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Running Title: Chimeric HER2 DNA overcome HER2 tolerance of cancer patients

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

Financial Support

This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro to MG (AIRC IG, n. 9366), to FC (AIRC IG, n. 5377); to FN (AIRC 5 x 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" (BIOTHER), Progetti strategici su tematiche di interesse regionale o sovra regionale (IMMONC) ; Ministero dell'Istruzione e della Ricerca (MIUR), Progetti di Rilevante Interesse Nazionale (PRIN 2009); University of Torino-Progetti di Ateneo 2011: Mechanisms of REsistance to anti-angiogenesis regimens THErapy (grant Rethe-ORTO11RKTW).; Fondazione Ricerca Molinette Onlus, the University of Torino and the Compagnia di San Paolo (Progetti di Ricerca Ateneo/CSP)

Key Words: HER2, DNA vaccine, dendritic cells, breast cancer, pancreatic cancer

TRANSLATIONAL RELEVANCE

DNA vaccines combining human and heterologous HER2 sequences have been shown to be efficacious in transgenic mouse models, but no data are available in humans. This work assessed the feasibility of using DNA coding for chimeric rat/human HER2 as a tool to overcome the tolerance of T cells from HER2-overexpressing cancer patients (CP). While dendritic cells from CP transfected with DNA plasmids coding for human HER2 do not activate T cells from CP, those transfected with chimeric rat/human HER2 induced antigen-specific perforin and IFN-γ-production by T cells, able to inhibit *in vivo* HER2+ tumor growth. The efficacy of chimeric plasmids relies on the ability to circumvent the suppressor effects by Treg cells and/or IL-10 and TGF-β1.

These results provide for the first time the proof of concept that chimeric HER2 DNA plasmids can be used as effective vaccine for all HER2-overexpressing CP, with the advantage of being MHC independent.

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 tolerance of T cells from HER2-overexpressing cancer patients.

Experimental Design: Dendritic cells (DCs) generated from HER2-overexpressing breast (n=24) and pancreatic (n=14) cancer patients 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 *in vivo* growth.

Results: Specific sustained proliferation and IFN-γ production by cancer patients CD4 and CD8 T cells was observed after stimulation with autologous DC transfected with chimeric rat/human HER2-expressing plasmids. Instead, T cells from healthy donors (n=20) could be easily stimulated with autologous DCs transfected with any human, rat or chimeric rat/human HER2 portions. Chimeric HER-2-transfected DCs from HER2-overexpressing cancer patients were also able to induce a sustained T cell response that significantly impeded $HER2^{+}$-tumor *in vivo* growth. The efficacy of chimeric plasmids in overcoming tolerance to HER2 relies on the ability to circumnvent suppressor effects by Treg cells and/or IL-10 and TGF-β1.

Conclusions: These results provide the proof of concept that chimeric rat/human HER2 DNA plasmids can be used as effective vaccines for all HER2-overexpressing cancer patients, independently of their MHC.

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, for example, 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, suggesting compensatory activity via alternative signaling pathways (5,6). Therefore, active immunotherapy against HER2 might provide an alternative strategy.

Vaccination studies in mouse models using HER2/neu peptides have been successful in inhibiting tumor growth (7). In humans, however, whilst specific immunological responses have been elicited with HER2 peptides, effective clinical results have not yet been obtained (8). The principal reason for this is that HER2/neu is a self-antigen and thus tolerogenic. Based on 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 (9). However low avidity self-specific T cells can be isolated from tolerant hosts and there are reports that such cells can be activated, expanded, and involved in antitumor responses (10).

A lot of effort has been spent in trying to overcome self-tolerance towards tumor antigens, and considerable success has been achieved using heteroclitic or heterologous peptides (11-13). The use of heterologous peptides may also be advantageous in case of patients with HER2-overexpressing tumors due to the presence of critical amino acid substitutions that markedly improve their immunogenicity and induce activation of non-

tolerized, cross-reactive and low affinity T cell clones. 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 (14,15). However, no data on their potential efficacy in humans are currently available. Compared to peptide-based vaccines, DNA vaccination has been shown to be more advantageous (16). 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 MHC independent (for a review see 17,18). 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 (19).

Here we evaluated the feasibility of using DNA coding for chimeric rat/human HER2 as a tool to counteract the tolerance of T cells from HER2-overexpressing cancer patients (CP). We transfected monocyte-derived dendritic cells (DCs) from CP and healthy subjects (HS) 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-overexpressing CP. Their ability relies on the activation of a significant lower number of Treg cells and lower production of IL-10 and TGFβ1 that results in the rescue from HER2 tolerance.

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 patients with HER2+ tumors, with the advantage of being MHC-independent.

Materials and Methods

Human specimens

Human peripheral blood leukocytes (PBL) were isolated by Ficoll-Hypaque (Lonza) gradient centrifugation from heparinized venous blood of HS (n=20) provided by the local Blood Bank (Torino, Italy), and CP (n=38), not previously treated with radio- or chemo-therapy. CP included patients with pancreatic adenocarcinoma (PDAC, n=14) or breast cancer (BC, n=24) recruited at the Centro Oncologico Ematologico Subalpino (COES), AO Città della Salute e della Scienza di Torino, Torino, Italy, with informed consent. Blood samples were immediately processed after drawing. Tumors from PDAC and BC patients were evaluated for HER2 positivity by immunohistochemistry (IHC). Only patients bearing $HER2^+$ tumors that were classified as 3+ or 2+ by IHC were included in the study (Supplementary Table S1). Patients with a 0-1 IHC score (n=5) were used as a HER2 negative control group (Supplementary Table S2). To determine human leukocyte antigen (HLA)-A2 positivity, PBL were incubated with anti-HLA-A2-PE mAb (clone BB7.2, BD Pharmingen) and expression was evaluated by flow cytometry.

Cell cultures

Monocyte-derived DC generation was conducted as previously described (20). 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 x 10^6/ml. At day 3, one third of supernatants was collected and replaced with fresh complete medium plus IL-7 (10 ng/ml, PeproTech).
The human pancreatic cancer cell line CF-PAC1 and the human ovarian carcinoma cell line SKOV-3-A2 (derived from SKOV-3 cells transduced by lentiviral vector with HLA-A2

gene), positive for the expression of HER2 and HLA-A2, were cultured in DMEM medium (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 pVAX1 was the backbone for all the DNA constructs used for transfection of DCs. All four plasmids code for the extracellular and transmembrane domains of HER2, as previously described (14). HuHuT codes for the fully human, and RRT for the fully rat HER2 molecule. RHuT codes for the first two extracellular domains of rat HER2 and the remaining part of human HER2. Conversely, HuRT codes for the first two 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 100µl of electroporation buffer (DC transfection kit, Amaxa, Lonza) and mixed with 5µg of plasmid DNA. Electroporation was performed using the Nucleofector program U-002 (Amaxa, Lonza). After electroporation, cells were immediately transferred to 2ml 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 Ab4 or Ab5 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 manifacturer's instruction. T2 cells were loaded with 10 µg/ml of the HLA-$A2^+$ immunodominant p369-377 E75 (KIFGSLAFL) or p654-662 GP2 (IISAVVGIL) peptides (PRIMM), for 6 hours at 37°C in serum-free medium. A total of 2.5 x 10^4 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 HS 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. ΔMFI values were calculated by subtracting the fluorescence of control isotypes.

