of these professional APCs should eliminate T-cell activation. However, adherent cells (including dendritic cells and macrophages) are routinely removed from the PBMCs before their coculture with vaccine cells, and in some experiments, CD19<sup>+</sup> B cells were also removed without affecting T-cell activation.

(c) Extensive *in vivo* studies using genetically marked vaccine cells conclusively demonstrated that the vaccine cells directly activate T lymphocytes (12–14). Therefore, it is unlikely that vaccine efficacy is attributable to leakage of tumor antigen, resulting in endocytosis by professional APCs for presentation by cross-priming.

The vaccines described here are based on the premise that tumor cells will be destroyed by CD8<sup>+</sup> T cells with help from CD4<sup>+</sup> T cells. Tumor-specific CD8<sup>+</sup> T cells could be activated either by interacting with MHC class I/peptide complexes of the genetically modified vaccine cells or by cross-presentation of class I-restricted epitopes by professional APCs. In either case, the activated CD8<sup>+</sup> T cells would be specific for MHC class I-restricted tumor peptides and for wild-type tumor cells. Although the vaccines described here are potent activators of CD4<sup>+</sup> T cells, vaccine cell expression of a MHC class I allele shared with the patient's lymphocytes may facilitate an even stronger immune response by capitalizing on the close proximity of CD4<sup>+</sup> and CD8<sup>+</sup> T cells during their activation. A MHC class I allele could be expressed in the vaccines by retroviral transduction. Alternatively, for an allele such as HLA-A2, which is expressed by approximately 50% of the Caucasian population, an HLA-A2<sup>+</sup> tumor cell line could be used as the “base” vaccine. Additional experiments assessing activation of CD8<sup>+</sup> T cells by the vaccines generated in this study *versus* MHC class I-matched or -engineered vaccines will be necessary to address this issue.

A significant technical obstacle in generating the MHC class II cell-based vaccines has been to routinely achieve high level expression of the desired MHC class II alleles in human tumor cells. Because many human tumor cells and cell lines can be problematic to maintain in culture, standard transfection and electroporation techniques did not result in reproducible class II expression.<sup>5</sup> In contrast, transduction using a bicistronic retrovirus encoding the DR $\alpha$  and DR $\beta$  chain genes separated by an IRES routinely yielded high-level HLA-DR expression in a high proportion of transductants. The efficiency of the current retroviruses appears to be attributable to the placement of the DR $\alpha$  and DR $\beta$  genes flanking the IRES, because a previous study using a retroviral construct encoding pig DQ $\alpha$  and DQ $\beta$  genes run off of separate promoters and without an IRES produced only low-level, DQ-expressing cells (51). It is likely the IRES construct will be universally useful, because similar retroviruses encoding other HLA-DR alleles also reproducibly yield high-level MHC class II expression in additional human tumor lines.<sup>6</sup>

The potency of the MHC class II vaccines for activating CD4<sup>+</sup> T cells

<sup>5</sup> S. Dissanayake and J. Bosch, unpublished results.

<sup>6</sup> J. Thompson and M. Pohl, unpublished results.

to tumor-encoded antigens suggests that these vaccines may have therapeutic efficacy for cancer patients. For example, the cell-based vaccines could be administered *in vivo* to patients with disseminated metastatic disease. Alternatively, they could be used *ex vivo* to activate patients' T cells for subsequent adoptive transfer. In either case, these vaccines provide a novel and potent approach for activating tumor-specific CD4<sup>+</sup> T cells and merit further clinical development and testing.

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# Activation of Tumor-specific CD4<sup>+</sup> T Lymphocytes by Major Histocompatibility Complex Class II Tumor Cell Vaccines: A Novel Cell-based Immunotherapy

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## ABSTRACT

Mouse tumor cells transfected with syngeneic MHC class II and costimulatory molecule genes are therapeutic vaccines in mice, provided they do not coexpress the class II-associated invariant chain (Ii). We demonstrated previously that the vaccine cells present tumor peptides via the endogenous antigen presentation pathway to activate CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Because of their efficacy in mice, we are translating this vaccine strategy for clinical use. To obtain MHC class II<sup>+</sup>CD80<sup>+</sup>Ii<sup>−</sup> human tumor cells, we developed retroviruses encoding HLA-DR and CD80. The HLA-DR virus encodes the DR $\alpha$  and DR $\beta$ 0101 chains using an internal ribosomal entry site to coordinate expression. SUM159PT mammary carcinoma and Mel 202 ocular melanoma cells transduced with the retroviruses DRB1/CD80 express high levels of DRB0101 and CD80 on the cell surface in the absence of Ii. Irradiated SUM159PT/DR1/CD80 vaccines stimulate proliferation of non-HLA-DRB0101 peripheral blood mononuclear cells and present an exogenous DR1-restricted tetanus toxoid (TT) peptide, indicating that the transduced DRB0101 is functional. SUM159PT/DR1/CD80 vaccines were further transduced with a retrovirus encoding the TT fragment C gene, as a model tumor antigen. These cells stimulate IFN- $\gamma$  release from TT-primed human DRB0101 peripheral blood mononuclear cells, demonstrating their ability to present “endogenous” tumor antigen. Depletion and antibody blocking experiments confirm that MHC class II-restricted, endogenously synthesized epitopes are presented to CD4<sup>+</sup> T cells. Therefore, the MHC class II vaccines are efficient antigen-presenting cells that activate tumor-specific MHC class II-restricted, CD4<sup>+</sup> T lymphocytes, and they are a novel and potential immunotherapeutic for metastatic cancers.

## INTRODUCTION

A key goal of cancer vaccine development is to generate therapeutic reagents that provide protection against development and outgrowth of metastatic tumor cells. Because metastatic disease for many tumors appears at varied intervals after diagnosis of primary tumor, the most effective vaccines will provide long-term immune memory. We (1, 2) and others (3–5) have focused on the critical role of CD4<sup>+</sup> T cells in cancer vaccines, because these cells, in conjunction with CD8<sup>+</sup> T lymphocytes, are likely to provide maximal antitumor immunity with long-term immunological memory.

To better activate tumor-specific CD4<sup>+</sup> T cells, we have designed cell-based vaccines that facilitate the presentation of MHC class II-restricted tumor peptides to responding CD4<sup>+</sup> T cells (2). We have reasoned that tumor cells present a variety of MHC-restricted peptides that are potential tumor antigens, and that if they constitutively express MHC class I molecules and are transduced with syngeneic MHC

class II and costimulatory molecules, they could function as antigen-presenting cells (APCs) for MHC class I- and class II-restricted tumor peptides. This approach is appealing for several reasons: (a) identification of specific tumor antigen epitopes is not required; (b) multiple class I- and class II-restricted epitopes will be presented concurrently; and (c) CD4<sup>+</sup> T cells may be activated to novel MHC class II-restricted tumor epitopes not presented by professional APCs.

To test our approach, cell-based vaccines were generated from three independent mouse tumors that constitutively express MHC class I molecules and do not express MHC class II molecules (mouse Sal sarcoma, B16 melanoma, and 4T1 mammary carcinoma). The mouse tumor cells were transfected with syngeneic MHC class II  $\alpha$ - and  $\beta$ -chain genes and with costimulatory molecule (CD80) genes. This vaccine approach was adapted for two reasons:

(a) In conventional immunity, activation of CD4<sup>+</sup> T cells requires the uptake of soluble antigen by professional APCs and the cross-presentation of the processed antigen to specific CD4<sup>+</sup> T cells. If antigen is limiting, as it may be when tumor burden is low, available antigen may not be sufficient for the activation of tumor-specific CD4<sup>+</sup> T cells. Our vaccine design bypasses the requirement for professional APCs and soluble tumor antigen because the genetically modified tumor cell vaccines function as the APC.

