Induction of specific human cytotoxic T cells using dendritic cells transduced with an adenovector encoding rat epidermal growth factor receptor 2

KALATHIL SURESH[1,2], ELIZABETH SCHEID[2], LAURENCE KLOTZ[1], VASUNDARA VENKATESWARAN[1], JACK GAULDIE[2] and RONAN FOLEY[2]

[1]Division of Urology, Sunnybrook Health Sciences Centre, University of Toronto, Toronto; [2]Centre for Gene Therapeutics, Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

Received March 23, 2011; Accepted May 18, 2011

DOI: 10.3892/ijo.2011.1124

Abstract. This study demonstrates the ability to generate antigen-specific cytotoxic T cells (CTLs) against HER2 using a xenoantigenic immune stimulation strategy. Dendritic cells (DCs) were transduced with an adenovirus vector incorporating full-length cDNA for rat (xenoantigen) epidermal growth factor receptor 2 (Adv-HER2). Stimulation of autologous T cells with Adv-HER2 infected DCs led to enhanced HER2-specific reactivity as assessed by quantitative real-time polymerase chain reaction (qRT-PCR) for T cell IFN-γ mRNA. In ELISPOT and intracellular cytokine staining (ICS) assays, $CD8^+$ CTLs induced by Adv-HER2 transduced DCs released IFN-γ following stimulation with irradiated autologous DCs infected with Adv-HER2 or loaded with a human prostate cancer cell line (LNCaP) lysate. DCs pulsed with HER2 peptides were less stimulatory than Adv-HER2 transduced DCs. HER2 DC induced CTL lysed $HER2^+$ $HLA\text{-}A2^+$ tumor cells (MCF-7); significantly reduced lysis occurred in $HER2^+$ $HLA\text{-}A2^-$ tumor cells (SKOV-3), and the NK cell sensitive cell line K-562.

Introduction

Development of therapeutic cancer vaccines capable of inducing an immune response against weakly immunogenic tumor-associated antigens remains a challenge. Identification of optimal tumor antigens recognized by host immune effector cells is essential. Human epidermal growth factor receptor 2 (HER2), human telomerase reverse transcriptase (hTERT) and carcinoembryonic antigen (CEA) are expressed in a variety of cancers and may be effective targets for anti-cancer vaccine therapies (1-5). HER2 is a transmembrane glycoprotein with tyrosine kinase activity. HER2 is genetically amplified and over-expressed in epithelial cancers including breast, ovarian, lung, prostate and colorectal carcinoma (6). HER2 over-expression contributes to cancer progression and is associated with a poor prognosis in breast cancer patients (7,8). Pre-existent antibodies and T cells reactive to HER2 have been observed in this patient population (1,9). This reinforces the suitability of HER2 as a target for immunotherapeutic strategies and has led to the investigation of HER2 vaccination in clinical studies, with many of these trials utilizing HER2 peptide vaccines (10). These early trials provide evidence that antigen-specific immune responses can be generated. However, successful CD8 T cell responses were often short-lived; for example immunization with a HER2 HLA-A2 peptide (p369-377) with GM-CSF as an adjuvant induced a low and transient $CD8^+$ T cell response (11). In an effort to enhance the weak response to immunization with MHC class I epitopes alone, vaccines incorporating HER2 class II peptides have also been investigated (12,13). Despite good evidence of immune response following peptide vaccination, studies investigating peptide vaccines for breast cancer therapy have demonstrated limited clinical efficacy. An alternative strategy to the direct delivery of antigens involves the *ex vivo* loading of antigen onto autologous DCs. DCs take up antigen (Ag) and subsequently present processed peptides on MHC class I and class II molecules to $CD8^+$ and $CD4^+$ T cells, respectively (14,15). Strategies based upon the activation of MHC class I restricted $CD8^+$ T cells by cellular vaccines composed of autologous DCs loaded *ex vivo* with tumor associated antigens have been investigated in clinical studies (16-18). These studies utilized peptide-pulsed-DCs; in an effort to increase the immunogenicity of DC vaccines other groups have employed DCs transfected *ex vivo* with viral vectors containing full-length genes coding for tumor antigens. Using this approach measurable tumor specific immune responses have been seen in preclinical and clinical studies (19-22). It has been suggested that vaccination with a xenoantigenic form of the tumor antigen could be advantageous in that

Correspondence to: Dr Ronan Foley, Juravinski Hospital, 2nd Floor Laboratory, 711 Concession St, Hamilton, Ontario, L8V 1C3, Canada
E-mail: foleyr@hhsc.ca

Present address: [3]Center for Genetics & Pharmacology, Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, USA

Key words: human breast cancer, HER2, CTL, IFN-γ, immunotherapy

this could help to overcome self tolerance in patients (23,24). In this study we demonstrate that autologous DCs that are transduced with a novel Ad vector incorporating a xenoantigenic (rat) form of HER2 stimulate specific cytotoxic T cells from HLA-A2$^+$ healthy donors that are able to recognize a variety of HER2$^+$ targets *in vitro*.

Materials and methods

Ethics statement. Blood samples were obtained from consenting donors. Approval for this work was received from the McMaster Research Ethics Board and the Sunnybrook Research Ethics Board.

Normal donors and blood samples. Heparinized peripheral blood samples were obtained from 14 normal healthy HLA-A2$^+$ or HLA-A2$^-$ donors.

