Chimeric Rat/Human HER2 Efficiently Circumvents HER2 Tolerance in Cancer Patients

Sergio Occhipinti1,3, Laura Sponton1,3, Simona Rolla1,3, Simona Caorsi4, Anna Novarino5, Michela Donadio5, Sara Bustreo5, Maria Antonietta Satolli6, Carla Pecchioni6, Cristina Marchini7, Augusto Amici7, Federica Cavallo1, Paola Cappello1,3, Daniele Pierobon1,3, Francesco Novelli1,3, and Mirella Giovarelli1,3

Abstract

Purpose: Despite the great success of HER2 vaccine strategies in animal models, effective clinical results have not yet been obtained. We studied the feasibility of using DNA coding for chimeric rat/human HER2 as a tool to break the unresponsiveness of T cells from patients with HER2-overexpressing tumors (HER2-CP).

Experimental Design: Dendritic cells (DCs) generated from patients with HER2-overexpressing breast (n = 28) and pancreatic (n = 16) cancer were transfected with DNA plasmids that express human HER2 or heterologous rat sequences in separate plasmids or as chimeric constructs encoding rat/human HER2 fusion proteins and used to activate autologous T cells. Activation was evaluated by IFN-γ ELISPOT assay, perforin expression, and ability to halt HER2+ tumor growth in vivo.

Results: Specific sustained proliferation and IFN-γ production by CD4 and CD8 T cells from HER2-CP was observed after stimulation with autologous DCs transfected with chimeric rat/human HER2 plasmids. Instead, T cells from healthy donors (n = 22) could be easily stimulated with autologous DCs transfected with any human, rat, or chimeric rat/human HER2 plasmid. Chimeric HER2-transfected DCs from HER2-CP were also able to induce a sustained T-cell response that significantly hindered the in vivo growth of HER2+ tumors. The efficacy of chimeric plasmids in overcoming tumor-induced T-cell dysfunction relies on their ability to circumvent suppressor effects exerted by regulatory T cells (Treg) and/or interleukin (IL)-10 and TGF-β1.

Conclusions: These results provide the proof of concept that chimeric rat/human HER2 plasmids can be used as effective vaccines for any HER2-CP with the advantage of being not limited to specific MHC.

Clin Cancer Res; 20(11); 1–12. © 2014 AACR.

Introduction

The ErbB-2 (neu in rat and HER2 in humans) tyrosine kinase receptor is an oncoantigen overexpressed by a variety of tumors (1). The driving role of HER2 in epithelial oncogenesis as well as its selective overexpression on malignant tissues makes it an ideal target for immunotherapy (2). Passive immunotherapy with monoclonal antibodies (mAb) such as trastuzumab, and receptor tyrosine kinase inhibitors, that is, lapatinib, are HER2-targeted therapies currently used for the treatment of HER2-overexpressing breast cancers (3, 4). Unfortunately, the therapeutic efficacy of both these therapies is abolished by primary and acquired tumor resistance, probably due to the emergence of compensatory activities via alternative signaling pathways (5, 6). Active immunotherapy against HER2 might thus represent an alternative strategy.

HER2/neu is a self-antigen and thus tolerogenic. On the basis of HER2-transgenic mouse models, it is now evident that this tolerogenicity causes deletion or inactivation of reactive high-avidity T cells against neu, thereby leading to self-tolerance (7). Nevertheless, low-avidity self-specific T cells can be isolated from tolerant hosts and there are reports that such cells when activated and expanded by a vaccine can give an antitumor response (8). Similarly, expansion of specific T cells has been elicited in patients following vaccination with subdominant (9) or heteroclitic peptides (10, 11); however, clinical responses have not yet been obtained (12), suggesting that additional mechanisms besides central tolerance contribute to T-cell dysfunction in patients with cancer.
A large body of data have been accumulated in the last decade about the role of regulatory T cells (Treg) in inducing T-cell peripheral tolerance and about the "exhaustion" of T cells with expression of inhibitory checkpoint receptors typical of patients with chronic infections or cancer (13).

Several studies have reported increased frequencies of Treg in blood, draining lymph nodes, and tumor tissues, associating an impaired immune response to cancer with a high frequency and/or hyperactivity of Treg (14). The majority of these Treg arise from tumor-induced conversion of conventional CD4⁺ T cells and differ from thymus-derived natural Treg that play a crucial role in regulating the immune response and in maintaining immune homeostasis in healthy individuals (for a review, see ref. 15).

To overcome tumor-driven T-cell dysfunction in patients with cancer, the use of heterologous peptides may be advantageous. In case of patients with HER2-overexpressing cancers (HER2-CP), the use of heterologous peptides characterized by the presence of critical amino acid substitutions that markedly improve their immunogenicity may induce activation of nontolerized, nonexhausted, and self-reactive low-affinity T-cell clones (16). These, in turn, release cytokines that enhance immune recognition in a paracrine way and eventually activate autoreactive B cells.

