Dendritic cells enhance the activity of human MUC1-stimulated mononuclear cells against breast cancer

Zhenyao Wang,[1,2] Monte D. Hall,[1,2] Kathleen A. Rewers-Felkins,[1,3] Imelda S. Quinlin[1,3] and Stephen E. Wright[1,2,3,*]

[1]Departments of Internal Medicine and Biomedical Sciences; Texas Tech University Health Sciences Center Schools of Medicine and Pharmacy; Amarillo, TX USA; [2]Department of Life, Earth and Environmental Sciences; West Texas A & M University; Canyon, TX USA; [3]Harrington Cancer Center; Amarillo, TX USA

Dendritic cells (DCs) are among the most potent antigen-presenting cells (APCs), stimulating peripheral blood mononuclear cells (PBMCs) to generate antigen-specific cytotoxic T lymphocytes (CTLs). The objectives of this study were to determine if interleukin (IL)-4 is beneficial or detrimental for the generation of human DCs in vitro and to understand whether DCs generated in vitro in the presence or absence of IL-4 stimulate the killing of adenocarcinoma cells by CTLs in vivo. Mucin 1 (MUC1), a glycoprotein found on the surface of adenocarcinoma cells was used to load DCs. MUC1-loaded DCs generated in the absence of IL-4 were superior to their counterparts produced with IL-4 in stimulating PBMCs to kill human breast cancer MCF-7 cells in vitro. A corollary in vivo protection experiment was performed by injecting immunodeficient NOD-SCID mice with MCF-7 cells s.c. and MUC1-loaded CTLs, PBMCs, or DCs generated in the absence of IL-4, i.p. Mice that received CTLs and MUC1-loaded DCs on days 0, 2, 4, 9, 14 and 19 were completely protected against the development of MCF-7-derived tumors, while other schedules conferred lower protection. Therefore, tumor antigen-loaded DCs enhance the efficacy of adoptive CTL transfer, and should thus be considered for combinatorial immunotherapeutic regimens.

Introduction

The current treatments for breast cancer such as chemotherapy and radiotherapy cause many unwanted side effects, including the emergence of secondary neoplasms and the senescence of normal cells, mostly because these interventions are not specific for cancer cells.[1] The development of highly specific anticancer therapies has indeed been one of the major goals of cancer research.[2] Immunotherapy constitutes one of such approaches. In this setting, the immune system can either be stimulated actively, by vaccination, or adoptively, by the transfer of one of more of its components, including antibodies and various cell types. The cellular components of the immune system, which can be stimulated in culture, are generated from peripheral blood mononuclear cells (PBMCs). Among these, cytotoxic T lymphocytes (CTLs) are able to recognize antigens that are unique to cancer cells and hence exert specific cytotoxic functions.[3]

Many tumor cells express indeed tumor-associated antigens (TAAs), which can be recognized by specific CTLs. Multiple antigenic peptides derived from TAAs can be presented to CTLs by antigen-presenting cells (APCs), including dendritic cells (DCs), which constitute the active drivers of the immune response. Mucin 1 (MUC1) is one such TAAs that has been identified as a breast cancer cell-specific epitope.[4–6] Mucins are polymorphic, O-linked glycosylated proteins expressed on the surface of ductal epithelial cells. MUC1 is characterized by 25–100 tandem repeats of a 20 amino acid sequence.[5,6] The glycosyltransferases that glycosylate mucin are often defective in adenocarcinomas, which are neoplasms that affect secretory organs. As a result, adenocarcinoma cells not only are characterized by altered protein trafficking but also express a variant of MUC1 that is not normally glycosylated, rendering it recognizable as a TAA by CTLs.[7]

In line with this notion, human CTLs[3,8–10] and B cells[11] that are specific for cancer-associated MUC1 have previously been
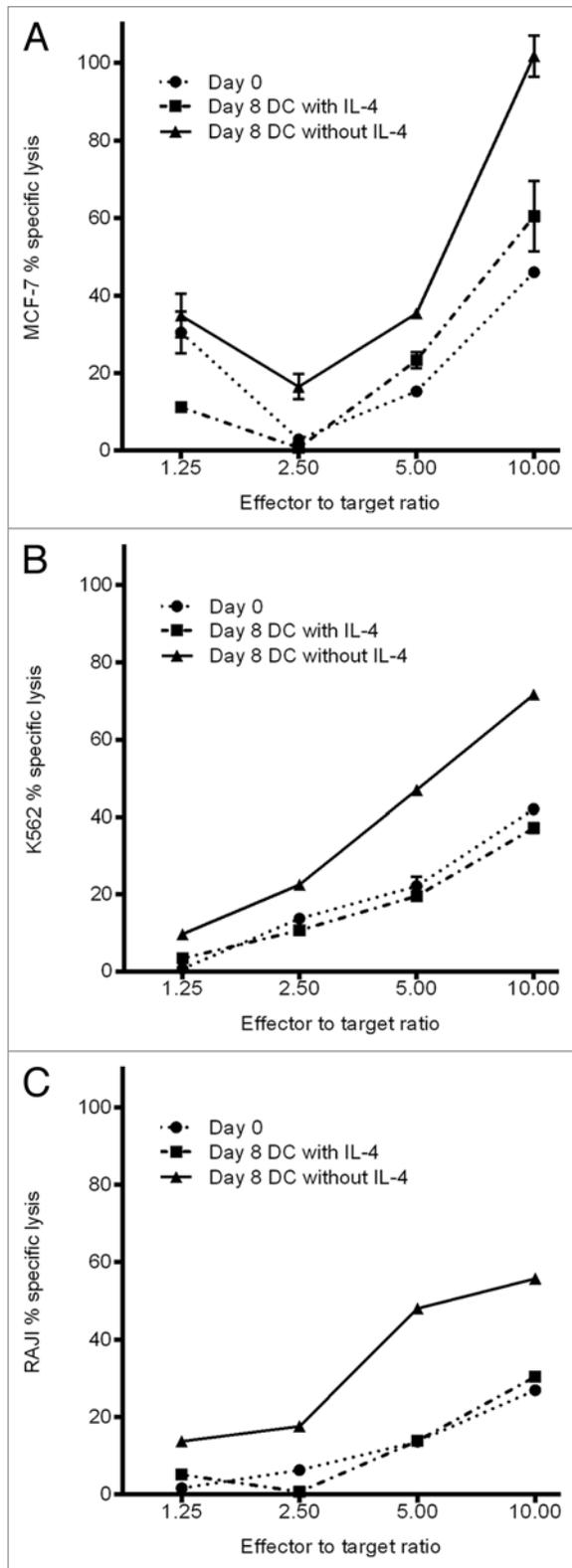
Figure 1. Influence of interleukin-4 (IL-4) on the generation of dendritic cells for the production of cytotoxic T lymphocytes. (**A–C**) Dendritic cells (DCs) were generated and matured with or without interleukin-4 (IL-4). The lytic potential of cytotoxic T lymphocytes (CTLs) stimulated by these DCs was tested on day 8 by XTT assays (MCF-7 cells, **A**) or alamarBlue® assays (K562 and RAJI cells, **B and C**). Bars indicate SEM.