FACS analysis of cell surface molecules on transfected DCs was carried out using the following mAb 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, PBL were stained with αCD4-PerCP (clone OKT4), αCD25-PE (clone BC96) (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, PBL were stained with αCD4-PerCP and αCD8-PE (clone HIT8a) (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-co-culture with transfected DCs, were resuspended in AIM-V, and restimulated with 1μg/ml coated αCD3 (clone OKT3, Biolegend) and 1μg/ml soluble αCD28 (clone CD28.2, Biolegend) in the presence of 10μg/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 min. After treatment with Fixation and Permeabilization buffer, cells were stained with αIFNγ-FITC (clone B27) and αperforin-APC (clone dG9) mAb (Biolegend) for 30 min at 4° C. 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 ^{51}Cr-release assay was performed at Effector-to-Target ratios of 50:1, 25:1, 12:1 and 6:1. CF-PAC1 and SKOV-3-A2 target cells were labeled with 50µl ^{51}Cr sodium chromate (PerkinElmer) in 5% CO_2 for 1 hour at 37°C, washed twice and added to wells of 96-well plates (5 x 10^3 cells/well) with effector T cells recovered from 7-day co-cultures with transfected DCs. Assays were performed in triplicate in a final volume of 200µl of RPMI 1640 with 10% heat-inactivated certified FBS. After 4 hour incubation, 50µl 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 ([experimental cpm - spontaneous cpm]/ [maximal cpm - spontaneous cpm]) x 100. Spontaneous release was always < 20% of maximal release.

Apoptosis assay

An Annexin V-FITC staining assay was performed to measure apoptosis in SKOV-3-A2 cells, seeded in 24-well plates (5 x 10^4/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 PI (BD) for 15 min at room temperature. Positive cells were detected with flow cytometry.

Cytokine analysis

Supernatants collected at day 3 of T cell-DC co-culture were analyzed by ELISA for the presence of IL-10 and TGFβ (eBioscience) and IFN-γ (Biolegend) following the manufacturer's instruction.

Mice

NOD-SCID IL2Rγ^{null} (NSG; 6-week-old female) mice were bred under sterile conditions in our animal facilities. One x 10^6 SKOV-3-A2 tumor cells were injected subcutaneously (s.c.) in the left flank and tumor growth was measured twice a week with a caliper in two perpendicular diameters. Ten days after tumor challenge, 10^7 differently *in vitro*-activated T cells were injected in the tail vein. The appearance of a tumor diameter of 5 mm was considered as death event.

Immunohistochemistry

Tumors were harvested at necropsy, fixed in 10% formalin and dehydrated in 70% ethanol. The fixed samples were then embedded in paraffin and four sequential serial sections/tumor were obtained. Sections were processed for IHC using αCD8 (clone C8/144B, Roche), αCD4 (clone 4B12) or αKi-67 (clone Mib-1) mAb which were applied using the Ultra BenchMark automated stainer (Ventana, Roche). Images were acquired using 20x magnification and 4 fields/sample were pseudo-randomly selected. Percentage of positive nuclei were 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, Torino, Italy (Prot. No. 0085724 and 0012068). Written informed consent was received from each participant prior to inclusion in the study and specimens were de-identified prior to 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, 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's multiple comparison post-test when needed. Kaplan-Mayer survival curves were evaluated with both the Log Rank Mantel-Cox and the Gehan-Breslow-Wilcoxon test. Only p values <0.05 were considered to be significant.

RESULTS

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

Mature DCs (mDCs) were generated *in vitro* from $CD14^+$ monocytes of HS and CP (Supplementary Table S1), as previously reported (26). $CD14^+$ cells derived from CP express higher amounts of IL-4Rα and lower levels of HLA-DR molecules compared to those from HS (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 (21).

Despite these differences in their precursors, mDCs from both CP and HS expressed similarly high levels of the maturation markers and co-stimulatory molecules CD83, CD80, CD86, CD40 and HLA-DR (Supplementary Fig. S1, B and C).

Nucleofection of the four plasmids self HuHuT, chimeric HuRT and RHuT, heterologous RRT always gave a range of 35-45% positive mDCs from both HS and 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 CP display similar features and potential stimulatory capacity as those from HS.

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 HS and HER2-overexpressing 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 HS stimulated with self HuHuT- or chimeric HuRT- and RHuT-DCs displayed higher proliferative ability compared to those stimulated with empty-DCs, while heterologous RRT-DCs had no effect

(Fig. 1, A and B). By contrast, only chimeric RHuT-DCs induced proliferation of CD8 T cells from HER2-overexpressing CP (Fig. 2, A and B).

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 HS T cells with HuHuT- or chimeric HuRT- and RHuT-DCs compared to those with empty-DCs. Chimeric RuHT-DCs also triggered an increase in the expression of perforin (Fig. 1C). By contrast, only chimeric RHuT-DCs from HER2-overexpressing CP led to the expression of both IFN-γ and perforin by CD8 T cells; transfection with the other plasmids had no effect (Fig. 2C). 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-$A2^+$ HS and CP recovered from the different co-cultures were stimulated with HLA-$A2^+$ matched T2 cells loaded with immunodominant HER2-derived E75 (22) and GP2 (23) peptides. Compared to control empty-DCs, self HuHuT- and chimeric RHuT-DCs from HS were able to activate a significant number of IFN-γ-releasing T cells in response to both peptides, while chimeric HuRT-DCs only in response to the GP2 peptide (Fig. 1D). By contrast, in CP, only chimeric RHuT-DCs were able to elicit peptide-specific IFN-γ production (Fig. 2D).

Moreover, T cells activated by HuHuT-, HuRT- and RHuT-DCs from HS were able to kill $HER2^+$ CF-PAC1 and SKOV-3-A2 tumor cells, as evaluated by a 4-hour ^{51}Cr release assay (Fig. 1E). In T cells from HER2-overexpressing CP, only stimulation with the chimeric RHuT-DCs led to destruction of the tumor cells (Fig. 2E).

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

Chimeric HER2-transfected DCs from HER2-overexpressing CP elicit a Th1 response

In order to activate a stronger and longer-lasting antitumor response, vaccines must not only elicit cytotoxic CD8 T cells but also Th1 cells (24). We first evaluated CD4 T cells in *in vitro* proliferation. All self HuHuT-, chimeric HuRT- and RHuT- and heterologous RRT-DCs from HS triggered proliferation of autologous CD4 T cells to similar levels, as evaluated by Ki--67 staining (Fig. 3, A and B). Conversely, only chimeric HuRT- and RHuT-DCs from HER2-overexpressing CP stimulated a significantly higher proliferation of $CD4^+$ T cells compared to empty-DCs, while heterologous RRT- and self HuHuT-DCs had no effect (Fig. 3, A and C). After 7 days of co-culture, activated T cells were restimulated with anti-CD3/CD28 mAb and analyzed for IFN-γ expression. Only chimeric HuRT- and RHuT-DCs from CP triggered a higher percentage of IFN-γ-producing CD4 T cells compared to empty-DCs (Fig. 3E). By contrast, DCs from HS transfected with the different self HuHuT, chimeric HuRT and RHuT and heterologous RRT plasmids all resulted in a similar increase in IFN-γ-producing CD4 T cells compared to empty-DCs (Fig. 3D).