Chimeric vaccines containing both self-human HER2 and heterologous rat neu DNA sequences induced a more potent cellular and humoral antitumor immunity than self-sequence alone (17, 18). However, no data on their potential efficacy in humans are currently available. Compared with peptide-based vaccines, DNA vaccination has been shown to be more advantageous (19). Indeed, DNA vaccines offer a precise strategy for delivering antigens to the immune system as they can be expressed on cell surfaces or, more commonly, as peptides in association with the MHC class I or II molecules, and their application is not limited to one or few specific MHC molecules (for a review, see refs. 20, 21). A first pilot clinical trial from Norell and colleagues demonstrated promising feasibility, safety, and tolerability of vaccination with DNA coding for the full-length HER2 molecule in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-2 in patients with advanced breast cancer already receiving trastuzumab but with limited clinical effects (22).

Here, we evaluated the feasibility of using DNA coding for chimeric rat/human HER2 as a tool to counteract the dysfunction of T cells from HER2-CP. We transfected monocyte-derived dendritic cells (DCs) from HER2-CP and healthy subjects with DNA plasmids coding for human, rat, or chimeric rat/human HER2. Only DCs transfected with the chimeric plasmids were able to elicit a specific anti-HER2 response by T cells from HER2-CP. Their ability relies on the activation of a significant lower number of Treg cells and lower production of IL-10 and TGF-β that result in the rescue from tumor-induced immune dysfunction.

In conclusion, these results provide the proof of concept that vaccination with chimeric rat/human HER2 DNA plasmids could be an effective therapeutic option for all HER2-CP, with the advantage of being not limited to specific MHC.

Materials and Methods

Human specimens

Human peripheral blood leukocytes (PBL) were isolated by Ficoll-Hypaque (Lanza) gradient centrifugation from heparinized venous blood of healthy subjects (n = 22) provided by the local Blood Bank (Torino, Italy), and patients with cancer (n = 44), not previously treated with radio- or chemotherapy. Patients with pancreatic adenocarcinoma (PDAC; n = 16) or breast cancer (n = 28) recruited at the Centro Oncologico Ematologico Subalpino (COES), AO Città della Salute e della Scienza di Torino, Torino, Italy, with informed consent were included in the study. Blood samples were immediately processed after drawing. Tumors from patients with PDAC and breast cancer were evaluated for HER2 positivity by immunohistochemistry (IHC). Patients bearing HER2⁺ tumors that were classified as 3+ or 2+ by IHC were classified as HER2-CP (Supplementary Table S1). Patients with tumors with a 0 to 1 IHC score, that is, HER2-negative (n = 5), were used as a control group (CTRL-CP; Supplementary Table S2). To determine human leukocyte antigen (HLA)-A2 positivity, PBLs were incubated with anti-HLA-A2-PE mAb (clone B7/2, BD Pharmingen), and expression was evaluated by flow cytometry.

Cell cultures

Monocyte-derived DC generation was conducted as previously described (23). TNF-α (50 ng/mL) and IL-1β (50 ng/mL, PeproTech) were added for the final 24 hours to induce DC maturation. CD14-depleted PBL were stored in liquid nitrogen until use. Thawed lymphocytes (>80% viability and >50% recovery) were cultured for...
7 days with autologous transfected DCs at 20:1 ratio in RPMI-1640 medium with 10% heat-inactivated human serum AB (Lonza) at 2 × 10^3/mL. At day 3, one third of supernatants was collected and replaced with fresh complete medium plus II-7 (10 ng/mL, PeproTech).

The human pancreatic cancer cell line CI-PEC1 and the human ovarian carcinoma cell line SKOV-3-A2 (derived from SKOV-3 cells transduced by lentiviral vector with HLA-A2 gene), both positive for the expression of HER2 and HLA-A2, and the human lung cancer cell line A549, positive for the expression of HER2 but HLA-A2 negative, were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) with 10% FBS, penicillin G (50 U/mL), and streptomycin (50 μg/mL). T2 cells, a TAP-deficient B-cell/T-cell hybrid cell line that express HLA-A2 but lack antigenic peptides, were cultured in RPMI-1640 with 20% FBS.

Plasmids and nucleofection
Plasmid pWAX1 was the backbone for all the DNA constructs used for transfection of DCs. All 4 plasmids code for the extracellular and transmembrane domains of HER2 as previously described (17). HuHuT codes for the fully human, and Rat for the fully rat HER2 molecule. RHuT codes for the first 2 extracellular domains of rat HER2 and the remaining part of human HER2. Conversely, HuRT codes for the first 2 extracellular domains of human HER2 and the remaining part of rat HER2. Large-scale preparation of the plasmids was carried out using EndoFree Plasmid Maxi kits (Qiagen). Mature DCs were harvested on day 6 of culture, resuspended in 100 μL of electroporation buffer (DC transfection kit, Amaxa, Lonza) and mixed with 5 μg of plasmid DNA. Electroporation was performed using the Nucleofector program U-002 (Amaxa, Lonza). After electroporation, cells were immediately transferred to 2 mL of complete media and cultured at 37°C. Efficiency of transfection was analyzed by flow cytometry after 6 hours following transfection. Transfected DCs were fixed, permeabilized, and stained with mouse α-rat- or α-human-HER2 mAb (Calbiochem) followed by αmouse-PE (BD Biosciences).