described. Previous studies have also shown that anti-MUC1 CTL-driven cytotoxicity is MHC unrestricted.[3,8–10] It has been hypothesized that, because of its highly repetitive, multivalent structure,[3,8–10] MUC1 can bind, crosslink and thus signal via the T-cell receptor (TCR) in the absence of MHC presentation. Therefore, tumor-associated mucins may be effective targets for CTL-based immunotherapy.

DCs are among the most potent APCs of the immune system. They efficiently stimulate antigen-specific T-cell responses[12] and, as such, can be considered ideal candidates for cancer immunotherapy in combination with CTLs. T cells have previously been primed in vivo (in SCID mice) with DCs,[13] viruses,[14] antigens in liposomes[15] or DNA.[16] The addition of peptides during DC maturation results in their efficient loading onto MHC Class I complexes.[17] DC loaded with TAAs, e.g., MUC1, can elicit specific tumor-reactive T cells, e.g., breast cancer-specific CTLs. In addition, a prolonged antigen stimulation in vivo enhances the cytotoxic activity of CTLs.[18] This may be explained by the observation that DC re-stimulation maintains protective memory CTLs in a murine model of viral infection.[19]

We have previously used MUC1 to stimulate PBMCs, resulting in the specific killing of human breast cancer cells in vitro[3] and in vivo.[20] The present study was performed to determine the effects of human DCs on the CTL-mediated lysis of human breast cancer cells. We first wished to compare DCs generated in vitro in the presence or in the absence of interleukine (IL)-4, which is incorporated into the differentiation protocol to reduce the development of macrophages.[21,22] The rationale for excluding IL-4 is that this cytokine favors the development of T_H2, at the expense of T_H1, responses.[23–25] This is due to the fact that CCL3/MIP1α and CCL4/MIP1β are not induced in human primary monocytes when granulocyte-macrophage colony-stimulating factor (GM-CSF) is combined with IL-4,[26] and these chemokines are required for DCs to polarize T_H1 responses.[27] In addition, DCs generated with GM-CSF alone have been shown to efficiently stimulate CTLs.[28] DCs generated in the absence of IL-4 were then used to prime CTLs for use in vivo. The ability of MUC1 loaded-DCs to enhance the cytotoxic activity of CTLs against human tumor cells was evaluated in immunodeficient NOD-SCID mice.

Results

Generation of DCs from PBMCs in the presence or in the absence of IL-4. In order to determine the optimal method for generating DCs that would be able to stimulate T lymphocytes, PBMCs were allowed to differentiate in vitro in the presence or in the absence of IL-4 and then compared for their ability to prime tumor-specific cytotoxic responses. PBMCs stimulated with MUC1-loaded DCs generated in the absence of IL-4 exerted a MCF-7-specific lytic activity of 100% at 10:1 effector:target (E:T) ratio. Conversely, when PBMCs were stimulated with MUC1-loaded DCs generated in the presence of IL-4, they were able to exert a tumor cell-specific lytic activity of only 60% (**Fig. 1A**). On the same day and at the same E:T ratio, MUC1-loaded DCs generated in the absence of IL-4

non-specifically killed 72% erythroleukemia K562 cells and 56% B-cell lymphoma RAJI cells. Conversely, PBMC stimulated with MUC1-loaded DCs generated in the presence of IL-4 exerted a non-specific lytic activity of 37% and 30% against K562 and RAJI cells, respectively (**Fig. 1B and C**). These data indicate that PBMCs stimulated with MUC1-loaded DCs generated in the absence of IL-4 have a higher specificity than PBMCs stimulated with MUC1-loaded DCs generated in the presence of IL-4. Therefore, MUC1-loaded DCs differentiated in the absence of IL-4 were used to generate CTLs for in vivo studies.

Generation of CTLs for in vivo studies. CTLs generated from PBMCs stimulated with MUC1-loaded DCs generated in the absence of IL-4 used for in vivo experiments exhibited a high specific lysis of MCF-7 cells on day 8 (47%) vs. day 0 (28%, $p < 0.05$), while they mediated minimal cytotoxicity against both K562 (8.5%) and RAJI (4.7%) cells on day 8 (**Fig. 2**). These data suggest that the cytotoxic activity mediated by CTLs stimulated with MUC1-loaded DCs produced in the absence of IL-4 is specific and not due to natural killer (NK) or lymphokine-activated killer (LAK) cells.