(b) Because each vaccine cell expresses both MHC class I and class II molecules and their associated tumor peptides, a given vaccine cell could be an APC for both MHC class I- and class II-restricted tumor antigen epitopes and concurrently activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. If CD4<sup>+</sup> and CD8<sup>+</sup> T cells are simultaneously activated by the same APC and are in close proximity to each other, then the transfer of “help” from the activated CD4<sup>+</sup> to the CD8<sup>+</sup> T cell should be highly efficient (2, 6), thereby maximizing the therapeutic effect. CD4<sup>+</sup> T cell “help” could be provided to CD8<sup>+</sup> T cells via the classical mechanism of soluble cytokine production or by the alternative mechanism of up-regulation of CD40 on the vaccine cells (APCs; Ref. 7). Regardless of the mechanism of help, the activated CD4<sup>+</sup> T cells do not need to directly interact with wild-type tumor cells or with professional APCs, because their only role is to provide help to CD8<sup>+</sup> T cells.

Extensive studies using a variety of mouse tumor models have shown that immunization/immunotherapy with the MHC class II plus CD80-modified vaccines induces a potent antitumor immunity against wild-type tumor that confers prophylactic protection (1), delays or eliminates growth of primary solid tumors (8), reduces both experimental (9) and spontaneous metastasis, and extends survival (10, 11). Immunization studies using genetically marked vaccine cells have demonstrated that the vaccine cells themselves are the relevant APCs *in vivo* (12–14), and that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are required for the optimal antitumor effect (8, 11). Therefore, by circumventing the traditional cross-presentation pathway for activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, these genetically modified cancer vaccines induce a potent tumor-specific immunity against wild-type tumor cells.

The efficacy of the vaccines depends on their ability to present endogenously synthesized, MHC class II-restricted tumor antigen epitopes to activate CD4<sup>+</sup> T cells. Presentation of endogenous antigen

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is favored when levels of the class II-associated accessory molecule, invariant chain (Ii), are limiting (reviewed in Ref. 15). Because MHC class II and Ii are coordinately regulated and Ii expression blocks vaccine efficacy (14, 16, 17), we have used tumor cells that do not constitutively express MHC class II (or Ii) as the “base” line for the vaccines.

Because of its therapeutic efficacy in mice, we are translating this vaccine approach for the treatment of human cancers. Our experimental design is to express syngeneic MHC class II and costimulatory molecules in established human tumor cell lines that constitutively express MHC class I molecules and do not constitutively express MHC class II or Ii. To achieve this goal, we are using retroviral transduction to express HLA-DR and CD80 molecules in two human tumor lines, an ocular melanoma (Mel 202) and a mammary carcinoma (SUM159PT). The resulting HLA-DRB0101-transduced cells stably express high plasma membrane levels of functional HLA-DRB0101, as measured by immunofluorescence, activation of allogeneic peripheral blood mononuclear cells (PBMCs), and presentation of a DR1-restricted peptide. To ascertain that the transductants activate CD4<sup>+</sup> T lymphocytes to endogenously synthesized antigens, we have shown that tetanus toxoid (TT) fragment C-transduced vaccine cells activate TT-specific HLA-DRB0101-restricted CD4<sup>+</sup> T cells. Therefore, human tumor cells genetically modified by gene transfer to express syngeneic MHC class II and costimulatory molecules express functional HLA-DR molecules and may serve as useful therapeutics for activating tumor-specific CD4<sup>+</sup> T lymphocytes of cancer patients.

## MATERIALS AND METHODS

**Construction of Retroviral Vectors.** For the pLNCX2/DR1 construct, DRA cDNA in the RSV.5 vector (18) was PCR amplified including 5' *NheI* and 3' *XhoI* restriction sites: DRA 5' primer, TGTCGCTAGCATGGCCATA-AGTGGAGT; and DRA 3' primer, ACTGCTCGAGTTACAGAGGCCCTGCGTT. The PCR product was cloned into the pCR2.1-TA vector (Invitrogen, Carlsbad, CA), excised with *NheI* and *EcoRI*, and inserted into the multiple cloning site (MCS)-A of *NheI*- and *EcoRI*-digested pIRES plasmid (Clontech, Palo Alto, CA). DRB0101 in the RSV.5 vector (18) was PCR amplified including 5' *XmaI* and 3' *NotI* sites and subcloned into the 5' *XmaI* and 3' *NotI* sites of the MCS-B of the pIRES vector: DRB0101 5' primer, AG-TACCCGGGATGGTGTGTCTGAAGCTC; and DRB01013' primer, TAG-TGCGGCCGCTCAGCTCAGGAATCCTGTG. PCR conditions for both DRA and DRB0101 amplifications were: denature at 94°C for 2 min, denature at 94°C for 1 min, anneal at 60.9°C or 62.9°C (DRA and DRB0101, respectively) for 1 min, extend at 72°C for 3 min (High Fidelity Taq; Roche, Basel, Switzerland); repeat the last three steps 30 times and extend at 72°C for 7 min. The resulting construct is pIRES/DR1 (Fig. 1A).

The pLNCX2 retroviral vector (Clontech) was modified to include a linker containing an *AvrII* site in the MCS. To make the linker, equimolar amounts of the oligonucleotides (5'-GATCTCGAGCTCCTAGGAATTGTTGGCCGAGGC-3' and 3'-AGCTCGAGGATCCTTAACAAACCGGCTCCGCCGG5'-) were mixed, heated at 95°C for 5 min, and then incubated at 22°C for 1 h. The resulting linker was ligated to *BglII*- and *NotI*-digested pLNCX2. The resulting construct is pLNCX2/*AvrII*.

The DRA-IRES-DRB0101 fragment of the pIRES/DR1 was digested with *NheI* and *NotI* and gel purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA) and then ligated to *AvrII*- and *NotI*-digested pLNCX2/*AvrII*. The final MHC class II construct is pLNCX2/DR1 (Fig. 1A).

For the pLHCX/CD80(HPH) construct, pLHCX (hygromycin resistance; Clontech) was modified to include a 5' *BamHI* site and a 3' *HindIII* site by inserting an oligonucleotide linker between the *HindIII* and *ClaI* sites of the MCS. The original *HindIII* in the vector was deleted by insertion of the linker. *XhoI*, *HpaI*, *AvrII*, and *NotI* restriction sites were included in the linker for future cloning purposes. The linker sequence was: L1, 5'-AGCTGCTCGAGT-TAACGGATCCTAGGAAGCTTGC GGCCGCAT-3'; and L2, 5'-CGAT-GCGGCCGCAAGCTTCCTAGGATCCGTTAACTCGAGC-3'.