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

T cells from HER2-overexpressing CP activated by chimeric HuRT- and RHuT-DCs impede $HER2^+$ tumor growth *in vivo*.

Next we evaluated whether T cells from HER2-overexpressing CP activated *in vitro,* with self or chimeric HER-2-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- and RHuT-DCs were able to delay tumor growth, whilst 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- 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- and HuRT-DCs, respectively, were still alive, compared to 0% of mice injected with T cells activated by empty- 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- and HuRT-DCs (% of Ki-67^+ cells 32,3$\pm$2.6 and 37.6$\pm$5.8, respectively, versus 63.9+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, while 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 counteracting tumor growth. Low or no T cell infiltration was evident in tumors from the other treated groups.

Overall these data demonstrated that DCs transfected with chimeric RHuT and HuRT plasmids activate T cells able to impede the growth of established tumors *in vivo*, via different mechanisms, either mediated by both CD8 and CD4 or only CD4 T cells, respectively. CD4 T cell-mediated inhibition of tumor growth is clearly independent of perforin, while cytokine secretion, such as IFN-γ, contributes to the impairment of tumor growth (25). Higher levels of IFN-γ were indeed detected in supernatants derived from co-cultures with chimeric HuRT- and RHuT-DCs compared to the other co-cultures with self

or heterologous HER2 (Fig. 5A), consistent with the higher intracytoplasmatic expression of IFN-γ in both CD4 and CD8 T cells (Fig. 2C and 3E).

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. 5, B 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. 5 D).

These results suggest that the antitumor response elicited by chimeric RHuT- 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-overexpressing CP against HER2 is dependent on IL-10 and TGFβ1 production.

Many publications have already reported an expansion of regulatory cells in the peripheral blood of cancer patients (26,27). As we stimulated T cells with transfected DCs, it is conceivable that regulatory cells, already expanded in CP (Supplementary Fig. S3A), could also be activated and expanded (28). 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 four different plasmids (Supplementary Fig. S3B). Therefore we evaluated the ability of Treg cells purified from the PBL of HER2-overexpressing 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 to those with empty-DCs (Supplementary Fig. S3C).

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 (29) and TGFβ1 (30). 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-, self HuHuT-, chimeric RHuT- and heterologous RRT-DCs from HS, self HuHuT-DCs from HER2-overexpressing CP induced a significantly higher production of IL-10 compared to empty-DCs. Interestingly, chimeric HuRT-DCs from both HS and CP stimulated high levels of IL-10 secretion (Fig. 6A). In cells from HS, DCs transfected with all four DNA plasmids induced the production of similar levels of TGFβ1, but in cells from CP, self HuHuT-DCs stimulated higher secretion of TGFβ1 compared to the empty-DCs (Fig. 6B). In conclusion, an increase of both IL-10 and TGFβ1 were detected in co-cultures of T cells from HER2-overexpressing CP with HuHuT-DCs.

To assess whether IL-10 and TGFβ1 production had a role in inhibiting the CD8 and CD4 T cell response against human HER2, lymphocytes from CP were activated with self HuHuT-DCs in the presence of anti-IL-10 and/or anti-TGFβ1 neutralizing mAb. Neutralization of both cytokines restored the ability of HuHuT-DCs to induce not only IFN-γ and perforin expression by CD8 T cells (Fig. 6C), but also a specific response against the immunodominant E75 and GP2 peptides (Fig. 6D) as well as RHuT-DCs (Fig. 2D). In addition, the ability of CD4 T cells to produce IFN-γ was also rescued (Fig. 6E). Overall, these data strongly suggest that the presentation of self HER2 could promote suppressive mechanisms, such as IL-10 and TGFβ1 production that impair antigen-specific CD8 and CD4 T cell activation.

Based on our results we hypothesized that, in HER2-overexpressing CP, HER2-specific regulatory cells are expanded and that self HuHuT-DCs could stimulate these cells

to produce IL-10 and TGF-β1. To clarify this point, DCs generated from CP with breast and pancreatic adenocarcinoma negative for HER2 expression, (Supplementary Table S2) were transfected and co-cultured with autologous lymphocytes. In this case self HuHuT-DCs did not suppress the production of IFN-γ and perforin by CD8 T cells, (Supplementary Fig. S4A) similarly to what was observed in co-cultures from HS (Fig. 1A). Moreover, self HuHuT-DCs were also able to expand CD4 T cells expressing IFN-γ, as for chimeric HuRT- and RHuT-DCs and heterologous RRT-DCs (Supplementary Fig. S4B). Notably, self HuHuT-DCs from CP bearing HER2-negative tumors did not induce suppressive mechanisms such as IL-10 (Supplementary Fig. S4C) and TGFβ1 production (Supplementary Fig. S4D).

These data indicate an increase of HER2-specific regulatory cells in HER2-overexpressing CP as a result of antigen overexpression that can be restimulated by the total self sequence of HER2.

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-overexpressing CP and efficiently circumvent tolerance mechanisms. No T cell response against HER2 was induced by autologous DCs transfected with DNA plasmids coding for self or fully heterologous HER2. By contrast, both self HuHuT- and chimeric RHuT-DCs from HS, as well as those from patients bearing HER2-negative tumors, 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 immunological responses and some clinical benefits in disease-free breast cancer patients (31). 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 Th reactivity (32,33). Indeed, vaccination of cancer patients with both Th epitopes and MHC class I binding motifs elicited enhanced HER2 peptide-specific CTL expansion and provided durable responses detectable more than 1 year after the final vaccination (34).

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 (16). 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 by CD4 and CD8 T cells from HER2-overexpressing CP.

CD4 Th cells provide critical signals for priming and maintenance of effector T cells (32). Moreover, $CD4^+$ Th1 cells can directly mediate tumor inhibition through cytokine secretion, such as IFN-γ, which may induce cytotoxic and cytostatic effects on tumor cells (35) as well as their senescence (36). Indeed, chimeric-transfected DCs from HER2-overexpressing 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 (37). The presence of these cells in cancer patients 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 (38,39).

Vaccination with a xenogeneic antigen has been reported to be effective in overcoming the immunological tolerance to self proteins (40). Results obtained from transgenic mouse models demonstrated that vaccination with DNA plasmids coding for xenogeneic HER2 elicited a strong immunological response without cross-reaction (13). 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 (14,15). Thus, the self sequence ensures the specificity of the immune response, while 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 associated with worse outcomes (41). Treg cells inhibit primary T-cell activation and are paradoxically expanded by tumor vaccines coding

for self sequences (42-45). Indeed, we show that HuHuT-DCs did not affect Treg expansion, but elicited their stronger suppressive ability. It is possible that DCs transfected with self HuHuT presented the immunodominant peptides recognized by Treg cells. By contrast, the combination of heterologous sequences, as present in chimeric RHuT and HuRT, counteracts this phenomenon by presenting additional non-self peptides, activating new Th cells able to release cytokines that rescue bystander anergic T and B lymphocytes

The weakly-induced suppressive machinery, such as IL-10 and TGFβ1 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 TGFβ1 in HuHuT-DC co-cultures from HER2-overexpressing CP, but not from HER2 negative CP or HS. 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 (46) and TGFβ1 (47) in suppressing an antigen-specific CD8 T cell response in cancer patients and in inhibiting anti-tumor immune responses. Whether these cytokines affect the stimulatory capacity of DCs, or directly affect antigen-specific T cell activation still needs to be determined.