PBMCs stimulated with MUC1-loaded DCs differentiated in the absence of IL-4 and used for in vivo experiments produced increased amounts of T_H1 cytokines. Tumor necrosis factor α (TNFα) rose from 0 pg/mL on day 0 to 19.7 pg/mL on day 3, and 146.9 pg/mL on day 8. Interferon γ (IFNγ) rose from 8.4 pg/mL on day 0 to 117 pg/mL on day 3 and 406 pg/mL on day 8. GM-CSF rose from 0 pg/mL on day 0 to 107.8 pg/mL on day 3 and 159.4 pg/mL on day 8. There was no production from day 0 through day 8 of IL-10, which may be either a T_H1 or a T_H2 cytokine and is produced by inducible regulatory T lymphocytes (**Fig. 3**).[29]

In vivo experiments. Animal experiments were performed in order to determine if DCs administered in vivo can enhance tumor cell killing (**Fig. 4**). MUC1-loaded PBMCs or DCs were used since CTLs alone may not survive in vivo, or may become unresponsive after five days in the absence of stimulation.[18] MCF-7 cells were injected in mice on day 0. A first group of mice mice (Group 1) received MCF-7 cells alone. Alternatively, unloaded DCs (Group 2) or MUC1-loaded DCs produced in the absence of IL-4 (Group 3) were injected i.p. on days 0, 2, 4, 9, 14 and 19 to ascertain if either of these cell types would enhance the antitumor activity of PBMCs in vivo. A fourth group of mice received only CTLs on day 0, as a control for the remaining experimental groups receiving in vivo stimulation. Finally, Groups 5–8 received CTLs on day 0 plus APCs on two different schedules. MUC1-loaded PBMCs were injected i.p. on days 0 and 14 (Group 5) or on days 0, 2, 4, 9, 14 and 19 (Group 6) as a control for the administration of DCs. In addition, MUC1-loaded DCs produced in the absence of IL-4 were injected i.p. on days 0 and 14 (Group 7) or on days 0, 2, 4, 9, 14 and 19 (group 8) to determine the optimal schedule for DC administration.

Tumors were allowed to develop for 31 d after the inoculation of MCF-7 cells on day 0 (**Fig. 4**). Ninety-one % (21/23) of mice belonging to Group 1, which received MCF-7 cells alone, developed tumors. A statistically significant difference was evident in
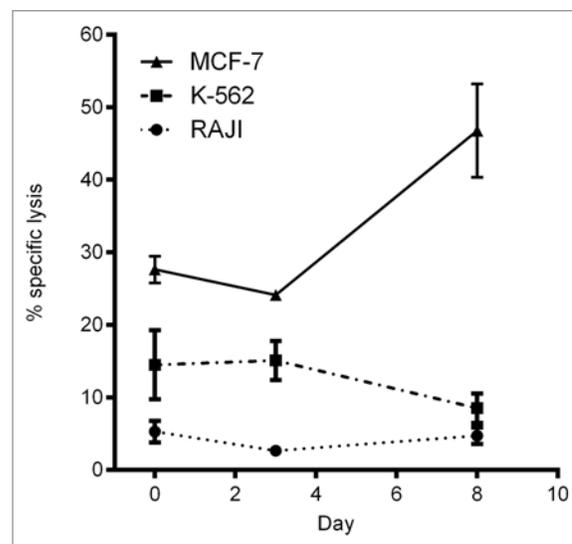

Figure 2. Lysis of specific and non-specific cell targets by cytotoxic T lymphocytes used for in vivo experiments. Mucin 1 (MUC1)-loaded dendritic cells generated in the absence of interleukin-4 (IL-4) were added to peripheral blood mononuclear cells (PBMCs) at 1:100 ratio on days 0 and 7. The lytic potential of the cells was evaluated by XTT assays (MCF-7 cells) and alamarBlue® assays (K562 and RAJI cells) at an effector:target (E:T) ratio of 10:1. Bars indicate SEM, and results are from two independent experiments.
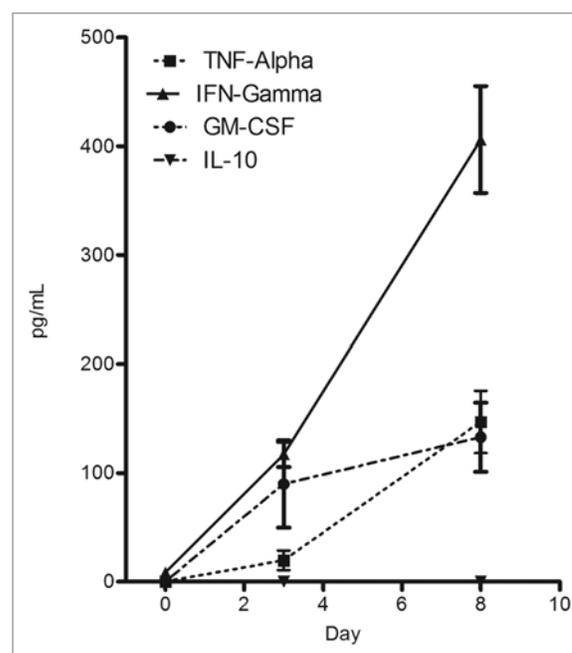

