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Immunization with a vaccine that combines the expression of MUC1 and B7 co-stimulatory molecules prolongs the survival of mice and delays the appearance of mouse mammary tumors

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Abstract

Human epithelial mucin (MUC1) is expressed by many carcinomas, including breast cancer cells. This breast cancer-associated antigen has been widely used for immunotherapy, despite the fact that cellular immune responses to MUC1 are impaired in breast cancer patients and MUC1 transgenic animals. Previously, we found that immunogenicity to MUC1 was also impaired in BALB/c mice injected with a mammary tumor cell line (410.4) expressing human MUC1. We suggested that one reason for its weak immunogenicity was the lack of expression of B7 molecules by 410.4 cells. Recognition of antigenic epitopes in conjunction with MHCI/II by the T-cell receptor without co-stimulation by B7/CD28 association resulted in T-cell anergy. Therefore, we attempted to enhance protective anti-MUC1-specific immunity in mice using B7 co-stimulatory molecules as a component of the MUC1 vaccine. We also compared cell-based with DNA-based vaccination strategies. One group of mice was vaccinated with an irradiated, 410.4 syngeneic mammary tumor cell line co-expressing human MUC1 and CD80 or CD86 co-stimulatory molecules, and a second group of mice was vaccinated with plasmids encoding MUC1 and CD80 or CD86. These mice along with appropriate controls were challenged with mammary tumor cell line 4T1, which expresses MUC1. There were significant inhibition on rates of tumor growth and survival in mice vaccinated with irradiated 410.4/MUC1 cells co-expressing either CD80 or CD86 molecules, compared to non-vaccinated animals. In addition, there were also significant delays in the appearance of measurable tumors and their growth in mice vaccinated by gene-gun immunization with plasmids encoding MUC1 and CD80 or CD86.

Abbreviations: MUC1 – human epithelial mucin; 410.4 – mammary tumor cell line isolated from a single spontaneously arising mammary tumor in a BALB/c fC3H mouse; 4T1 – thioguanine-resistant variant derived from 410.4 cells without mutagen treatment; 410.4/MUC1 – 410.4 cells expressing MUC1; 410.4/MUC1/CD80 (CD86) – 410.4/MUC1 cells expressing CD80 (or CD86) molecule; 4T1/MUC1 – 4T1 cells expressing MUC1; VNTR – variable number of tandem repeats; B7 – CD80 and CD86 co-stimulatory molecules; MUC1/Tg – MUC1 transgenic animals; DMEM – Dulbecco-modified Eagle's essential medium; D10 – DMEM containing low glucose and supplemented with 5% fetal bovine serum, 5% newborn calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate; pCD80 – plasmid encoding CD80 molecule; pCD86 – plasmid encoding CD86 molecule; pcMUC1 – plasmid encoding MUC1; pcMUC1/CD80 (CD86) – mixture of plasmids pcMUC1 and pCD80 (or pCD86); s.c. – subcutaneously; i.m. – intramuscularly; RMA – Rauscher virus-induced T-cell lymphoma; EL4 – T-cell lymphoma induced by 9,10-dimethyl-1,2-benzanthracene; MC38 – murine colon adenocarcinoma induced by methylcholntren; B16-F10 – spontaneous murine melanoma

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Introduction

Immune protection against breast cancer and other types of neoplastic disease requires the generation of potent cellular immune responses against unique tumor antigens expressed by the malignant cells. One such antigen is epithelial mucin (MUC1), a well-characterized breast cancer-associated antigen. MUC1 is a type I transmembrane glycoprotein expressed by human epithelial cells; it is composed of a variable number (20–125) of 20 amino acid tandem repeats (VNTR) and is heavily glycosylated [1–3]. In patients, MUC1 is expressed in an aberrant form not only by breast cancer cells, but also by carcinomas of the lung, ovary, colon and pancreas [4, 5]. More specifically, breast cancer-associated MUC1 is under-glycosylated. Its expression is dramatically increased compared to normal tissues. As a result, malignant cell epitopes are exposed to the immune system [6, 7]. These features of the tumor-associated antigen MUC1 make it a promising candidate for immunotherapy in breast cancer patients [1, 3–9].

Most tumor cells fail to elicit anti-tumor cellular immune responses, even though they express determinants that are potentially antigenic. Breast cancer cells expressing MUC1 are also poorly immunogenic. Only low frequencies of MHC-unrestricted and MHC-restricted cytotoxic lymphocytes and low titers of MUC1-specific antibodies were found in breast cancer patients [10–16]. A possible reason for this lack of immunogenicity and the impaired subsequent immune response is that MUC1 and other tumor antigens are self-antigens, and the immune system has safety mechanisms for preventing self-tissue antigen recognition and autoimmune responses. Among such mechanisms are: (i) low level expression of MHC class I antigens [17]; (ii) lack of expression of B7 (CD80/CD86) co-stimulatory molecules [18]; (iii) T-cell growth suppression [19, 20]; (iv) ineffective MUC1 processing and presentation by dendritic cells or other antigen presenting cells [21]; (v) immune switching from effective cellular to ineffective humoral responses [22].

