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The Novel C24D Synthetic Polypeptide Inhibits Binding of Placenta Immunosuppressive Ferritin to Human T Cells and Elicits Anti–Breast Cancer Immunity *In Vitro* and *In Vivo*[1]

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Abstract

Immune tolerance mechanisms supporting normal human pregnancy are exploited by breast cancer and other malignancies. We cloned from human placenta and breast cancer cells the novel human immunomodulator named placenta immunosuppressive ferritin (PLIF). PLIF is composed of a ferritin heavy chain–like domain and a novel cytokine-like domain, named C48. Both intact PLIF and C48 inhibit T cell proliferation. Blocking PLIF by specific antibodies in a tolerant breast cancer model in nude mice resulted in tumor cell apoptosis and rejection. This prompted us to study active immune preventive strategies targeting PLIF activity. Currently, we report on the design and synthesis of the novel C24D polypeptide, which inhibits the binding of PLIF to T cells and therefore inhibits the immune suppressive effect of PLIF. The effect of C24D on the generation of anti–breast cancer cytotoxic T lymphocytes (CTLs) was studied *in vitro* in cultures of MCF-7 (HLA-A2$^+$) or T47D (HLA-A2$^-$) breast cancer cells incubated with peripheral blood mononuclear cells (PBMCs) from healthy blood donors. We found that C24D treatment exclusively induced development of CTLs. On reactivation by their specific target cells, the CTLs secreted interferon-γ and induced target apoptosis. Anti–MCF-7 CTLs were cross-cytotoxic to MDA-MB-231 (HLA-A2$^+$) triple-negative breast cancer but not to T47D. Moreover, C24D treatment *in vivo* inhibited the growth of MCF-7 tumors engrafted in immune-compromised nude mice transfused with naïve allogeneic human PBMCs. Our results demonstrate that C24D treatment breakdown breast cancer induced tolerance enabling the initiation of effective anti-tumor immune response.

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Abbreviations: CTLs, cytotoxic T lymphocytes; E:T, effector/target; FITC, fluorescein isothiocyanate; IFN-γ, interferon-γ; IL, interleukin; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PLF, placental type isoferritin; PLIF, placenta immunosuppressive ferritin; TNBC, triple-negative breast cancer; TNF-α, tumor necrosis factor–α

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[2] In memory of Prof. Chaya Moroz who passed away just before this paper was submitted. Prof. Chaya Moroz dedicated her entire life to science and inspired many investigators.

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Introduction

A multitude of immunomodulatory properties of the feto-maternal interface (placenta) has evolved to allow the survival of the immunologic distinct fetus without an attack from the maternal immune system. A substantial body of literature exists describing the mechanisms used by cancer cells to escape apoptosis while evading a host immune response. These studies have advanced the field of cancer immunology and passive or active immunotherapy [1–6].

The similarities between the mechanisms involved in feto-maternal and tumor-associated immunologic tolerance are intriguing and suggest a common pattern. Our research strategy, which has spanned the last three decades, searched for mechanisms of cancer immune tolerance based on the parallels in proliferation and immune privilege between cancer and pregnancy. Investigation of the similarities between tolerogenic systems within the tumor microenvironment and the feto-maternal interface led to fundamental clinical and experimental milestone discoveries, listed as follows: A tumor-associated antigen that cross-reacted with human ferritin blocked the surface of T cells and induced immune suppression in patients with Hodgkin's disease [7] and breast cancer [8]; the development of CM-H9 monoclonal antibody (mAb) specific to placental and tumor-derived isoferritins that did not react with normal human ferritin [9] enabled the discovery of placental type isoferritin (PLF) secretion during normal human gestation and the high correlation between low or deficient PLF and abortions [10]. The further use of CM-H9 mAb confirmed that PLF acted as a diagnostic biomarker for patients with early breast malignancy [11]. Finally, we cloned the novel *placenta immunosuppressive ferritin* (*PLIF*) gene from human placenta and from breast cancer cells and discovered its unique expression, protein structure, and function in the regulation of specific cell-mediated immunity [12].

PLIF is a protein composed of 165 amino acids, of which 117 match the ferritin heavy chain sequence, whereas the 48 C-terminal amino acids (C48) represent a novel amino acid sequence. C48 exhibits the immunosuppressive bioactivity of PLIF [12]. C48-PLIF binds to macrophages and activated T cells, inducing high levels of interleukin-10 (IL-10), and acts as a regulatory cytokine governing the balance between Th1 and Th2 cytokines [12–14]. During normal pregnancy, PLIF expression is elevated as early as day 11, remains elevated throughout gestation, and declines at term. PLIF is low in missed abortions, early pregnancy failures, and intrauterine growth restriction [10,15–18]. Furthermore, it is a physiological down-regulator of cell-mediated immune reactivity during pregnancy [19,20].

Experimental neutralization of PLIF in pregnant mice by antibodies inhibited placental and fetal growth and modulated the cytokine network toward a TH1-type immune response [21].

PLIF is upregulated and expressed in the majority of human breast primary tumors [22] and human breast cancer cell lines (T47D and MCF-7) [12] but not in benign breast disease [11]. Both breast cancer cells and syncytiotrophoblasts of the developing placenta secrete PLIF. It creates a microenvironment supportive of immunologic privilege and systemic alterations in immunity, particularly with respect to a helper T cell type 2 polarization [14,16].

Experiments were performed in a mouse model to restore T cell cytotoxic activity and reject breast cancer by neutralizing C48-PLIF and its immunosuppressive cytokines (IL-10). We have previously shown that blocking PLIF by anti–C48-PLIF antibodies in nude mice engrafted with MCF-7 breast cancer cells and human peripheral blood mononuclear cells (PBMCs) resulted in tumor regression associated with tumor cell apoptosis. This treatment affected the cytokine network from TH2 to TH1 type, leading to the breakdown of PLIF-induced immune tolerance [23]. It was thus demonstrated that blocking C48-PLIF could be an effective specific strategy to treat PLIF-secreting breast cancers.

In the current study, we introduce the novel molecule C24D, designed and synthesized as a PLIF antagonist. We show that C24D polypeptide competitively inhibits the binding of C48-PLIF to T cells. Anti-tumor cytotoxic T lymphocytes (CTLs) were generated from naïve human lymphocytes treated with C24D and stimulated *in vitro* with allogeneic whole non-manipulated MCF-7 and T47D tumor cells. On reactivation with their targets, all CTL lines secreted high levels of interferon-γ (IFN-γ) and induced tumor cell apoptosis. Furthermore, we demonstrated *in vivo* that C24D treatment inhibited the growth of MCF-7 tumors engrafted in nude mice that were immune-compromised with naïve human PBMCs.

Our accumulated data indicate that C24D may represent a novel strategy in breast cancer treatment through breakdown of the immune tolerance common in tumor diseases.

Methods

C24D Polypeptide and Recombinant C48 Protein

C24D polypeptide synthesis was performed by the fully automated Applied Biosystems Peptide Synthesizer Model 433A. This was arranged by special order according to a submitted amino acid sequence and is of 99.5% purity.