Human CD80 was excised from the pREP10/B7.1 vector with *BamHI* and

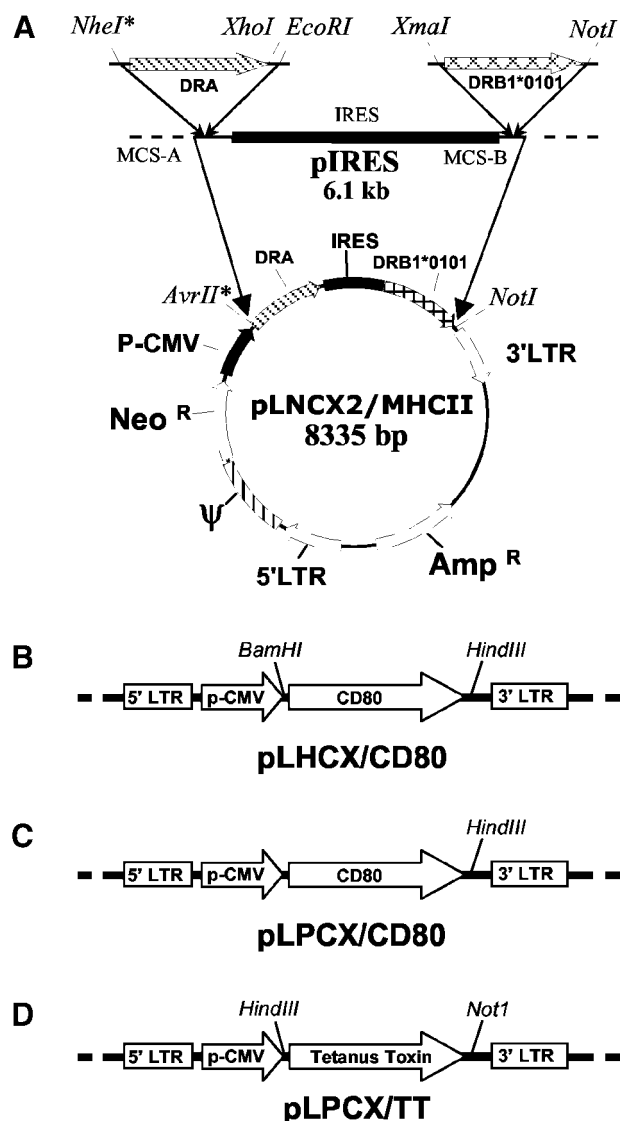


Fig. 1. Retroviral constructs made and used in these studies. A, the pLNCX2/MHC II construct contains the DRA and DRB0101 cDNAs flanking an IRES and under the control of the cytomegalovirus (CMV) promoter and contains the *G418* resistance gene. B and C, the pLHCX/CD80 and pLPCX/CD80 constructs encode the human *CD80* gene and contain the hygromycin or puromycin resistance genes, respectively. D, the pLPCX/TT construct encodes the TT fragment C gene and contains the puromycin resistance gene. LTR, long terminal repeat.

*HindIII* and inserted into the modified pLHCX vector using the *BamHI* and *HindIII* sites (Fig. 1B).

For the pLPCX/CD80 (Puro) construct, the *CD80* gene was excised from pREP10/B7.1 by digestion with *BglII* and *HindIII* and ligated into pLPCX digested with *BamHI* and *HindIII*. The *BamHI* and *BglII* sites were deleted during this process (Fig. 1C).

For the pLPCX/TT construct, TT fragment C DNA was PCR amplified from pCR Blunt (19) to include an ATG start codon and *HindIII* site at the 5' end and a *BamHI* site at the 3' end: 5' primer sequence, CCGCCGAGCT-TGCCACCATGAAAAACCTTGATTGTT; and 3' primer sequence, CTGT-TCGGATCCTTAGTCGTTGGTCCAA. PCR conditions were: denature at 94°C for 5 min, denature at 94°C for 1 min, anneal at 55°C for 1 min, extend at 72°C for 1 min (*Taq*DNA polymerase; Invitrogen); repeat the last three steps 35 times and extend at 72°C for 10 min. The resulting PCR product was inserted into the TA cloning vector, pGEM-T-Easy (Invitrogen). The modified TT fragment C gene was then excised with *HindIII* and *BamHI* and inserted into the mammalian expression vector pCDNA3.1/Zeo(+) (Invitrogen). A *HindIII*-*NotI* fragment containing the TT fragment C gene was then excised from pCDNA3.1/Zeo(+) and subcloned into the *HindIII*-*NotI* site of the MCS of pLPCX(Puro) to produce the pLPCX/TT vector.

Table 1 Tumor cell vaccines (transductants) used in these studies

| Cell line        | HLA-DRB0101 | CD80 | TT <sup>a</sup> | Drug selection                                           |
|------------------|-------------|------|-----------------|----------------------------------------------------------|
| SUM/DR1          | +           |      |                 | G418 <sup>b</sup>                                        |
| SUM/CD80         |             | +    |                 | HPH <sup>c</sup>                                         |
| SUM/TT           |             |      | +               | Puro <sup>d</sup>                                        |
| SUM/DR1/CD80     | +           | +    |                 | G418 <sup>b</sup> + Puro <sup>d</sup>                    |
| SUM/DR1/CD80/TT  | +           | +    | +               | G418 <sup>b</sup> + HPH <sup>c</sup> + Puro <sup>d</sup> |
| SUM/DR1/TT       | +           |      | +               | G418 <sup>b</sup> + Puro <sup>d</sup>                    |
| SUM/CD80/TT      |             | +    | +               | HPH <sup>c</sup> + Puro <sup>d</sup>                     |
| Mel 202/DR1      | +           |      |                 | G418 <sup>b</sup>                                        |
| Mel 202/CD80     |             | +    |                 | HPH <sup>c</sup>                                         |
| Mel 202/DR1/CD80 | +           | +    |                 | G418 <sup>b</sup> + Puro <sup>d</sup>                    |

<sup>a</sup> Tetanus toxin fragment C.<sup>b</sup> 600 µg/ml.<sup>c</sup> 200 µg/ml.<sup>d</sup> 0.2 µg/ml.<sup>e</sup> 75 µg/ml.

**Cells.** Media for all cell lines contained 1% gentamicin, 1% penicillin/streptomycin (all from BioSource, Rockville, MD), and 2 mM Glutamax (BRL/Life Sciences, Grand Island, NY). All cells and T-cell activation assays were cultured at 37°C in 5% CO<sub>2</sub>. SUM159PT was obtained from the Michigan Breast Cell/Tissue Bank<sup>3</sup> and was maintained in Ham's F-12 medium with 10% heat-inactivated FCS (Hyclone, Logan, UT), 1 µg/ml hydrocortisone, and 5 µg/ml insulin (both from Sigma, St. Louis, MO). Mel 202 (20) was grown in RPMI 1640 (BioSource, Rockville, MD) with 10% FCS, 0.01 M HEPES (Invitrogen, Grand Island, NY), and 5 × 10<sup>-5</sup> M β-mercaptoethanol (J. T. Baker, Inc., Phillipsburg, NJ). Transductants were grown in the same medium as their parental cells, supplemented with G418 (Sigma), puromycin (Clontech, Palo Alto, CA), or hygromycin (Calbiochem, San Diego, CA; see Table 1 for dosages), depending on their transgenes. Sweig and Jurkat cells were obtained from the American Type Culture Collection and were maintained in Iscove's modified Dulbecco's medium (BioSource) supplemented with 10% fetal clone I (FBP; Hyclone). EBV B cells were grown in RPMI 1640 with 10% FCS and 0.01 M β-mercaptoethanol. Peripheral blood mononuclear cells (PBMCs) were grown in Iscove's modified Dulbecco's medium with 5% human AB serum (Gemini Bio-Products, Woodland, CA). All cell lines and procedures with human materials were approved by the Institutional Review Boards of the participating institutions.

**Retrovirus Production.** 293T cells (obtained from the Harvard Gene Therapy Institute) were plated in a 6-cm dish at 9 × 10<sup>5</sup> cells/4 ml of 293T medium [DMEM (BioSource, Rockville, MD), 1% gentamicin, 1% penicillin/streptomycin, 1% Glutamax, and 10% heat-inactivated FCS] and cultured at 37°C. Twenty h later, the growth medium was replaced with 4 ml of 37°C Iscove's modified Dulbecco's medium containing 25 mM HEPES (BioSource), 1% Glutamax, and 10% heat-inactivated FCS. Three h later, the 293T cells were transfected with pLNCX2/DR0101, pLHCX/CD80, pLPCX/CD80, or pLPCX/TT plasmids (8 µg) plus pMD.MLV gag.pol (6 µg) and pMD.G (2 µg) using CaPO<sub>4</sub> (21). Twelve to 16 h after transfection, medium was replaced with 293T growth medium containing 10 mM HEPES. Virus was collected 48 h later and either used immediately or stored at -80°C.