Figure 3. Cytokine production by cytotoxic T lymphocytes used in protection experiments. The quantification of cytokines (pg/mL) produced by peripheral blood mononuclear cells (PBMCs) stimulated with mucin 1 (MUC1)-loaded dendritic cells generated in the absence of interleukin-4 (IL-4) was performed with commercial ELISA kits. Bars indicate SEM, and results are from two independent experiments.
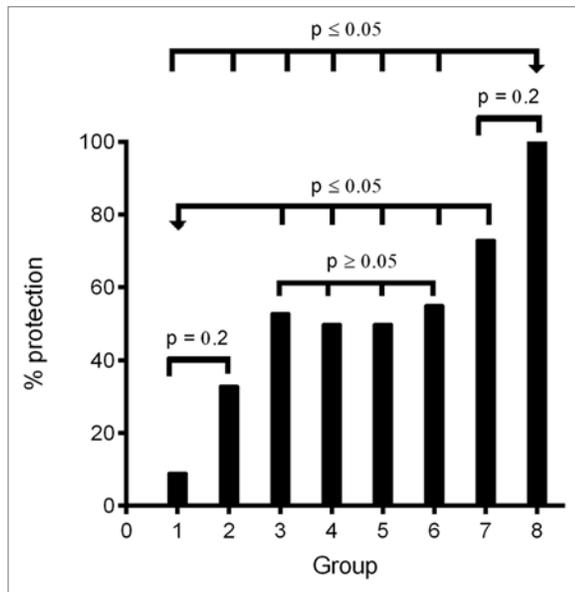
Figure 4. Enhancement of cytotoxic T lymphocyte activity against MCF-7 tumor cells in vivo. On day 0, NOD-SCID mice were injected with 5×10^6 MCF-7 cells s.c., then assigned to either of the following groups: Group 1, no other treatment; Group 2, 5×10^7 peripheral blood mononuclear cells (PBMCs) + 5×10^5 unloaded dendritic cells (DCs) on days 0, 2, 4, 9, 14, 19; Group 3, 5×10^7 PBMCs + 5×10^5 mucin 1 (MUC1)-loaded PBMCs on days 0, 2, 4, 9, 14, 19; Group 4, 5×10^7 cytotoxic T lymphocytes (CTLs); Group 5, 5×10^7 CTLs + 5×10^5 MUC1-loaded PBMCs, on days 0, 14; Group 6, 5×10^7 CTLs + 5×10^5 MUC1-loaded PBMCs, on days 0, 2, 4, 9, 14,19; Group 7, 5×10^7 CTLs + 5×10^5 MUC1-loaded DCs generated in the absence of interleukin-4 (IL-4), on days 0, 14; Group 8, 5×10^7 CTLs + 5×10^5 MUC1-loaded DCs generated in the absence of IL-4, on days 0, 2, 4, 9, 14, 19. p values are reported and results are from two independent experiments with cells from two subjects.

the number of mice developing tumors in Group 1 vs. all other groups ($p < 0.05$), with the single exception of Group 2, which received PBMCs on day 1 and unloaded DCs on days 0, 2, 4, 9, 14 and 19, exhibiting only 33% protection (2 tumor-free mice out of 6, **Fig. 4**). This indicates that all interventions excluding the administration of unloaded DCs were effective. Mice from Group 3, which received PBMCs on day 0 and MUC1-loaded DCs produced in the absence of IL-4 on days 0, 2, 4, 9, 14 and 19, exhibited 53% protection (8 tumor-free mice out of 15), suggesting that PBMCs may be stimulated in vivo to kill tumor cells by MUC1-loaded DCs developed in the absence of IL-4. Mice from Group 4, which received CTLs only on day 0, were protected to 50% (5 tumor-free mice out of 10). This suggests that CTLs generated in vitro exhibit similar cytotoxic activity as PBMCs stimulated in vivo (Group 4). Mice from group 5, which received CTLs on day 0 and MUC1-loaded PBMCs on days 0 and 14, were protected to 50% (5 tumor-free mice out of 10). Thus, MUC1-loaded PBMCs on days 0 and 14 do not enhance the protection given by CTLs alone. Mice from group 6, which received CTLs on day 0 and MUC1-loaded PBMCs on days 0, 2, 4, 9, 14 and 19, were protected from tumor development to 56% (5 tumor-free mice out of 9), indicating that MUC1-loaded PBMCs over additional days do not enhance the protection

conveyed by CTLs alone. Mice from group 7, which received CTLs on day 0 and MUC1-loaded DCs produced in the absence of IL-4 on days 0 and 14, exhibited a protection of 73% (8 tumor-free mice out of 11). This implies that MUC1-loaded DCs produced in the absence of IL-4 given on days 0 and 14 enhance tumor cell killing by CTLs. Finally, mice from group 8, which received CTLs on day 0 and MUC1-loaded DCs generated in the absence of IL-4 on days 0, 2, 4, 9, 14 and 19, were fully protected from tumor development (11 tumor-free mice out of 11). This implies that the administration of MUC1-loaded DCs generated in the absence of IL-4 over additional days further increase the cytotoxic activity of CTLs. A statistically significant difference was observed between the number of mice developing tumors in Group 8 vs. all other experimental groups ($p < 0.05$), with the single exception of Group 7, which received the same cells in a less intensive schedule (**Fig. 4**). All results are representative of two experiments with cells from two subjects.

Discussion

The results of our study support the concept that DCs generated in the absence of IL-4[30,31] are superior in activating CTLs to kill tumor cells. T_H2 responses driven by DCs generated in the presence of IL-4 may explain their reduced ability to stimulate T lymphocytes.[23–28] Our findings also indicate that non-stimulated PBMCs as well as unloaded DCs are ineffective in protecting against tumor growth in the absence of T lymphocytes, and that loaded DCs can stimulate PBMCs in vivo to generate CTLs that exert cytotoxic functions. In fact, T lymphocytes activated in vivo were as effective as those generated in vitro. MUC1-loaded PBMCs were ineffective in activating CTLs to prevent tumor formation. On the other hand, MUC1-loaded DCs generated in the absence of IL-4 administered on days 0, 14 (Group 7) or on days 0, 2, 4, 9, 14 and 19 (Group 8) resulted in enhanced protection as compared with other appraoches. There was no statistically significant difference between animals from Group 7 and Group 8, but the latter, which received DCs over six days, was completely protected from the development of tumors. This indicates that repeated and/or extended stimulations of CTLs with DCs constitutes an optimal setting for the elicitation of antitumor responses.

The fact that mice inoculated with MCF-7 cells were protected from tumor growth when MUC1-loaded DCs produced in the absence of IL-4 were injected along with CTLs suggests that these DCs enhance the cytotoxic activity of CTLs in vivo and are required to obtain a complete antitumor protection. Similar observations have recently been reported with murine DCs.[32] The present study is novel in that human cells were used.