Recombinant C48 protein was produced in *Escherichia coli*, as previously described [12].

Cells and Antibodies

HD-MAR T cell line established from a patient with Hodgkin's lymphoma [1] was kindly provided by Hanna Ben-Bassat (The Hebrew University Hadassah Medical School, Jerusalem, Israel). HD-MAR cells were grown and maintained in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. The human breast cancer cell lines employed were obtained from American Type Culture Collection (Rockville, MA). MCF-7, T47D, and MDA-MB-231 human breast cancer cell lines were maintained in monolayer cultures in fetal calf serum and antibiotics. For passages, confluent monolayer cultures were trypsinized with trypsin-EDTA solution (0.25% and 0.05%, respectively), washed once, and seeded in culture medium.

PBMCs were isolated from blood buffy coats obtained from non-identified blood bank donors supplied by MDA Blood Bank (Tel Hashomer, Israel) and were approved by the local ethics committee of Beilinson Medical Center (Petah Tikva, Israel). Buffy coats were layered onto Lymphoprep solution (Nycomed, Oslo, Norway) and spun at 2000 rpm for 20 minutes. The interface layer was collected, washed twice, counted, and resuspended in phosphate-buffered saline (PBS, pH 7.4) to the desired cell concentration.

Anti-C24D Ig was prepared by immunizing outbred mice with C24D polypeptide in PBS mixed with complete Freund's adjuvant at 1:1 ratio (vol/vol), on days 1, 7, and 21. The mice were bled on day 28 and Igs were isolated by salt precipitation. Anti-C48 mAb was prepared, as previously described [9]. This mAb reacted with C48-PLIF but did not cross-react with C24D. Anti-CD3 mAb (OKT3) was purchased from Ortho Biotech Inc (Horsham, PA). phycoerythrin (PE)–anti-CD14$^+$, fluorescein isothiocyanate (FITC)–anti-CD4$^+$ and PE–anti-CD8$^+$ were purchased from Becton Dickinson (San Jose, CA). FITC–anti-mouse IgG was purchased from Dako (Carpinteria, CA).

Detection of C48 and C24D Binding to HD-MAR by Flow Cytometry

For C48 and C24D binding experiments, HD-MAR T cells were incubated with C48, C24D or both for 20 minutes under 5% CO_2, at room temperature, and 90% humidity, followed by two washes with PBS containing 1% BSA and 0.05% sodium azide. The cells were then incubated for 20 minutes on ice with anti-C48 mouse mAb or mouse anti-C24 IgG followed by two washes, as above. After incubation with FITC–anti-mouse IgG for 20 minutes on ice, the cells were washed twice with PBS containing 0.05% sodium azide and analyzed with a BD flow cytometer.

Breast Cancer Cells and PBMC Co-Cultures In Vitro for Primary Activation

MCF-7 or T47D cells were transferred to RPMI 1640 and human AB serum (10%). PBMCs (1×10^6) were added to MCF-7 or T47D (0.1×10^6) at an effector/target (E:T) ratio of 10:1, followed by treatment with C24D (30 μg/ml) at 0, 24, and 48 hours. The cell co-cultures without C24D treatment served as a control. The cultures were subjected to microscopic evaluation on days 5 and 7 of the experiment.

Development of Specific Anti–Breast Cancer CTLs

On day 7 of co-culture, the medium was replaced by fresh RPMI 1640 containing 10% human AB serum and IL-2 (5 ng/ml). This growth medium of the cultures was refreshed three times a week. The lymphocytes were harvested after 4 weeks for further studies.

Tumor Cell Cytotoxicity Assays

Alamar Blue assay for quantitative analysis of cell viability was performed, as previously described [24]. In brief, Alamar Blue was directly added to culture medium. At various time intervals, the redox reaction in which Alamar Blue was reduced by the cells was measured by absorbance readings at 540 and 630 nm.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction, which is an indicator of cellular metabolic activity, was measured as previously described [25]. In brief, breast cancer cells were incubated for 5 hours with lymphocytes at different E:T ratios. After incubation for 3 hours, 50 μl of 0.25% (wt/vol) solution of MTT in PBS was added and further incubated for 2 hours. The non-adherent cells were removed and the remaining adherent tumor cells were dissolved in a mixture of DMSO (Sigma-Aldrich, Rehovot, Israel), 5% (wt/vol) sodium dodecyl sulfate and 1% (vol/vol) 1 N hydrochloric acid. The absorption at 570/650 nm was measured with a plate reader (FLUOstar; BMG LABTECH, Offenburg, Germany).

Data Analysis

Cell viability was calculated with regard to the untreated breast cancer cultures alone (control) [y_0], which was set at 100% viability. A lysis control [y_{100}], wherein the cells were treated with 0.5% Triton X-100, was set at 0% viability. This was found to be sufficient to induce 100% cell death. Mean values from eight wells were determined [25].

Annexin V Test for Apoptosis

Apoptotic cell death of breast cancer cells was performed using Annexin V–based apoptosis kit [26]. In brief, 5×10^5 tumor cells were mixed with 1.7×10^5 CTLs (E:T ratio of 1:3) in 0.4 ml of RPMI 1640 containing 10% human AB serum at various time periods. The cells were further reacted with FITC–Annexin V and propidium iodide, using the Annexin V Apoptosis Detection Kit FITC (eBioscience, San Diego, CA), according to the manufacturer's instructions.

Gating of tumor cells from CTL–tumor cell mixture for apoptosis analysis was first determined using anti-CD3 staining of the culture. Negatively stained large cells were gated as tumor cell population. We noticed that the same gating pattern was obtained with light-scatter analysis of the culture and so we used this latter assay in our further experiments for determination of tumor population. The percentage of FITC-conjugated Annexin V–positive cells was analyzed by flow cytometry (Becton Dickinson).

Quantitative image-based apoptotic index measurement using multispectral imaging flow cytometry was acquired on the ImageStream multispectral imaging flow cytometer (Amnis Corporation, Seattle, WA) [27].

Cytokine Production Evaluation

ELISA kits for the human cytokines IFN-γ and tumor necrosis factor–α (TNF-α) were purchased from R&D Systems Incorporation (Minneapolis, MN). These kits were used according to the manufacturer's instructions to quantify the indicated cytokine production in the supernatants harvested from CTLs and target tumor cell mixed cultures at different time periods.

In Vivo Studies

Athymic BALB/c nude mice were purchased from Harlan (Rehovot, Israel). The mice were housed in a barrier facility. All procedures were approved by the Institutional Animal Care Committee of Tel Aviv University (Tel Aviv, Israel). The mice were inoculated subcutaneously with MCF-7 human breast cancer cells (5×10^6), in the presence of an estrogen source (slow release 60-day estrogen pellets, 0.72 mg). Human PBMCs (50×10^6/0.5 ml PBS) were injected intravenously after transplantation of MCF-7 tumor cells, as previously described [23]. In part of the groups, mice did not receive PBMCs to assess direct effect of C24D on tumor growth.