**Retroviral Transduction.** Tumor cells were plated in 6-well plates at 1.2-3 × 10<sup>5</sup> cells/3 ml growth medium/well. Approximately 16 h after plating, when cells were in log phase, growth medium was replaced with 500 µl of viral supernatant mixed with 500 µl of 293T medium containing 4 µg/ml polybrene (Sigma) and 10 mM Hepes. Cells were incubated for 5-6 hrs at 37°C, washed twice with excess PBS and maintained in growth medium for 2 days before adding G418, puromycin, and/or hygromycin.

**Peptides, Antibodies, Reagents, and Immunofluorescence.** TT p2 peptide TT<sub>830-844</sub> (QYIKANSKFIGITEL; Ref. 22) was synthesized at the University of Maryland Biopolymer Laboratory. Formaldehyde-inactivated TT was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY).

Monoclonal antibodies [mAbs; HLA-DR-FITC, CD80-phycoerythrin (PE), and anti-TT], streptavidin-PE, FITC-isotype, and PE-isotype controls were purchased from BD PharMingen (San Diego, CA). Biotinylated HLA-DR0101 was purchased from One Lambda, Inc. (Canoga Park, CA). Rat anti-mouse IgG-FITC was purchased from ICN (Costa Mesa, CA), and CD4-FITC, CD8-FITC, and anti-human IgG-FITC were purchased from Miltenyi Biotech (Au-

burn, CA). Human IgG-FITC was purchased from Cappel (West Chester, PA). Culture supernatants of hybridomas W6/32 (pan HLA-A,B,C), L243 (pan anti-HLA-DR), 28.14.8 (anti-H-2L<sup>d</sup>, D<sup>b</sup>), and PIN1 (anti-Ii) were purified on protein A or protein G affinity columns as described previously (1). Tumor cells and PBMCs were stained for cell surface markers (MHC class I, class II, CD80, CD4, CD8, and immunoglobulin) or fixed and stained for internal markers (Ii, TT) by direct or indirect immunofluorescence as described previously (1, 16). PBMCs were collected by venipuncture from HLA-typed healthy donors and isolated using Histopaque 1077 separation medium as described previously (20). For some experiments, PBMCs were provided by Dr. D. Mann (University of Maryland Baltimore). PBMCs were stored at 1 × 10<sup>7</sup> cells/ml at -80°C until used.

**Western Blots.** Western blot analyses were performed as described (14) using 10% SDS-PAGE. Blots were incubated with PIN1.1 mAb (0.003 µg/ml) followed by sheep anti-mouse HRP at a 1:10,000 dilution (Amersham).

**Allogeneic T-Cell Activation.** Responder PBMCs (1 × 10<sup>5</sup>/well) were cultured in triplicate with 5 × 10<sup>3</sup> or 1 × 10<sup>4</sup> irradiated (CS-137 irradiator; Kewaunee Scientific, Statesville, NC) stimulator SUM159PT (50 Gy) or 5 × 10<sup>5</sup> allogeneic PBMCs (40 Gy) per well in 200 µl/well of culture medium (RPMI, 10% FCS, 1% penicillin/streptomycin, 2 mM Glutamax, and 0.01 M β-mercaptoethanol) in flat-bottomed 96-well microtiter plates (Corning, Inc., Corning, NY). Cells were incubated at 37°C in 5% CO<sub>2</sub> for 6 days and pulsed with [<sup>3</sup>H]thymidine (2 µCi/well) during the final 18 h, after which the cells were harvested onto glass fiber filter mats using a Packard Micromate 196 cell harvester (Downers Grove, IL). Filter mats were sealed into plastic bags with 5 ml of betaplate scintillation fluid (Perkin-Elmer, Gaithersburg, MD) and counted using a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer). Samples were run in triplicate.

Stimulation Index (SI)

$$= \frac{(\text{cpm of transduced experimental tumor cells} + \text{allogeneic PBMC}) - (\text{cpm of transduced tumor cells alone})}{\text{cpm of allogeneic PBMCs alone}}$$

Responder PBMCs do not express DRB0101.

**TT-boosted PBMCs.** DRB0101 PBMCs (2 × 10<sup>7</sup> cells/4 ml/well) were cultured with 1 µg/ml of exogenous TT (Accurate, Westbury, NY) in 6-well tissue culture plates (Corning). After 5 days of culture, nonadherent cells were harvested, washed twice with Iscove's modified Dulbecco's medium, and replated in culture medium with 20 units/ml of recombinant human interleukin 2 (R&D Systems, Minneapolis, MN) at 1 × 10<sup>6</sup> cells/2 ml/well in 24-well plates (Corning). Remaining nonadherent cells were harvested 7 days later, and live cells were isolated using Histopaque-1077 separation medium. TT-activated, nonadherent cells were maintained in culture medium without exogenous interleukin 2 overnight and used the following day.

**Antigen Presentation Assays for Endogenous TT and Exogenous TT Peptides.** Irradiated (50 Gy) stimulator cells (1 × 10<sup>4</sup> or 2.5 × 10<sup>4</sup> cells/well) were cocultured in triplicate with adherent cell-depleted, TT-primed DRB0101 responder PBMCs (5 × 10<sup>4</sup> cells/well) in 200 µl/well in flat-bottomed 96-well microtiter plates (Corning). After 2 days of culture, supernatants were collected and assayed by ELISA for IFN-γ according to the manufacturer's instructions (Endogen, Woburn, MA). For the antibody blocking experiments, 1 × 10<sup>4</sup> stimulator cells were incubated with 10 µg/ml or 12.5 pg/ml of L243 (anti-HLA-DR) or 28.14.8 (isotype-matched irrelevant mAb) in 100 µl/well for 45 min before the addition of responder PBMCs. Values are the averages of triplicate points with their SDs.

For exogenous TT peptide p2 presentation, assays were as for endogenous antigen presentation, except soluble TT peptide p2 (22, 23) was added at the beginning of the 2-day culture period, and antigen-presenting cells not transduced with TT were used.

**CD4, CD8, and CD19 Cell Depletions.** Adherent cell-depleted, TT-primed PBMCs were depleted for CD4<sup>+</sup>, CD8<sup>+</sup>, or CD19<sup>+</sup> cells using magnetic beads, LD columns, and the QuadroMACS separation system according to the manufacturer's instructions (Miltenyi Biotech). Purity of depleted fractions was confirmed by flow cytometry.

**HLA-DR Nomenclature.** The PBMCs used in these studies were HLA typed by PCR; hence, they are known to be HLA-DRB0101. The HLA-DR gene used in these studies was sequenced and identified as HLA-DRB0101 and

<sup>3</sup> Internet address: www.cancer.med.umich.edu/breast\_cell/umbnkbdb.htm.

is abbreviated as "DR1" in the names of the transductants. The TT p2 peptide has been identified as a DR1-restricted epitope; however, its DR1 subtype is not known.

## RESULTS

**Construction of Retroviruses Encoding HLA-DR $\alpha$  Plus HLA-DR $\beta$ , CD80, and TT Fragment C.** To generate human tumor cells expressing high levels of MHC class II molecules, retroviruses encoding HLA-DR $\alpha$  plus HLA-DR $\beta$  genes have been generated. The HLA-DRB0101 allele was selected because it is one of the more common alleles in the Caucasian population and is a frequently used restriction element (24, 25). A novel bicistronic retroviral vector that drives coordinate expression of approximately equimolar amounts of HLA-DR $\alpha$  and HLA-DR $\beta$  was developed using the pLNCX2(neo) retroviral backbone. DR $\alpha$  and DR $\beta$ 0101 cDNAs (18) were cloned upstream and downstream, respectively, of the internal ribosomal entry site (IRES) of the vector pIRES. The DRA-pIRES-DRB segment was then excised from the pIRES vector and ligated into the pLNCX plasmid to yield the pLNCX/DR $\beta$ 1 plasmid (Fig. 1A). This construct will produce a single-chain mRNA driven by the cytomegalovirus promoter in which DR $\alpha$  is translated by a CAP-dependent mechanism and DR $\beta$  is translated via the IRES in a CAP-independent manner.