Cell separation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation from peripheral blood samples (Pharmacia Biotech, NJ, USA). CD14$^+$ monocytes were enriched using MACS (Miltenyi Biotech, Germany) isolation kits according to the manufacturer's instructions (purity >95% CD14$^+$). T cells were negatively selected using a Rosettesep™ T cell enrichment kit as described by the manufacturer (Stem Cell Technologies, Vancouver, Canada). T cell purity was tested by flow cytometry (CD3$^+$) using FITC conjugated anti-CD3 monoclonal antibody and was consistently >95%.

Generation of dendritic cells. Positively selected (CD14$^+$) monocytes were grown in DC growth and expansion medium (DC-3 Stemgenix, Buffalo, NY, USA) containing 10 μg/ml gentamicin and glutamine with 100 ng/ml GM-CSF (Leukine, Immunex, Richmond, CA) and 20 ng/ml IL-4 (Stem Cell Technologies). On day 5, 10 ng/ml TNF-α (R&D, Minneapolis, MN) and 100 ng/ml IFN-γ (Stem Cell Technologies) were added. On day 7 DC cultures were infected with Adv-HER2/dl-70-3 or mock infected. After 24 h cells were harvested and assessed for viability and expression of DC maturation markers and HER2 expression.

Tumor cell lines. MCF-7, SKOV-3, LNCaP and K562 (ATCC) were cultured in RPMI-1640 or DMEM supplemented with 10% FBS (Sigma).

Recombinant adenovirus-HER2 transduction of DCs. Adv-HER2 is a recombinant type 5 human E1 substituted E3 deleted adenovirus vector containing the full length sequence for rat HER2 (25). Adenovector dl70-3 (26) was used as a control. DCs were infected on day 7 of culture at a MOI of 200 and cultured for an additional 24 h before harvest.

Flow cytometry analysis. FACS analysis was used to determine the purity of monocytes and T cells, to characterize the DCs, and for the detection of IFN-γ. The following monoclonal antibodies (mAbs) were used: FITC-conjugated CD80, CD3, CD8, CD14; PE-conjugated CD86, HLA-DR, CCR7, CD4, IFN-γ and PC5-conjugated CD83 (Becton-Dickinson, USA). Cells were analyzed using FACScan (Becton-Dickinson).

T cell co-cultures. CD3$^+$ autologous T cells from HLA-A2$^+$ or HLA-A2$^-$ donors (1x10^5 cells/ml) were co-cultured with DCs at a DC:T cell ratio of 1:10 in RPMI-1640 medium supplemented with 10% human AB serum (Biowhittaker, Walkersville, MD, USA), 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 5x10^{-5} M β-mercaptoethanol (BDH, UK). DCs were one of three types: i) DCs transduced with Adv-HER2, ii) dl-70-3 infected DCs, or iii) un-infected DCs. DCs were added to autologous T cells on days 0 and 7. On day 2, 5 ng/ml IL-7 (Stem Cell Technologies, Vancouver, Canada) and 50 U/ml IL-2 (Sigma-Aldrich, St. Louis, MO) were added. On day 14, T-cells were harvested for ^{51}Cr release, ELISPOT and intracellular staining, or were stimulated with irradiated autologous Adv-HER2 DCs, Adv-dl70-3 DCs or untransduced DCs for 3 h before RNA extraction.

IFN-γ ELISPOT assay. Cytotoxic T cells generated *in vitro* using Adv-HER2 DCs were added to anti-human IFN-γ antibody coated 96-well plates at a concentration of 2x10^5 cells/well. (Human IFN-γ ELISPOT kit, R&D Inc, USA). Autologous irradiated Adv-HER2 DCs, Ad dl-70-3 DCs, and untransduced DCs were added at a DC to T cell ratio of 1:10. A biotin-labeled anti-human IFN-γ antibody was used for detection. Spots were counted after an incubation period of 36 h using an automated reader (CTL-ImmunoSpot Analyzer, Cellular Technology Ltd., USA).

Intracellular cytokine staining (ICS) assay. Intracellular cytokine staining was performed according to the manufacturer's protocol. Day 4 DCs were pulsed with LNCaP tumor lysate at 37˚C for 4 h followed by TNF-α and IFN-γ for 24 h. DCs were then washed and incubated at 1:10 ratio with Adv-HER2 DC stimulated autologous T cells or un-transduced DC stimulated T cells. After 3 h Brefeldin-A (10 μg/ml) was added with further incubation for 6 h at 37˚C. Cells were stained with FITC-labeled anti-CD8 (BD Pharminogen) for 30 min at 4˚C, washed and fixed (BD Cytofix/Cytoperm™). After permeabilization (BD Perm/Wash™) cells were stained with anti-IFN-γ PE for 30 min at 4˚C and flow cytometry was performed using LSRII (Becton-Dickinson).

Quantitative real-time PCR measurement of IFN-γ. Total RNA was extracted from T cells (3x10^6) (RNeasy Protect Mini Kit Qiagen Inc., Canada). DNA was digested using an RNAse-free DNAse Kit (Qiagen Inc.). Purified RNA was analyzed using an Agilent 2100 Bioanalyser (Agilent Technologies, Canada) and reverse transcribed to cDNA (Sensiscript reverse transcription kit, Qiagen Inc.). Quantitative real-time PCR (qRT-PCR) was performed using gene specific TaqMan probes and primers on an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) as described earlier (27).