Treatment of Tumor-Bearing Mice with C24D

In each experiment, mice were divided into groups of five. C24D dissolved in PBS (pH 7.2) was injected intraperitoneally to each mouse daily, at an optimal dose of 60 μg/0.2 ml. Control mice received daily injections of PBS.

Gross and Microscopic Pathology

Tumors were excised, measured and fixed in phosphate-buffered formalin (pH 7.0). The paraffin-embedded blocks, tissue sections and immunohistochemistry staining were performed by the Department of Pathology, Rabin Medical Center (Petah Tikva, Israel). For immunohistochemical staining, slides were incubated with anti-LCA primary antibodies (BioGenex, Fremont, CA) at previously determined optimal concentrations and developed with a biotinylated secondary antibody, followed by conjugated streptavidin-peroxidase (Zymed Laboratories, San Francisco, CA), according to the manufacturer's instructions. The stained slides were counterstained with Mayer's Hematoxylin and Eosin (H&E) and mounted with aqueous mounting solution.

Statistical Analysis

Results were presented as means ± SD. Comparisons were made by Student's *t* test. A *P* value of less than or equal to .05 was considered statistically significant.

Results

The C24D Molecule

C24D is a dimer of identical polypeptide chains cross-linked at the N-terminal end with the amino acid cysteine followed by amino acids homologous to amino acids 25 to 48 at the C-terminus of C48 protein [12]. The molecular weight of the synthetic C24D molecule is 6418.8 with 99.5% purity.

C24D Binds to Human T Cells (HD-MAR) and Competitively Inhibits the Binding of C48-PLIF

HD-MAR is a human T cell line ($CD3^+$, $CD4^-$, $CD8^-$) that constitutively binds PLIF [28,29]. FACS analysis using anti-C24D and anti-C48 antibodies revealed the binding of both C24D and C48 to the surface of human HD-MAR cells (Figure 1, *A, B, E* and *F*). Yet, C24D inhibited the binding of C48 to HD-MAR cells in a concentration-dependent manner (Figure 1, *C, D* and *F*). This was demonstrated by the significant lower Mean Fluorescence Intensity (MFI) measured when HD-MAR cells were preincubated with C24D for 20 minutes at room temperature and then with C48, compared to cells without C24D (Figure 1*F*). These results indicate that C24D compete with C48 in binding to HD-MAR cells and thus inhibits C48-PLIF subsequent binding. It is worth mentioning that although C24D binds to HD-MAR cells and compete with C48 and PLIF binding, it does not exert their immunomodulatory activity (data not shown). Thus, C24D may represent a competitive inhibitor for PLIF in its biologic systems. This prompted our attempts to investigate whether C24D would break up the PLIF-induced immune suppression in anti–breast cancer immunity.

C24D Treatment of PBMCs Cultured with MCF-7 and T47D Human Breast Cancer Cells Resulted in Activation of Specific Anti-Tumor CTLs

A schematic outline for the use of C24D to develop anti–breast cancer immune response and specific CTLs from PBMCs of blood bank donors is portrayed in Figure 2*A*. MCF-7 and T47D tumor cells grow as a monolayer in tissue culture plates. We found that the addition of PBMCs to tumor cell cultures at a ratio of 10:1 for 7 days did not affect their growth. However, the addition of C24D to the cultures resulted in tumor cell lysis. An example of a microscopic view is shown for MCF-7 and PBMC co-cultures (Figure 2, *B* and *C*). It is noteworthy that C24D did not bind to the tumor cells. It had no direct anti-tumor effect when added to the tumor cell cultures without PBMCs (not shown). Using the Alamar Blue assay for quantitative analysis of tumor cell viability, it was evident that 25% to 35% of the tumor cells were lysed in both T47D and MCF-7 tumor-PBMC co-cultures treated with C24D but not in the untreated cultures (Figure 2*D*). Furthermore, PBMCs remained adherent in the lytic areas of the C24D-treated co-cultures (Figure 2*C*). The adherent PBMC-derived cells were identified as $CD3^+$ and $CD14^+$ cell types (not shown). Furthermore, cells cultured for 4 weeks in IL-2 supplemented growth medium yielded six lymphoid