Because of the critical role of costimulatory molecules in the activation of naive T cells (26), we have also generated retroviral plasmids encoding human CD80 (hCD80). The *hCD80* gene was excised from the pREP10/B7.1 plasmid and ligated into the retroviral vector pLHCX(HPH) or pLPCX(Puro) to form the pLHCX/CD80 (Fig. 1B) or pLPCX/CD80 (Fig. 1C) plasmids, respectively.

To monitor presentation of endogenously synthesized antigen, a retroviral plasmid encoding the TT fragment C was generated. The TT fragment C gene was excised from the pCR Blunt plasmid, an ATG start codon was inserted at its 5' end, and the resulting construct was ligated into the pLPCX(Puro) vector to form the pLPCX/TT retroviral plasmid (Fig. 1D). All retroviral plasmids were packaged in 293T cells, and supernatants containing infectious retroviruses were harvested and used to transduce target tumor cells.

**Transduced Human Tumor Cells Express Cell Surface HLA-DRB0101 and CD80 and Internal TT.** The human ocular melanoma cell line Mel 202 and the mammary carcinoma cell line SUM159PT were transduced with different combinations of the pLNCX2/DR1, pLHCX/CD80, pLPCX/CD80, and pLPCX/TT retroviruses. The resulting transductants are shown in Table 1. SUM159PT and Mel 202 tumors were chosen because they do not constitutively express MHC class II molecules and hence should not express Ii, which we have shown previously inhibits presentation of MHC class II-restricted endogenous antigens (14, 17). To assess the magnitude and stability of transgene expression, transductants were tested by immunofluorescence and flow cytometry 1 week after being placed on drug selection (see Table 1 for drug selection conditions for each transductant line) and intermittently for 6 months thereafter. As shown in Fig. 2, Mel 202 and SUM159PT transductants express high levels of cell surface HLA-DR (L243 mAb), CD80 (CD80-PE mAb), and internal TT (polyclonal anti-TT ab), as measured at 6 months after transduction. HLA-DR-expressing Mel 202 and SUM159PT cells were also biotinylated, and the cell extracts were immunoprecipitated with anti-HLA-DR mAbs to assure proper structural conformation of cell surface-expressed, transduced class II molecules. Both lines displayed high levels of SDS-stable MHC class II  $\alpha\beta$  dimers, indicating proper conformation and peptide binding.<sup>4</sup> The parental lines and transduc-

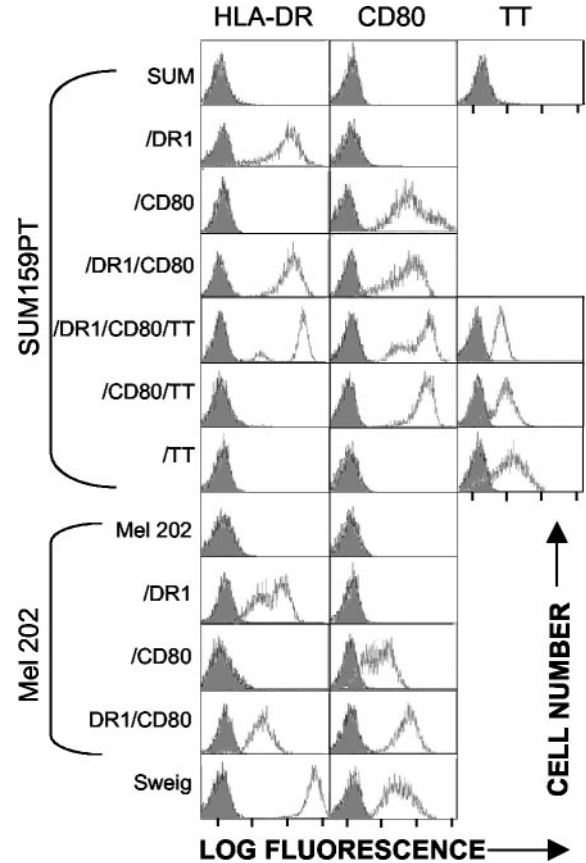


Fig. 2. SUM159PT and Mel 202 transductants express MHC class II HLA-DR and CD80 on the cell surface and TT internally. Live transductants were stained by direct immunofluorescence for plasma membrane HLA-DR (L243-FITC) or CD80 (CD80-PE). Fixed cells were stained by indirect immunofluorescence for internal TT (TT mAb plus fluorescent conjugate). Gray peaks denote staining with fluorescent conjugate alone or isotype control; white peaks represent staining with directly coupled primary antibody or primary antibody plus fluorescent conjugate. These data are from one of three to five independent experiments.

tants were also stained for MHC class I molecules (W6/32 mAb). All lines showed strong class I expression, with transductants displaying levels roughly comparable with their parental lines (data not shown).

To ascertain that the MHC class II expression is allele specific, SUM/DR1 and SUM/DR1/CD80 cells were stained for cell surface expression of HLA-DR1 using the HLA-DR1-specific mAb. As shown in Fig. 3, pLNCX2/DR1-transduced SUM cells express high levels of DR1 and only stain at background levels with an irrelevant HLA-DR2-specific mAb. Therefore, SUM/DR1/CD80, SUM/DR1, SUM/CD80, SUM/DR1/CD80/TT, Mel 202/DR1, Mel 202/CD80, and Mel 202/DR1/CD80 transductants express high levels of the transduced *HLA-DR*, *CD80*, and/or *TT* genes as measured by antibody reactivity and immunofluorescence.

**SUM159PT and Mel 202 Cells Do Not Express Invariant Chain.** Because coexpression of Ii inhibits endogenous antigen presentation by MHC class II vaccine cells (14, 17), SUM159PT and Mel 202 cells were tested to ascertain that they do not express Ii. Cells were permeabilized, stained with the Ii-specific mAb PIN-1, and analyzed by flow cytometry. As shown in Fig. 4A, neither tumor line contains Ii, whereas the human B cell line, Sweig, which constitutively expresses Ii, is strongly positive. To further confirm the absence of Ii, detergent extracts of SUM159PT, Mel 202, Ii-positive Sweig, and Ii-negative Jurkat cells were electrophoresed by SDS-PAGE and analyzed by Western blotting for Ii expression. As shown in Fig. 4B, neither SUM159PT, Mel 202, Mel 202/DR1/CD80, nor SUM/DR1/

<sup>4</sup> V. Clements, unpublished results.

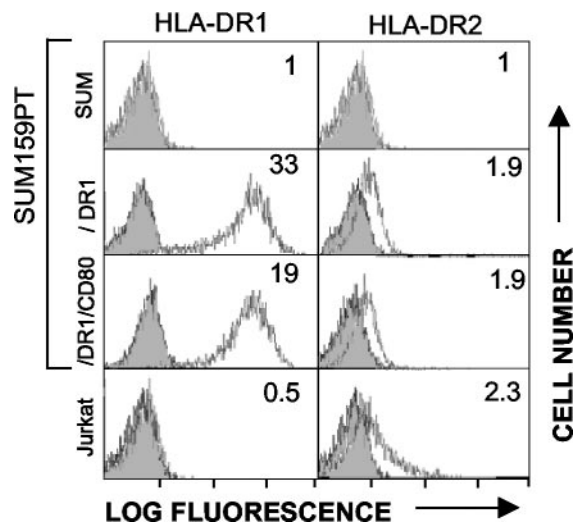


Fig. 3. SUM/DR1 and SUM/DR1/CD80 transductants express HLA-DR1 at the cell surface. Live cells were stained by indirect immunofluorescence for plasma membrane HLA-DR1 (mAb HLA-DR1 biotin) or with an irrelevant Ab (HLA-DR2-biotin) plus an avidin-PE conjugate. Jurkat is a DR1<sup>-</sup> cell line. Gray peaks denote staining with fluorescent conjugate without primary antibody; white peaks represent staining with primary antibody plus fluorescent conjugate. Numbers in the upper right-hand corner of each profile are the mean channel fluorescence for the antibody stained peak. These data are from one of two to five independent experiments.