The development of an anti-MUC1 vaccine that can successfully induce anti-tumor cellular immune responses capable of leading to tumor-rejection requires the 'breaking' of immune tolerance to the molecule [23]. One means of accomplishing this is to modify breast cancer cells to co-express CD80 and/or CD86 molecules. Current evidence suggests that B7 molecules that bind CD28 provide an antigen-nonspecific signal, which, along with an antigen-specific signal, is crucial for T-cell activation [18, 24, 25]. Recognition of a foreign epitope in conjunction with MHCI/II by the antigen-specific T-cell receptor without additional co-stimulation provided by B7/CD28 association results in T-cell anergy [18, 26, 27]. Thus engineering of autologous tumor cells to express B7 should enhance cellular immunity to tumor-associated antigens expressed by the malignant cells. In fact, it was directly demonstrated that the expression of B7 resulted in an enhancement of cellular immune responses to different tumor antigens [28, 29], including MUC1 [30]. Indeed, one of us reported that tumorigenicity of 410.4 mammary tumor cells (derived from a spontaneous carcinoma in a BALB/cfC3H mouse) co-expressing mouse CD80 molecules and human MUC1 was impaired in MUC1 transgenic (MUC1/Tg) mice naturally tolerant to this antigen [30]. The goal of the present study was to enhance anti-MUC1 tumor immunity by vaccination of BALB/c mice with either killed mammary tumor cells (410.4) co-expressing MUC1 and CD80 (or CD86) molecules or with DNA encoding MUC1 and CD80 (or CD86) molecules. Importantly, for the challenge experiments we used the 4T1 subline of 410.4 mammary tumor cells, because the pathogenic properties of this mouse tumor model resembles human breast cancer [31–33].

Materials and methods

Mice

Female, 6–8-week-old BALB/c mice were from the Jackson Laboratory (Bar Harbor, Maine, USA). The animals were housed in the pathogen-free facility of the Institute for Brain Aging and Dementia at the University of California, Irvine. This facility is accredited for animal care by the American Association of Laboratory Animal Care, and the experiments were performed in accordance with the approved institutional protocol and the guidelines of the Institutional Animal Care and Use Committee.

Tumor cell lines

Two mammary tumor cell lines provided by Dr F. Miller (Karmanos Cancer Institute, Detroit, Michigan, USA) [31–34] were used. 410.4 is a mammary tumor cell line originally isolated from a single spontaneously arising mammary tumor in a BALB/c fC3H mouse. 4T1 cells are a thioguanine-resistant variant derived from 410.4 cells without mutagen treatment. The cells were cultured (37 °C, 10% CO_2) in Dulbecco-modified Eagle's essential medium (DMEM) containing low glucose and supplemented with 5% fetal bovine serum, 5% newborn calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (D10) (Life Technologies, Inc.).

Plasmid DNA preparation

Previously, it was found that both mouse and human B7 molecules similarly enhanced cellular immune responses to various antigens in mice. Thus, plasmids encoding human CD80 (pCD80) and CD86 (pCD86) were prepared [39] and used in this study. An expression plasmid designated pcMUC1 was prepared by cloning the entire human MUC1 cDNA sequence containing 32 VNTR (from Dr S. Gendler, Mayo Clinic, Scottsdale, Arizona, USA) into a pSRαpuro1+ vector [40] by digestion with *Spe*I and *Xho*I restriction enzymes following ligation into the same sites on a vector.

Development of breast cancer cell lines expressing MUC1, MUC1 plus CD80 or MUC1 plus CD86

Both 410.4 and 4T1 cells were transfected with pcMUC1 plasmid or a control vector using Lipofectamine Plus Reagent (Life Technologies, Inc.), according to the manufacturer's instructions. After 48 h incubation, the cells were transferred to D10 medium supplemented with 25 μg/ml puromycin (Sigma, St. Louis, Missouri, USA) and maintained in culture for 3 weeks. To isolate high MUC1 producer cells, both cell lines (410.4 and 4T1) transfected with MUC1 were stained with HMFG2 anti-MUC1 monoclonal antibody (gift from Dr J. Taylor-Papadimitriou, Imperial Cancer Fund, London, UK) [41] followed by incubation with anti-mouse IgG conjugated with FITC (BD PharMingen, San Diego, California, USA). After incubation cells were sorted using a MoFlo Cell Sorter (Cytomation, Colorado, USA), and mammary tumor cell lines with the highest mean expression of MUC1 (410.4/MUC1 and 4T1/MUC1) were collected for further propagation in D10 medium supplemented with 25 μg/ml of puromycin.

To modify 410.4/MUC1 cells to express CD80 or CD86 co-stimulatory molecules we essentially followed the same procedure as above, except that cells were not sorted. Briefly, 410.4/MUC1 cells were transfected with pCD80 or pCD86 and propagated for 3 weeks in D10 medium. To select double transfectants D10 medium was supplemented with 600 μg/ml of Geneticin (Life Technologies, Inc.) and 25 μg/ml of puromycin. The resulting 410.4/MUC1/CD80 and 410.4/MUC1/CD86 cell lines were analyzed by FACS. Cell lines were stained with FITC-labeled anti-CD80 or anti-CD86 antibodies according to manufacturer's recommendations (BD PharMingen). In addition, we detected the expression of MHC class I and II molecules by the 410.4, 4T1, 410.4/MUC1, 410.4/MUC1/CD80, 410.4/MUC1/CD86, and 4T1/MUC1 cell lines. To detect MHC class I molecules these cell lines were stained with anti-H-2K^d monoclonal antibodies, followed by incubation with goat anti-mouse IgG-FITC-labeled polyclonal antibodies (BD PharMingen). For detection of MHC class II molecules monoclonal anti-I-A/I-E antibody directly conjugated with FITC (BD PharMingen) was used. Flow cytometry analyses were performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, California, USA). The data were analyzed with CellQuest software (Becton Dickinson).