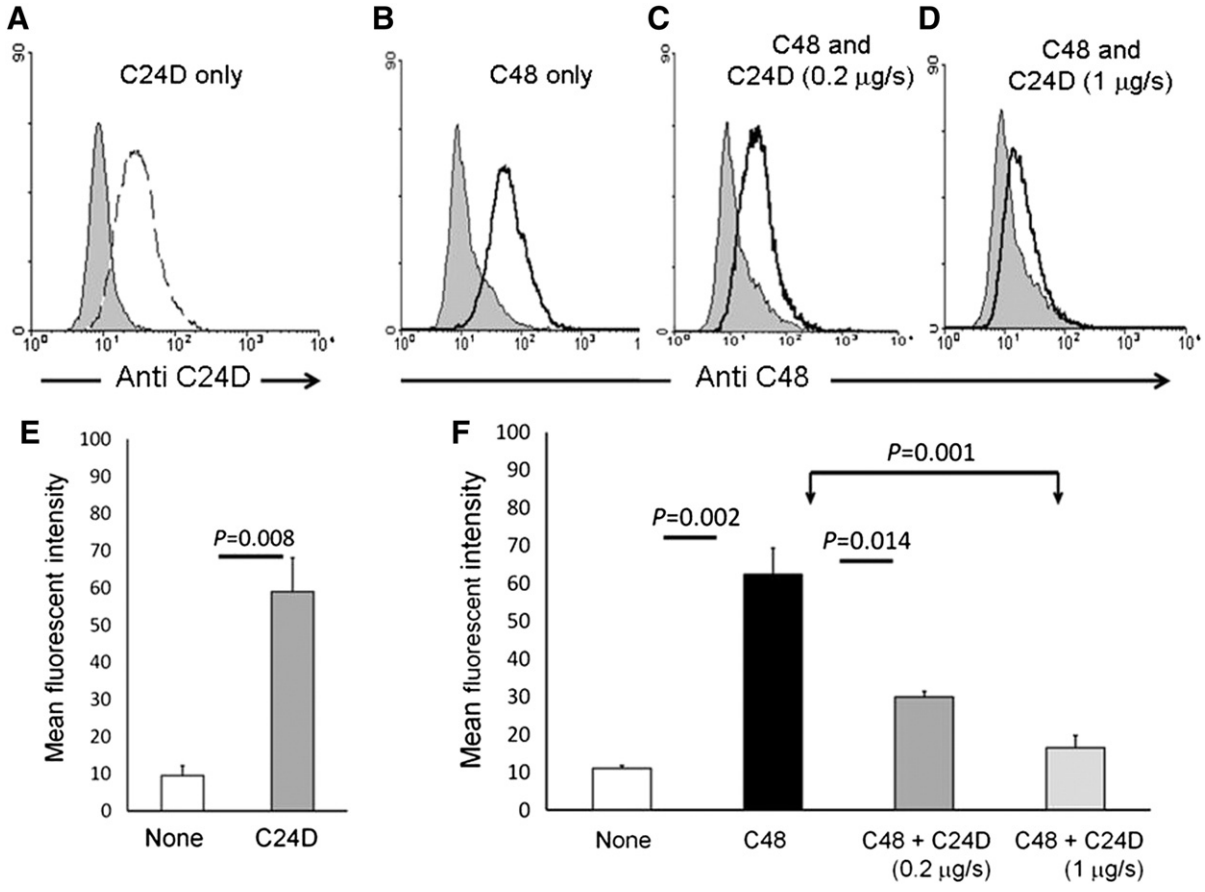

Figure 1. C24D inhibits the binding of C48-PLIF protein to T lymphocytes. Representative histograms for FACS analysis of binding of C24D (A) and C48 (B) to HD-MAR T cells detected by anti-C24D IgG and anti-C48 mAb, respectively. C48 binding to HD-MAR cells is inhibited by 0.2 μg per sample of C24D (C). C48 binding to HD-MAR cells is inhibited by 1 μg per sample of C24D (D). The above results are further demonstrated as MFI value ± SD ($n = 3$) of C24D binding (E) and binding of C48 to HD-MAR cells and its inhibition by C24D (F).

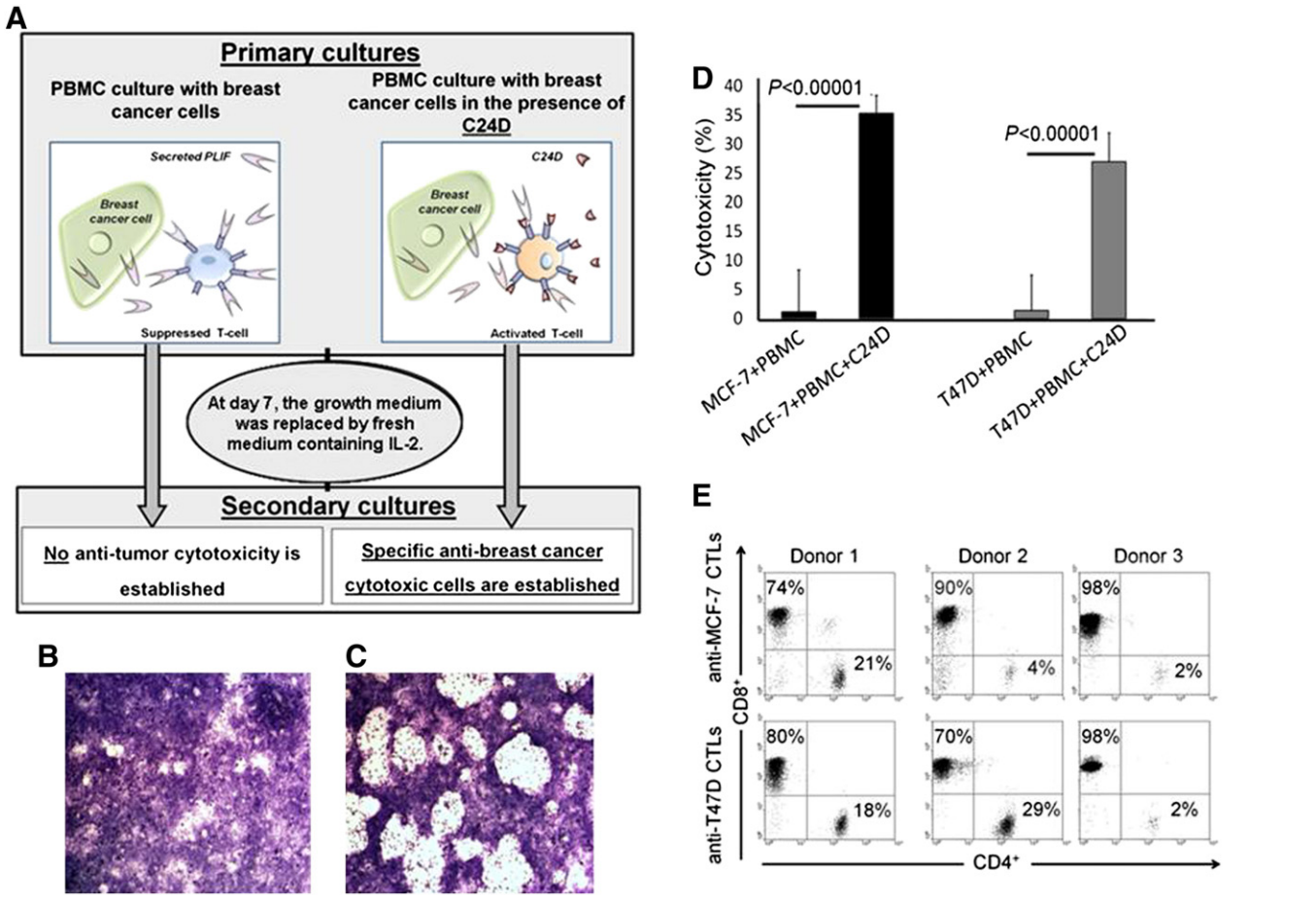

Figure 2. *In vitro* development of anti-tumor cytotoxic T cells following C24D treatment. (A) A schematic illustration describing the basic concept of this study, i.e., blocking C48-PLIF by C24D on T cells results in the development of specific anti-tumor CTLs. The two stages of the experiments are exhibited: In primary cultures, human PBMCs are co-cultured with human breast cancer cell lines at an E:T ratio of 10:1. The mixed cultures are either untreated (control) or treated with C24D at 0, 24 and 48 hours and incubated for 7 days. The culture medium is replaced on day 7 with growth medium supplemented with human IL-2. The cultures are further incubated for 4 weeks and harvested. Highly cytotoxic cells were obtained from PBMCs incubated with tumor cells in presence of C24D. In contrast, there is no anti-tumor cytotoxic activity from PBMCs incubated with tumor cells without C24D. (B and C) Giemsa staining of MCF-7 tumor cells co-cultured for 7 days with control PBMCs (B) or with C24D-treated PBMCs (C). (D) Alamar Blue cytotoxicity assay performed on day 7 on MCF-7 and T47D tumor cells cultured with their respective control or C24D-treated PBMCs (E:T ratio of 10:1). Histograms represent mean value ± SD ($n = 6$). (E) Flow cytometry analysis of T cells in secondary culture shows a predominance of $CD8^+$ over $CD4^+$ T cells. PBMCs from three different donors are presented.

cell lines from three blood donors, each activated with MCF-7 and T47D human breast cancer cells. FACS analyses revealed that the majority cells of the six harvested C24D-treated lymphocyte lines were $CD8^+$ (Figure 2*E*). A low yield of lymphocytes was harvested from the control (C24D–untreated tumor–PBMC co-cultures), which were mostly $CD4^+$ (not shown). It is noteworthy that no residual tumor cells were recovered from the harvested lymphocyte cultures.