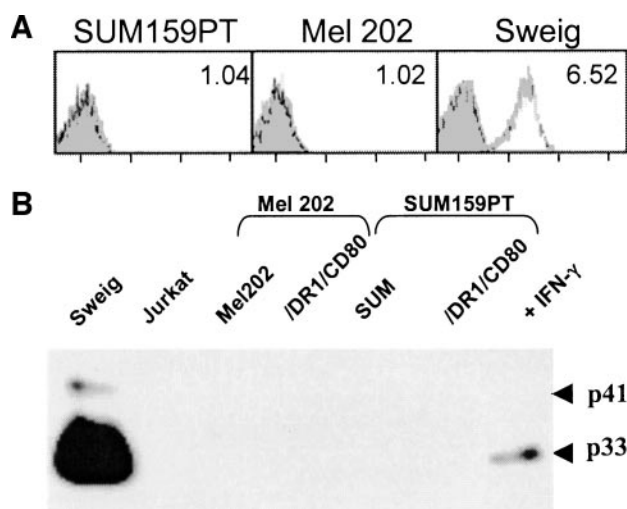


Fig. 4. SUM159PT and Mel 202 cells do not express Ii chain. **A**, fixed cells were stained by indirect immunofluorescence for Ii (mAb PIN1.1; white peaks) or with fluorescent conjugate alone (gray peaks). These data are representative of three independent experiments. **B**, uninduced or IFN- $\gamma$ -treated (+ IFN $\gamma$ ) cells were detergent lysed, electrophoresed on 10% SDS-PAGE gels under reducing conditions, and transferred to nitrocellulose. Blots were stained for Ii with the mAb PIN1.1. Sweig and Jurkat cells are Ii<sup>+</sup> and Ii<sup>-</sup> cell lines, respectively. These data are from one of two to three independent experiments.

CD80 cells contain detectable Ii, although Ii expression is inducible in SUM159PT cells by a 48-h treatment with IFN- $\gamma$ . Therefore, SUM159PT and Mel 202 tumor cells do not constitutively express Ii; therefore, Ii will not be present in the transduced vaccine cells to inhibit binding and presentation of endogenously synthesized peptides.

**HLA-DRB0101 Transductants Stimulate HLA-DR Allogeneic PBMCs.** Coculture of cells expressing functional HLA-DR molecules with allogeneic CD4<sup>+</sup> T lymphocytes results in T-cell proliferation (27). Therefore, to determine whether the HLA-DRB0101 molecules expressed by the transduced tumor cell vaccines are functional, we cocultured the various transductants with allogeneic PBMCs. Responder non-HLA-DRB0101 PBMCs were mixed with various

numbers of irradiated transductants, and proliferation was assessed by measuring the SI at the end of 6 days of culture. Irradiated allogeneic PBMCs were used as a positive control. As shown in Fig. 5A, SUM/DR1/CD80 cells induce high SI, whereas SUM, SUM/CD80, or SUM/DR1 transductants produce only background levels. Therefore, the cell-based vaccines activate allogeneic PBMCs, provided they coexpress DRB0101 and CD80.

**Transduced Tumor Cells Present an HLA-DR1-restricted TT Peptide.** TT peptide p2 is an HLA-DR1-restricted epitope (22). If the HLA-DRB0101 molecules of the transductants are properly conformed and functional, when pulsed with the TT p2 peptide, the transductants should activate TT-specific HLA-DRB0101 lymphocytes. Because the TT-specific CD4<sup>+</sup> T-cell precursor frequency in peripheral blood of the DRB0101 donor was low (data not shown), the HLA-DRB0101 PBMCs were boosted *in vitro* with TT to expand the number of TT-reactive T cells. TT-booster PBMCs were incubated at various ratios with tumor cell transductants pulsed with various quantities of TT p2 peptide to determine whether the transductants present this HLA-DR1-restricted epitope. T-cell activation was assessed by measuring IFN- $\gamma$  release. As shown in Fig. 5B, SUM/DR1/CD80 tumor cells activate the TT-specific T cells as or more efficiently than EBV-transformed HLA-DR1 B cells (DR1-EBV B cells), whereas HLA-DR1-negative parental SUM cells do not activate. Therefore, SUM/DR1/CD80 tumor cells are effective APCs for an HLA-DR1-restricted epitope, further demonstrating that the transduced MHC class II molecules are functional.

**HLA-DR1/CD80 Tumor Cell Transductants Present Endogenous TT and Activate TT-specific T Lymphocytes.** We have generated the DR1/CD80 transductants to use as cancer vaccines to immunize patients and activate their T lymphocytes to tumor-encoded

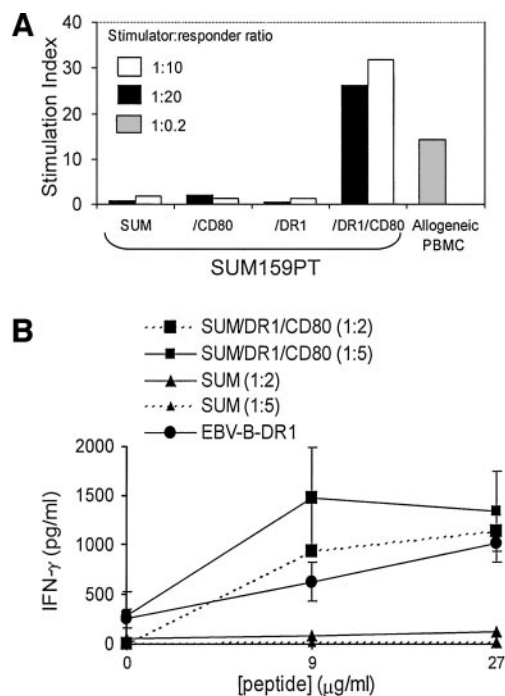


Fig. 5. SUM/DR1/CD80 cells induce proliferation of allogeneic T cells and present a DR1-restricted peptide to DRB0101 PBMC. **A**, irradiated SUM transductants expressing HLA-DRB0101 and/or CD80 or allogeneic PBMCs were cocultured with non-DRB0101 PBMCs at various ratios of APCs to responder lymphocytes. Proliferation was assessed by measuring the SI after 6 days of culture. These data are from one of three independent experiments. **B**, SUM, SUM/DR1/CD80, or DRB0101-expressing EBV B cells were pulsed with the DR1-restricted TT peptide, p2, and cocultured with TT-primed DRB0101 PBMCs at various ratios of APCs to PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN- $\gamma$ . These data are from one of three independent experiments. Bars, SD.

