



# Cytotoxic Activity and Memory T Cell Subset Distribution of *in vitro*-Stimulated CD8<sup>+</sup> T Cells Specific for HER2/neu Epitopes

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Minimal residual disease remaining after resection of primary tumors can lead to tumor recurrence and metastasis, increasing mortality and morbidity rates among cancer patients. Thus, there is a need for new technologies for recognition and elimination of single cancer cells remaining in a patient's body after radiation therapy, chemotherapy, or surgical resection. Effector CD8<sup>+</sup> T cells, also commonly known as cytotoxic T lymphocytes (CTLs), play a key role in antitumor cellular immunity and, when properly activated, are able to effectively destroy tumor cells. The aims of this study were to obtain CD8<sup>+</sup> CTLs specific for the HER2/neu epitopes E75 and E88 and to assess the cytotoxic activity and composition of these cells in terms of the distribution of memory T-cell subsets. We obtained HER2-specific CD8<sup>+</sup> T cells and assessed T cell subset distribution among them including naive T cells (T<sub>N</sub>), central memory T cells (T<sub>CM</sub>), effector memory T cells (T<sub>EM</sub>), stem cell-like memory T cells (T<sub>SCM</sub>) and terminally-differentiated T cells (T<sub>EMRA</sub>) via eight-color flow cytometry. HER2-specific CTLs were largely (~40–50%) represented by T<sub>SCM</sub> cells, a population capable of mounting pronounced antitumor immune responses due to a combination of effector function and self-maintenance. In comparison with activated peripheral blood mononuclear cells (PBMCs) and bulk CD8<sup>+</sup> T cells, HER2-specific CTLs exhibited greater cytotoxicity against the HER2-expressing human breast adenocarcinoma cell line MCF-7 and produced higher levels of IFN-γ in response to tumor cells. We also showed the presence of HER2-specific CTLs in healthy individuals and increase in them in HER2-positive breast cancer patients. Collectively, our results suggest that HER2-specific CD8<sup>+</sup> T cells isolated using this approach could be used for adoptive T-cell transfer to eliminate tumor cells and prevent metastasis and relapse in patients with HER2-overexpressing cancers.

**Keywords:** CD8<sup>+</sup> T cells, cytotoxicity, memory T cell subsets, HER2/neu, stem cell-like memory T cells

## INTRODUCTION

Every arm of the immune system is involved in the antitumor immune response. Both CD8<sup>+</sup> cytotoxic T-lymphocytes (CTLs) and CD4<sup>+</sup> T-helper cells play important roles in anti-tumor immunity by secreting perforin and granzyme, inducing FasL/TRAIL-mediated cell death, or secreting effector cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha

(TNF- $\alpha$ ) (1–3). Both T-helper cell and CTL frequencies are associated with better survival in cancer patients (4, 5). However, CD8<sup>+</sup> CTLs have received greater attention in the field of cancer Immunotherapy. When properly activated by antigen-presenting cells (APCs) displaying antigenic peptides in complex with major histocompatibility complex (MHC) class I molecules, CTLs are able to specifically recognize and destroy malignant cells (6). Therefore, CD8<sup>+</sup> T-lymphocytes are used more often for adoptive T-cell transfer studies (5). It is clear that the ability to directly mediate cytotoxicity is one of the main reasons that CD8<sup>+</sup> T-cells are preferred for adoptive T-cell therapy. However, cytotoxicity is not the only requirement for adoptively-transferred cells; the ability of transferred CTLs to maintain themselves is equally important for the induction of a stable response to T-cell therapy. The viability and proliferation of T cells directly correlate with the antitumor activity of adoptive T-cell therapy (7–9). In this regard, the presence of memory T-cells in the transferred T-cells is of particular importance (10).