Our results provide the proof of concept that chimeric rat/human HER2 DNA constructs are able to overcome tolerance of T cells from HER2-overexpressing 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.

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LEGENDS TO FIGURES

Figure 1. HuHuT-, HuRT- and RHuT-DCs from HS elicit anti-HER2 CD8 response. (A) Representative expression of Ki-67 on CD8+ T cells after 7 day of co-culture with empty- (○), HuHuT- (□), HuRT- (△), RHuT- (◇) or RRT-DCs (▽). Results from one of four experiments are shown. (**B**) Percentage of Ki-67+ on CD8+ T gated cells from 4 HS. * $p<0.05$ compared to empty-DCs. (**C**) IFN-γ and perforins intracellular staining of CD8 T cells. After 7 day of co-culture T cells were restimulated with αCD3/CD28 mAb (1μg/ml). Graphs show percentage of IFN-γ+ (left panel) and perforins+ (right panel) cells in CD8+ T gated cells from eight HS. *$p<0.05$, ** $p<0.001$ compared to empty-DCs. (**D**) IFN-γ ELISPOT assay performed after 7 days of culture with transfected DCs from HS (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 to empty-DCs. (**E**) Cytotoxicity assay. After 7 day co-cultures, recovered T cells were tested in a 4 h ^{51}Cr release assay at different Effector:Target ratios against CF-PAC1 or SKOV-3-A2 cells. Percentage of specific lysis was determined as described in Methods. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$ compared to empty-DCs.

Figure 2. RHuT-DCs from HER2-overexpressing CP elicit anti-HER2 CD8 T cell response. (A) Representative expression of Ki-67 on CD8+ T cells after 7 day of co-culture with empty- (○), HuHuT- (□), HuRT- (△), RHuT- (◇) or RRT-DCs (▽). Results from one of six experiments are shown (**B**) Percentage of Ki-67+ on CD8+ T gated cells from six HER2-overexpressing CP. * $p<0.05$. compared to empty-DCs (**C**) Percentage of IFNγ+ (left panel) and perforins+ (right panel) on CD8+ T gated cells from HER2-overexpressing CP (n=12). *** $p<0.0001$ compared to empty-DCs. (**D**) IFN-γ ELISPOT assay performed after 7 days co-culture with transfected DCs from HER2-overexpressing

CP (n=13). IFN-γ release was evaluated in response to T2 cells pulsed with E75 or GP2 peptides. *** p<0.0001 compared to empty-DCs. (**E**) ^{51}Cr release assay against CF-PAC1 or SKOV-3-A2 cells. ** p<0.001, *** p<0.0001 compared to empty-DCs.

Figure 3. Chimeric HuRT- RHuT-DCs from HER2-overexpressing CP elicit a Th1 response. Proliferation and IFN-γ expression of CD4+ T cells after 7 day co-culture with transfected DCs. **(A)** Representative expression of Ki-67 on CD4+ T gated cells of HS (upper row) and HER2-overexpressing CP (lower row). Graphs show percentage of Ki-67+ gated on CD4+ T cells from four HS (**B**) and seven HER2-overexpressing CP (**C**). IFN-γ-intracellular staining of CD4 T cells. After 7 day of co-cultures with transfected DCs, recovered T cells were restimulated with αCD3/CD28 mAb (1μg/ml). Graphs show percentage of IFNγ+ on CD4+ T gated cells from five HS (**D**) and eight HER2-overexpressing CP (**E**). * p<0.05, ** p<0.001, *** p<0.0001 compared to empty-DCs.

Figure 4. T cells from HER2-overexpressing CP activated in vitro with HuRT- and RHuT-DCs are able to inhibit HER2+ tumor growth. One x 10^6 SKOV-3-A2 cells were injected s.c. in the left flank of NSG mice. Mice were injected i.v. with 10^7 in vitro activated T cells with empty- (○, n=5), HuHuT- (□, n=5), HuRT- (△, n=5), RHuT-DCs (◇, n=7) or PBS (●, n=12) at day 10 after tumor challenge. **(A)** Tumor growth was monitored weekly and expressed as tumor volume. ** p<0.001, *** p<0.0001 (**B**) Kaplan-Meier survival analysis of untreated and treated mice. Tumor diameter of 5 mm was considered as lethal event. * p<0.05, ** p<0.001 compared to untreated group. (**C**) Representative immunohystochemical staining of tumor sections from mice injected with T cells recovered from co-cultures with transfected DCs, for Ki-67 (upper row), CD8 (middle row) or CD4 (lower row) expression. The percentage of positive cells on total cells evaluated in each single mouse is reported as mean±SEM (n=3 per group). * p<0.05, ** p<0.001, *** p<0.0001 compared to empty-DCs.

Figure 5. HuRT- and RHuT-DCs from HER2-overexpressing CP elicit enhanced IFN-γ production. (A) IFN-γ production analyzed by ELISA on supernatants of co-cultures of T cells from CP with autologous transfected DCs (n=15) collected at day 3. *** p<0.0001. compared to empty-DCs. **(B)** Representative AnnexinV/PI assay of SKOV-3-A2 cells cultured for 48 h with supernatants derived from DC co-cultures. (**C)** Graphs show the percentage of AnnexinV+PI+ SKOV-3-A2 cells cultured for 48 h with supernatants derived from co-cultures of three different CP. ** p<0.001 compared to empty-DCs**. (D)** AnnexinV/PI assay of SKOV-3-A2 cells cultured for 48 h with indicated concentration of human rIFN-γ.

Figure 6. IL-10 and TGFβ1 neutralization both restore the ability of HuHuT-DCs to activate anti-HER2 T cell responses from HER2-overexpressing CP. IL-10 **(A)** and TGFβ1 **(B)** production analyzed by ELISA on supernatants from co-cultures of T cells from HS (white dots, n=9) and CP (grey dots, n=13) with autologous transfected DCs, collected at day 3. * P<0.05, *** P<0.0001 compared to empty-DCs. **(C)** and **(D**) DCs from HER2-overexpressing CP were transfected with HuHuT (□) or empty plasmid (○), and cultured with autologous T cells in the presence of neutralizing mAb for IL-10 and/or TGFβ1 or control isotypes. (**C**) Percentage of IFNγ+ and perforin+ cells in CD8+ T gated cells (left and right panels). * P<0.05. compared to empty-DCs (**D**) IFNγ response evaluated by ELISpot assay in response to T2 cells loaded with E75 and GP2 peptides was compared to that elicited by RHuT-DCs. * P<0.05, ** P<0.001 compared to empty-DCs. (**E**) Percentage of IFNγ+ cells in CD4+ T gated cells. * P<0.05 compared to empty-DCs.