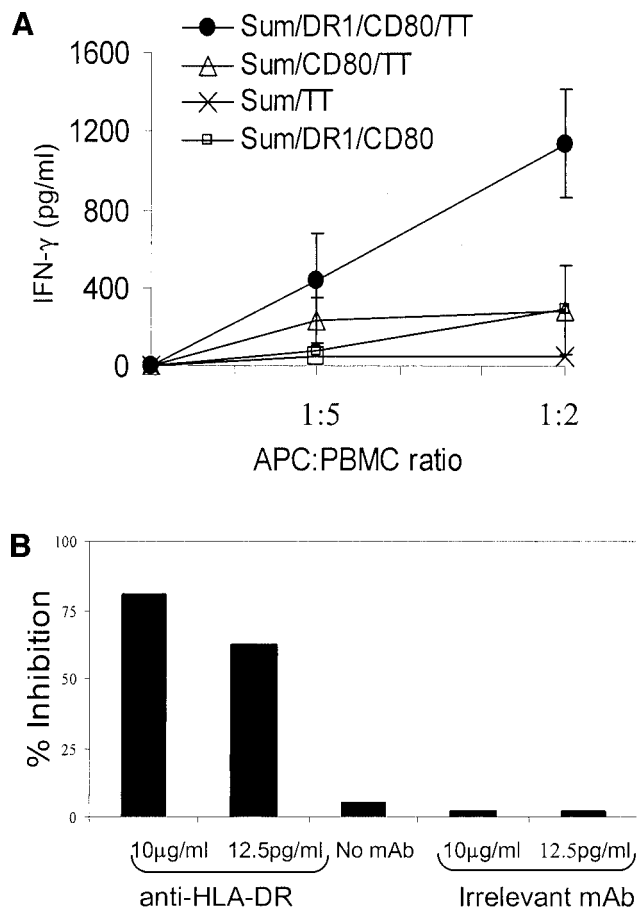


Fig. 6. SUM/DR1/CD80/TT cells activate HLA-DR-restricted DRB0101 PBMCs to tumor-encoded TT. A, irradiated SUM transductants were cocultured with TT-primed DRB0101 PBMCs at various ratios of APCs to responder PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN- $\gamma$ . These data are from one of three independent experiments; bars, SD. B, HLA-DR-specific (L243) or irrelevant (28-14-8) mAb was added to culture wells containing irradiated SUM/DR1/CD80/TT cells before addition of TT-primed responder DRB0101 PBMCs at a ratio of 1:2 APCs to responder cells. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN- $\gamma$ . These data are from one of two independent experiments.

tumor peptides. To achieve this goal, the transductants must not only express functional HLA-DR molecules, but the DR molecules must also bind and present endogenously synthesized tumor peptides. To determine whether the transductants have this capability, we tested SUM/DR1/CD80/TT cells as APCs for endogenously encoded TT. Because the TT construct does not contain a signal sequence, TT protein will reside in the cytoplasm and serve as a “model” tumor antigen for a cytoplasmically localized tumor antigen.

Adherent cell-depleted HLA-DRB0101 PBMCs were boosted *in vitro* with TT as per the experiment of Fig. 5B and cocultured at various ratios with irradiated transduced SUM cells. Activation was assessed by measuring IFN- $\gamma$  release. As shown in Fig. 6A, SUM/DR1/CD80/TT tumor cells activate a potent T-cell response, whereas SUM transductants without DRB0101 (SUM/CD80/TT), without TT (SUM/DR1/CD80), or without DRB0101 and CD80 (SUM/TT) do not activate. Because SUM/TT and SUM/CD80/TT cells do not activate, TT is not being released into the culture medium and being presented by other APCs in the PBMC population. Therefore, tumor cells transduced with *HLA-DRB0101*, *CD80*, and *TT* genes are effective APCs for endogenously encoded molecules.

To further analyze whether the presentation of endogenous TT is DR1 restricted, anti-HLA-DR mAb (L243) was added at various concentrations at the beginning of the assay. As shown in Fig. 6B, in

the presence of the highest dose of antibody, T-cell activation is inhibited >80%, whereas an irrelevant isotype-matched mouse H-2L<sup>d</sup>-specific mAb does not inhibit.

#### DR1/CD80/TT Tumor Cells Activate CD4<sup>+</sup> T Lymphocytes.

To identify the PBMCs that are specifically activated by the vaccine cells, adherent cell-depleted, TT-primed DRB0101 PBMCs were depleted for CD4<sup>+</sup> or CD8<sup>+</sup> T cells or for B cells and then used as responding cells in antigen presentation assays with SUM/DR1/CD80/TT transductants. T and B cells were depleted by magnetic bead separation. To ascertain the efficiency of the depletions, PBMCs before and after depletion were tested by flow cytometry for the percentage of CD4<sup>+</sup>, CD8<sup>+</sup>, and immunoglobulin<sup>+</sup> (B) cells. As shown in Fig. 7A, antibody depletion eliminated 98–99% of the target lymphocytes. The relatively high percentage of CD4<sup>+</sup> T cells and low percentage of CD8<sup>+</sup> T cells in the undepleted, TT-boosted population probably reflects the preferential activation of CD4<sup>+</sup> T cells during the *in vitro* boosting process.

After T- and B-cell depletion, the resulting PBMCs were cocultured with irradiated vaccine cells and endogenous TT presentation assessed by ELISA. As shown in Fig. 7B, CD4-depleted PBMCs stimulated with SUM159/DR1/CD80/TT vaccine cells are not activated, as measured by IFN- $\gamma$  release. In contrast, CD8-depletion did not affect IFN- $\gamma$  release. Likewise, depletion of CD19<sup>+</sup> cells did not affect IFN- $\gamma$  release, demonstrating that cross-priming by B cells is not occurring. Stimulation of undepleted PBMCs with SUM159/DR1/CD80 APCs also did not cause IFN- $\gamma$  release, demonstrating that

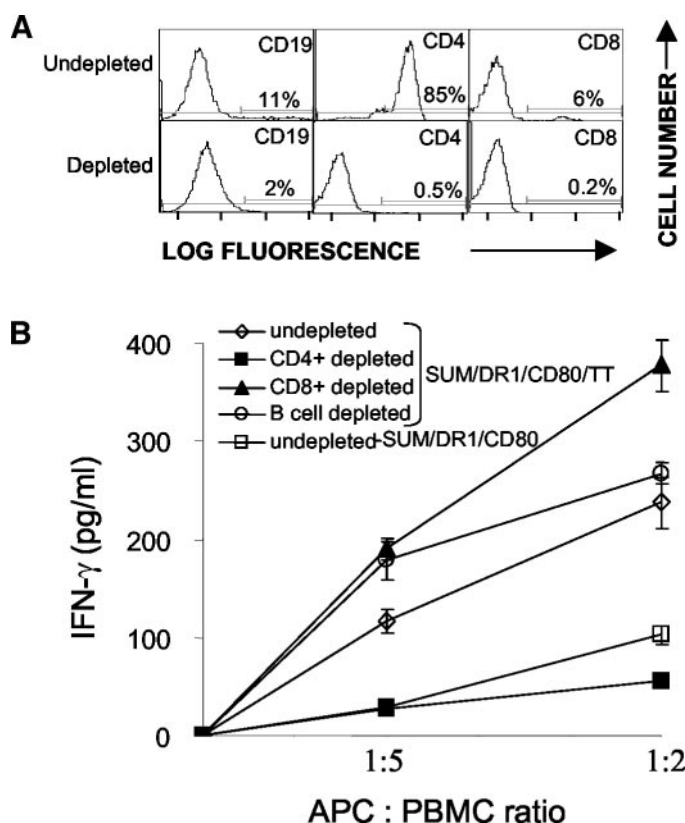


Fig. 7. SUM/DR1/CD80/TT cells activate CD4<sup>+</sup> T lymphocytes to tumor-encoded antigen. A, DRB0101 PBMCs were primed *in vitro* with TT, and separate aliquots were depleted for CD4<sup>+</sup>, CD8<sup>+</sup>, or CD19<sup>+</sup> cells. The resulting cells were stained by direct immunofluorescence for these populations. Values in the lower right-hand corners of each profile represent the percentage of the indicated cells. B, irradiated SUM/DR1/CD80/TT or SUM/DR1/CD80/transductants were cocultured with CD4, CD8, or CD19-depleted, or not depleted, TT-primed DRB0101 PBMCs at various ratios of APCs to responder PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN- $\gamma$ . These data are from one of two to four independent experiments; bars, SD.