ELISPOT assay
After 7 days of co-culture, HLA-A2–restricted CD8+ T-cell activation was detected by the IFNγ ELISPOT assay (BD Bioscience), following manufacturer’s instruction. T2 cells were loaded with 10 μg/mL of the HLA-A2+ immunodominant p369–377 E75 (KIFGSLAFI) or p654–662 GP2 (HISAVVIGIL) peptides (PRIMM) for 6 hours at 37°C in serum-free medium. A total of 2.5 × 10^5 recovered T cells were seeded in 96-well ELISPOT assay plates (Millipore) at 10:1 ratio with E75- or GP2-loaded or unloaded T2 cells in AIM-V medium (Invitrogen) for 24 hours. Spots were counted with a computer-assisted image analysis system, Transtec 1300 ELISPOT Reader (AMI Bioline). The number of specific spots was calculated by subtracting the number of spots produced in the presence of unloaded T2 cells and spontaneously produced spots.

Flow cytometry
PBL from healthy subjects and CP were stained with αCD14-APC (clone M5E2), αHLA-DR-PerCP (clone L243; Biolegend), and αIL-4Rα-PE (clone 25463, R&D System) mAb to characterize the phenotype of CD14+ monocytes. Matched isotype controls were included for each sample. Changes in mean fluorescence intensity (MFI) values were calculated by subtracting the fluorescence of control isotypes.

Fluorescence-activated cell-sorting (FACS) analysis of cell surface molecules on transfected DCs was carried out using the following mAbs: αCD80-PE (clone 2D10), αCD86-PE (clone IT2.2), αCD40-PE (BD Biosciences), αCD83-PE (clone HB15e), and αHLA-DR-PerCP (Biolegend). To detect Treg cells, PBLs were stained with αCD4-PerCP (clone OKT4), αCD25-PE (clone BC961 Biolegend) mAbs on the cell surface, treated with Fixation and Permeabilization buffer (eBioscience), and stained with αFoxp3-FITC (clone 236A/E7) mAb (eBioscience).

To detect proliferating cells, PBLs were stained with αCD4-PerCP and αCD8-PE (clone H71/8a; Biolegend) mAbs on the cell surface, treated with Fixation and Permeabilization buffer, and stained with αKi-67-APC (clone Ki67) mAb (Biolegend).

For intracellular staining, 10^6 lymphocytes recovered after 7 days of co-culture with transfected DCs were resuspended in AIM-V and restimulated with 1 μg/mL coated αCD3 (clone OKT3, Biolegend) and 1 μg/mL soluble αCD28 (clone CD28.2, Biolegend) in the presence of 10 μg/mL BrefeldinA (Sigma) at 37°C for 6 hours. Cells were washed twice, and incubated with αCD8-PE and αCD4-PerCP mAb (Biolegend) at 4°C for 30 minutes. After treatment with Fixation and Permeabilization buffer, cells were stained with αIFNγ-FITC (clone B27) and α perforin-APC (clone dG9) mAb (Biolegend) for 30 minutes at 4°C.

To determine IL-10- and TGF-β–producing cells, lymphocytes were cultured with transfected DCs with or without Brefeldin A, respectively. Intracellular staining with αIL-10-PE (clone JES3-19F1, Biolegend) or surface staining with αLAP1-APC (clone TW4-2F8, Biolegend) were performed. Stained cells were acquired on a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences). Cells were gated according to their light scatter properties to exclude cell debris.

In vitro cytotoxicity assay
The 51Cr-release assay was performed at effector-to-target ratios of 50:1, 25:1, 12:1, and 6:1. CI-PEC1, SKOV-3-A2, and A549 target cells were labeled with 50 μL 51Cr sodium chromate (PerkinElmer) in 5% CO2 for 1 hour at 37°C, washed twice, and added to wells of 96-well plates (5 × 10^3 cells per well) with effector T cells recovered from 7-day co-cultures with transfected DCs. Assays were performed in triplicate in a final volume of 200 μL of RPMI-1640 with 10% heat-inactivated certified FBS. After 4-hour incubation, 50 μL of supernatants were collected on Lumaplate (PerkinElmer), and radioactivity was measured with a TopCount Scintillation Counter (Packard Biosciences). The percentage
of specific lysis was calculated by \((\text{experimental cpm} - \text{spontaneous cpm}) / \text{(maximal cpm} - \text{spontaneous cpm})\) × 100. Spontaneous release was always less than 20% of maximal release.

**Apoptosis assay**

An Annexin V-fluorescein isothiocyanate (FITC) staining assay was performed to measure apoptosis in SKOV-3-A2 cells, seeded in 24-well plates (5 × 10^4 per well), and exposed to different doses of human rIFN-γ (PeproTech) or supernatants derived from DC/T-cell co-cultures for 48 hours. Cells were then collected by trypsinization, washed twice with PBS, and stained with Annexin V-FITC and propidium iodide (PI; BD) for 15 minutes at room temperature. Positive cells were detected with flow cytometry.

**Cytokine analysis**

Supernatants collected at day 3 of DC/T-cell co-cultures were analyzed by ELISA for the presence of IL-10, TGF-β (both from eBioscience), and IFN-γ (Biolegend) following the manufacturer’s instruction.