Cytotoxicity assays. Standard ^{51}Cr release assay was performed as previously described (27). T cells harvested 5 days after the last antigenic stimulation were used as effector cells in a 4-h ^{51}Cr release assay. Target cells were MCF-7, K-562 or SKOV-3 tumor cells. Target cells were labeled with $Na^{51}Cro_4$ (Amersham, Canada) (100 μCi/10^6 cells) washed, and incubated with effector cells in duplicate at different effector: target ratios, in v-bottomed 96-well plates for 4 h. A 25-μl volume

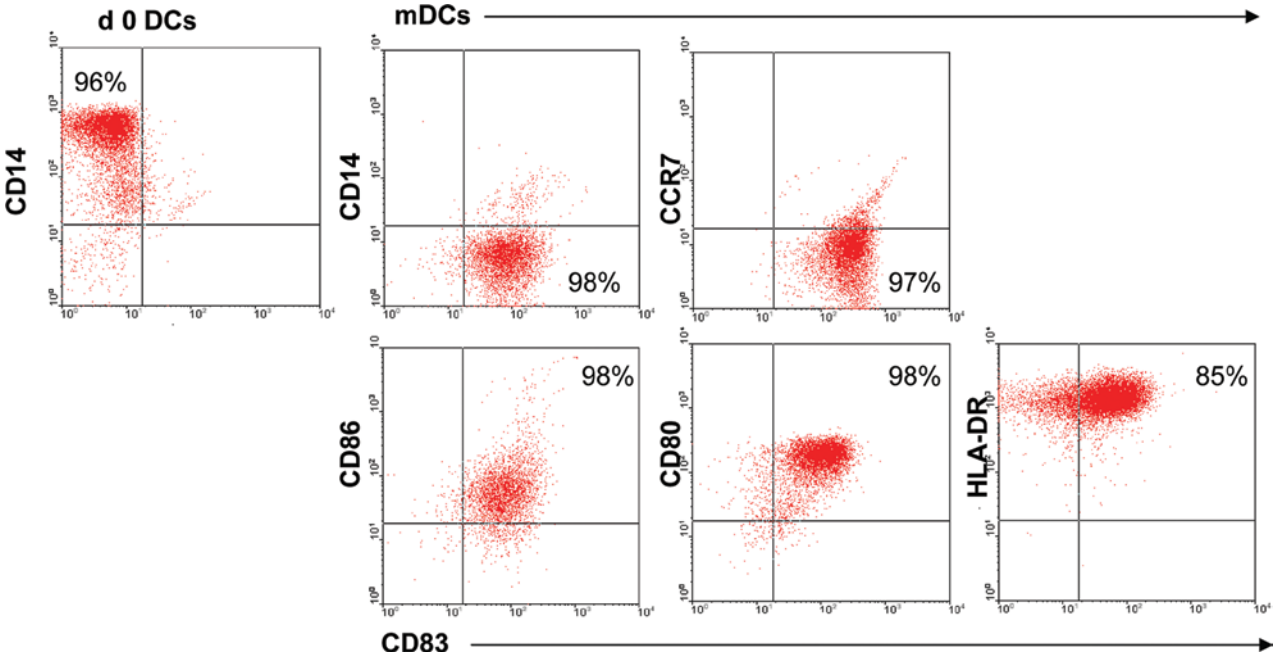

Figure 1. Representative phenotypes of d-0 DC and d-7 transduced DCs. CD14+ monocytes were expanded in serum-free medium containing GMCSF and IL-4, followed by treatment with TNF-α and IFN-γ. On d-7 mature DCs were transduced with Adv-HER2 at a MOI of 200. After a 24-h incubation at 37˚C DCs were subjected to three color flow cytometry analysis as described in Materials and methods The density plots show fluorescence values on gated large cells expressing DC related markers, CD14, HLA-DR, CCR-7, CD80, CD83 and CD86. Values of FL-1 height (upper left hand corner) Fl 2 or FL 3 heights (lower right hand corner) are shown from one representative experiment.

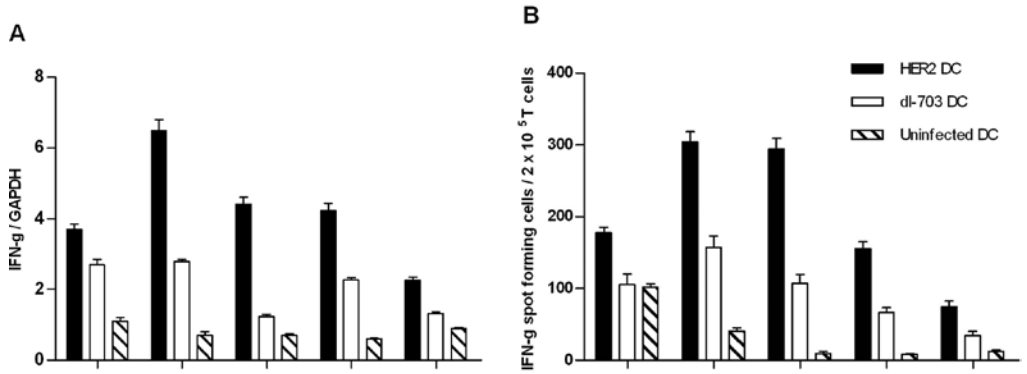

Figure 2. Correlation between HER2 specific T cell IFN-γ/GAPDH mRNA ratio and ELISPOT. GAPDH normalized IFN-γ mRNA/IFN-γ protein levels in autologous T lymphocytes following stimulation with d-7 DCs transduced with Adv-HER2, dl-70-3 or un-transduced control DCs. DCs were co-cultured with purified autologous T cells and q-RTPCR analysis (A) and ELISPOT IFN-γ assay (B) was carried out as described in Materials and methods. Results represent independent experiments from 5 healthy controls and values are the mean ± SE of replicates.