Animals, immunizations and tumor challenge

Three groups of BALB/c mice were used. The first group of animals was immunized subcutaneously (s.c.) at the base of the tail with the cell-based vaccines, the second group was injected intramuscularly (i.m.) with DNA vaccines, and the third group was injected into the skin with plasmids by use of a gene gun. Animals from the first group were injected with 50 μl of a suspension of 2×10^6 irradiated (3500 rads from a ^{60}Co source) 410.4/MUC1 ($n = 5$), 410.4/MUC1/CD80 ($n = 6$), or 410.4/MUC1/CD86 ($n = 5$) cells three times with a two week interval in between. Mice from the second group were injected into the hind leg with 50 μg of pcMUC1 ($n = 6$) or with a mixture of pcMUC1 (50 μg) with pCD80 (50 μg) ($n = 6$) or pcMUC1 (50 μg) plus pCD86 (50 μg) ($n = 6$) three times with a two-week interval in between. As a control for both the first and the second groups, BALB/c mice ($n = 5$) were injected three times biweekly with 100 μl of PBS (s.c.) and 50 μg of vector (i.m.). The third group of animals was injected with plasmids by use of a gene-gun. Gene gun immunizations were performed on the shaved abdomens of mice using the Helios Gene Gun (Bio-Rad, Hercules, CA) as described [42]. The mice were injected with 2 μg of pcMUC1 ($n = 5$), 2 μg of pcMUC1 mixed with 2 μg pCD80 ($n = 5$) or 2 μg pcMUC1 mixed with 2 μg pCD86 ($n = 5$) three times with a two week interval in between. As a control, BALB/c mice ($n = 5$) were injected the same way with 4 μg of vector.

Three weeks after the last immunization, mice from the first and the second groups were challenged with live 1×10^6 4T1/MUC1 cells (50 μl in PBS) injected into the mammary fat pad. The third group of mice was challenged by injection into the mammary fat pad with 2×10^5 4T1/MUC1 cells. Tumor volumes were determined by two-dimensional measurement and calculation three-times weekly using the formula $(a \times b^2)/2$, where a represents the largest diameter and b the smallest diameter of the tumor. The time of appearance (latency period) was designated as the presence of a tumor with a volume in excess of 0.1 cm^3. In addition, we determined the time of appearance of a tumor volume of 0.5 cm^3. The experiments were terminated when the mouse appeared moribund or the tumors reached approximately 2 cm^3, the maximum size recommended by the Institutional Animal Care and Use Committee, University of California, Irvine guidelines for tumor research using a mouse model. To determine tumor growth rate, we analyzed scatter plots in the near linear periods of tumor growth.

Statistical analysis

Results on the average times of appearance of tumor nodules (latency period) or the times when tumors reached approximately 0.5 cm^3 as well as survival times were examined using an analysis of variance (ANOVA) and post-hoc comparisons. When the ANOVA indicated statistically significant differences between groups, the data were further analyzed with a post-hoc Fischer Probable Least Significant Differences (PLSD) test to determine specific differences between groups. All three groups of experimental mice with appropriate control animals were analyzed separately. Kaplan–Meier curves were generated for period of survival. P-values less than 0.05 were considered significant.