**Mice**

NOD-SCID IL2Rαnull (NSG; 6-week-old female) mice were bred under sterile conditions in our animal facilities. A total of 1 × 10^6 SKOV-3-A2 tumor cells were injected subcutaneously in the left flank and tumor growth was measured twice a week with a caliper in 2 perpendicular diameters. Ten days after tumor challenge, 10^7 differently sized tumors were harvested at necropsy, fixed in 10% formalin, and dehydrated in 70% ethanol. The fixed samples were processed for IHC and then stained with antibodies specific to CD4, CD8, Ki-67, and p53 (clone Mib-1) mAb that were applied using the Ultra BenchMark automated stainer (Ventana, Roche). Images were acquired using 200× magnification and 4 fields per sample were pseudo-randomly selected. Percentage of positive nuclei was quantified by measuring the percentages of Ki-67+, CD8+, CD4+ cells, respectively, among the total mononuclear cells.

**Immunohistochemistry**

Tumors were harvested at necropsy, fixed in 10% formalin, and dehydrated in 70% ethanol. The fixed samples were then embedded in paraffin and 4 serial sections per tumor were obtained. Sections were processed for IHC using ωCD8 (clone C8/144B, Roche), ωCD4 (clone 4B12), or ωKi-67 (clone MiB-1) mAb that were applied using the Ultra BenchMark automated stainer (Ventana, Roche). Images were acquired using 200× magnification and 4 fields per sample were pseudo-randomly selected. Percentage of positive nuclei was quantified by measuring the percentages of Ki-67+, CD8+, CD4+ cells, respectively, among the total mononuclear cells.

**Ethics statement**

The human studies were conducted according to the Declaration of Helsinki principles. Human investigations were performed after approval of the study by the Scientific Ethics Committee of AO Città della Salute e della Scienza di Torino (Prot. No. 4.2/2012). Written informed consent was received from each participant before inclusion in the study and specimens were deidentified before analysis. All animal studies were performed in accordance with EU and institutional guidelines approved by the Bioethics Committee for Animal Experimentation of the University of Torino, Torino, Italy (Prot. No. 4.2/2012).

**Statistical analysis**

Statistical analyses were performed using Prism 5.0 GraphPad Software, and results are expressed as the mean ± SEM. One-way ANOVA was performed, followed by Dunnett multiple comparison post-test when needed. Kaplan–Meier survival curves were evaluated with both the log-rank Mantel–Cox and the Gehan–Breslow–Wilcoxon test. Only \(P < 0.05\) was considered to be significant.

**Results**

Only chimeric RHuT-DCs are able to elicit a specific anti-HER2 response by CD8 T cells from HER2-CP

Mature DCs (mDCs) were generated in vitro from CD14+ monocytes of healthy subjects and HER2-CP (Supplementary Table S1), as previously reported (23). CD14+ cells derived from HER2-CP express higher amounts of IL-4Rα and lower levels of HLA-DR molecules than from healthy subjects (Supplementary Fig. S1A). These data are in line with current notions indicating an expansion of a monocyte population with a myeloid-derived suppressor cell–like phenotype that correlates with tumor growth (24).

Despite these differences in their precursors, mDCs from both HER2-CP and healthy subjects expressed similarly high levels of the maturation markers and co-stimulatory molecules CD83, CD80, CD86, CD40, and HLA-DR (Supplementary Fig. S1B and S1C).

Nucleofection of the 4 plasmids (self HuHuT, chimeric HuRT, and RHuT, heterologous Rat) always gave a range of 35% to 45% positive mDCs from both healthy subjects and HER2-CP (Supplementary Fig. S2A), thus showing high reproducibility (Supplementary Fig. S2B). DCs transfected with pVAX1 plasmid (empty-DCs) were used as control. These results indicate that mDCs generated from HER2-CP display similar features and potential stimulatory capacity as those from healthy subjects.

To assess the ability of self versus heterologous and chimeric DNA plasmids to induce a specific anti-HER2 CD8+ T-cell response, mDCs generated from healthy subjects and HER2-CP were transfected with the different plasmids specified above and used to stimulate autologous T cells. After 7 days of co-culture, CD8 T cells from healthy subjects stimulated with self HuHuT-DCs or chimeric HuRT-DCs and RHuT-DCs displayed higher proliferative ability compared with those stimulated with empty-DCs, whereas heterologous Rat-DCs had no effect (Supplementary Fig. S3A and S3B). In contrast, only chimeric RHuT-DCs induced proliferation of CD8 T cells from HER2-CP (Supplementary Fig. S4A and S4B).

Activated T cells were then restimulated with anti-CD3/anti-CD28 mAb and analyzed for IFN-γ and perforin expression. A similar increase in the percentage of IFN-γ–producing CD8 T cells was obtained from co-culture of healthy subjects T cells with HuHuT-DCs or chimeric HuRT-DCs and RHuT-DCs compared with those with empty-DCs. Chimeric RuHT-DCs also triggered an increase in the
expression of perforin (Supplementary Fig. S3C). In contrast, only chimeric RHuT-DCs from HER2-CP led to the expression of both IFN-γ and perforin by CD8 T cells; transfection with the other plasmids had low or no effect (Supplementary Fig. S4C). The concomitant expression of IFN-γ and perforin in CD8 T cells implies their potential cytotoxic ability.