of supernatant was counted (Packard TopCount NXT™) and the specific lysis was calculated using the formula: % lysis = (experimental release-spontaneous release/maximal release-spontaneous release) x 100. Spontaneous and maximal release were determined in the presence of RPMI medium or 1N HCL, respectively.

Results

Generation and maturation of DCs. Phenotypic analysis of DCs included a complete immunophenotypic evaluation of CD14, CD80, CD83, CD86, CCR7 and HLA-DR on days 0 and 8. Treatment of DCs with TNF-α and IFN-γ resulted in the enhanced expression of co-stimulatory molecules. Markers consistent with mature DCs (CD80, CD83, CD86, HLA-DR) were homogeneously expressed by day 8, with concomitant down-regulation in the expression of CD14 (Fig. 1).

Adv-HER2 transduced DCs induced elevated levels of HER2 specific T cell IFN-γ mRNA in HLA-A2⁻/HLA-2⁺ donors. We investigated whether Adv-HER2 transduced DCs were able to induce HER2 specific T cells *in vitro*. T cell derived IFN-γ specific mRNA induced by HER2 antigen was measured by real-time qRT-PCR. T cells co-cultured with adv-HER2 transduced DCs for 2 cycles were tested for HER2 specific IFN-γ mRNA upon incubation for 3 h with irradiated autologous Adv-HER2 transduced DCs, dl-70-3 transduced DCs, un-transduced DCs, or T cells alone. T cells stimulated with Adv-HER2 transduced DCs expressed significantly increased copies of IFN-γ gene transcripts than T cells stimulated with Ad dl-70-3 transduced

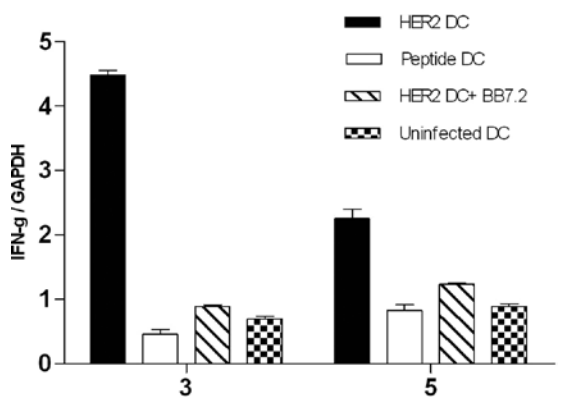

Figure 3. HER2 specific q-RTPCR T cell IFN-γ response in HLA-A2$^+$ healthy donors 3 and 5. GAPDH normalized IFN-γ mRNA levels in autologous T lymphocytes following stimulation with day-7 DCs transduced with Ad-rat-HER2, peptide pulsed DCs, dl-70-3 or untransduced control DCs. DCs were cocultured with purified autologous T cells and qRT-PCR analysis was carried out as described in Materials and methods. Inhibition of T cell response followed by blocking of HLA-A2 molecules on target cells is also shown. Results represent independent experiments from 2 healthy controls and values are the mean of replicates.

DCs or un-transduced DCs (3-9-fold increase, p≤0.05 and 0.002 respectively) (Fig. 2A). No measurable IFN-γ mRNA was detected from unstimulated T cells.

Adv-HER2 transduced DCs elicit HER2 specific T cell IFN-γ protein in HLA-A2$^-$/HLA-A2$^+$ donors. T cells induced by Adv-HER2 transduced DCs were able to release IFN-γ on recall stimulation *in vitro*. CD3$^+$ T cells stimulated with autologous Adv-HER2 transduced DCs for two 7-day cycles of stimulation showed HER2 specific T cell IFN-γ release on recall stimulation with irradiated autologous Adv-HER2 transduced DCs in ELISPOT assay. As depicted in Fig. 2B, T cells from donors released high levels of IFN-γ upon stimulation with Adv-HER2 transduced DCs as compared to T cells stimulated with dl-70-3 transduced DCs or un-transduced DCs (p≤0.05 and 0.002 respectively). Moreover qRT-PCR T cell IFN-γ results correlated with the results of ELISPOT T cell IFN-γ assay.

HER2 specific T cell IFN-γ mRNA transcription and protein release were inhibited by blocking antibody BB7-2. HER2 specific CTL from one donor responded with a 9-fold increase of specific IFN-γ mRNA when challenged with Adv-HER2 DCs *ex vivo* (Fig. 3). Adv-HER2 DC induced elevated IFN-γ mRNA expression and protein release were completely inhibited by anti-HLA-A2 monoclonal antibody BB7-2, indicating that HLA-A2 is the restriction element in this *in vitro* situation (Figs. 3 and 5A).