PBMC activation is TT specific. Therefore, CD4<sup>+</sup> PBMCs are activated by the vaccine cells, and the activation is mediated by direct presentation of endogenously synthesized TT by the genetically modified tumor cell transductants.

## DISCUSSION

Recent animal studies and some clinical trials have indicated that the use of genetically engineered tumor cells as vaccines may have therapeutic efficacy for the treatment of cancer (28–30). Parallel studies have recognized the critical role played by CD4<sup>+</sup> T cells in orchestrating the host immune response against cancer and have developed methods to activate CD4<sup>+</sup> T cells (2–5, 31–33). Because CD4<sup>+</sup> T cells play a central role in enhancing antitumor immunity, our laboratory has focused on facilitating the activation of these cells. We have hypothesized that tumor cells that constitutively express MHC class I molecules do not contain Ii and are genetically modified to express syngeneic MHC class II molecules, and costimulatory molecules will function as APCs for endogenously synthesized MHC class I- and class II-restricted tumor antigen epitopes. If used as immunogens in tumor-bearing individuals, such cells will serve as “vaccines” to activate tumor-specific CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes that will facilitate regression of wild-type tumor (2, 6). Because the efficacy of these vaccines against wild-type primary tumors and experimental and spontaneous metastatic disease has been demonstrated in multiple mouse models (8, 10, 11, 34, 35), the goal of this study was to translate this strategy for clinical use.

Activation of tumor-specific CD4<sup>+</sup> T cells by the cell-based vaccines is based on the supposition that the MHC class II molecules of the vaccine cells bind peptides synthesized within the tumor cells and directly present these peptides to CD4<sup>+</sup> T lymphocytes. This mode of presentation is different from that of professional APCs that typically bind peptides derived from endocytosed, exogenously synthesized antigens (36). This fundamental difference is attributable to the absence of the MHC class II-associated accessory molecule, Ii, in the vaccine cells. If APCs express Ii, Ii binds to newly synthesized MHC class II molecules, thereby preventing the binding of endogenously derived peptides and favoring the binding of exogenously synthesized peptides (37). However, in the absence of Ii, MHC class II molecules bind peptides derived from endogenously synthesized antigens (14). Because the MHC class II and Ii genes are coordinately regulated and coordinately induced by IFN- $\gamma$  (38), professional APCs and tumor cells that constitutively express MHC class II genes and/or are induced by IFN- $\gamma$  are unlikely to be APCs for endogenously synthesized tumor antigens. Studies with Ii<sup>+</sup> and Ii<sup>−</sup> MHC class II<sup>+</sup> tumor cells support this concept and demonstrate that the most efficacious vaccines are MHC class II<sup>+</sup>Ii<sup>−</sup> (14, 16, 17).

Early studies suggested that expression of MHC class II molecules without coexpression of Ii produces reduced levels of class II molecules that are improperly conformed and unable to function as antigen presentation elements (39–41). More recent studies have demonstrated that the Ii dependency of MHC class II molecules is allele specific (42, 43), and that many MHC class II alleles do not require Ii expression for stability or antigen presentation function (44). The studies reported here demonstrating efficient antigen presentation by MHC class II<sup>+</sup>Ii<sup>−</sup> tumor cell vaccines add HLA-DR0101 to the list of MHC class II alleles whose expression and function are independent of Ii coexpression.

In addition to the absence of Ii for maximal vaccine efficacy, the studies reported here demonstrate that optimal vaccine activity requires coexpression of CD80 for delivery of a costimulatory signal. This observation agrees with extensive mouse and human studies showing the requirement for costimulation for optimal T-cell activa-

tion (reviewed in Ref. 26), as well as many studies that showed that CD80 expression facilitates tumor rejection (45–47).

Several lines of evidence support the hypothesis that the MHC class II tumor cell-based vaccines activate CD4<sup>+</sup> T cells by direct antigen presentation of endogenously encoded tumor antigens, rather than by cross-priming or indirect presentation via host-derived APCs, as suggested by other investigators for other cell-based vaccines and/or tumor cells (48–50):

(a) If tumor-encoded antigens were presented by host-derived APCs such as B cells or other APCs in the PBMCs, then SUM/DR1/TT, SUM/TT, and SUM/CD80/TT cell lines should be just as effective APCs as are SUM/DR1/CD80/TT. However, only SUM/DR1/CD80/TT vaccine cells activate PBMCs.

(b) If professional APCs, rather than the tumor cell vaccines, are the relevant APCs, then removal of these professional APCs should eliminate T-cell activation. However, adherent cells (including dendritic cells and macrophages) are routinely removed from the PBMCs before their coculture with vaccine cells, and in some experiments, CD19<sup>+</sup> B cells were also removed without affecting T-cell activation.

(c) Extensive *in vivo* studies using genetically marked vaccine cells conclusively demonstrated that the vaccine cells directly activate T lymphocytes (12–14). Therefore, it is unlikely that vaccine efficacy is attributable to leakage of tumor antigen, resulting in endocytosis by professional APCs for presentation by cross-priming.

The vaccines described here are based on the premise that tumor cells will be destroyed by CD8<sup>+</sup> T cells with help from CD4<sup>+</sup> T cells. Tumor-specific CD8<sup>+</sup> T cells could be activated either by interacting with MHC class I/peptide complexes of the genetically modified vaccine cells or by cross-presentation of class I-restricted epitopes by professional APCs. In either case, the activated CD8<sup>+</sup> T cells would be specific for MHC class I-restricted tumor peptides and for wild-type tumor cells. Although the vaccines described here are potent activators of CD4<sup>+</sup> T cells, vaccine cell expression of a MHC class I allele shared with the patient's lymphocytes may facilitate an even stronger immune response by capitalizing on the close proximity of CD4<sup>+</sup> and CD8<sup>+</sup> T cells during their activation. A MHC class I allele could be expressed in the vaccines by retroviral transduction. Alternatively, for an allele such as HLA-A2, which is expressed by approximately 50% of the Caucasian population, an HLA-A2<sup>+</sup> tumor cell line could be used as the “base” vaccine. Additional experiments assessing activation of CD8<sup>+</sup> T cells by the vaccines generated in this study *versus* MHC class I-matched or -engineered vaccines will be necessary to address this issue.

A significant technical obstacle in generating the MHC class II cell-based vaccines has been to routinely achieve high level expression of the desired MHC class II alleles in human tumor cells. Because many human tumor cells and cell lines can be problematic to maintain in culture, standard transfection and electroporation techniques did not result in reproducible class II expression.<sup>5</sup> In contrast, transduction using a bicistronic retrovirus encoding the DR $\alpha$  and DR $\beta$  chain genes separated by an IRES routinely yielded high-level HLA-DR expression in a high proportion of transductants. The efficiency of the current retroviruses appears to be attributable to the placement of the DR $\alpha$  and DR $\beta$  genes flanking the IRES, because a previous study using a retroviral construct encoding pig DQ $\alpha$  and DQ $\beta$  genes run off of separate promoters and without an IRES produced only low-level, DQ-expressing cells (51). It is likely the IRES construct will be universally useful, because similar retroviruses encoding other HLA-DR alleles also reproducibly yield high-level MHC class II expression in additional human tumor lines.<sup>6</sup>

The potency of the MHC class II vaccines for activating CD4<sup>+</sup> T cells

<sup>5</sup> S. Dissanayake and J. Bosch, unpublished results.

<sup>6</sup> J. Thompson and M. Pohl, unpublished results.

to tumor-encoded antigens suggests that these vaccines may have therapeutic efficacy for cancer patients. For example, the cell-based vaccines could be administered *in vivo* to patients with disseminated metastatic disease. Alternatively, they could be used *ex vivo* to activate patients' T cells for subsequent adoptive transfer. In either case, these vaccines provide a novel and potent approach for activating tumor-specific CD4<sup>+</sup> T cells and merit further clinical development and testing.

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