Our results indicate that DCs loaded with a TAA (MUC1) in vitro and administered to animals along with CTLs can initiate a protective antitumor immune response that is superior to that elicited by either intervention alone. This suggests that MUC1-loaded DCs generated in the absence of IL-4 boost the efficacy of the adoptively transferred CTLs, perhaps by increasing the survival of T cells. The survival of T cells in vivo is critical for the success of adoptive cell transfer[33] and it has previously been shown

that DCs extend the viability of T cells in this setting.[19] Cytokines such as IL-21,[34] IL-7[35] or IL-15[36] also enhance the survival of T cells and thus might replace DCs. The clinical relevance of this approach was demonstrated by a complete remission achieved by pancreatic cancer patients upon the infusion of DCs and CTLs.[37] These results need to be confirmed in other clinical trials. Tumor antigen-loaded DCs, IL-21, IL-7 or IL-15 or other interventions that would enhance the survival of CTLs, such the provision of co-stimulatory signals to TCRs or the use of chimeric TCRs,[38] should be considered for CTL-based adoptive immunotherapy.

Materials and Methods

Human cells. Human cells were obtained in accordance with the Helsinki Declaration of 1975 and the Texas tech University Health Sciences Center Institutional Review Board from expired subjects. PBMCs from two different subjects were used. PBMC were not HLA typed, as we[3,20,39] and others[40] have found that the cytotoxicity of MUC1-specific CTLs can be non-MHC restricted. Cells used for each experiment were from one subject. In vitro experiments and one of the in vivo experiments were from subject 1. The other in vivo experiment was from subject 2.

Breast cancer MCF-7 (ATCC HTB-22), erythroleukemia K562 (ATCC CCL-243) and B-cell lymphoma RAJI (ATCC CCL-86) cell lines were obtained from, and cultured as recommended by, the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco-BRL, Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 1% bovine insulin (Gibco) and 1% L-glutamine (Gibco). K562 and RAJI were cultured in RPMI-1640 (Gibco-BRL, Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1% L-glutamine. All the cells were maintained in a 37°C humidified and 5% CO_2 atmosphere.

MUC1 peptide. The MUC1-mtr$_1$ peptide GNNAPPAHGVNNAPDNRPAP (S2,12-N; T3,11,16-N)[5] was custom synthesized by American Peptide Co., Inc.

Generation and maturation of dendritic cells. DCs were generated from PBMCs of breast adenocarcinoma patients obtained by apheresis,[41] as previously published, with a few modifications.[31] Briefly, PBMCs were cultured in 225 cm² tissue culture flasks at 2 × 10⁶ cells/mL in 75 mL of AIM-V® serum-free lymphocyte medium (Gibco BRL, Life Technologies, Inc.) and incubated at 37°C and 5% CO_2 for 1 h. After a microscopic confirmation of adherence, the non-adherent fraction was removed and 75 mL of fresh AIM-V® serum-free lymphocyte medium was added (day 0). Non-adherent cells were centrifuged and placed in a second set of 225 cm² tissue culture flasks for adherence. After 1 h of incubation, the non-adherent fraction of the second set of flasks was removed and 75 mL of fresh AIM-V® serum-free lymphocyte medium were added. Both sets of flasks were incubated at 37°C and 5% CO_2. MUC1 (1 μg/mL final concentration) was added on day 0 of culture. Human recombinant GM-CSF (500 IU/mL final concentration, Biosource International Inc.) was added on days 0, 3 and 7. Human recombinant TNFα (10 ng/mL final concentration, Biosource International Inc.), as well as

polyriboinosinic polyribocytidylic acid (50 μg/mL final concentration, Sigma Chemical Co.)[30] was added on day 7. IL-4 (15 ng/mL final concentration, Biosource International Inc.)[31] was added to one set of the flasks on days 0, 3 and 7, while the other set did not receive any IL-4. It has been shown that this method induces the expression of CD83 and co-stimulatory molecules.[30] On day 9, mature DCs were counted and their viability was assessed by trypan blue exclusion test (Invitrogen Corporation).

Generation of CTLs for protection experiments. CTLs were generated from PBMCs and grown in a gas-permeable hydrophobic bag for eight days. PBMCs were cultured at 2 × 10⁶ cells/mL in AIM-V® serum-free lymphocyte medium and maintained in a 37°C humidified and 5% CO_2 atmosphere. IL-2 (Cetus, GenWayBio, Inc.) was added twice per week at 100 IU/mL. PBMCs were stimulated with MUC1 (1 μg/mL final concentration)[42] or MUC1-loaded DCs generated in the absence of IL-4 (1:100 ratio) on days 0 and 7 of culture. The culture medium was not changed, but added on days 4 and 7 if needed, to maintain 2 × 10⁶ cells/mL.[39] Cell and supernatant samples were collected on days 0, 3 and 8 for cytotoxicity assays and cytokine assays. PBMCs stimulated under these optimized conditions[41] are primarily CD4⁺ T lymphocytes, with a lower amount of CD8⁺ T lymphocytes.[3] PBMCs from both subjects were stimulated separately and used individually in animal experiments.

MUC1 loading of PBMCs. PBMC were loaded with MUC1 (1 μg/mL final concentration) and maintained in a flask at 37°C humidified and 5% CO_2 atmosphere for two hours.

Cytotoxicity assays. In order to evaluate the ability of CTLs to lyse specific, as well as non specific target cell lines in vitro, two cytotoxicity assays were used during this study: the XTT assay and the alamarBlue® assay. Based on previous unpublished observations, the XTT assay works better with adherent cell lines, while the alamarBlue® assay works better with hematopoietic cells. MCF-7 cells express hypoglycosylated MUC1,[8] and were used as target cell line in a XTT assay (Roche Diagnostics Corp.). K562 cells, a NK/LAK-sensitive target,[43] and RAJI cells, a NK-relatively resistant/LAK-sensitive target,[44] were used as target cell lines in alamarBlue® assays (Biosource International Inc.).

Cell lines were seeded into separate 96-well tissue culture plates. Five thousands target cells were added to each well, except the background wells. Washed effector CTLs were added to each well in three E:T ratios: 10:1, 5:1 and 2.5:1. Effector cells alone were seeded at the corresponding numbers per well in background wells. Six wells were set up with the target cells only. CTLs from each subject were analyzed separately, in triplicate wells for each E:T ratio.