HER2 specific T cells from HLA A2$^+$ donors lysed HLA matched tumor cells expressing HER2 protein. As shown in Figs. 4 and 5B, CTL induced by Adv-HER2 transduced DCs efficiently lysed HER2$^+$ HLA-A2$^+$ MCF-7 tumor cells in a dose-dependent manner. This cytolytic activity was abolished by treatment of MCF-7 target cells with blocking antibody BB7.2 (Fig. 5B). The same effectors failed to lyse HER2 over-expressing HLA-A2-SKOV-3 tumor cells (Fig. 4). The inability of the effector CTL to lyse the NK cell sensitive K-562 targets demonstrates that

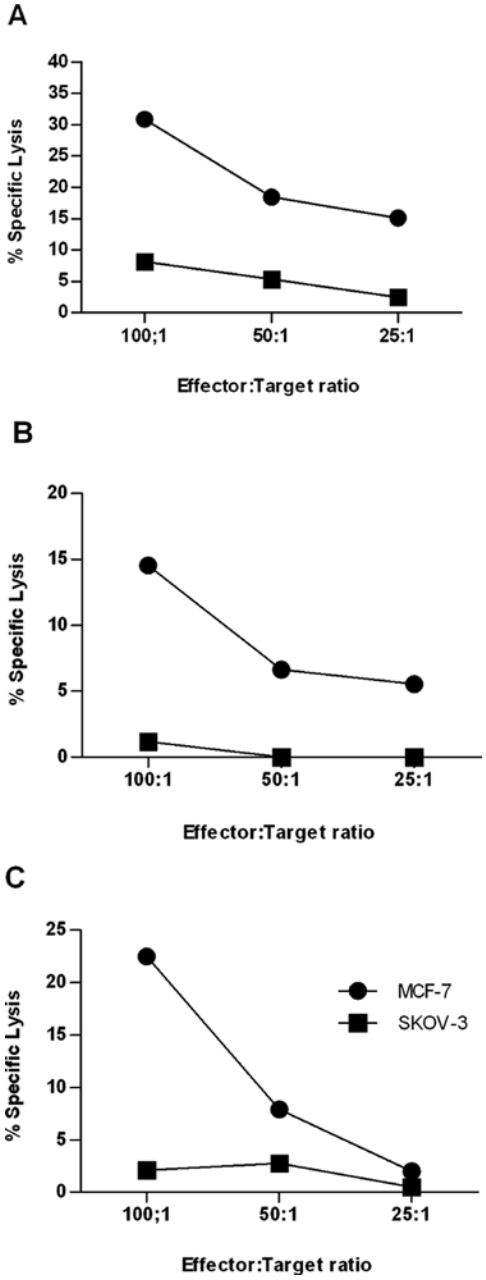

Figure 4. HER2 specific CTL response from three HLA-A2$^+$ healthy controls assessed by Chromium release assay. HER2 specific CTL were generated as described in Materials and methods and cytolytic potential was tested against HER2 expressing tumor cells in a 4-h chromium release assay. Effector CTL demonstrated low or negligible activity against HLA-A2 negative SKOV-3 tumor cells. Results represent independent experiments from 3 HLA-A2$^+$ controls and values are the mean of replicates.

the CTL lacked natural killer activity (Fig. 5B). HER2 specific IFN-γ production by CTL from HLA-A2$^+$ donors correlated with the cytolysis of MCF-7 targets assessed by the chromium release cytotoxicity assay (Fig. 5).

Adv-HER2 transduced DC stimulated T cells recognized LNCaP lysate loaded DCs and produced IFN-γ on recall stimulation. We tested whether HER2 DC stimulated T cells would recognize HER2 antigen on LNCaP prostate cancer cells by recall stimulation assay. Previously stimulated T cells were analyzed for IFN-γ secretion in response to exposure to autologous DCs

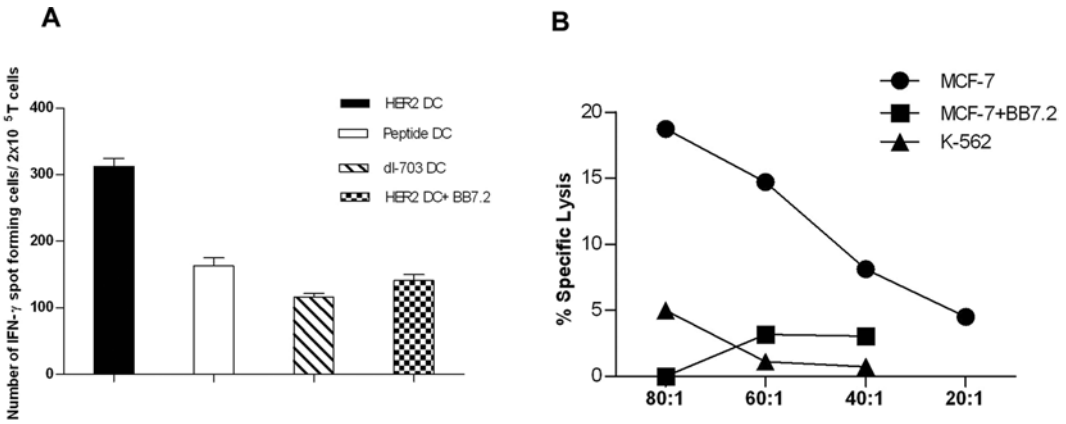

Figure 5. MHC restricted, HER2 specific T cell IFN-γ response in HLA-A2$^+$ healthy donor quantitated by ELISPOT. Inhibition of T cell response followed by blocking of HLA-A2 molecules on target cells is also shown. Values are the mean ± SEM for triplicates (A). Dose-dependent cytolysis of MCF-7 tumor cells by CTLs from the same donor as assessed by Chromium release assay. Non-reactivity with K-562 target cells and inhibition of HER2 specific cytolysis followed by blocking of HLA-A2 molecules on the MCF-7 targets are also shown (B).