Anti–MCF-7 and Anti-T47D CTLs Are Highly Cytotoxic and Tumor Type Restricted

Successful experimental cancer immunotherapy requires representative cell lines that can be used as target cells for vaccines and verification of the efficacy of activated CTLs. Cytotoxic T cells recognize short peptide sequences presented by the HLA class I molecules on the tumor cell surface [30]. MCF-7 and T47D breast cancer cells, although share at least seven identified tumor antigens, have different HLA class I molecules and thus expected to induce non–cross-reactive CTL clones [31].

In a microscopic view, we showed that the PBMCs pre-activated with whole tumor cells without C24D (control) did not affect their target cells (Figure 3, *A* and *D*). In contrast, following C24D treatment, the cytolytic activity of CTLs raised against each of MCF-7 (Figure 3*B*) and T47D (Figure 3*E*) cell lines was tumor target restricted and not cross-reactive with the other cell line (Figure 3, *C* and *F*). These CTLs were highly cytotoxic to their specific target cells, leading to cytolysis of up to 90% of the corresponding tumor cells at E:T ratios of 1:1 after a 5-hour incubation (Figure 3, *G* and *H*).

Anti–MCF-7 and Anti-T47D CTLs Secrete High Levels of IFN-γ and Induce Apoptosis of Tumor Cells on Reactivation with Their Respective Target Cells

When anti–MCF-7 and anti-T47D CTLs were reactivated for 5 hours with their corresponding cancer cells, they secreted increasing levels of IFN-γ proportional to the number of effector cells (Figure 4, *A* and *B*). In contrast, secretion was not detected when CTLs were

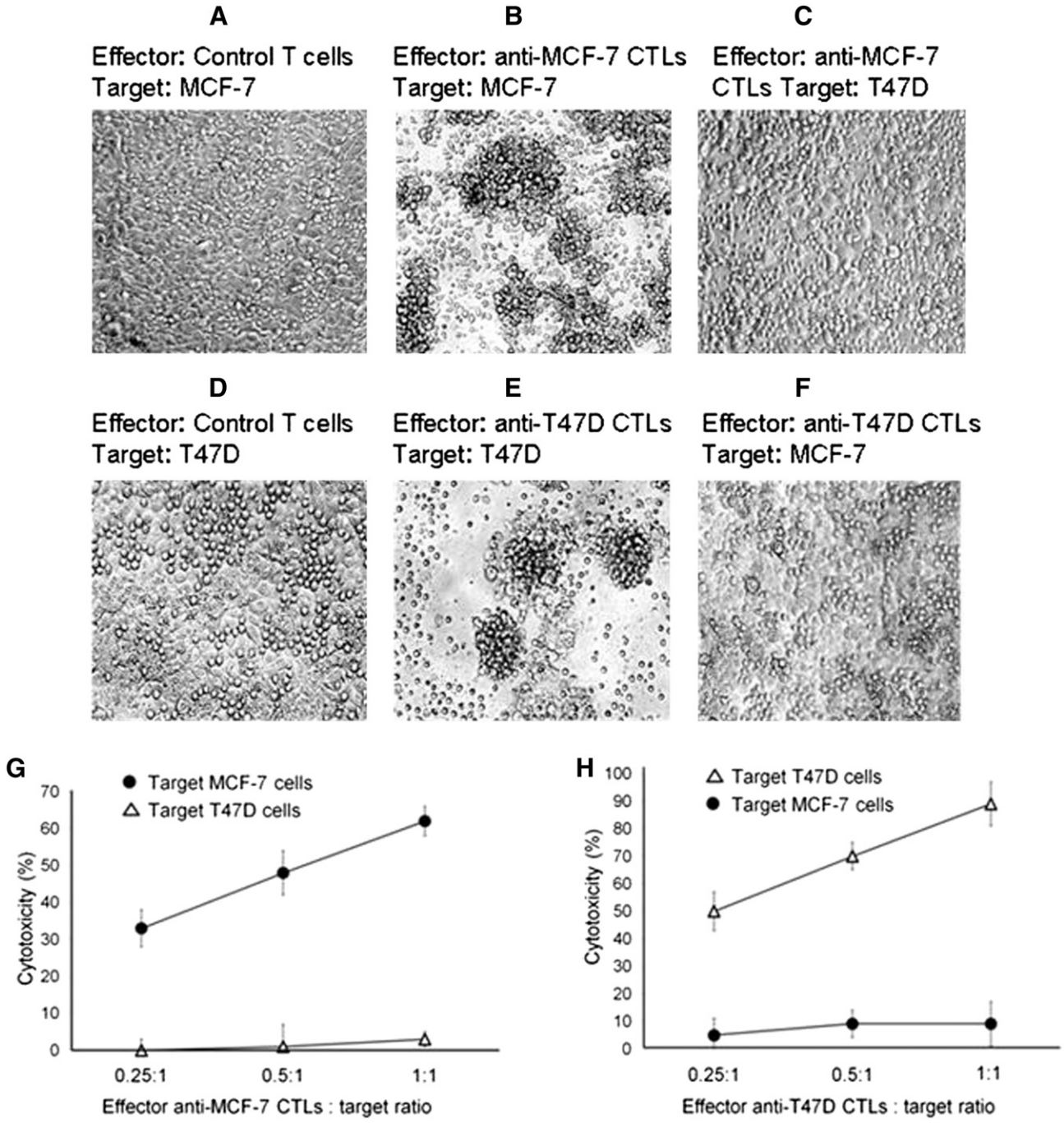

Figure 3. C24D-induced CTLs are highly cytotoxic to their specific tumor cells. (A–F) Representative light microscopy images of MCF-7 and T47D tumor cells co-cultured for 5 hours with control T lymphocytes or induced CTLs. (G and H) E:T cell ratio–dependent cytotoxicity detected by MTT cytotoxicity assay (5-hour incubation). Data represent mean value ± SD (n = 3).

cross-activated with another, HLA class I unmatched, breast cancer cell line. Furthermore, the high levels of IFN-γ secreted by the CTLs increased following extension of reactivation time, still in a tumor-target specific manner (Figure 4*C*). The cytokine secretion was accompanied by enhanced apoptotic death of MCF-7 and T47D tumor cells co-cultured with their respective CTLs (Figure 4, *D* and *E*). Apoptosis determined by FACS analysis of Annexin V and PI-labeled cells showed that anti–MCF-7 CTLs (Figure 4*D*) and anti-T47D CTLs (Figure 4*E*) induced target cell–specific apoptosis. The above data indicate the functional restriction and specificity of the C24D-developed CTLs.

Anti–MCF-7 CTLs Are Cross-Cytotoxic to the MDA-MB-231 Cell Line

To examine whether the lack of cross-reactivity between anti–MCF-7 and anti-T47D CTLs is referred to HLA-A type incompatibility, we tested the ability of anti–MCF-7 CTLs to react against MDA-MB-231, a breast cancer cell line that share the same HLA-A type with MCF-7 (HLA-A*0201). MDA-MB-231 (HLA-A*0201) is a triple-negative breast cancer (TNBC), an aggressive breast cancer subtype, not expressing estrogen receptor, progesterone receptor, and HER2/*neu* that represents a major clinical challenge [32].