The tumor-associated antigen HER2/neu is a member of the human epidermal growth factor receptor family and is expressed during embryogenesis as well as on normal tissue cells of adult organisms (11, 12). Overexpression of HER2/neu occurs in various malignant carcinomas, which has made it a convenient target for immunotherapy. Many studies and approaches have been designed to inhibit the proliferation of HER2-positive tumor cells, some of which have been applied in clinical practice (13–19). Despite the success of several of these approaches, expression of HER2/neu continues to be associated with aggressive cancers, poor clinical prognoses and deteriorating survival rates (20). Therefore, studies of HER2/neu therapeutics are clearly still relevant (12, 21–24).

We have previously shown that a CTL response against HER2-expressing MCF-7 cells can be induced in a culture of activated peripheral blood mononuclear cells (PBMCs) using dendritic cells (DCs) transfected with a polyepitopic DNA construct encoding antigenic HER2/neu peptides (19). However, the potency of the immune response generated was low. We hypothesized that an insufficient number of HER2/neu-specific effector cells were contained in the mixed culture of PBMCs. In an attempt to increase the potency of the antitumor immune response, we decided to purify enriched populations of activated HER2/neu-specific CTLs. In the present study, we obtained HER2-specific CTLs using transfected DCs and HLA Streptamer technology and investigated the effector properties of the resulting cells, as well as their composition in terms of the distribution of memory T-cell subsets.

## MATERIALS AND METHODS

### DNA Constructs

A pMax-2 polyepitopic DNA construct encoding multiple copies of each of the two target HER2/neu epitopes in different molecular contexts was produced and designated as the “HER2 plasmid.” The two target immunogenic epitopes were HER2/neu p369–377 (E75, KIFGSLAFL) and HER2/neu p689–697 (E88, RLLQETELV).

A control plasmid DNA construct (pDNA5-BC-C) encoding the non-immunogenic peptide Ag51 was produced and designated as the “P5 plasmid.”

Both plasmids were designed and described in more detail previously (23).

### Donors and Patients

PBMCs were obtained from 18 HLA-A\*02-positive healthy donors (age 34 to 60 years, median 42 years), as well as four HLA-A\*02-positive patients with a confirmed diagnosis of HER2-positive breast cancer receiving treatment at Oncology Department No. 3, Novosibirsk City Clinical Hospital No. 1 (age 29 to 65 years, median 55 years). The presence of the HLA-A\*02 allele was assessed by HLA genotyping, which was carried out at the stage of selecting donors and patients.