The specificity of the CD8 T-cell response against human HER2 was assessed by IFN-γ ELISPOT assay. Lymphocytes from HLA-A2+ healthy subjects and HER2-CP recovered from the different co-cultures were stimulated with HLA-A2+–matched T2 cells loaded with immunodominant HER2-derived E75 (25) and GP2 (26) peptides. The chimeric HER2 construct RHuT codes for both E75 and GP2 peptides whereas HuRT only for E75. Compared with control empty-DCs, self HuHuT-DCs and chimeric RHuT-DCs from healthy subjects were able to activate a significant number of IFN-γ–releasing T cells in response to both peptides whereas chimeric HuRT-DCs only in response to the GP2 peptide (Fig. 1A). In contrast, in CP, only chimeric RHuT-DCs were able to elicit peptide-specific IFN-γ production (Fig. 2A).

Figure 1. HuHuT-DCs, HuRT-DCs, RHuT-DCs, and Rat-DCs from healthy subjects elicit anti-HER2 CD8 response. A, activation of CD8+ T cells from healthy subjects after 7 days of co-culture with empty-DCs (○), HuHuT-DCs (□), HuRT-DCs (△), RHuT-DCs (○), or Rat-DCs (▼). IFN-γ ELISPOT assay performed after 7 days of culture with transfected DCs from healthy subjects (n = 17). IFN-γ release was evaluated in response to T2 cells pulsed with E75 or GP2 peptides. Values of peptide-specific spots were calculated by subtracting the number of spots against unloaded T2 from the number of spots against peptide-loaded T2. ***, P < 0.001; ****, P < 0.0001 compared with empty-DCs. B, cytotoxicity assay: after 7 days co-cultures, recovered T cells were tested in a 4-hour 51Cr release assay at different effector:target ratios against CF-PAC1, SKOV-3-A2, or A549 cells. Percentage of specific lysis was determined as described in Materials and Methods. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 compared with empty-DCs.

Figure 2. RHuT-DCs from HER2-CP elicit anti-HER2 CD8 T-cell response. Activation of CD8+ T cells from CP after 7 days of co-culture with empty-DCs (○), HuHuT-DCs (□), HuRT-DCs (△), RHuT-DCs (○), or Rat-DCs (▼). A, IFN-γ ELISPOT assay performed after 7 days of co-culture with transfected DCs from HER2-CP (n = 13). IFN-γ release was evaluated in response to T2 cells pulsed with E75 or GP2 peptides. ***, P < 0.0001 compared with empty-DCs. B, 51Cr release assay against CF-PAC1, SKOV-3-A2, or A549 cells. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 compared with empty-DCs.
Moreover, T cells activated by HuHuT-DCs, HuRT-DCs, RHuT-DCs, and Rat-DCs from healthy subjects were able to kill HER2⁺ CF-PAC1 and SKOV-3-A2 tumor cells, as evaluated by a 4-hour ⁵¹Cr release assay (Fig. 1B). In T cells from HER2-CP, stimulation with the chimeric RHuT-DCs seems to be more efficient in inducing cytotoxic activity (Fig. 2B). No lysis was observed against A549 (HER2⁺, HLA-A2⁺) control tumor cells.

Overall, these data suggest that DCs transfected with the chimeric plasmid RHuT were able to overcome tumor-induced dysfunction of T cells from HER2-CP and to induce a specific anti-HER2 CD8 cytotoxic response.

**Chimeric HER2-transfected DCs from HER2-CP elicit a T₃1 response**

To activate a stronger and longer lasting antitumor response, vaccines must not only elicit cytotoxic CD8 T cells but also T helper (T₃1) cells (27). We first evaluated CD4 T cells in *in vitro* proliferation. All self HuHuT-DCs, chimeric HuRT-DCs, and RHuT-DCs and heterologous Rat-DCs from healthy subjects triggered proliferation of autologous CD4 T cells to similar levels, as evaluated by Ki-67 staining (Fig. 3A and B). Conversely, only chimeric HuRT-DCs and RHuT-DCS from HER2-CP stimulated a significantly higher proliferation of CD4⁺ T cells compared with

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**Figure 3.** Chimeric HuRT-DCs and RHuT-DCs from HER2-CP elicit a T₃1 response. Proliferation and IFN-γ expression of CD4⁺ T cells after 7 days of co-culture with transfected DCs. A, representative expression of Ki-67 on CD4⁺ T gated cells of healthy subjects (top row) and HER2-CP (bottom row). Graphs show percentage of Ki-67⁺ on CD4⁺ T cells from 4 healthy subjects (B) and 7 HER2-CP (C). IFN-γ intracellular staining of CD4 T cells. After 7 days of co-culture with transfected DCs, recovered T cells were restimulated with antiCD3/CD28 mAb (1 μg/mL). Graphs show percentage of IFN-γ⁺ on CD4⁺ T gated cells from 5 healthy subjects (D) and 8 HER2-CP (E). *P < 0.05; **P < 0.01; ***P < 0.001; ******P < 0.0001 to empty-DCs.
empty-DCs, heterologous Rat-DCs, and self HuHuT-DCs (Fig. 3A and C). After 7 days of co-culture, activated T cells were restimulated with anti-CD3/CD28 mAb and analyzed for IFN-\(\gamma\) expression. Chimeric HuRT-DCs and RHuT-DCs from HER2-CP triggered a higher percentage of IFN-\(\gamma\)-producing CD4 T cells compared with the other groups (Fig. 3E). In contrast, DCs from healthy subjects transfected with the different plasmids all resulted in a similar increase in IFN-\(\gamma\)-producing CD4 T cells compared with empty-DCs (Fig. 3D).