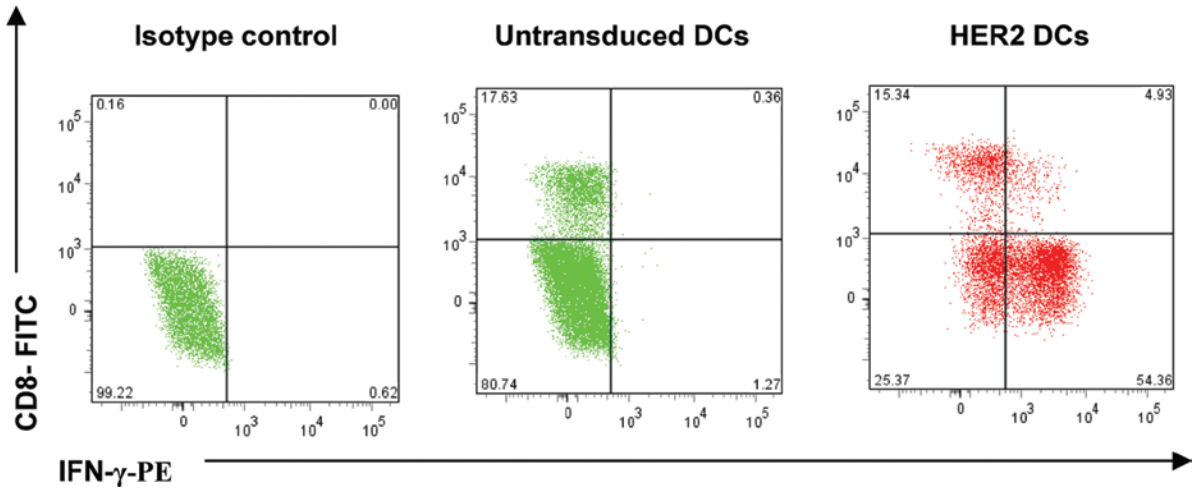

Figure 6. Intracellular staining of IFN-γ in T cells stimulated with Adv-HER2 transduced DCs. Autologous T cells were stimulated with Adv-HER2 DCs or untransduced DCs for two cycles at a 7-day interval. Stimulated T cells were then analyzed for IFN-γ production in response to encounter with LNCaP tumor cell lysate loaded DCs as described in Materials and methods. T cells were then labeled with anti-CD8 FITC and anti-IFN-γ PE monoclonal antibodies and analyzed by flow cytometry. Values presented in the upper right quadrants represent the percentage of IFN-γ secreting T cells within the CD8$^+$ population.

loaded with LNCaP tumor cell lysate. The results of IFN-γ secretion assays from healthy volunteers are depicted in Fig. 6. The frequency of IFN-γ secreting CD8$^+$ T cells showed individual variation and was in some cases as high as 4.9%. A non-CD8$^+$ T cell population comprised of mainly CD4$^+$ T cells also elicited a potent IFN-γ response on recall stimulation. T cells stimulated with untransduced DCs produced relatively low levels of IFN-γ. T cell IFN-γ production was abrogated when blocking antibody was added to the DC T cell culture.

Discussion

Breast cancer is a relatively common malignancy in women (28). Metastatic breast cancer is currently incurable with a median survival of 18-24 months (29). New therapies such as immunotherapy that have the potential to improve immune responses without the toxicity associated with standard therapies are of current clinical interest. The recognition of putative epithelial cancer antigens, such as HER2, has prompted the development of strategies for inductive and adoptive immunotherapy. Traditional direct or *in vivo* vaccination approaches comprised of tumor antigen/peptide mixed with adjuvant have generally not been effective (11,30,31). Another way of inducing an immune response to specific TAA is to use DCs loaded with tumor antigen delivered as peptide or as DNA via recombinant viruses. The advantage of a therapeutic strategy using autologous DCs transduced by viral vectors is that DCs manipulated in this way are capable of presenting a broad range of epitopes which may lead to improved immunogenicity (32). Both preclinical and pilot clinical studies suggest that HER2 can indeed be immunogenic (33-35). Expression of an entire gene encoding a tumor antigen obviates the need for identification of HLA restricted CTL epitopes and allows endogenous processing of all available epitopes for presentation on MHC molecules. DCs transfected with Adv-HER2 serve as an autologous endogenous antigen presenting system which can stimulate both CD4$^+$ T

cells and $CD8^+$ T cells. Pre-clinical studies have demonstrated that vaccination with DCs modified with a recombinant adenovirus (rAdv) or lentivirus to express HER2 and IL-12 conferred tumor protection in transplantable HER2 expressing tumor models (25,36). Adenoviral vectors expressing xenogenic human HER2 proved capable of breaking immune tolerance in a mouse model (37).