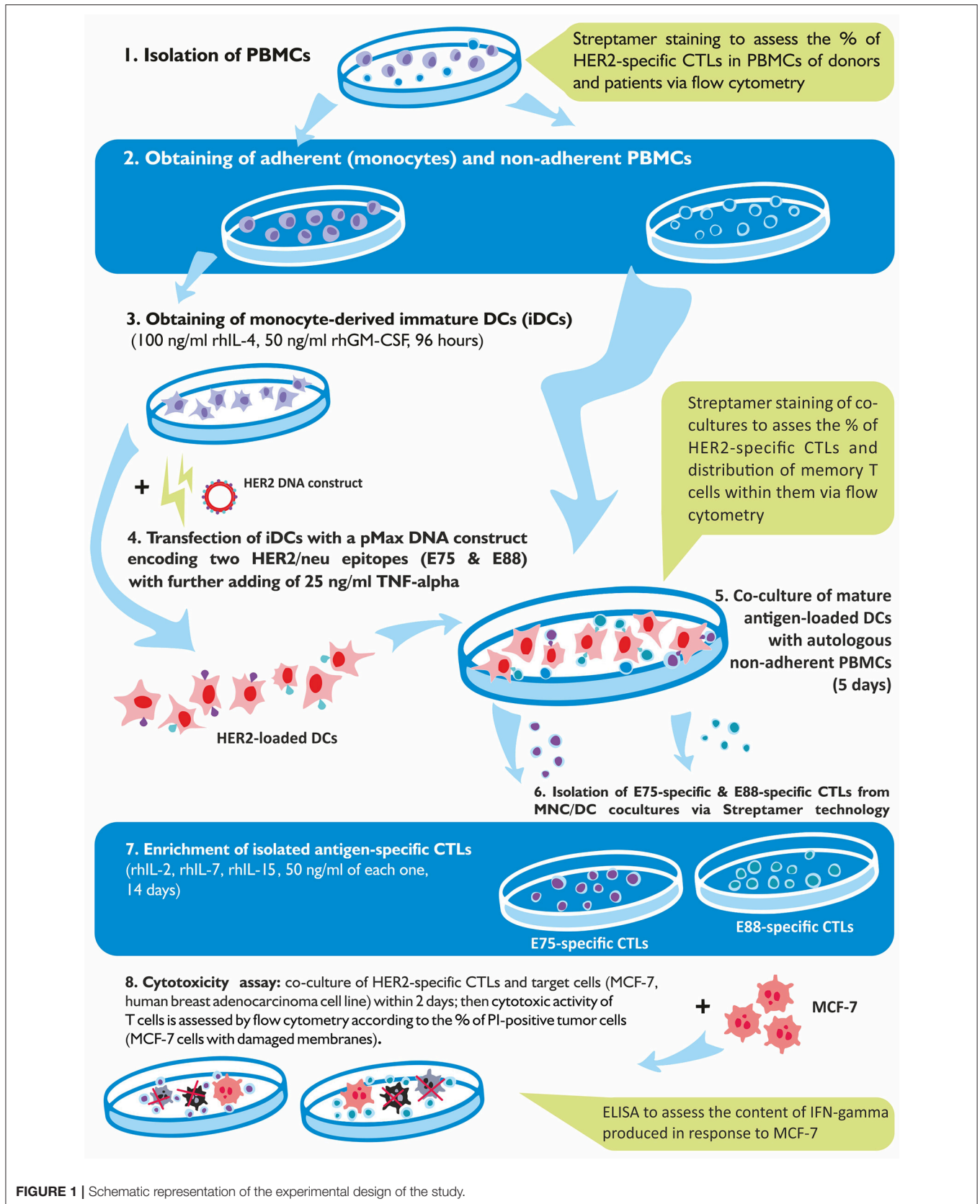
Voluntary informed consent was obtained from all donors and patients. This study was approved by the local Research Institute of Fundamental and Clinical Immunology (RIFCI) ethics committee. The experimental design of the study is shown in **Figure 1**.

PBMCs were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50  $\mu$ M mercaptoethanol, 25 mM HEPES, 80  $\mu$ g/mL gentamicin and 100  $\mu$ g/mL ampicillin (hereinafter called the culture medium). HER2-specific CTLs were obtained using a protocol we developed earlier (22) with a number of methodological changes described later. After magnetic separation, the epitope-specific CD8<sup>+</sup> T cell population was cultured in the presence (50 ng/mL each) of IL-2, IL-7 and IL-15 (Biozol, Germany) for 2 weeks in order to enrich the purified cells and stimulate memory T-cell proliferation.

### Isolation of Adherent PBMCs

Adherent PBMCs were isolated by incubation for 30 min on plastic Petri dishes (Nunc, Denmark) containing 10 mL of culture medium at 37°C under a humidified atmosphere containing 5% CO<sub>2</sub>. After incubation, the medium containing non-adherent PBMCs was transferred into a clean centrifuge tube. Next, 10 mL of RPMI-1640 medium was added to the Petri dishes and adherent PBMCs were removed from the bottom of the Petri dishes with a scraper (Sigma-Aldrich, USA). Suspensions containing adherent and non-adherent fractions were centrifuged at 1,500 rpm for 10 min. After centrifugation, the cell pellets were resuspended in culture medium for further manipulations.