This evidence suggests that DCs from HER2-CP transfected with both the chimeric HER2 plasmids are able to trigger a Th1 response.

T cells from HER2-CP activated by chimeric HuRT-DCs and RHuT-DCs impede HER2\(^+\) tumor growth in vivo

Next, we evaluated whether T cells from HER2-CP activated in vitro, with self or chimeric HER2-transfected DCs, were able to counteract growth of HER2\(^+\) cancer cells in vivo, in a therapeutic setting. Immunodeficient NSG mice were challenged subcutaneously in the left flank with 10\(^6\) SKOV-3-A2 cells. After 10 days, when mice were already displaying established palpable tumors, they were injected with 10\(^7\) in vitro--activated lymphocytes in the tail vein.

T cells activated by chimeric HuRT-DCs and RHuT-DCs were able to delay tumor growth, whereas mice treated with T cells activated by self HuHuT-DCs developed tumors with the same kinetics as mice receiving T cells activated by empty-DCs or PBS only (Fig. 4A). Moreover, T cells from co-cultures with HuRT-DCs and RHuT-DCs significantly improved overall survival (Fig. 4B). After 50 days following tumor challenge, 57% and 20% of mice injected with T cells activated by chimeric RHuT-DCs and HuRT-DCs, respectively, were still alive, compared with 0% of mice injected with T cells activated by empty-DCs or self HuHuT-DCs.

Lower tumor growth was consistent with a lower Ki-67 expression in tumors from mice injected with T cells recovered from co-cultures with RHuT-DCs and HuRT-DCs (% of Ki-67\(^+\) cells 32.3\(\pm\)2.6 and 37.6\(\pm\)5.8, respectively, vs. 63.9\(\pm\)1.5 in tumors from mice injected with T cells from co-cultures with HuHuT-DCs) and further supports the notion that these T cells are able to impede tumor growth (Fig. 4C). Moreover, immunohistochemical analysis showed that tumors from mice injected with T cells from co-cultures...
with RHuT-DCs displayed high levels of CD4 and CD8 infiltration throughout the tumor mass, whereas those receiving T cells from co-cultures with HuRT-DCs displayed only high amounts of CD4, concentrated at the periphery of the tumor growing area (Fig. 4C), suggesting a key role of these cells in countering tumor growth. Low or no T-cell infiltration was evident in tumors from the other treated groups.

Overall, these data demonstrate that DCs transfected with chimeric RHuT and HuRT plasmids activate T cells able to impede the growth of established tumors in vivo, and this effect seems to correlate with tumor infiltration of CD8 and/or CD4 T cells. CD4 T-cell–mediated inhibition of tumor growth is clearly independent of perforin, whereas cytokine secretion, such as IFN-γ, contributes to the impairment of tumor growth (28). Higher levels of IFN-γ were indeed detected in supernatants derived from co-cultures with chimeric HuRT-DCs and RHuT-DCs compared with the other co-cultures with self or heterologous HER2 (Fig. 5A), consistent with the higher intracytoplasmic expression of IFN-γ in both CD4 and CD8 T cells (Supplementary Fig. S4C and S3E).

To verify the role of IFN-γ in the inhibition of in vivo tumor growth, SKOV-3-A2 tumor cells were cultured for 48 hours with supernatants derived from the different co-cultures. Supernatants from both chimeric RHuT- and HuRT-DCs co-cultures induced higher percentages of apoptotic cells in comparison to those from empty-DC co-cultures (Fig. 5B and C). The addition of IFN-γ neutralizing mAb to the supernatants abrogated this effect. Moreover, when SKOV-3-A2 cells were cultured for 48 hours with increasing concentrations of recombinant human IFN-γ, from 0.5 to 8 ng/mL, a dose response apoptotic induction was observed (Fig. 5D).

These results suggest that the antitumor response elicited by chimeric RHuT-DCs and HuRT-DCs may be, in part, mediated by IFN-γ. However, the more potent antitumor response induced by co-culture with RHuT-DCs seems to also involve perforin-expressing CD8 T cells.

The inability of self HuHuT-DCs to activate T cells from HER2-CP against HER2 is dependent on IL-10 and TGF-β1 production

Many publications have already reported an expansion of tumor-induced regulatory cells in the peripheral blood of patients with cancer (29, 30). As we stimulated T cells with transfected DCs, it is conceivable that regulatory cells, already expanded in HER2-CP (Supplementary Fig. S5A).
could also be activated and expanded (31). However, we did not observe any differences in the percentage of CD4⁺ CD25⁺ FoxP3⁺ Treg cells after 7 days of co-culture with autologous DCs transfected with the 4 different plasmids (Supplementary Fig. S3B). Therefore, we evaluated the ability of Treg cells purified from the PBL of HER2-CP and cultured with differently transfected DCs to suppress the activation of CD4⁺ CD25⁻ autologous T cells. Treg cells co-cultured with HuHuT-DCs displayed a significantly higher suppressive activity compared with those with empty-DCs (Supplementary Fig. S5C).