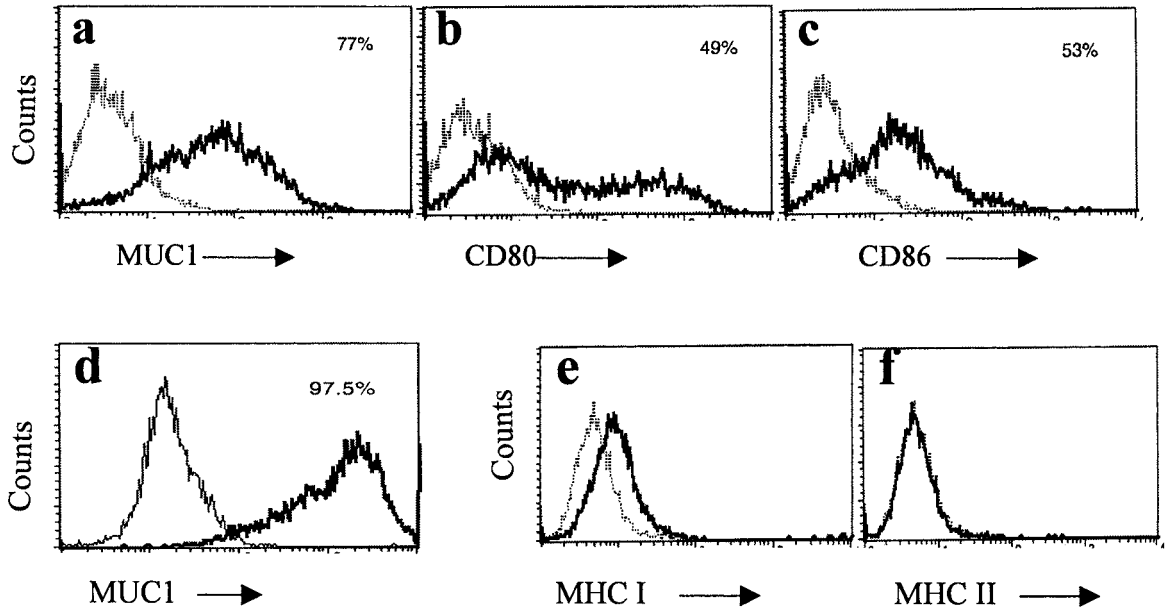

Figure 1. a–c. Expression MUC1, MUC1 and CD80, or MUC1 and CD86 molecules by modified 410.4 mouse mammary tumor cell lines (see Materials and methods for details). a) *Bold line* – MUC1 expression was detected after incubation of 410.4 cells transfected with pcMUC1 with anti-MUC1 monoclonal antibodies (HMFG-2) followed by incubation with anti-mouse-Ig-FITC antibodies. *Light line* – the same cells were incubated with secondary antibodies only. b) *Bold line* – 410.4/MUC1 cells were transfected with pCD80, and the expression of CD80 was confirmed by staining with FITC-labeled anti-CD80 antibodies. *Light lines* – the same cells stained with isotype control FITC-labeled antibodies. c) *Bold line* – 410.4/MUC1 cell line was transfected with pCD86, and the expression of CD86 was confirmed by staining with FITC-labeled anti-CD86 antibodies. *Light lines* – the same cells stained with isotype control FITC-labeled antibodies. d) Expression of MUC1 molecules by modified 4T1 mouse mammary tumor cell line (see Materials and methods for details). *Bold line* – MUC1 expression was detected as described in Figure 1a; *Light line* – the same cells stained with secondary antibodies. e–f) 410.4 mouse mammary tumor cells expressing a low amount of MHC class I molecules (e) and do not express MHC class II molecules (f). Expression of MHC class I was analyzed with anti-H-2K^d antibodies followed by staining with goat anti-mouse IgG conjugated with FITC (*bold line*). For detection of MHC class II, anti-I-A/IE antibodies directly conjugated with FITC were used (*bold line*). Control cells were stained with isotype matched control Ig (*light lines*).

Results

Generation of mouse mammary tumor cell lines expressing MUC1, MUC1 and CD80 or CD86 co-stimulatory molecules

To obtain 410.4 mouse mammary tumor cells with high expression of MUC1 brightly stained 410.4/MUC1 cells were separated from cells staining with lesser intensity. After propagation of the sorted cells in selective medium, we generated a cell line in which 77% of the cells expressed MUC1 (Figure 1a). To generate 410.4 cell lines that co-expressed both MUC1 and CD80 or CD86 co-stimulatory molecules, we transfected 410.4/MUC1 cells with either pCD80 or pCD86, followed by selection in medium containing both geneticin and puromycin. Cytofluorometric analyses reveled that both 410.4/MUC1/CD80 and 410.4/MUC1/CD86 cell lines expressed MUC1 along with equal amounts of CD80 or CD86 molecules (Figure 1a–c). These cell lines were used as cell-based vaccines for immunization of the experimental animals. In addition, these transfection studies demonstrated that plasmids encoding MUC1, CD80, and CD86 were functional *in vitro* and therefore could be used for DNA immunization *in vivo* (Figure 1a–d).

In order to use 4T1 cells for challenge experiments the cell line was first modified to express MUC1. After sorting by FACS and propagation in selective medium, about 98% of the 4T1/MUC1 cells expressed MUC1 (Figure 1d). In addition, the expression of MHC class I and II molecules by 410.4 and 4T1 cells and their transfected sublines was determined. These cells expressed a very low quantity of MHC class I molecules, and did not express MHC class II molecules (Figure 1e–f). Importantly, the same results were obtained with 410.4/MUC1, 410.4/MUC1/CD80, 410.4/MUC1/CD86, 4T1 and 4T1/MUC1 cell lines (data not shown) indicating that co-expression of MUC1 and B7 did not affect the expression of MHC class I or II molecules.

Immunization with 410.4/MUC1/CD80 or 410.4/MUC 1/CD86 cell-based vaccines inhibits tumor growth in mice challenged with 4T1/MUC1 cells

Balb/c mice were immunized s.c. in the base of the tail with 2×10^6 irradiated (35 Gy) 410.4/MUC1, 410.4/MUC1/CD80 or 410.4/MUC1/CD86 cell sublines. To detect inhibition of tumor growth, vaccinated mice were challenged by injection into the mammary fat pads of 1×10^6 4T1 cells that expressed MUC1. Control animals also received the same number of 4T1/MUC1 cells. The time of tumor growth to a volume of 0.5 cm^3 along with the rate of tumor growth were determined. When the times of tumor nodules those exceeding a volume of 0.5 cm^3 were greater and tumor growth rates were less than in controls, these were considered as evidence of immunity to the MUC1-expressing neoplastic cells.

The results indicated that mice injected with 4T1/MUC1 cells developed tumors in both the vaccinated and control groups. No significant differences in latency periods between the groups was detected. However, the mean time