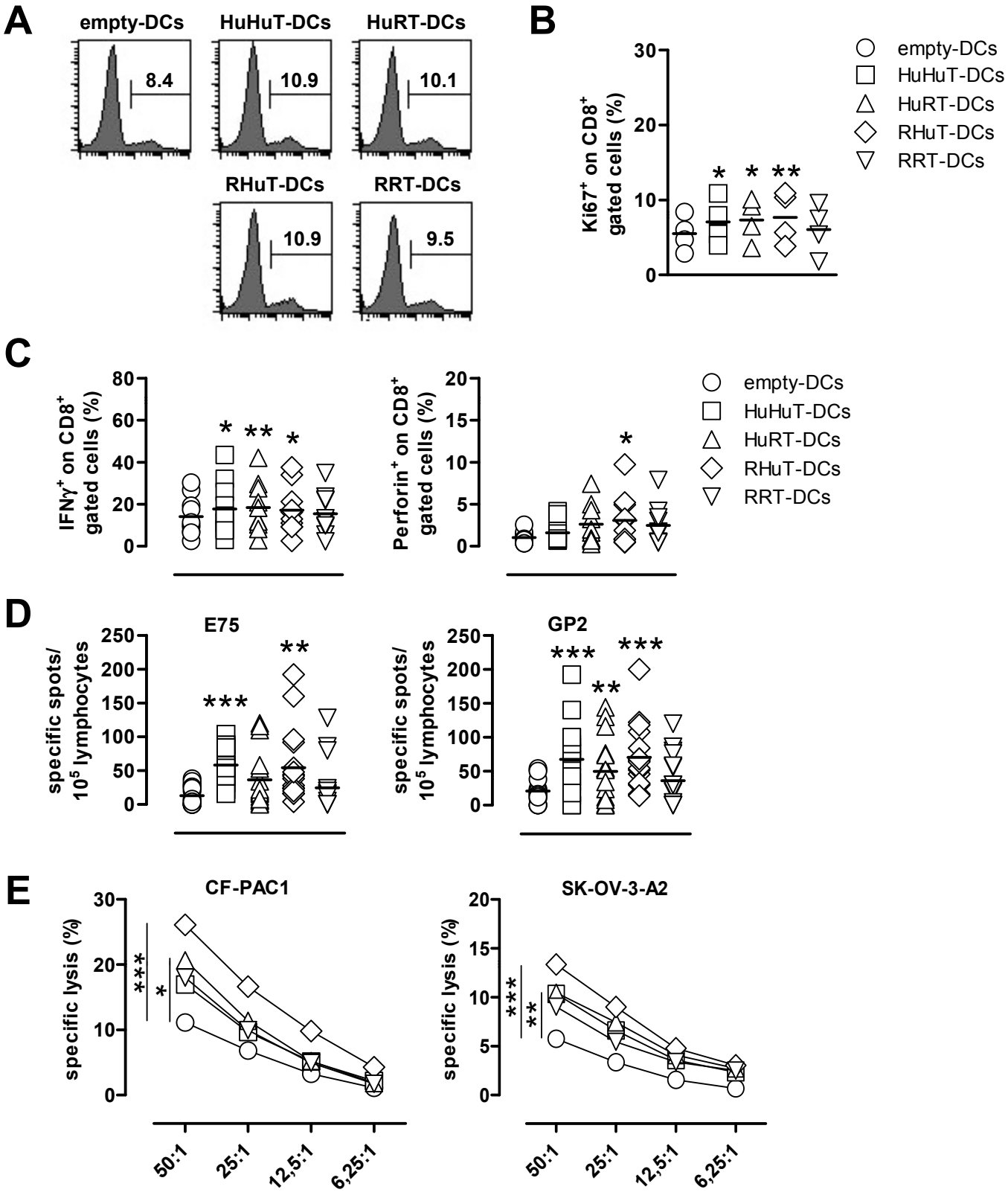

A
empty-DCs
HuHuT-DCs
HuRT-DCs
RHuT-DCs
RRT-DCs
8.4
10.9
10.1
10.9
9.5
B
Ki67+ on CD8+ gated cells (%)
empty-DCs
HuHuT-DCs
HuRT-DCs
RHuT-DCs
RRT-DCs
C
IFNγ+ on CD8+ gated cells (%)
Perforin+ on CD8+ gated cells (%)
D
E75
GP2
specific spots/ 10^5 lymphocytes
E
CF-PAC1
SK-OV-3-A2
specific lysis (%)
50:1
25:1
12,5:1
6,25:1