The inability of DCs transfected with self HuHuT to induce an effective response of TH1 and CD8 T cells from HER2-CP could be attributed to soluble factors released by immune cells namely IL-10 (32) and TGF-β1 (33). We evaluated the presence of these cytokines in the supernatants of co-cultures. While comparably low levels of IL-10 were detected in co-cultures with empty-DCs, self HuHuT-DCs, chimeric RHuT-DCs, and heterologous Rat-DCs from healthy subjects, self HuHuT-DCs from HER2-CP induced a significantly higher production of IL-10 compared with empty-DCs. Interestingly, chimeric HuRT-DCs from both healthy subjects and HER2-CP stimulated high levels of IL-10 secretion (Fig. 6A). In cells from healthy subjects, DCs transfected with all 4 DNA plasmids induced the production of similar levels of TGF-β1, but in cells from HER2-CP,
self HuHuT-DCs stimulated higher secretion of TGF-β1 compared with the empty-DCs (Fig. 6B). In conclusion, an increase of both IL-10 and TGF-β1 was detected in cocultures of T cells from HER2-CP with HuHuT-DCs.

To assess whether IL-10 and TGF-β1 production had a role in inhibiting the CD8 and CD4 T-cell response against human HER2, lymphocytes from HER2-CP were activated with self HuHuT-DCs in the presence of anti-IL-10 and/or anti-TGF-β1 neutralizing mAb. Neutralization of both cytokines increased the ability of HuHuT-DCs to induce IFN-γ and perforin expression by CD8 T cells (Fig. 6C) and also a specific response against the immunodominant E75 and GP2 peptides (Fig. 6D) as well as RHuT-DCs (Fig. 2A). In addition, the ability of CD4 T cells to produce IFN-γ was also increased (Fig. 6E). Overall, these data strongly suggest that the presentation of self HER2 could promote suppressive mechanisms such as IL-10 and TGF-β1 production that impair antigen-specific CD8 and CD4 T-cell activation.

On the basis of our results, we hypothesized that in HER2-CP, HER2-specific tumor-induced regulatory cells are expanded and that self HuHuT-DCs could stimulate these cells to produce IL-10 and TGF-β1. To verify this hypothesis, we evaluated whether transfected DCs stimulated Treg cells to secrete these cytokines. Interestingly, HuHuT-DCs elicited higher expression of IL-10 (Fig. 6F) and TGF-β1–associated LAP (Fig. 6G) in CD4+Foxp3+ T cells compared with empty-DCs and DCs transfected with the other constructs. To further clarify this point, DCs generated from CTRL-CP with breast cancer and PDAC negative for HER2 struct. To further clarify this point, DCs generated from HER2-CP with empty-DCs and DCs transfected with the other constructs were co-cultured and autologous lymphocytes. In this case, self expression (Supplementary Table S2) were transfected and CTRL-CP with breast cancer and PDAC negative for HER2 struct.

Discussion

In the current study, we demonstrated, for the first time, that DNA plasmids coding for chimeric rat/human HER2 are able to elicit an effective immune response by T cells from HER2-CP and efficiently circumvent T-cell dysfunction. No T-cell response against HER2 was induced by autologous DCs transfected with DNA plasmids coding for self or fully heterologous HER2. In contrast, both self HuHuT-DCs and chimeric RHuT-DCs from healthy subjects, as well as those from CTRL-CP, in which there are no HER2-specific negative regulatory mechanisms, showed a similar induction of HER2-specific CD8 T-cell response.

Anti-HER2 vaccines consisting of MHC class I–restricted peptides demonstrated the ability to elicit immunologic responses and some clinical benefits in disease-free patients with breast cancer (34). However, the efficacy of the immune response required for antigen-specific tumor inhibition depends not only on correct antigen presentation by DCs and activation of cytotoxic CD8 T cells but also on the magnitude of CD4 T11 reactivity (35, 36). Indeed, vaccination of patients with cancer with both T11 epitopes and MHC class I–binding motifs elicited enhanced HER2 peptide–specific cytotoxic T lymphocyte (CTL) expansion and provided durable responses detectable more than 1 year after the final vaccination (37).

Nevertheless, HLA restriction limits the potential number of patients who can receive these vaccines, and the use of DNA plasmids coding for tumor antigens has therefore been shown to be advantageous (19). Vaccines able to induce both CD8 and CD4 responses, and hence CTL and humoral immunity, are considered better than vaccines able to induce just one response.

Here, we demonstrated that different combinations of rat/human HER2 sequences induce anti-HER2 immune responses through different mechanisms, suggesting that the position of heterologous moieties is determinant for overcoming tolerance to HER2 or exhaustion of CD4 and CD8 T cells (38). Moreover, CD4+ T11 cells can directly mediate tumor inhibition through cytokine secretion, such as IFN-γ, which may induce cytotoxic and cytostatic effects on tumor cells as well as their senescence (39). Indeed, chimeric-transfected DCs from HER2-CP elicited enhanced T-cell IFN-γ secretion that induced apoptosis of cancer cells.

In recent years, a number of reports have identified Treg cells specific for a range of different tumor antigens in human cancer, including HER2 (40). The presence of these cells in patients with cancer raises serious concerns about the potential of cancer vaccines to expand not only effector but also regulatory cells. Many cancer vaccines have failed to induce significant clinical benefits, despite the induction of seemingly potent tumor antigen–specific responses (41, 42).