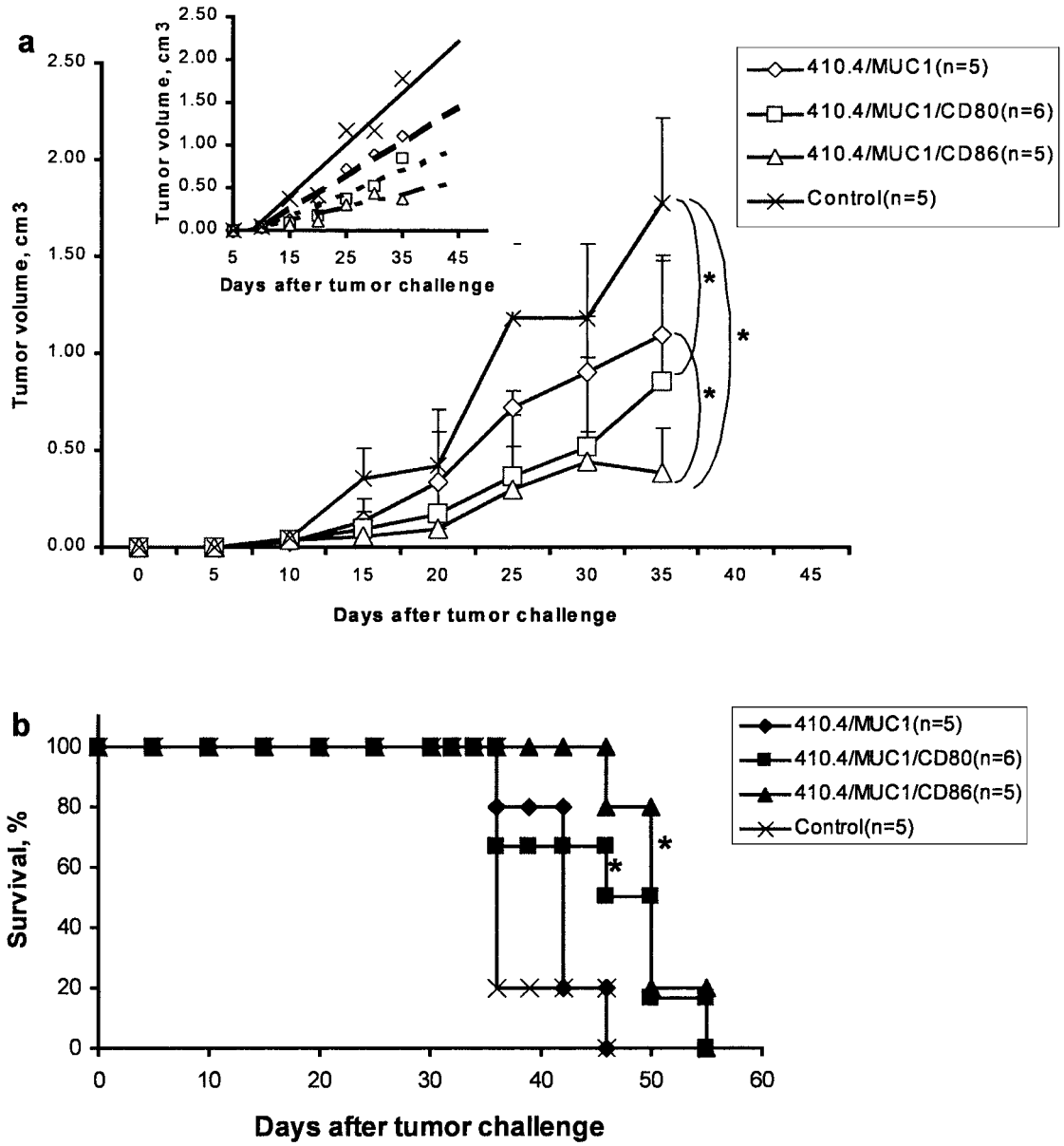

Figure 2. Vaccination with irradiated 410.4 cell lines expressing MUC1 and CD80 or MUC1 and CD86 significantly prolonged the time of tumor growth to the volume of 0.5 cm^3 (a) and lower tumor growth rates (inset) and survival (b) of Balb/c mice challenged with 4T1/MUC1 cells. Immune and control mice were challenged three weeks after the last immunization with 1×10^6 4T1/MUC1 cells (see details in Materials and methods). The data represent mean tumor volume ± SD in each group of mice. The end point of the line represents the death or termination of the mice. Significant differences in a) $^*P < 0.05$; in b) $^*P < 0.05$.

of tumor growth to the volume of 0.5 cm^3 was significantly delayed in the groups vaccinated with 410.4/MUC1/CD80 or 410.4/MUC1/CD86 cells ($F[6, 32] = 2.735$, $P = 0.0293$) (Figure 2a). Post-hoc analysis with the Fischer PLSD indicated that the 410.4/MUC1/CD86 group had a statistically significant later mean onset time to a tumor volume of 0.5 cm^3 and slower growth than all other groups ($P < 0.05$), with the exception of the animals receiving 410.4/MUC1/CD80 cells. The mean time of tumor nodules growth to a volume of 0.5 cm^3 in animals receiving 410.4/MUC1/CD80 cells differed statistically from the control group of mice ($P < 0.05$). Once measurable tumors appeared, the rate of tumor growth in groups vaccinated with 410.4/MUC1/CD80 or 410.4/MUC1/CD86 was compared. It was significantly less in the experimental groups ($P < 0.05$) than in the control group injected with equivalent numbers of 4T1/MUC1 mammary tumor cells (Figure 2a). These results correlated with the period of survival. Mice immunized with 410.4/MUC1/CD80 or 410.4/MUC1/CD86 cells survived significantly longer after tumor challenge than non-vaccinated mice challenged with 4T1/MUC1 cells ($F[6, 32] = 3.344$, $P = 0.0113$) (Figure 2b). The mean survival time of mice immunized with 410.4/MUC1/CD86 cells was significantly longer than that of mice in any of the other groups, with the exception of mice immunized with 410.4/MUC1/CD80 cells ($P < 0.05$). Mice immunized with 410.4/MUC1/CD80 cells also survived significantly longer than control non-vaccinated mice injected with 4T1/MUC1 tumor cells ($P < 0.05$). On day 45, when mice in the control group (non-vaccinated) and mice immunized with

410.4/MUC1 cells died from tumor growth, 50% of mice immunized with 410.4/MUC1/CD80 and 80% of mice immunized with 410.4/MUC1/CD86 were alive. These data indicated that a cell-based vaccine that combined the co-stimulatory molecules (CD80 and CD86) along with the expression of MUC1 was more effective in inhibiting the growth of mammary tumor cells *in vivo* than a cell-based vaccine that included MUC1 alone.

DNA vaccination i.m. does not prevent tumor growth in mice challenged with 4T1/MUC1 cells

Previously we found that immunization i.m. with a mixture of plasmids encoding gp120 of HIV-1 and B7 molecules resulted in co-expression of the molecules in the same cell [35, 36]. Now mice were immunized i.m. with pcMUC1, pcMUC1 plus pCD80 or pcMUC1 plus pCD86. As a control, the same group of animals that was used as a control for the cell-based vaccination was used. Three weeks after the last booster injection, the mice were challenged by inoculation of 1×10^6 4T1/MUC1 cells into their mammary fat pads. Compared to unvaccinated control mice, there were no differences in the mean times of first appearance of measurable tumor nodules and subsequent growth in mice immunized with pcMUC1, pcMUC1/CD80, or pcMUC1/CD86 (Figure 3a, $P > 0.4$). DNA immunization i.m. also did not affect the survival of mice challenged with 1×10^6 4T1/MUC1 tumor cells, compared to the control group (Figure 3B). Contrary to our expectations, the expression of CD80 or CD86 co-stimulatory molecules was ineffective in delaying tumor appearance and preventing tumor growth when the appropriate plasmids were delivered via i.m. immunization along with DNA encoding MUC1 immunogen.