In order to identify the optimal conditions for purifying adherent PBMCs, we previously conducted an analysis of six variations of the method in which incubation time and bottom pretreatment were variables. These variations involved cultivation in untreated or bovine serum albumin (BSA)-pretreated Petri dishes for 30, 60, or 120 min. The effectiveness of each strategy was assessed by labeling adherent cells with phycoerythrin (PE)-conjugated anti-human CD14 antibody (Becton Dickinson, USA) and counting the relative number of CD14<sup>+</sup> cells on a BD FACSVers flow cytometer.



**FIGURE 1** | Schematic representation of the experimental design of the study.

## Selection of Transfection Method

To determine the optimal method for plasmid delivery to DCs, we compared three transfection methods: (i) magnet-assisted transfection, (ii) electroporation and (iii) nucleofection of monocyte-derived DCs from healthy donors. A PMaxGFP plasmid encoding green fluorescent protein (GFP) was used for the comparison. Transfection efficiency was assessed based on the number of GFP-positive cells 24 h post-transfection using flow cytometry. DCs were analyzed by flow cytometry in the region of large granular leukocytes.

The nucleofection procedure was carried out using the Amaxa Human Dendritic Cell Nucleofector Kit (Lonza, Switzerland) according to the manufacturer's protocol and a Nucleofector 2b instrument. Magnet-assisted transfection of DCs was carried out using magnetic "MATra-A" nanoparticles and a universal 26 × 26 cm magnetic board for magnetic transfection (PromoKine, USA) according to the manufacturer's protocol. Electroporation was performed using Bio-Rad Gene Pulser disposable cuvettes (0.2 cm electrode gap) and a BTX ECM 830 electroporator with a voltage of 260 V and a pulse duration of 5 ms.

## Transfection of Immature DCs

Nucleofection was chosen for DC loading according to the results of the selection experiment. The HER2 plasmid was used for generation of HER2-loaded DCs (DC<sub>HER2</sub>). The P5 plasmid was used as a transfection control, yielding p5-loaded DCs (DC<sub>p5</sub>).

## Maturation of Transfected DCs and Activation of HER2-Specific T Cells

DC<sub>HER2</sub> and DC<sub>p5</sub> cell cultures (1 × 10<sup>6</sup> cells/mL) were cultured in flasks with a surface area of 25 cm<sup>2</sup> in culture medium containing 25 ng/mL TNF-α for 24 h to stimulate maturation of DCs. After 24 h of incubation with TNF-α, the cell number and viability of transfected cultures were counted in a Goryaev chamber using erythrosine staining. Next, the antigen-loaded DCs were co-cultured with autologous non-adherent PBMCs to generate HER2-specific T cells. Co-culture (PBMC+DC) was performed at a PBMC:DC ratio of 10:1 in 48-well plates (Cellstar, USA) containing culture medium (1 mL/well) for 4 days.

Co-cultures were labeled with antigen-specific Streptamers (IBA, Germany) to determine the frequencies of E75- and E88-specific CTLs. A detailed staining protocol has been previously described (23). Co-cultures were also analyzed for frequency of memory T-cell subsets as well as a number of effector functions.

## Phenotyping of HER2-Specific CTLs

Co-cultures of PBMCs and DCs stained with PE-conjugated Streptamers were labeled with the following monoclonal antibodies for flow cytometry: CD8-Brilliant-Violet-510, anti-human CD28-FITC, anti-human CD27-PerCP, anti-human CD95-PE-Cy7, anti-human CD127-APC, anti-human CD62L-APC-Cy7, anti-human CD45RA-Pacific-Blue. The prepared samples were analyzed on a BD FACSVerser. The lymphocyte gate was defined according to forward and side light scattering parameters. Then, CD8<sup>+</sup> cells and Streptamer<sup>+</sup>CD8<sup>+</sup> cells were gated from the lymphocyte gate events.