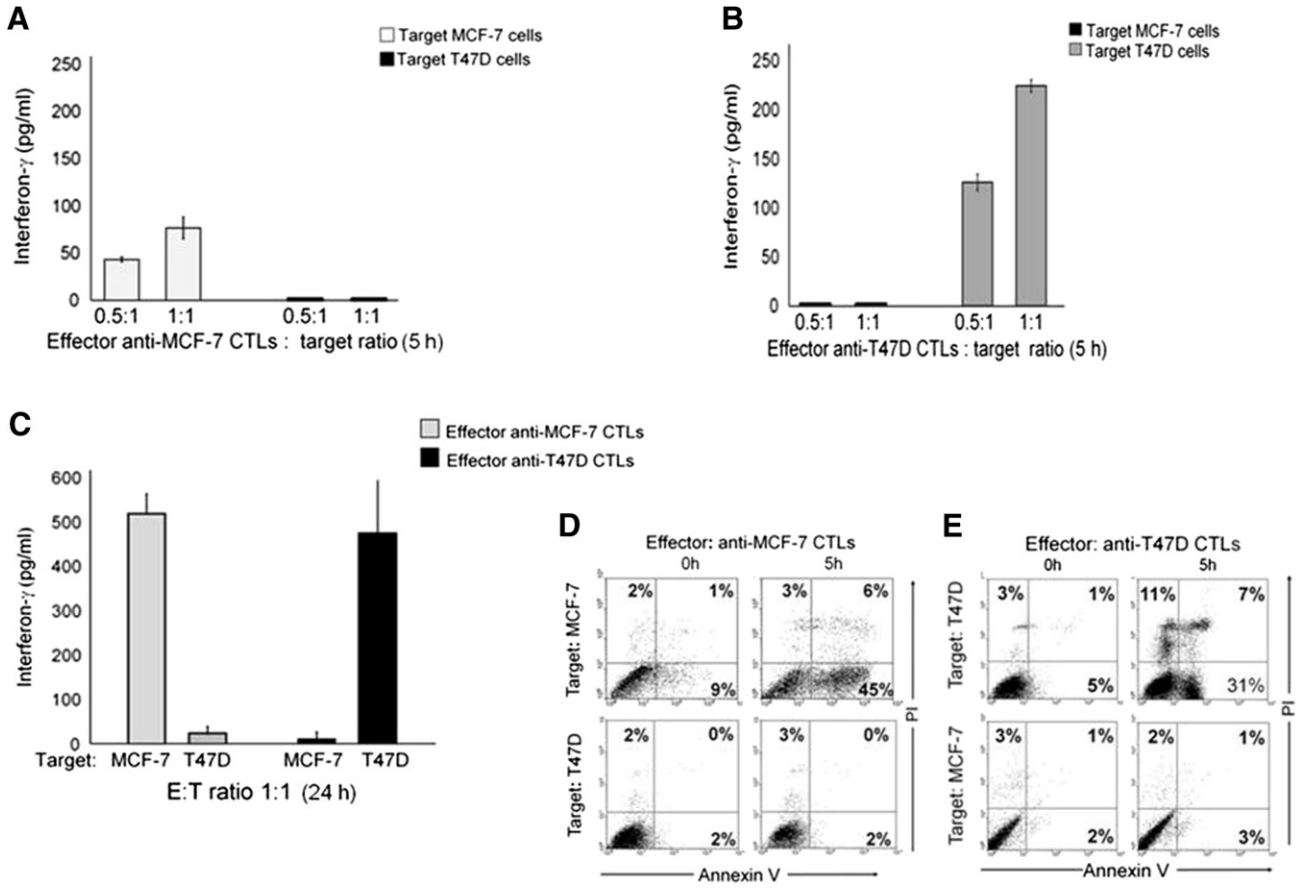

Figure 4. Activated CTLs secrete IFN-γ and initiate apoptosis of their specific targets. (A and B) IFN-γ concentrations in the supernatants obtained from 5-hour co-cultures of MCF-7 and T47D tumor cells with increasing CTL ratios (0.5:1 and 1:1). (A) Anti–MCF-7 CTLs. (B) Anti-T47D CTLs. (C) IFN-γ secreted by anti–MCF-7 CTLs and anti-T47D CTLs (E:T ratio of 1:1) determined after extended incubation (24 hours). All values are expressed as mean ± SD (n = 3). (D and E) Enhanced apoptotic death of MCF-7 and T47D tumor cells co-cultured with (D) anti–MCF-7 CTLs or (E) anti-T47D CTLs determined by FACS analysis (Annexin V and PI labeling).

Anti–MCF-7 CTL cytotoxicity against MDA-MB-231 cells is presented in a representative microscopic view (Figure 5*A*). We observe major lysis of MDA-MB-231 TNBC cells with the anti–MCF-7 CTLs (Figure 5*A*, *upper*) but not with the anti-T47D CTLs (Figure 5*A*, *lower*).

In a multispectral imaging flow cytometry, we measured the apoptosis of MDA-MB-231 cells stained with FITC–Annexin V (green) and PI (red) following reactivity with anti–MCF-7 CTLs at an E:T ratio of 1:1 (Figure 5*B*). The increasing rate of early (Annexin V^+) and late (PI^+) apoptosis is demonstrated in a quantitative time course (up to 5 hours) of an image-based apoptotic index (Figure 5*C*). It is noteworthy that no apoptosis was demonstrated with anti-T47D CTLs cross-activated with MDA-MB-231 (not shown). Furthermore, anti–MCF-7 CTLs exhibited broad reactivity against MDA-MB-231 and MCF-7 target cells in TNF-α and IFN-γ assays. Comparative time course measurements at incubations of 3 and 5 hours revealed the secretion of both TNF-α (Figure 5*D*) and IFN-γ (Figure 5*E*) by anti–MCF-7 CTLs reactivated with MDA-MB-231 TNBC. It is noteworthy that when anti–MCF-7 CTLs were reactivated with MDA-MB-231 tumor cells, both IFN-γ and TNF-α cytokines were secreted earlier and at significantly higher levels compared to the reactivation of their specific target MCF-7 cells (Figure 5, *D* and *E*). Anti-T47D CTLs did not secrete TNF-α or IFN-γ upon reaction with MDA-MB-231 due to HLA-A restriction, as expected (Figure 5, *D* and *E*).

Therapeutic Effect of C24D on MCF-7 Tumor Growth in Nude Mice

The *in vitro* effect of C24D treatment on the development of anti–MCF-7 CTLs documented above motivated us to investigate whether *in vivo* C24D treatment of human PBMCs reconstituted, tumor-bearing nude mice would affect the MCF-7 tumor growth. Nude mice were engrafted subcutaneously with MCF-7 tumor cells, transfused with human PBMCs, and treated by daily intraperitoneal injections of either C24D or PBS for 19 days. C24D treatment did not affect the mouse weight compared to the PBS-treated control group (Figure 6*A*) but markedly inhibited the tumor growth (P = .017; Figure 6, *B* and *C*). The stability of mice body weight in both groups along the experiment indicated lack of toxicity of C24D.