In the present study we demonstrate the ability of Adv-rat HER2 transduced DCs to induce HER2 specific autologous CTL response in healthy controls. In this case we have explored whether a xenoantigen enhances reactivity. We used highly sensitive qRT-PCR assay to quantify antigen specific IFN-γ transcripts levels in Adv-HER2 DC-induced T cells on recall stimulation. The combination of qRT-PCR and other independent functional assays like ELISPOT IFN-γ and conventional chromium release assay confirmed our results.

Adv-HER2 transduced DC-stimulated T cells from donors produced HER2 specific T cell IFN-γ protein or mRNA on recall stimulation. Interestingly, Adv-HER2 DC-stimulated $CD8^+$ T cells were also activated (i.e., produced IFN-γ) following exposure to DCs pulsed with a prostate cancer cell line LNCaP lysate. A population of non-$CD8^+$ T cells also elicited a remarkably high IFN-γ response on recall stimulation. This response could be attributed partly to $CD4^+$ T cells, or may represent non-specific T cell activation. Earlier studies have demonstrated the role of HER2 in the development and progression of prostate cancer (38) and it has been demonstrated that LNCaP cells express significantly high amount of HER2 molecule on their surfaces (38,39).

Our data indicate that rat HER2 transduced human DCs induced specific CTL that were capable of lysing HER2 expressing MCF-7 cells in an HLA-A2 restricted manner, demonstrating that this CTL epitope is naturally processed and presented. It is speculated that vaccinating with Adv-HER2 DCs could induce T cells of higher avidity, able to recognize endogenously processed peptide epitopes and subsequently lyse $HER2^+$ tumor cells. The specificity displayed by the effector CTL with respect to IFN-γ mRNA as well as protein production was confirmed by blocking HLA-A2 molecules on the target cells. Our results suggest that HER2 specific CTL from HLA-$A2^+$ donors did not lyse HLA-A2- HER2 over-expressing SKOV-3 cells in cytotoxicity assays. These results are in agreement with results reported by others (18). Non-reactivity of the effector CTL with K-562 targets rules out the involvement of NK or NKT cells in the cytolysis of MCF-7 targets.

In conclusion, Adv-HER2-transduced DCs induced specific cytotoxic T cells in healthy donors that can recognize and kill $HER2^+$ cell targets. Our studies indicate that DCs transduced with a viral rat-HER2 may also be beneficial and can be considered as a potential candidate for vaccination in patients with HER2 over-expressing tumors. Further studies to compare and contrast preclinical efficacy of rat HER2 to human HER2 are required.

Acknowledgements

This work was supported by a grant from the Canadian Breast Cancer Research Association (CBCRA Grant # 014450, http://www.breast.cancer.ca/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Disis ML, Knutson KL, Schiffman K, Rinn K and McNeel DG: Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. Breast Cancer Res Treat 62: 245-252, 2000.
2. Marshall JL: Novel vaccines for the treatment of gastrointestinal cancers. Oncology (Williston Park) 19: 1557-1565, 2005.
3. Murray JL, Przepiorka D and Ioannides CG: Clinical trials of HER-2/neu-specific vaccines. Semin Oncol 27: 71-75, 2000.
4. Nair SK, Heiser A, Boczkowski D, *et al*: Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells. Nat Med 6: 1011-1017, 2000.
5. Lesterhuis WJ, De Vries IJ, Schuurhuis DH, *et al*: Vaccination of colorectal cancer patients with CEA-loaded dendritic cells: antigen-specific T cell responses in DTH skin tests. Ann Oncol 17: 974-980, 2006.
6. Disis ML and Cheever MA: HER-2/neu oncogenic protein: issues in vaccine development. Crit Rev Immunol 18: 37-45, 1998.
7. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235: 177-182, 1987.
8. Slamon DJ and Clark GM: Amplification of c-erbB-2 and aggressive human breast tumors? Science 240: 1795-1798, 1988.
9. Disis ML and Cheever MA: HER-2/neu protein: a target for antigen-specific immunotherapy of human cancer. Adv Cancer Res 71: 343-371, 1997.
10. Disis ML, Grabstein KH, Sleath PR and Cheever MA: Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clin Cancer Res 5: 1289-1297, 1999.
11. Knutson KL, Schiffman K, Cheever MA and Disis ML: Immunization of cancer patients with a HER-2/neu, HLA-A2 peptide, p369-377, results in short-lived peptide-specific immunity. Clin Cancer Res 8: 1014-1018, 2002.
12. Knutson KL, Schiffman K and Disis ML: Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. J Clin Invest 107: 477-484, 2001.
13. Disis ML, Gooley TA, Rinn K, *et al*: Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. J Clin Oncol 20: 2624-2632, 2002.
14. Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL and Bhardwaj N: Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J Exp Med 188: 1359-1368, 1998.
15. Nouri-Shirazi M, Banchereau J, Fay J and Palucka K: Dendritic cell based tumor vaccines. Immunol Lett 74: 5-10, 2000.
16. Mackensen A, Herbst B, Chen JL, *et al*: Phase I study in melanoma patients of a vaccine with peptide-pulsed dendritic cells generated *in vitro* from CD34(+) hematopoietic progenitor cells. Int J Cancer 86: 385-392, 2000.
17. Thurner B, Haendle I, Roder C, *et al*: Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med 190: 1669-1678, 1999.
18. Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L and Brugger W: Induction of cytotoxic T-lymphocyte responses *in vivo* after vaccinations with peptide-pulsed dendritic cells. Blood 96: 3102-3108, 2000.
19. Kaplan JM, Yu Q, Piraino ST, Pennington SE, Shankara S, Woodworth LA and Roberts BL: Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. J Immunol 163: 699-707, 1999.
20. Wan Y, Emtage P, Zhu Q, Foley R, Pilon A, Roberts B and Gauldie J: Enhanced immune response to the melanoma antigen gp100 using recombinant adenovirus-transduced dendritic cells. Cell Immunol 198: 131-138, 1999.
21. Tsao H, Millman P, Linette GP, Hodi FS, Sober AJ, Goldberg MA and Haluska FG: Hypopigmentation associated with an adenovirus-mediated gp100/MART-1-transduced dendritic cell vaccine for metastatic melanoma. Arch Dermatol 138: 799-802, 2002.