Immunization by gene-gun of pcMUC1 mixed with pCD80 or pcCD86 vaccines suppresses tumor growth in mice challenged with 4T1/MUC1 cells

We attempted to stimulate anti-mammary tumor immunity by delivering plasmids encoding MUC1 and CD80 or MUC1 and CD86 with a gene gun. Control animals were injected with vector only. Three weeks after the last injection, the mice were challenged with live 2×10^5 4T1/MUC1 cells implanted into the mammary fat pad. Fewer 4T1/MUC1 cells were used for the challenge in order to prolong lives of tumor-bearing mice and create a more sensitive mouse tumor model. The latency period (0.1 cm^3) in mice treated by immunization with pcMUC1, pcMUC1 plus pCD80, or pcMUC1 plus pCD86 administered with the gene gun was significantly delayed ($F[3, 16] = 14.557$, $P < 0.0001$). The longest delay was in the group of mice injected with pcMUC1/CD80. Mice immunized with pcMUC1 and pcMUC1/CD86 also showed a delay in the development of measurable tumors relative to animals that received the vector control (Figure 4a). Post-hoc analysis with the Fischer PLSD indicated that the pcMUC1/CD80 group had a later onset of tumor appearance than the pcMUC1/CD86 and vector groups ($P < 0.05$), but this did not differ from the pcMUC1 group. Both the pcMUC1 and pcMUC1/CD86 groups showed delayed tumor growth relative to the vector control ($P < 0.05$).

The long-term survival of mice that received plasmid DNA administered by the gene gun treatment was significantly prolonged relative to that of the control group ($F[3, 16] = 23.069$, $P < 0.0001$) (Figure 4b). Mice injected with pcMUC1/CD80 had the longest delay in mortality or in growth of tumors to a volume of 2.0 cm^3 ($P < 0.001$). Mice injected with pcMUC1/CD86 survived significantly longer than mice injected with vector only ($P = 0.017$). In addition, mice injected with pcMUC1 also survived for significantly longer periods than mice injected with vector only ($P < 0.05$). Thus, gene gun immunization with plasmids encoding MUC1, MUC1/CD86 or MUC1/CD80 prolonged the survival of mice injected with mammary tumor cells (Figure 4b). On day 60, when mice injected with vector died of excessive tumor burden, 40% of mice vaccinated with pcMUC1/CD86 or pcMUC1 and 80% of mice vaccinated with pcMUC1 plus pCD80 were still alive. All mice from the latter group died 80 days after the challenge with 4T1/MUC1 tumor cells.

Discussion

Tumors avoid destruction by the immune system in various ways, including failure to express MHC class I molecules and co-stimulatory molecules, such as CD80/CD86, which are necessary to elicit a primary T-cell response. Failure to express these co-stimulatory molecules can lead to immune tolerance. Previously, we observed that expression of human CD86 provides important activation signals to murine T-cells following DNA vaccination with envelope proteins from HIV-1 [35, 36]. Others obtained similar results after immunization of mice with DNA encoding HIV-1 or influenza antigens and plasmids encoding mouse CD80 or CD86 [37, 38]. Thus, expression of either mouse or human B7 molecules was able to enhance cellular immune responses to viral antigens in mice. Previously, we analyzed the effect of CD80 molecules on the tumorogenecity of MUC1-expressing mammary tumor cells. We studied tumor growth in non-vaccinated MUC1/Tg mice injected with the live 410.4/MUC1/CD80 cells [30]. Here, we utilized CD80 or CD86 molecules as a component of the MUC1 vaccine, in order to enhance antigen specific anti-mammary tumor immunity. Two different vaccination strategies were used. In the first, the mice were immunized with irradiated mammary tumor cells modified to express MUC1, a known breast cancer antigen, and MUC1 together with CD80 or CD86. In the second, the mice were immunized directly with plasmids encoding MUC1 along with plasmids encoding CD80 or CD86 co-stimulatory molecules.

Mice vaccinated with irradiated 410.4 cells that expressed MUC1 and either CD80 or CD86 were challenged with 1×10^6 4T1 mammary tumor cells, modified to express MUC1. Previously, it was found that injection of as few as 1×10^5 4T1 cells into the mammary glands of $H2^d$ mice resulted in local growth of mammary tumors (100%