It is worth mentioning that C24D treatment of nude mice bearing MCF-7, without PBMC inoculation, did not influence tumor size compared to control PBS treatment (tumor size of 180 ± 27 cm and 164 ± 19 cm, respectively, $P > .5$, $n = 4$).

Histochemical analyses of representative tumors derived from PBMCs reconstituted immune-compromised mice treated with C24D exhibited a large area of tumor necrosis (Figure 6*D*, *upper right*), compared to minimal or no necrosis in the corresponding control mice (Figure 6*D*, *upper left*). Further, immunohistochemical staining with anti-$CD45^+$ antibodies was performed. Images obtained from the representative tumors are shown in Figure 6*D*

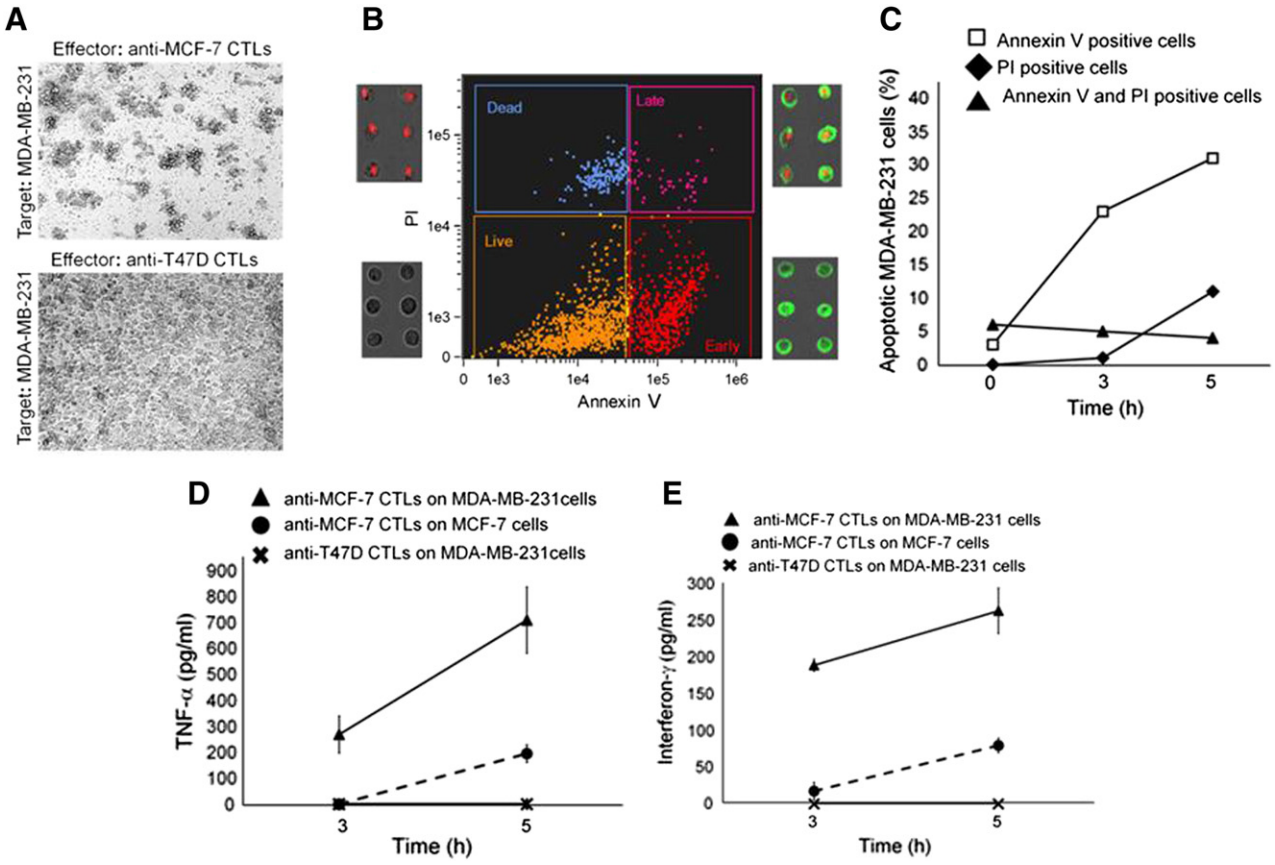

Figure 5. Anti–MCF-7 CTLs cross-activated by HLA-A2$^+$ MDA-BM-231 cells induced target cell apoptosis and TNF-α and IFN-γ secretion. (A) Representative light microscopy images of MDA-MB-231 tumor cells co-cultured for 7 hours with anti–MCF-7 CTLs or anti-T47D CTLs. (B) Stages of apoptotic death of MDA-MB-231 tumor cells co-cultured for 5 hours with anti–MCF-7 CTLs, determined by imaging flow cytometry: lower left—viable cells; lower right—early apoptosis; upper right—late apoptosis; upper left—dead cells (Annexin V and PI labeling). (C) Representative time course development of early and late apoptosis of MDA-MB-231 tumor cells by anti–MCF-7 CTLs. (D and E) Cytokine concentrations in the supernatants obtained from co-cultures of MDA-MB-231 and MCF-7 tumor cells with anti–MCF-7 CTLs or anti-T47D CTLs at 3 hours and 5 hours for (D) TNF-α and (E) IFN-γ. All values are expressed as mean ± SD (n = 3).

(*lower*). The specimen obtained from the C24D-treated group exhibited massive necrotic areas with intra-tumor infiltration of human CD45$^+$ cells (Figure 6*D*, *lower right*, brown staining), compared to lack of CD45$^+$ cells in the PBS-treated specimen (Figure 6*D*, *lower left*). It is therefore concluded that *in vivo* treatment of PBMC reconstituted mice with C24D enables anti–MCF-7 vaccination resulting in tumor growth inhibition.

Discussion

The unique aspect of this study is the finding that C24D is a novel PLIF-specific binding antagonist to T cells. C24D is able to break the *in vitro* and *in vivo* immune tolerance induced by PLIF-producing human breast cancer cells, resulting in tumor cell apoptosis and growth inhibition. Without blocking PLIF activity, no anti-tumor activation or tumor growth inhibition developed, indicating the major role of PLIF in the suppression of the anti-tumor immune response. It is worth mentioning here that C24D does not exert any direct anti-tumor effect.

We demonstrate here that tumor-specific CTLs were generated from PBMCs stimulated with, whole non-manipulated allogeneic, MCF-7 and T47D tumor cells only following treatment with C24D. Depending on the stimulator cell line, highly reactive CTL lines were developed from each donor. This specificity was reflected by the fact that CTLs raised against MCF-7 failed to react against T47D cells that differ in HLA-A haplotype but strongly reacted against MDA-MB-231 cells that share the same HLA-A haplotype with MCF-7 cells.