Vaccination with a xenogeneic antigen has been reported to be effective in overcoming the immunologic tolerance to self-proteins (43). Results obtained from transgenic mouse models demonstrated that vaccination with DNA plasmids coding for xenogenic HER2 elicited a strong immunologic response without cross-reaction (16). Chimeric rat/human HER2 plasmids were most effective in blunting immune tolerance to both rat and human HER2, suggesting that the presence of heterologous regions enhances immunogenicity against the antigen (17, 18). Thus, the self-sequence ensures the specificity of the immune response, whereas the xenogeneic part circumvents immune tolerance.

Increased levels of Treg cells were observed both in our cohort of patients and in patients with different malignancies and are often associated with worse outcomes (44). Treg...
cells inhibit primary T-cell activation and are paradoxically expanded by tumor vaccines coding for self-sequences (45–48). Indeed, we show that HuHuT-DCs did not affect Treg expansion, but elicited their stronger suppressive ability, probably due to their increased production of IL-10 and TGF-β1. It is possible that DCs transfected with self-HuHuT present the immunodominant peptides recognized by Treg cells or by exhausted T cells. In contrast, the combination of heterologous sequences, as present in chimeric RHuUT and HuRT, counteracts this phenomenon by presenting additional non-self-peptides, activating new TH cells able to release cytokines that rescue bystander dysfunctional CD8+ T and B lymphocytes specific for autologous HER2 epitopes. The chimeric benefit is even more evident considering that the heterologous rat sequence alone was not sufficient to circumvent tumor-induced unresponsiveness to HER2. This may be due to the repertoire of peptides generated by the processing of the complete rat protein. Most of these peptides are not shared with the HER2 sequence but are predicted to have a binding affinity to HLA-I superior or comparable to that of HER2 peptides and thus compete with them for presentation to CD8+ T cells, indeed reducing the anti-HER2 response.

The weakly induced suppressive machinery, such as IL-10 and TGF-β1 secretion, seemed to represent the success of chimeric variants in activating antitumor responses. We observed a higher production of the suppressive cytokines IL-10 and TGF-β1 in HuHuT-DC co-cultures from HER2-CP, but not from CTRL-CP or healthy subjects. Moreover, when we blocked the effects of these cytokines by adding neutralizing antibodies, the ability of self-sequences to activate both CD4 and CD8 responses was restored. These results further confirm the key role of IL-10 (49) and TGF-β1 (50) in suppressing an antigen-specific CD8 T-cell response in patients with cancer and in inhibiting antitumor immune responses.

Our results provide the proof of concept that chimeric rat/human HER2 DNA constructs are able to overcome tumor-induced dysfunction of T cells from HER2-CP and elicit an efficient anti-HER2 response that avoids the activation of regulatory mechanisms. Therefore, chimeric HER2 DNA plasmids, or DCs transfected with these plasmids, could represent a novel therapeutic approach for all patients with HER2-overexpressing cancer and introduce a new concept for designing anti-cancer vaccines.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Occhipinti, S. Rolla, P. Cappello, F. Novelli, M. Giovarelli
Development of methodology: S. Occhipinti, L. Sponton, C. Caorsi, F. Cavallo, C. Marchini, A. Amici, F. Cavallo, M. Giovarelli
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Occhipinti, L. Sponton, A. Novarino, M. Donadio, S. Bustreo, M.A. Satolli, D. Pierobon, M. Giovarelli
Analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis): S. Occhipinti, L. Sponton, C. Caorsi, A. Amici, D. Pierobon, F. Novelli, M. Giovarelli
Writing, review, and/or revision of the manuscript: S. Occhipinti, S. Rolla, P. Cappello, F. Cavallo, M. Giovarelli
Administrative, technical or material support (i.e. reporting or organizing data, constructing databases): S. Occhipinti, L. Sponton, A. Novarino, C. Pecchioni, C. Marchini, M. Giovarelli
Study supervision: M. Giovarelli

Grant Support
This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro to M. Giovarelli (AIRC IG, n. 9366), to F. Cavallo (AIRC IG, n. 5377 and 11675); to F. Novelli (AIRC 5 × 1000 [no. 12182] and IG [no. 5548 and 11643]). European Community, Seventh Framework Program European Pancreatic Cancer-Tumor-Microenvironment Network (EPC-TM-Net, no. 256974); Regione PIEMONTE: Ricerca Industriale “Converging Technologies” (BIOHER), Progetti strategici su tematiche di interesse regionale o sovra regionale (IMMONC); Ministero dell’Istruzione e della Ricerca (MIUR), Progetti di Rilevanza Nazionale (PRIN 2009); University of Torino-Progetti di Ateneo 2011: Mechanisms of Resistance to anti-angiogenesis regimens THERapy (grant Rehi-ORTO11RTK); Fondazione Ricerca Molinette-Onlus, the University of Torino and the Compagnia di San Paolo (Progetti di Ricerca Ateneo/CSP).

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Received September 27, 2013; revised February 6, 2014; accepted February 17, 2014; published OnlineFirst March 25, 2014.


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Sergio Occhipinti, Laura Sponton, Simona Rolla, et al.

Clin Cancer Res  Published OnlineFirst March 25, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-2663

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2014/03/25/1078-0432.CCR-13-2663.DC1

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