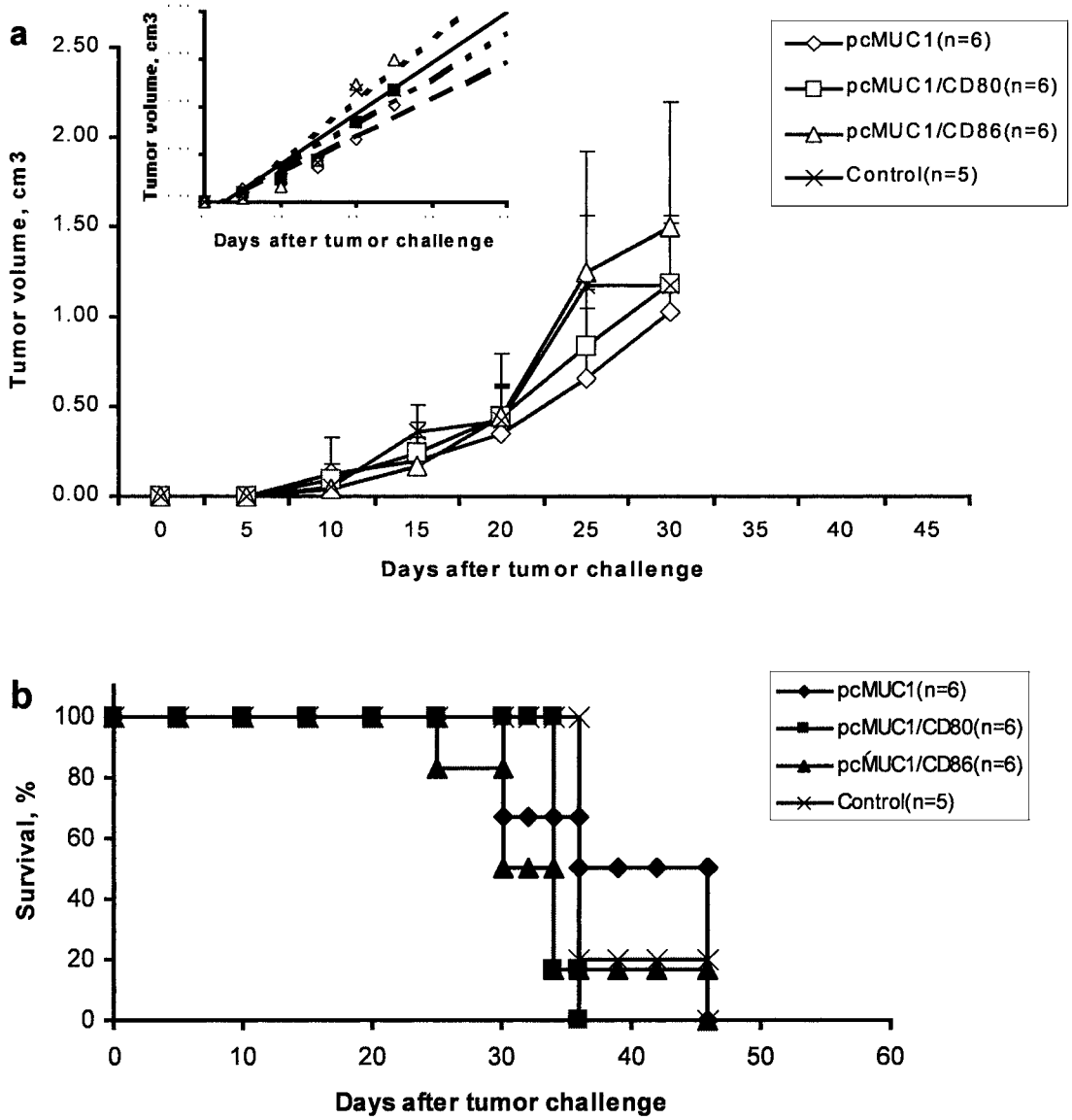

Figure 3. DNA vaccination i.m. with pcMUC1, pcMUC1 plus pCD80 or pcMUC1 plus pCD86 was ineffective in delaying appearance of tumors (a), suppression of tumor growth rate (inset), and prolongation of survival time (b) of Balb/c mice challenged with 4T1/MUC1 cells. Tumor measurements were performed as described in Figure 2. Data represent mean tumor volume ± SD in each group of mice. The end point of the line represents the death or termination of the mice.

of challenged mice had tumors, and the average time of onset of measurable tumor nodules was 36.6 ± 0.9 days) and metastasis to liver, lung, and bone [32, 33]. In the present study, we demonstrated that even under stringent conditions, when mice were challenged with ten-times higher numbers of 4T1/MUC1 cells, immunziation with the irradiated 410.4/MUC1 cell-based vaccine resulted in a slight delay in the onset of measurable tumors.

However, the expression of CD80 or CD86 molecules together with MUC1 on the surface of 410.4 cells had a highly significant effect on 4T1/MUC1 tumor growth and survival. Therefore, even under conditions when none of the mice survived the challenge with 1×10^6 highly tumorogenic 4T1/MUC1 cells, there was a significant effect from vaccination with irradiated 410.4 cells co-expressing MUC1 and either CD80 or CD86 co-stimulatory molecules.

Previously, immunization i.m. with a plasmid encoding MUC1 was found to have a significant effect on tumor growth and the survival of C57BL/6 mice challenged with RMA (Rauscher virus-induced T-cell lymphoma) [43], EL4 (T-cell lymphoma induced by 9,10-dimethyl-1,2-benzanthracene) [44] and MC38 (murine colon adenocarcinoma cells induced by methylcholntren) [45] tumor cells. In these studies mice were challenged with tumor cells induced by virus or chemicals. More recently, immunization i.m. with pcMUC1 was found to suppress the development of lung metastases in C57Bl/6 mice challenged with B16-F10 (spontaneous murine melanoma) cells expressing MUC1 [46]. In this study, we chose for the first time to test a DNA vaccination strategy in an experimental mouse model that closely mimics breast cancer in patients [31–33]. Animals vaccinated with pcMUC1, pcMUC1 mixed with pCD80 or pCD86 were challenged with 1×10^6 4T1/MUC1