XTT assay. The XTT assay was used to evaluate the ability of CTLs to kill the specific target cells, MCF-7 cells, per manufacturer's instructions. The XTT assay is a non-radioactive, colorimetric assay using the XTT labeling reagent, sodium 3'-[1-(phenylamino-carbonyl)-3,4- tetrazolium]–bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate,[45] which is cleaved into formazan dye only by metabolically active cells. It is used for the determination of cellular proliferation, viability and activation in response to growth factors, cytokines, nutrients and cytotoxicity.

The plate was set up as described above one day before the assay. Fifty μL of XTT labeling mixture (5 mL XTT labeling reagent plus 100 μL electron coupling reagent) were added to all wells of the plate. The plate was incubated in a 37°C humidified and 5% CO_2 atmosphere for four hours. The optical density (OD) was then measured by using a spectrophotometer (Dynatech MR 5000, Dynatech Laboratories Inc.). The plate was read at a wavelength of 450 nm with a reference wavelength of 630 nm, and background absorbance was subtracted. The maximum XTT was determined as the mean of the six wells containing only target cells and the minimum was determined as the mean of the six wells containing medium only. The nonspecific formation of formazan attributable to the presence of effector cells was determined from wells containing effector cells alone. The percent specific lysis (%SL) was calculated as follows:[46]

$$\%SL = \frac{OD_{(target-medium)} - OD_{(experimental\ wells\ -wells\ with\ corresponding\ number\ of\ effectors)}}{OD_{(target-medium)}} \times 100$$

Alamar Blue® assay. Alamar Blue® assay[47] was used to evaluate the ability of CTLs to kill the non-specific target cells K562 and RAJI, per manufacturer's instructions.

The internal environment of a proliferating cell is more reduced than that of a non-proliferating cell.[48] AlamarBlue®, which can be reduced by metabolic intermediates, is useful in monitoring cell proliferation because its reduction is accompanied by a measurable shift in color. As the alamarBlue® reacts with metabolic intermediates, it accepts electrons and changes in color from the oxidized indigo blue, non-fluorescing state to the reduced pink-fluorescing state.

Plates were set up as described above the same day of the alamarBlue® assay. Twenty μL of alamarBlue® reagent was added to each well. After overnight incubation in a 37°C humidified and 5% CO_2 atmosphere, the OD was read with a spectrophotometer at wavelengths of 570 and 630 nm.

The alamarBlue® reduction was evaluated by measuring absorbance spectrophotometrically and by calculating the percentage of alamarBlue® reduction, according to the manufacturer's recommendations.

Percentage difference in alamarBlue® reduction:

$$= \frac{(\varepsilon_{ox})\lambda_2 A\lambda_1 - (\varepsilon_{ox})\lambda_1 A\lambda_2 \text{ of test dilution}}{(\varepsilon_{ox})\lambda_2 A^0\lambda_1 - (\varepsilon_{ox})\lambda_1 A^0\lambda_2 \text{ of untreated positive growth control}} \times 100$$

In the formula, $\varepsilon_{ox}\lambda_1$ and $\varepsilon_{ox}\lambda_2$ represent the molar extinction coefficient of alamarBlue® at 570 and 630 nm, respectively, in the oxidized forms. $A\lambda_1$ and $A\lambda_2$ represent the absorbance of test wells at 570 and 630 nm, respectively. $A^0\lambda_1$ and $A^0\lambda_2$ represent

the absorbance of untreated positive growth control wells at 570 and 630 nm, respectively. The values of percentage difference in alamarBlue® reduction were corrected for background values of untreated positive growth controls.

ELISA cytokine assay. The amount of TNFα (BD PharMingen Inc.) IFNγ (BD PharMingen Inc.), IL-10 (BD PharMingen Inc.) and GM-CSF (Biosource International Inc.) present in the supernatants was determined by enzyme linked-immunosorbent assays, per manufacturer's instructions. The supernatants from the CTLs of each subject were analyzed separately, in triplicate instances.

In vivo protection experiments. Female NOD-SCID mice (Jackson Laboratory, Bar Harbor, ME, USA) of 6–12 weeks of age were injected s.c. in the back of the neck with 0.1 mL of 1:1 PBS:matrigel (Gibco BRL, Life Technologies, Inc.) containing 5×10^6 MCF-7 cells. Fifty million washed PBMCs or CTLs were injected i.p. alone or together with 5×10^5 unloaded DCs, MUC1-loaded DCs generated in the absence of IL-4 or MUC1-loaded PBMCs according to the schedules described in the legend to **Figure** 4. Control animals received PBS or the above mentioned cells individually. Each mouse was checked for tumor development three times per week for one month. Animal care was in accordance with institutional guidelines. Authors followed the Guidelines for Ethical Conduct in the Care and Use of Animals (http://www.apa.org/science/anguide.html) by the APA Board of Scientific Affairs Committee on Animal Research and Ethics.

Statistical analyses. Statistical significance for in vitro cytotoxicity assays and cytokine assays was determined by the Mann-Whitney rank sum test. Fisher's exact test was used to analyze data obtained from in vivo experiments. p values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Carroll PR, DeVita VT Jr. Cancer of the Prostate. Cancer: Principles and Practice of Oncology. Philadelphia: Lippincott Williams & Wilkins, 2001:1418-79.

2. Rosenberg SA. Progress in human tumour immunology and immunotherapy. Nature 2001; 411:380-4; PMID:11357146; http://dx.doi.org/10.1038/35077246.

3. Wright SE, Kilinski L, Talib S, Lowe KE, Burnside JS, Wu JY, et al. Cytotoxic T lymphocytes from humans with adenocarcinomas stimulated by native MUC1 mucin and a mucin peptide mutated at a glycosylation site. J Immunother 2000; 23:2-10; PMID:10687132; http://dx.doi.org/10.1097/00002371-200001000-00002.

4. Devine PL, McGuckin MA, Ramm LE, Ward BG, Pee D, Long S. Serum mucin antigens CASA and MSA in tumors of the breast, ovary, lung, pancreas, bladder, colon, and prostate. A blind trial with 420 patients. Cancer 1993; 72:2007-15; PMID:7689923; http://dx.doi.org/10.1002/1097-0142(19930915)72:6<2007::AID-CNCR2820720636>3.0.CO;2-U.