Figure 1

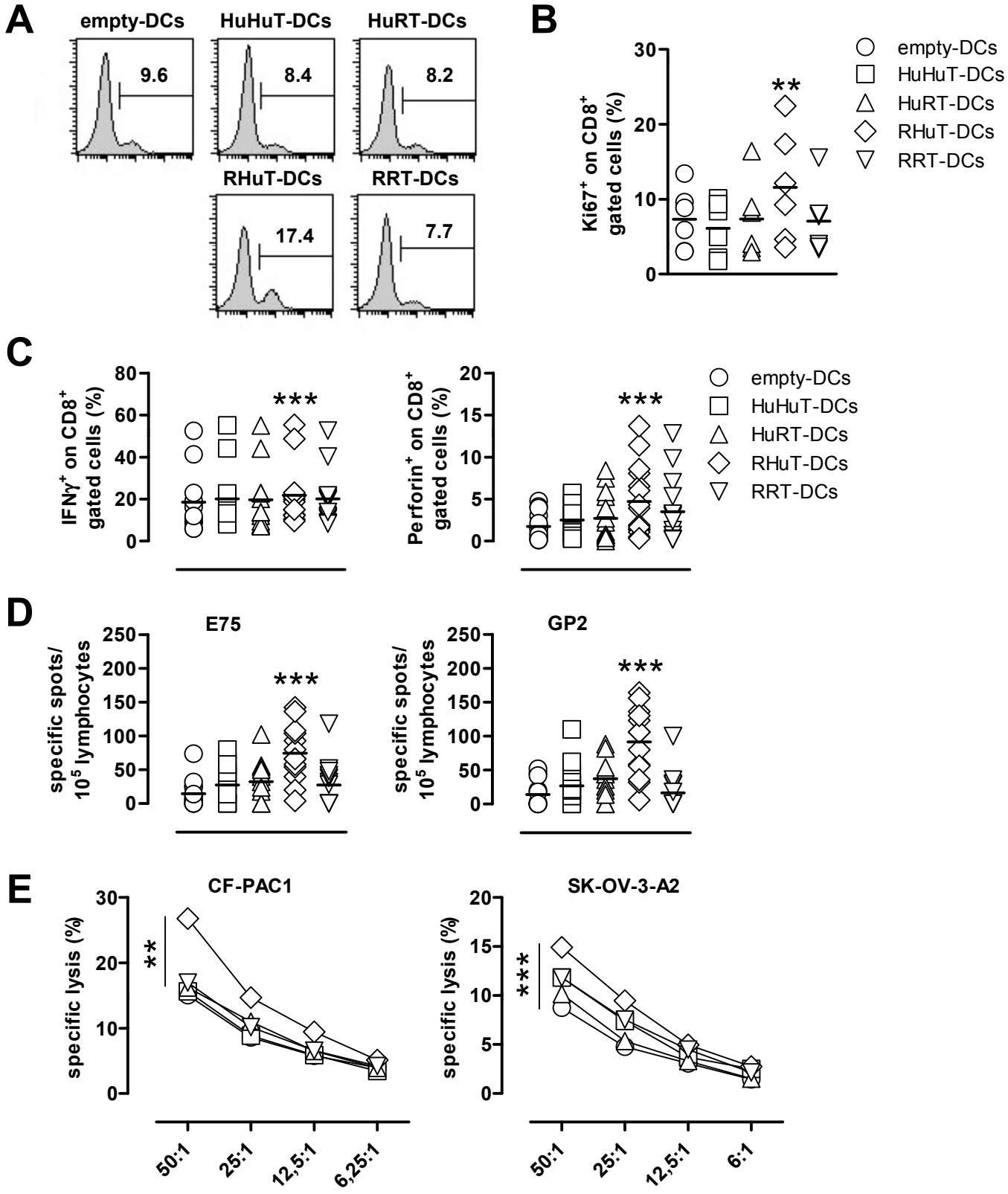

A
empty-DCs
HuHuT-DCs
HuRT-DCs
RHuT-DCs
RRT-DCs
9.6
8.4
8.2
17.4
7.7
B
Ki67+ on CD8+ gated cells (%)
**
empty-DCs
HuHuT-DCs
HuRT-DCs
RHuT-DCs
RRT-DCs
C
IFNγ+ on CD8+ gated cells (%)
***
Perforin+ on CD8+ gated cells (%)
***
D