The CTL lines (CD8$^+$) generated in the C24D-treated co-cultures secreted high levels of IFN-γ and TNF-α on reactivation with the corresponding tumor target. IFN-γ regulates various biologic programs that could participate in abrogating tumor growth [33,34]. One of the important anti-tumor mechanisms induced by IFN-γ is its capacity to inhibit cellular proliferation [35–37] and promote apoptosis.

Our results indicate that the therapeutic potential of C24D in tumor immune therapy is applicable in direct treatment of breast cancer–bearing patients leading to braking of PLIF-related immune suppression. Moreover, C24D is also applicable in allogeneic whole tumor cell–based vaccination strategies for the induction of anti-tumor immunity in cases of weak immunogenic tumors [38–41]. Actually, our results support these notions since whole MCF-7 tumor cells as a vaccine in the C24D-induced T cell activation resulted in high reactivity against the MCF-7 cells itself as well as against other breast cancer cell line sharing matched HLA-A haplotype with MCF-7 cells (i.e., MDA-MB-231).

PLIF, a physiological pregnancy-related protein, is upregulated and secreted by other malignant diseases [42] and in human immunodeficiency virus infection [43]. PLIF exerts its antigen-specific

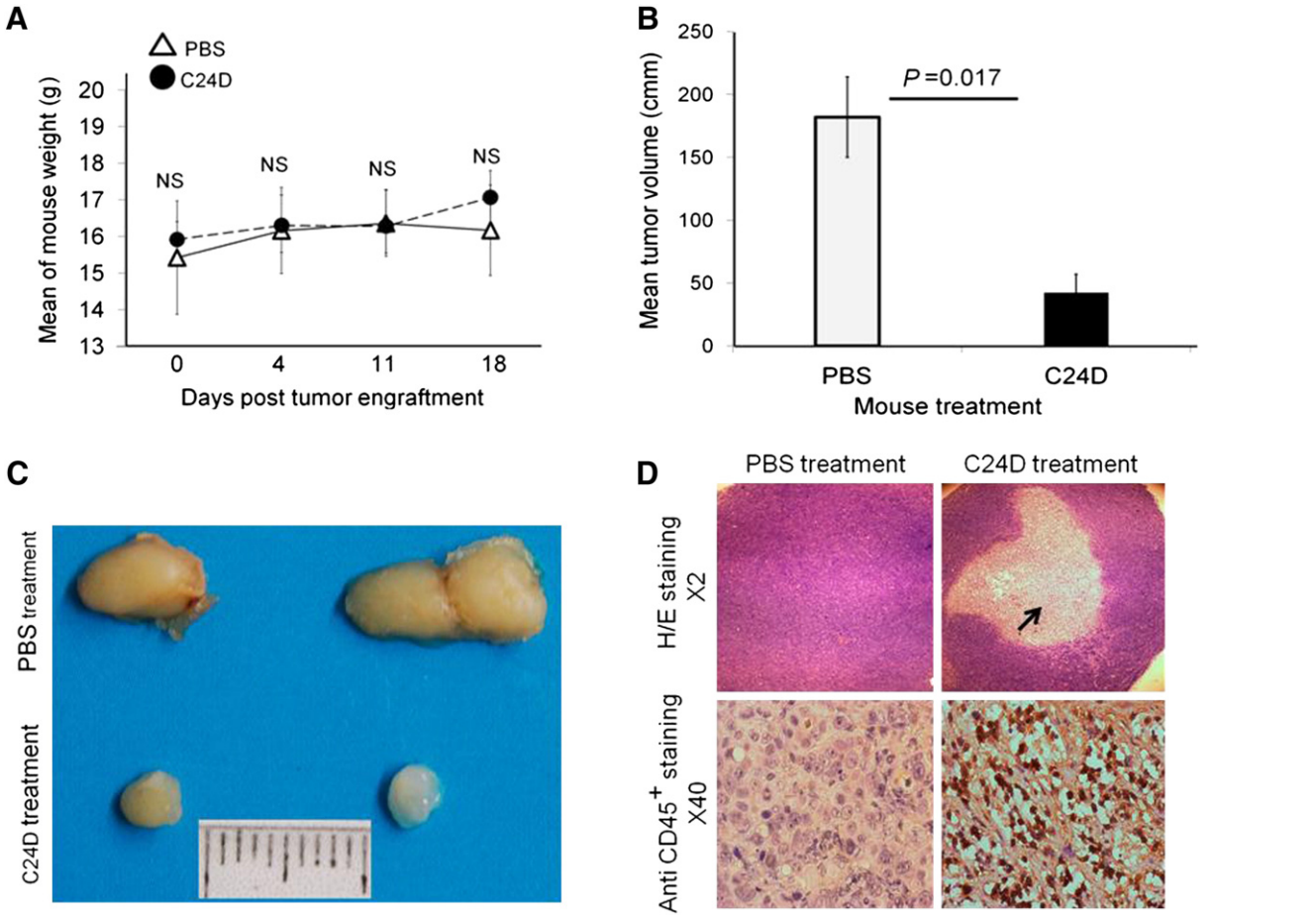

Figure 6. *In vivo* C24D treatment inhibits MCF-7 tumor growth in human PBMC transfused nude mice. (A–D) Immune-compromised nude mice engrafted with MCF-7 tumor cells were treated with C24D or PBS (control) for 18 days (five mice in each group). (A) Mean value of mice weight ± SD. (B) Mean volumes ± SD of tumors removed on day 19. (C) Photographs of removed tumors. (D) H&E staining (top panels) and anti-CD45$^+$ staining (bottom panels) of tumor sections. CD45$^+$ cell infiltration (stained brown, right bottom panel) is exhibited only in the tumor removed from C24D-treated mice. No infiltration of CD45$^+$ cells was seen in the tumor removed from control mice (left bottom panel).

immune suppression by binding only to the activated T cells [12–14]. In previous studies, we demonstrated that human C48-PLIF is an effective therapeutic immune suppressive agent enabling allogeneic non-manipulated murine bone marrow transplantation yielding long-lasting donor-specific immune tolerance [44]. In contrast, this study demonstrates that C24D competitively inhibit PLIF binding to T cells and restores T cell immunity, resulting in rejection of breast cancer cells.

To validate any vaccination strategy, it is important to define the most appropriate population for testing the vaccine and to determine the optimal combination with other existing therapies. The greatest expectations for cancer vaccines consider the use of immunotherapy in patients whose immune system and E:T ratio are favorable. To optimize the C24D activity and address questions regarding optimal treatment schedules, PLIF is an effective biomarker for diagnosis and follow-up in patients with breast cancer [11,45–48].

Conclusion

The current findings support our strategy to modulate PLIF-induced tumor-immune suppression by the novel C24D molecule and produce an anti–breast cancer vaccination in adoptive and active breast cancer immunotherapy.

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