The following subsets were identified within the CD8<sup>+</sup> and Streptamer<sup>+</sup>CD8<sup>+</sup> lymphocyte populations: (i) T<sub>EMRA</sub> (CD45RA<sup>+</sup>CD62L<sup>-</sup>); (ii) T<sub>EM</sub> (CD45RA<sup>-</sup>CD62L<sup>-</sup>); (iii) T<sub>CM</sub> (CD45RA<sup>-</sup>CD62L<sup>+</sup>); (iv) T<sub>N</sub> (CD45RA<sup>+</sup>CD62L<sup>+</sup>CD127<sup>+</sup>CD27<sup>+</sup>CD28<sup>+</sup>CD95<sup>-</sup>); and T<sub>SCM</sub> (CD45RA<sup>+</sup>CD62L<sup>+</sup>CD127<sup>+</sup>CD27<sup>+</sup>CD28<sup>+</sup>CD95<sup>+</sup>). Differentiation of T<sub>EMRA</sub>, T<sub>EM</sub>, and T<sub>CM</sub> cell subsets was then analyzed via expression of CD27 and CD28.

## Analysis of HER2-Specific CTL Effector Properties

The target cells used to assess the direct cytotoxicity of HER2-specific T-lymphocytes were human breast adenocarcinoma MCF-7 cells (Bank of Cell Cultures, the Institute of Cytology of the Russian Academy of Medical Sciences, Russia). Evaluation of direct cytotoxicity was carried out according to a previously described protocol (23). Briefly, carboxyfluorescein succinimidyl ester (CFSE)-labeled MCF-7 cells were co-cultured with HER2-specific T-lymphocytes in wells of 96-well round-bottom culture plate at a ratio of 1:10 for 48 h and then labeled with propidium iodide (PI) and analyzed on a BD FACSVerser.

To quantitate cytotoxicity, we used the following formula:

$$\% \text{Cytotoxicity} = \% \text{PI}^+ \text{CFSE}^+ \text{MCF-7}_{(\text{effector}+\text{target})} - \% \text{PI}^+ \text{CFSE}^+ \text{MCF-7}_{(\text{target})}$$

where % PI<sup>+</sup>CFSE<sup>+</sup>MCF-7<sub>(effector+target)</sub> was the percentage of non-viable MCF-7 cells among the total cells in a co-culture of effector cells and target cells and % PI<sup>+</sup>CFSE<sup>+</sup>MCF-7<sub>(target)</sub> was the percentage of target cells undergoing spontaneous death in the absence of effector cells.

IFN-γ production in response to target cells was evaluated in conditioned media using the commercial gamma-INTERFERON-IFA-BEST system (Vector-Best, Russia) according to the manufacturer's protocol. Conditioned media were harvested after 48 h of co-culture of E75- and E88-specific cells with MCF-7 cells.

## Statistical Analysis

Statistical analysis and graphical representations were done using Prism 6 (GraphPad Software). Statistical significance was determined using two-way ANOVA with Tukey's multiple comparisons test (when analyzed groups have passed D'Agostino & Pearson omnibus normality test) and nonparametric two-tailed Mann-Whitney test and Kruskal-Wallis or Friedman test with Dunn's multiple comparison test.

All the data are presented as median and interquartile interval.

## RESULTS

### Optimization of the Adherent PBMC Purification Method

We compared six isolation methods for adherent PBMCs (30-, 60-, and 120-min incubation on untreated or BSA-treated plastic) by estimating the number of cells expressing the monocyte marker CD14. The results showed that incubation on untreated surfaces for 30 min yielded the highest numbers of CD14<sup>+</sup>



cells from PBMCs of healthy donors (57.12% of CD14<sup>+</sup> cells, compared with 48.38 and 46.01% after 60- and 120-min incubation on untreated surfaces, respectively, and 53.30, 45.67, and 44.63% after 30-, 60-, and 120-min incubation on BSA-treated surface, respectively;  $p \leq 0.05$ ,  $n = 12$ , Kruskal-Wallis test with Dunn's multiple comparison test). Thus, to obtain adherent cells, we used a 30 min incubation on untreated plastic.