5. Gendler S, Taylor-Papadimitriou J, Duhig T, Rothbard J, Burchell J. A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. J Biol Chem 1988; 263:12820-3; PMID:3417635.

6. Lan MS, Batra SK, Qi WN, Metzgar RS, Hollingsworth MA. Cloning and sequencing of a human pancreatic tumor mucin cDNA. J Biol Chem 1990; 265:15294-9; PMID:2394722.

7. Guevara-Patiño JA, Engelhorn ME, Turk MJ, Liu C, Duan F, Rizzuto G, et al. Optimization of a self antigen for presentation of multiple epitopes in cancer immunity. J Clin Invest 2006; 116:1382-90; PMID:16614758; http://dx.doi.org/10.1172/JCI25591.

8.   Barnd DL, Lan MS, Metzgar RS, Finn OJ. Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. Proc Natl Acad Sci U S A 1989; 86:7159-63; PMID:2674949; http://dx.doi.org/10.1073/pnas.86.18.7159.

9.   Ioannides CG, Fisk B, Jerome KR, Irimura T, Wharton JT, Finn OJ. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. J Immunol 1993; 151:3693-703; PMID:7690810.

10. Jerome KR, Barnd DL, Bendt KM, Boyer CM, Taylor-Papadimitriou J, McKenzie IF, et al. Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. [UNITEDSTATES]. Cancer Res 1991; 51:2908-16; PMID:1709586.

11. Rughetti A, Turchi V, Ghetti CA, Scambia G, Panici PB, Roncucci G, et al. Human B-cell immune response to the polymorphic epithelial mucin. Cancer Res 1993; 53:2457-9; PMID:8495404.

12. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392:245-52; PMID:9521319; http://dx.doi.org/10.1038/32588.

13. Ronchese F, Hausmann B, Le Gros G. Interferon-gamma- and interleukin-4-producing T cells can be primed on dendritic cells in vivo and do not require the presence of B cells. [GERMANY]. Eur J Immunol 1994; 24:1148-54; PMID:8181524; http://dx.doi.org/10.1002/eji.1830240521.

14. Albert SE, McKerlie C, Pester A, Edgell BJ, Carlyle J, Petric M, et al. Time-dependent induction of protective anti-influenza immune responses in human peripheral blood lymphocyte/SCID mice. J Immunol 1997; 159:1393-403; PMID:9233636.

15. Ifversen P, Mårtensson C, Danielsson L, Ingvar C, Carlsson R, Borrebaeck CA. Induction of primary antigen-specific immune reponses in SCID-hu-PBL by coupled T-B epitopes. Immunology 1995; 84:111-6; PMID:7534259.

16. Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD Jr. DNA-based immunization by in vivo transfection of dendritic cells. Nat Med 1996; 2:1122-8; PMID:8837611; http://dx.doi.org/10.1038/nm1096-1122.

17. Luykx-de Bakker SA, de Gruijl TD, Scheper RJ, Wagstaff J, Pinedo HM. Dendritic cells: a novel therapeutic modality. Ann Oncol 1999; 10:21-7; PMID:10076717; http://dx.doi.org/10.1023/A:1008349920664.

18. Ochsenbein AF, Klenerman P, Karrer U, Ludewig B, Pericin M, Hengartner H, et al. Immune surveillance against a solid tumor fails because of immunological ignorance. Proc Natl Acad Sci U S A 1999; 96:2233-8; PMID:10051624; http://dx.doi.org/10.1073/pnas.96.5.2233.

19. Ludewig B, Oehen S, Barchiesi F, Schwendener RA, Hengartner H, Zinkernagel RM. Protective antiviral cytotoxic T cell memory is most efficiently maintained by restimulation via dendritic cells. J Immunol 1999; 163:1839-44; PMID:10438917.

20. Wright SE, Rewers-Felkins KA, Quinlin IS, Eldridge PW, Zorsky PE, Klug PP, et al. Adoptive immunotherapy of mucin1 expressing adenocarcinomas with mucin1 stimulated human peripheral blood mononuclear cells. Int J Mol Med 2002; 9:401-4; PMID:11891536.

21. Conti L, Gessani S. GM-CSF in the generation of dendritic cells from human blood monocyte precursors: recent advances. Immunobiology 2008; 213:859-70; PMID:18926300; http://dx.doi.org/10.1016/j.imbio.2008.07.017.

22. Arpa L, Valledor AF, Lloberas J, Celada A. IL-4 blocks M-CSF-dependent macrophage proliferation by inducing p21Waf1 in a STAT6-dependent way. Eur J Immunol 2009; 39:514-26; PMID:19130475; http://dx.doi.org/10.1002/eji.200838283.

23. Seder RA, Paul WE, Davis MM, Fazekas de St Groth B. The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. J Exp Med 1992; 176:1091-8; PMID:1328464; http://dx.doi.org/10.1084/jem.176.4.1091.

24. Croft M, Carter L, Swain SL, Dutton RW. Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. J Exp Med 1994; 180:1715-28; PMID:7525836; http://dx.doi.org/10.1084/jem.180.5.1715.

25. Boni A, Iezzi G, Degl'Innocenti E, Grioni M, Jachetti E, Camporeale A, et al. Prolonged exposure of dendritic cells to maturation stimuli favors the induction of type-2 cytotoxic T lymphocytes. Eur J Immunol 2006; 36:3157-66; PMID:17111344; http://dx.doi.org/10.1002/eji.200535597.

26. Wang J, Alvarez R, Roderiquez G, Guan E, Caldwell Q, Wang J, et al. CpG-independent synergistic induction of beta-chemokines and a dendritic cell phenotype by orthophosphorothioate oligodeoxynucleotides and granulocyte-macrophage colony-stimulating factor in elutriated human primary monocytes. J Immunol 2005; 174:6113-21; PMID:15879106.

27. Zou W, Borvak J, Marches F, Wei S, Galanaud P, Emilie D, et al. Macrophage-derived dendritic cells have strong Th1-polarizing potential mediated by beta-chemokines rather than IL-12. J Immunol 2000; 165:4388-96; PMID:11035076.