22. Boudreau JE, Bridle BW, Stephenson KB, *et al*: Recombinant vesicular stomatitis virus transduction of dendritic cells enhances their ability to prime innate and adaptive antitumor immunity. Mol Ther 17: 1465-1472, 2009.
23. Fong L, Brockstedt D, Benike C, Breen JK, Strang G, Ruegg CL and Engleman EG: Dendritic cell-based xenoantigen vaccination for prostate cancer immunotherapy. J Immunol 167: 7150-7156, 2001.
24. Prud'homme GJ: DNA vaccination against tumors. J Gene Med 7: 3-17, 2005.
25. Chen Y, Emtage P, Zhu Q, *et al*: Induction of ErbB-2/neu-specific protective and therapeutic antitumor immunity using genetically modified dendritic cells: enhanced efficacy by cotransduction of gene encoding IL-12. Gene Ther 8: 316-323, 2001.
26. Putzer BM, Hitt M, Muller WJ, Emtage P, Gauldie J and Graham FL: Interleukin 12 and B7-1 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression. Proc Natl Acad Sci USA 94: 10889-10894, 1997.
27. Suresh K, Fraser G, Scheid E, Leber B, Gauldie J and Foley R: Generation of *in vitro* B-CLL specific HLA class I restricted CTL responses using autologous dendritic cells pulsed with necrotic tumor lysate. Leuk Lymphoma 47: 297-306, 2006.
28. Salehi F, Turner MC, Phillips KP, Wigle DT, Krewski D and Aronson KJ: Review of the etiology of breast cancer with special attention to organochlorines as potential endocrine disruptors. J Toxicol Environ Health B Crit Rev 11: 276-300, 2008.
29. Eitan R, Gemignani ML, Venkatraman ES, Barakat RR and Abu-Rustum NR: Breast cancer metastatic to abdomen and pelvis: role of surgical resection. Gynecol Oncol 90: 397-401, 2003.
30. Salazar LG, Fikes J, Southwood S, *et al*: Immunization of cancer patients with HER-2/neu-derived peptides demonstrating high-affinity binding to multiple class II alleles. Clin Cancer Res 9: 5559-5565, 2003.
31. Zaks TZ and Rosenberg SA: Immunization with a peptide epitope (p369-377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu^{+} tumors. Cancer Res 58: 4902-4908, 1998.
32. Nanda NK and Sercarz EE: Induction of anti-self-immunity to cure cancer. Cell 82: 13-17, 1995.
33. Murray JL, Gillogly ME, Przepiorka D, *et al*: Toxicity, immunogenicity, and induction of E75-specific tumor-lytic CTLs by HER-2 peptide E75 (369-377) combined with granulocyte macrophage colony-stimulating factor in HLA-A2^{+} patients with metastatic breast and ovarian cancer. Clin Cancer Res 8: 3407-3418, 2002.
34. Hueman MT, Stojadinovic A, Storrer CE, *et al*: Analysis of naive and memory CD4 and CD8 T cell populations in breast cancer patients receiving a HER2/neu peptide (E75) and GM-CSF vaccine. Cancer Immunol Immunother 56: 135-146, 2007.
35. Czerniecki BJ, Koski GK, Koldovsky U, *et al*: Targeting HER-2/neu in early breast cancer development using dendritic cells with staged interleukin-12 burst secretion. Cancer Res 67: 1842-1852, 2007.
36. Mossoba ME, Walia JS, Rasaiah VI, *et al*: Tumor protection following vaccination with low doses of lentivirally transduced DCs expressing the self-antigen erbB2. Mol Ther 16: 607-617, 2008.
37. Ko HJ, Kim YJ, Kim YS, *et al*: A combination of chemoimmunotherapies can efficiently break self-tolerance and induce antitumor immunity in a tolerogenic murine tumor model. Cancer Res 67: 7477-7486, 2007.
38. Wang L, Liu B, Schmidt M, Lu Y, Wels W and Fan Z: Antitumor effect of an HER2-specific antibody-toxin fusion protein on human prostate cancer cells. Prostate 47: 21-28, 2001.
39. Di Lorenzo G, Autorino R, De Laurentiis M, Cindolo L, D'Armiento M, Bianco AR and De PS: HER-2/neu receptor in prostate cancer development and progression to androgen independence. Tumori 90: 163-170, 2004.