E75
GP2
specific spots/ 10^5 lymphocytes
***
***
E
CF-PAC1
SK-OV-3-A2
specific lysis (%)
**
***
50:1
25:1
12,5:1
6,25:1
50:1
25:1
12,5:1
6:1

Figure 2

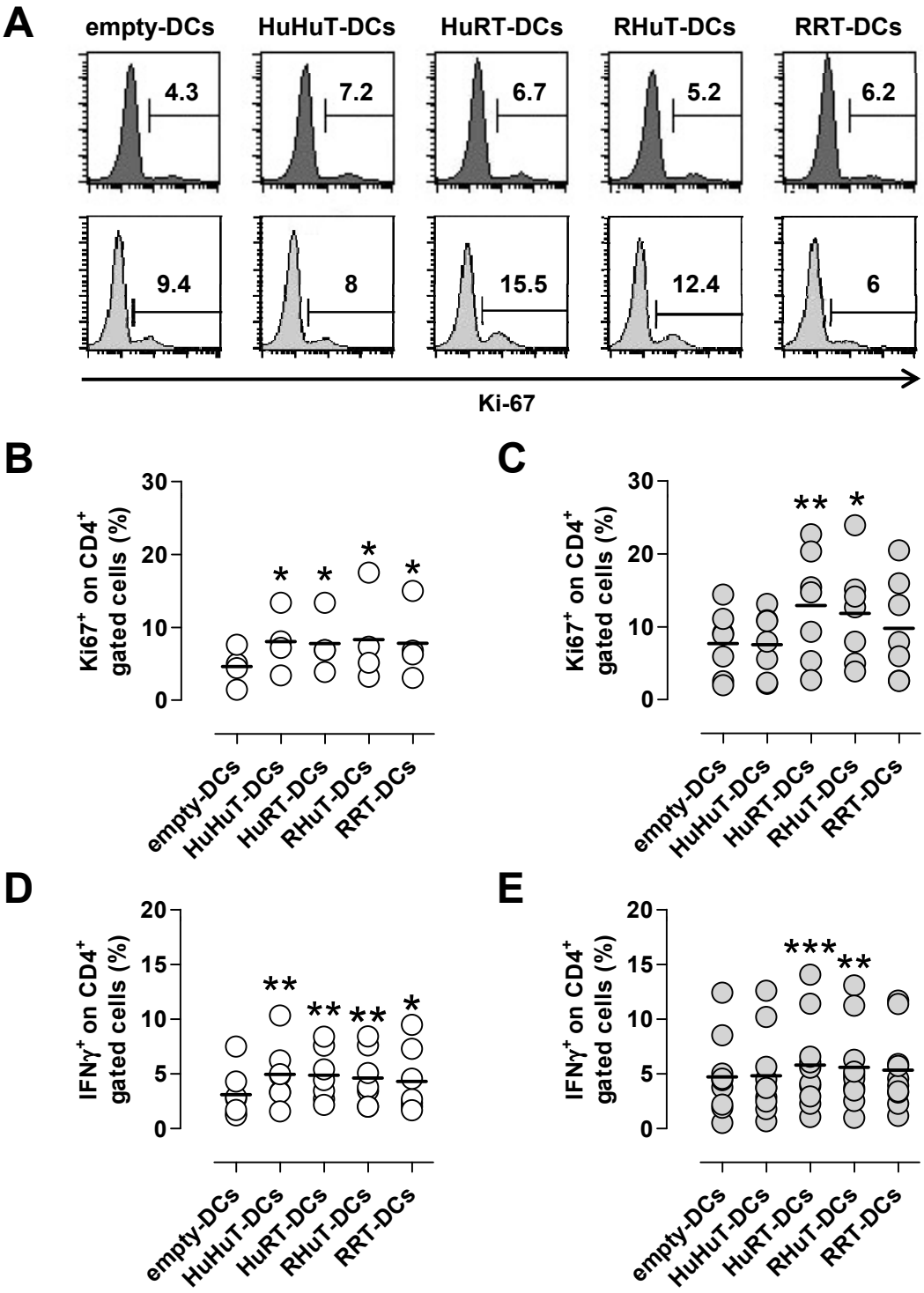
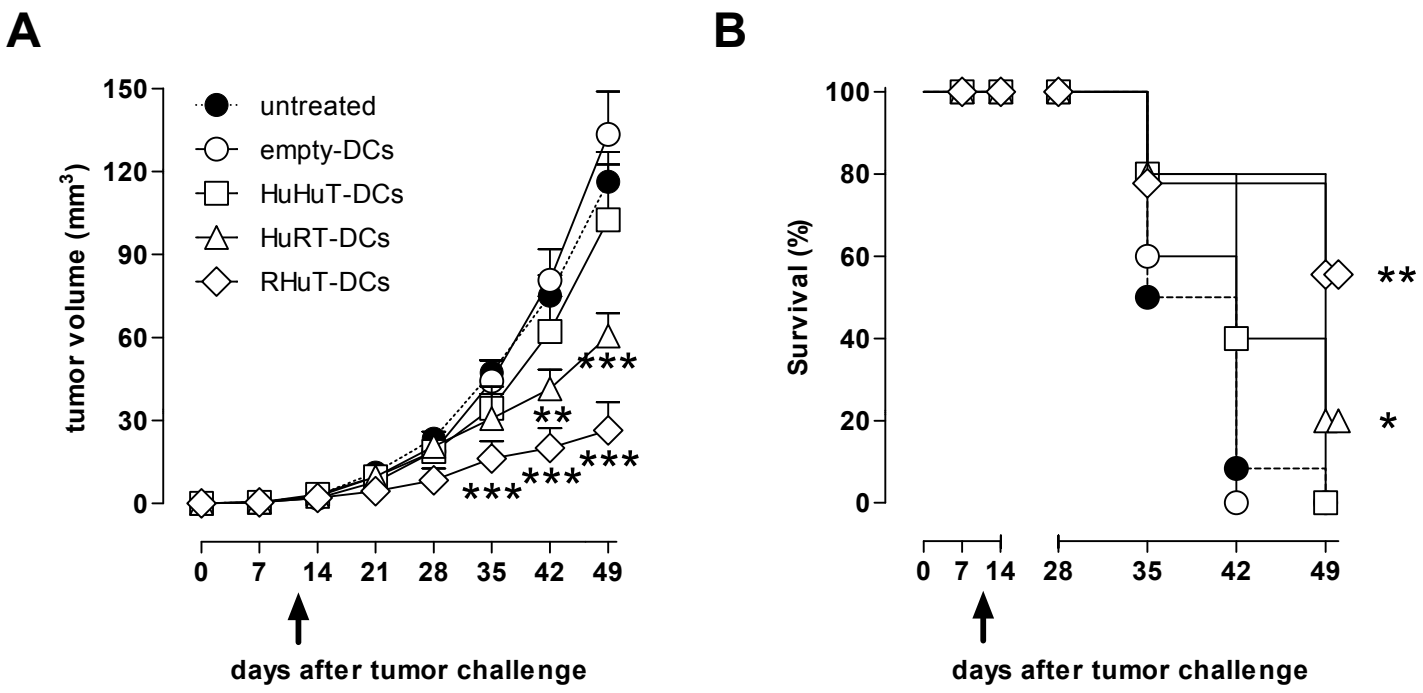
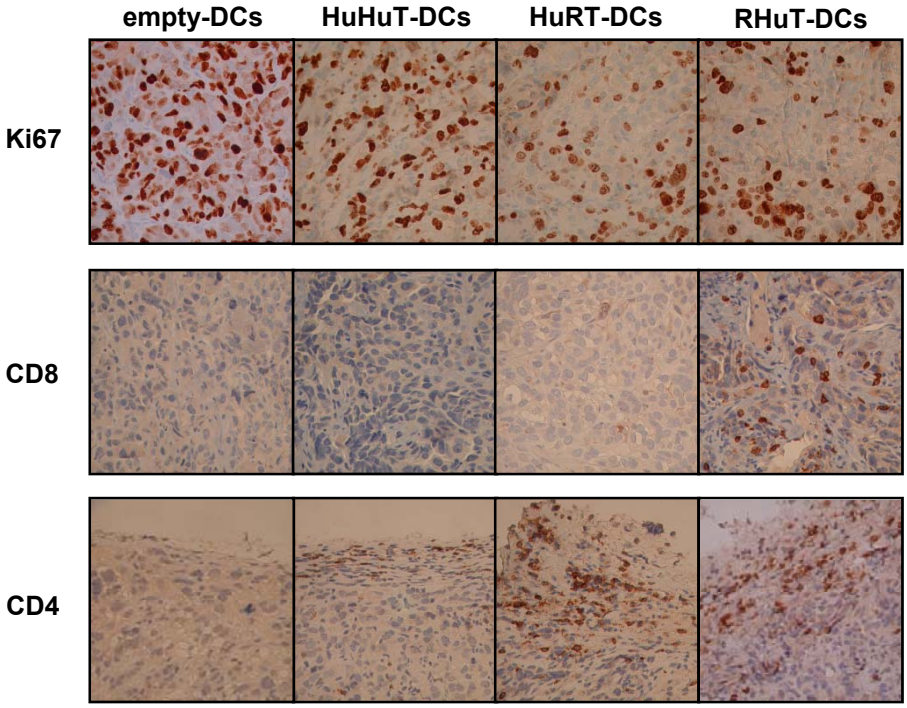