28. Chakraborty NG, Sporn JR, Tortora AF, Kurtzman SH, Yamase H, Ergin MT, et al. Immunization with a tumor-cell-lysate-loaded autologous-antigen-presenting-cell-based vaccine in melanoma. Cancer Immunol Immunother 1998; 47:58-64; PMID:9755879; http://dx.doi.org/10.1007/s002620050504.

29. Dobrzanski MJ, Rewers-Felkins KA, Samad KA, Quinlin IS, Phillips CA, Robinson W, et al. Immunotherapy with IL-10- and IFN-γ-producing CD4 effector cells modulate "Natural" and "Inducible" CD4 TReg cell subpopulation levels: observations in four cases of patients with ovarian cancer. Cancer Immunol Immunother 2012; 61:839-54; PMID:22083345; http://dx.doi.org/10.1007/s00262-011-1128-x.

30. Spisek R, Bretaudeau L, Barbieux I, Meflah K, Gregoire M. Standardized generation of fully mature p70 IL-12 secreting monocyte-derived dendritic cells for clinical use. Cancer Immunol Immunother 2001; 50:417-27; PMID:11726136; http://dx.doi.org/10.1007/s002620100215.

31. Thurner B, Röder C, Dieckmann D, Heuer M, Kruse M, Glaser A, et al. Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. J Immunol Methods 1999; 223:1-15; PMID:10037230; http://dx.doi.org/10.1016/S0022-1759(98)00208-7.

32. Lou Y, Wang G, Lizée G, Kim GJ, Finkelstein SE, Feng C, et al. Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells in vivo. Cancer Res 2004; 64:6783-90; PMID:15374997; http://dx.doi.org/10.1158/0008-5472.CAN-04-1621.

33. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J Clin Oncol 2008; 26:5233-9; PMID:18809613; http://dx.doi.org/10.1200/JCO.2008.16.5449.

34. Markley JC, Sadelain M. IL-7 and IL-21 are superior to IL-2 and IL-15 in promoting human T cell-mediated rejection of systemic lymphoma in immunodeficient mice. Blood 2010; 115:3508-19; PMID:20190192; http://dx.doi.org/10.1182/blood-2009-09-241398.

35. Sportès C, Hakim FT, Memon SA, Zhang H, Chua KS, Brown MR, et al. Administration of rhIL-7 in humans increases in vivo TCR repertoire diversity by preferential expansion of naive T cell subsets. J Exp Med 2008; 205:1701-14; PMID:18573906; http://dx.doi.org/10.1084/jem.20071681.

36. Bergamaschi C, Rosati M, Jalah R, Valentin A, Kulkarni V, Alicea C, et al. Intracellular interaction of interleukin-15 with its receptor alpha during production leads to mutual stabilization and increased bioactivity. J Biol Chem 2008; 283:4189-99; PMID:18055460; http://dx.doi.org/10.1074/jbc.M705725200.

37. Kondo H, Hazama S, Kawaoka T, Yoshino S, Yoshida S, Tokuno K, et al. Adoptive immunotherapy for pancreatic cancer using MUC1 peptide-pulsed dendritic cells and activated T lymphocytes. Anticancer Res 2008; 28(1B):379-87; PMID:18383873.

38. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med 2011; 3:95ra73; PMID:21832238; http://dx.doi.org/10.1126/scitranslmed.3002842.

39. Wright SE, Rewers-Felkins KA, Quinlin IS, Fogler WE, Phillips CA, Townsend M, et al. MHC-unrestricted lysis of MUC1-expressing cells by human peripheral blood mononuclear cells. Immunol Invest 2008; 37:215-25; PMID:18389441; http://dx.doi.org/10.1080/08820130801967874.

40. Alajez NM, Schmielau J, Alter MD, Cascio M, Finn OJ. Therapeutic potential of a tumor-specific, MHC-unrestricted T-cell receptor expressed on effector cells of the innate and the adaptive immune system through bone marrow transduction and immune reconstitution. Blood 2005; 105:4583-9; PMID:15746083; http://dx.doi.org/10.1182/blood-2004-10-3848.

41. Wright SE, Khaznadar R, Wang Z, Quinlin IS, Rewers-Felkins KA, Phillips CA, et al. Generation of MUC1-stimulated mononuclear cells using optimized conditions. Scand J Immunol 2008; 67:24-9; PMID:18021186.

42. Quinlin IS, Burnside JS, Dombrowski KE, Phillips CA, Dolby N, Wright SE. Context of MUC1 epitope: immunogenicity. Oncol Rep 2007; 17:453-6; PMID:17203187.

43. Kornbluth J, Flomenberg N, Dupont B. Cell surface phenotype of a cloned line of human natural killer cells. J Immunol 1982; 129:2831-7; PMID:6982944.

44. von Zons P, Crowley-Nowick P, Friberg D, Bell M, Koldovsky U, Whiteside TL. Comparison of europium and chromium release assays: cytotoxicity in healthy individuals and patients with cervical carcinoma. Clin Diagn Lab Immunol 1997; 4:202-7; PMID:9067656.

45. Roehm NW, Rodgers GH, Hatfield SM, Glasebrook AL. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods 1991; 142:257-65; PMID:1919029; http://dx.doi.org/10.1016/0022-1759(91)90114-U.

46. Jost LM, Kirkwood JM, Whiteside TL. Improved short- and long-term XTT-based colorimetric cellular cytotoxicity assay for melanoma and other tumor cells. J Immunol Methods 1992; 147:153-65; PMID:1548398; http://dx.doi.org/10.1016/S0022-1759(12)80003-2.

47. Ahmed SA, Gogal RM Jr., Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H] thymidine incorporation assay. J Immunol Methods 1994; 170:211-24; PMID:8157999; http://dx.doi.org/10.1016/0022-1759(94)90396-4.

48. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65:55-63; PMID:6606682; http://dx.doi.org/10.1016/0022-1759(83)90303-4.