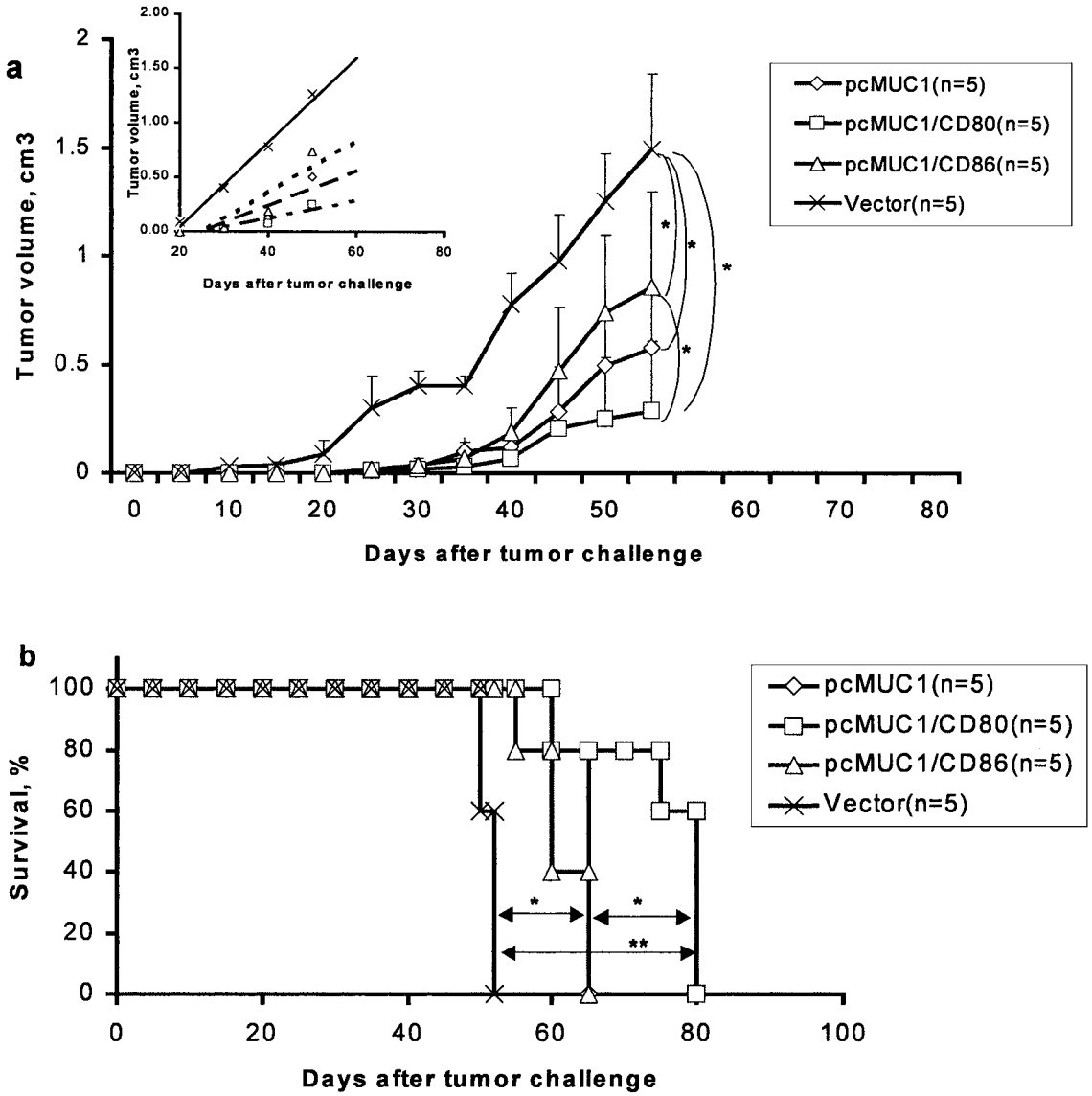

Figure 4. Gene-gun immunization with pcMUC1, pcMUC1 plus pCD80 or pcMUC1 plus pCD86 resulted in significant delay in the appearance of measurable tumors (a) and subsequent tumor growth rate (inset), as well as prolongation of the survival (b) of mice. The groups of vaccinated and control mice were challenged three weeks after the last immunization with 2×10^5 4T1/MUC1 cells (see details in Materials and methods). Tumor measurements and calculation of tumor volumes were performed as described in Figure 2. Data represent mean volume of tumor $\pm$ SD in each group of mice. Significant differences in a) $^{*}P < 0.05$; in b) $^{*}P =\leq 0.05$; $^{**}P < 0.001$.

cells. Under these stringent conditions, DNA immunization i.m. with plasmids encoding MUC1, MUC1/CD80, or MUC1/CD86 did not induce protection against the challenge with 4T1/MUC1 cells. However, when mice were immunized with only 2 μg of MUC1-expressing plasmid delivered via gene-gun, there was significant protection against the challenge with 2×10^5 4T1/MUC1 cells. The average times for the appearance of measurable tumors were significantly delayed and tumor growth rates were significantly less in mice vaccinated with pcMUC1, pcMUC1/CD80, or pcMUC1/CD86. Gene gun vaccination with pcMUC1, pcMUC1/CD86, and more profoundly with pcMUC1/CD80, also significantly prolonged survival of the animals. Thus, plasmids encoding CD80 co-stimulatory molecules increased the immunogenic properties of pcMUC1 delivered by gene gun, a technique that has been widely used in cancer vaccination studies [47–52].

In conclusion, we found that co-stimulatory molecules used as a vaccine component along with MUC1 antigen can delay the onset of mammary tumor appearance, inhibit the tumor growth rate, and prolong the survival of mice with experimental mouse breast cancer. In addition, both DNA immunization via gene gun delivery and cell-based vaccination strategies were feasible. The analyses of both humoral and cellular immune responses along with studying the prevention and eradication tumor growth and metastasis in F1 (H-$2^{k\times d}$) MUC1/Tg mice, tolerant to MUC1, will be subject of our next study. This will allow us to extend our studies to a more appropriate animal model, in which MUC1 will be a self-antigen.

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