A
empty-DCs
HuHuT-DCs
HuRT-DCs
RHuT-DCs
RRT-DCs
4.3
7.2
6.7
5.2
6.2
9.4
8
15.5
12.4
6
Ki-67
B
Ki67+ on CD4+ gated cells (%)
C
Ki67+ on CD4+ gated cells (%)
D
IFNγ+ on CD4+ gated cells (%)
E
IFNγ+ on CD4+ gated cells (%)

Figure 3



C



	Ki-67$^+$ cells (%)
empty-DCs	65,8±6,1
HuHuT-DCs	63,9±1,5
HuRT-DCs	37,6±5,6 **
RHuT-DCs	32,3±2,6 **

	CD8$^+$ cells (%)
empty-DCs	1,7±0,9
HuHuT-DCs	6,0±4,1
HuRT-DCs	2,5±2,5
RHuT-DCs	19,6±6,8 *

	CD4$^+$ cells (%)
empty-DCs	0,7±0,3
HuHuT-DCs	8,7±5,1
HuRT-DCs	29,0±3,8 ***
RHuT-DCs	20,0±1,2 **

Figure 4

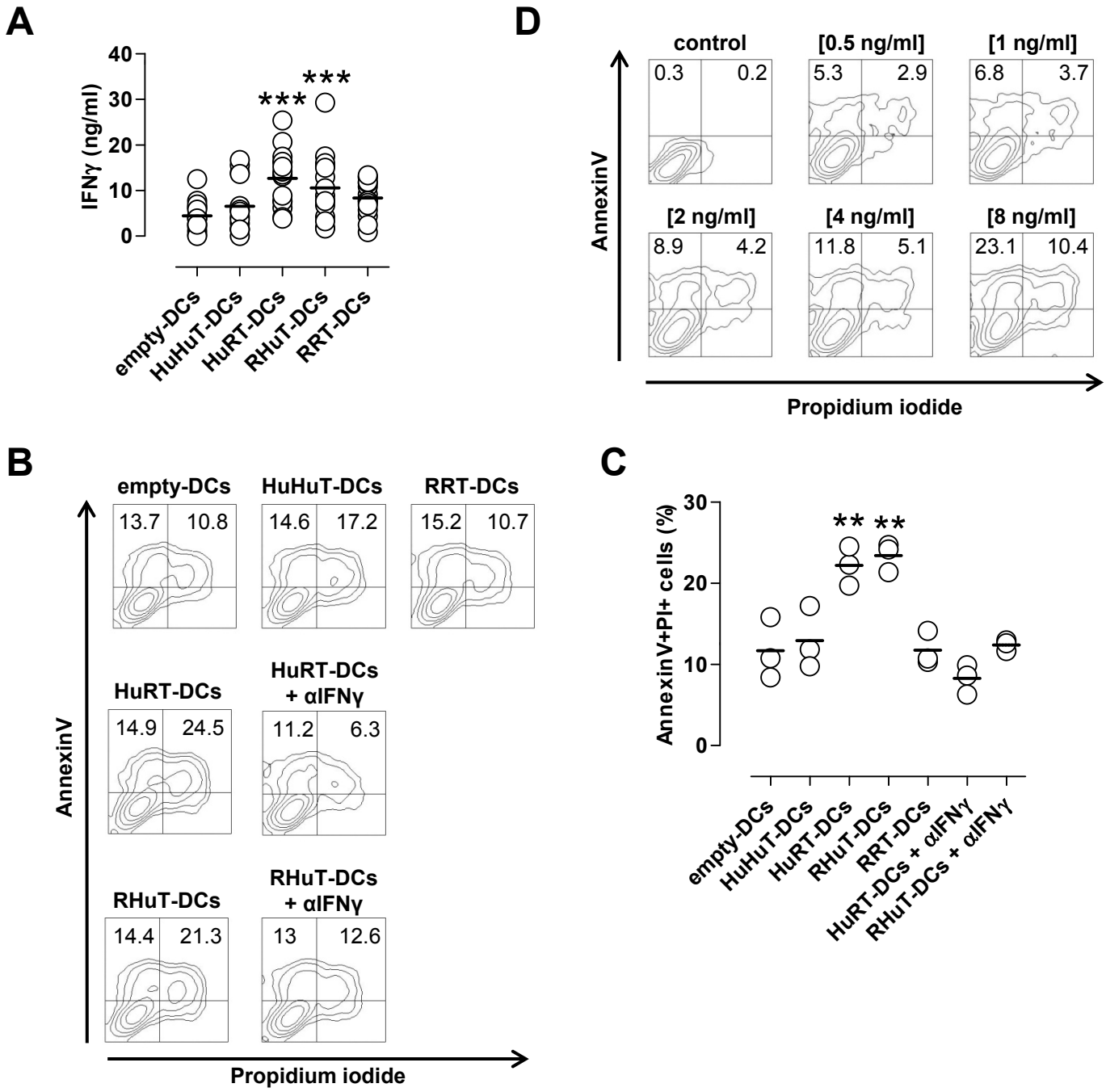

A
IFNγ (ng/ml)
40
30
20
10
0
***
***
empty-DCs
HuHuT-DCs
HuRT-DCs
RHuT-DCs
RRT-DCs
D
control
[0.5 ng/ml]
[1 ng/ml]
0.3
0.2
5.3
2.9
6.8
3.7
[2 ng/ml]
[4 ng/ml]
[8 ng/ml]
8.9
4.2
11.8
5.1
23.1
10.4
AnnexinV
Propidium iodide
B
empty-DCs
HuHuT-DCs
RRT-DCs
13.7
10.8
14.6
17.2
15.2
10.7
HuRT-DCs
HuRT-DCs + αIFNγ
14.9
24.5
11.2
6.3
RHuT-DCs
RHuT-DCs + αIFNγ
14.4
21.3
13
12.6
AnnexinV
Propidium iodide
C
AnnexinV+PI+ cells (%)
30
20
10
0
**
**
empty-DCs
HuHuT-DCs
HuRT-DCs
RHuT-DCs
RRT-DCs
HuRT-DCs + αIFNγ
RHuT-DCs + αIFNγ

Figure 5

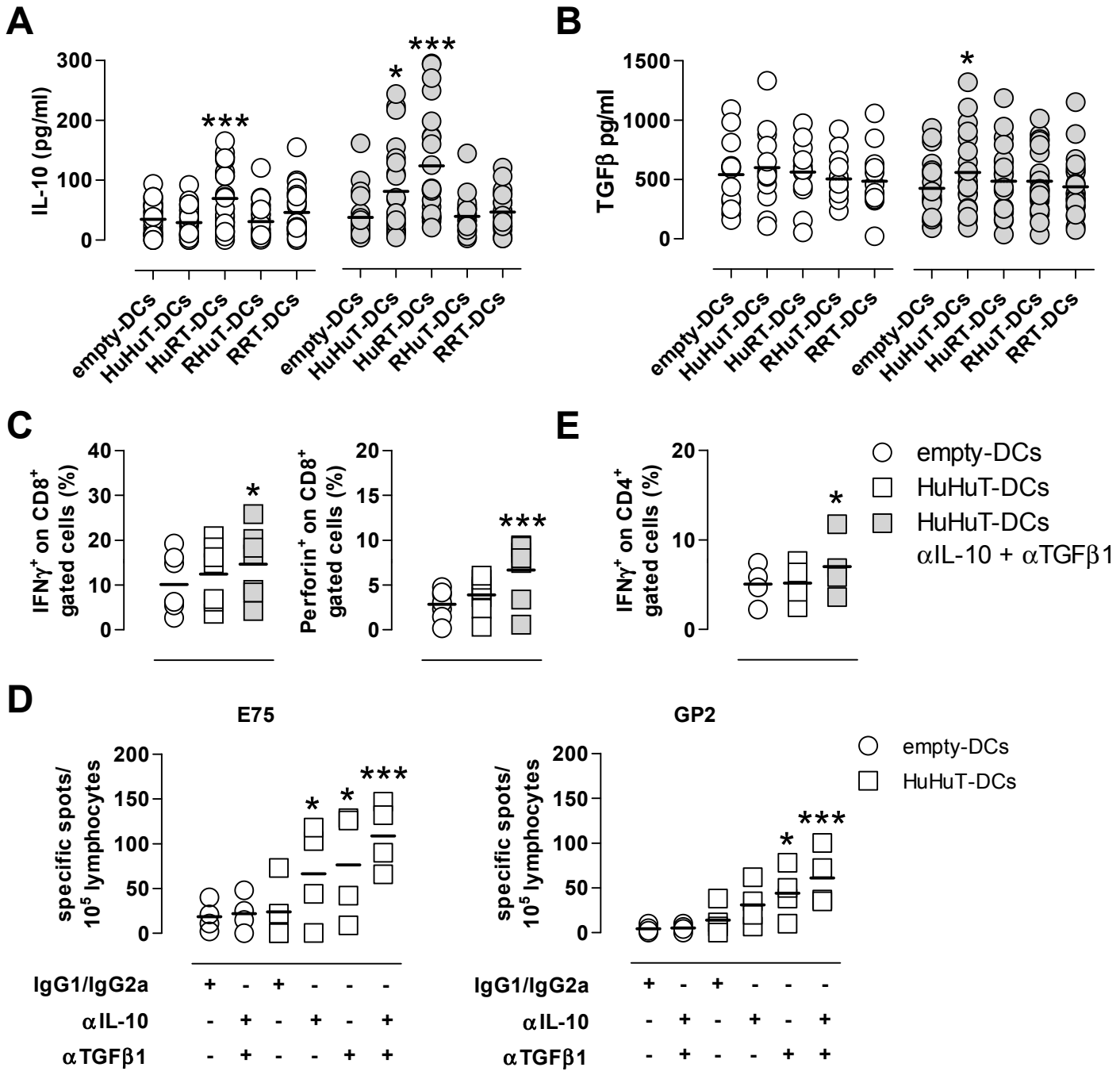

A
IL-10 (pg/ml)
300
200
100
0
***
*
***
empty-DCs
HuHuT-DCs
HuRT-DCs
RHuT-DCs
RRT-DCs
B
TGFβ pg/ml
1500
1000
500
0
*
C
IFNγ+ on CD8+ gated cells (%)
40
30
20
10
0
*
Perforin+ on CD8+ gated cells (%)
20
15
10
5
0
***
E
IFNγ+ on CD4+ gated cells (%)
20
10
0
*
empty-DCs
HuHuT-DCs
HuHuT-DCs
αIL-10 + αTGFβ1
D
E75
GP2
specific spots/ 10^5 lymphocytes
200
150
100
50
0
*
*
***
*
***
empty-DCs
HuHuT-DCs
IgG1/IgG2a + - + - - -
αIL-10 - + - + - +
αTGFβ1 - + - - + +

Figure 6