## DC Transfection

Comparison of DC transfection methods showed that the percentage of DCs expressing GFP following nucleofection significantly exceeded the percentage of GFP<sup>+</sup> DCs following magnet-assisted transfection or electroporation (41.75% GFP<sup>+</sup> DCs for nucleofection vs. 31.50% for magnet-assisted transfection and vs. 6.52% for electroporation,  $p = 0.0173$  and  $p = 0.0022$ , respectively Mann-Whitney test). Thus, nucleofection was used for antigen loading of DCs. The viability of the DCs transfected using nucleofection with HER2/p5 plasmid did not decrease below 80% for both DC<sub>HER2</sub> and DC<sub>p5</sub>, according to the cell viability assessment in a Goryaev chamber using erythrosine staining.

## The Frequency of E75- and E88-Specific CTLs Among PBMCs of Healthy Donors and Patients With HER2-Positive Breast Cancer

To confirm the *in vivo* formation of a specific immune response against HER2/neu, we examined the content of E75- and E88-specific CTLs among PBMCs from healthy donors and patients with HER2-overexpressing breast cancer.

We found that PBMCs from HER2-positive breast cancer patients contained significantly higher proportions of HER2-specific CTLs (both E75-specific and E88-specific) compared with those of healthy donors (Figure 2A).

This result indicated that in association with development of HER2/neu-overexpressing tumors, clonal expansion of HER2-specific T-lymphocytes occurs, confirming the formation of a specific T-cell response to this antigen.

## Frequencies of E75- and E88-Specific CTLs in Co-cultures of PBMCs and Antigen-Loaded DCs

Analysis of co-cultures of PBMCs and DCs transfected with the HER2 plasmid (PBMC+DC<sub>HER2</sub>) showed that co-culture resulted in an increase in the frequencies of E75-specific T-lymphocytes (average, 0.32%; median, 0.23%) and E88-specific T-lymphocytes (average, 0.44%; median, 0.41%). These frequencies significantly exceeded those observed from co-cultures of PBMCs and DCs transfected with the P5 plasmid (PBMC+DC<sub>p5</sub>) (Figure 2B).

This result confirmed that clonal expansion of epitope-specific T lymphocytes was directly related to lymphocyte activation by DCs transfected with the HER2 plasmid, and that epitope-specific T cells could not be activated non-specifically by DCs transfected with a control plasmid.

## Distribution of T Cell Subsets Among DC-Stimulated CTLs

DC-stimulated E75 and E88-specific CTLs and total CD8<sup>+</sup> T cell populations were analyzed by flow cytometry and classified according to phenotype into the following subpopulations: T<sub>N</sub>, T<sub>SCM</sub>, T<sub>CM</sub>, T<sub>EM</sub>, and T<sub>EMRA</sub> (25–28).

The typical contour plots showing the gating strategy for phenotyping the CD8<sup>+</sup> T cells and CD8<sup>+</sup> HER2-specific (Streptamer<sup>+</sup>) T lymphocytes, and the frequencies of the listed subsets are shown in Figure 3.

When comparing the frequencies of T-cell subsets among CD8<sup>+</sup> T cells, no significant differences were observed between non-*in vitro* activated CD8<sup>+</sup> T cells (Figure 3C, first column from left), DC<sub>p5</sub>-stimulated CD8<sup>+</sup> T cells (Figure 3C, second column from left) and DC<sub>HER2</sub>-stimulated CD8<sup>+</sup> T cells (Figure 3C, third column from left), except for T<sub>N</sub> cells, which significantly decreased among total CD8<sup>+</sup> T cells after co-culture with DC<sub>HER2</sub> cells. Thus, a trend toward CD8<sup>+</sup> T-lymphocyte activation was observed in DC<sub>HER2</sub>-activated CD8<sup>+</sup> T-lymphocytes compared with non-activated cells, which was manifested in a reduction of T<sub>N</sub> cells.

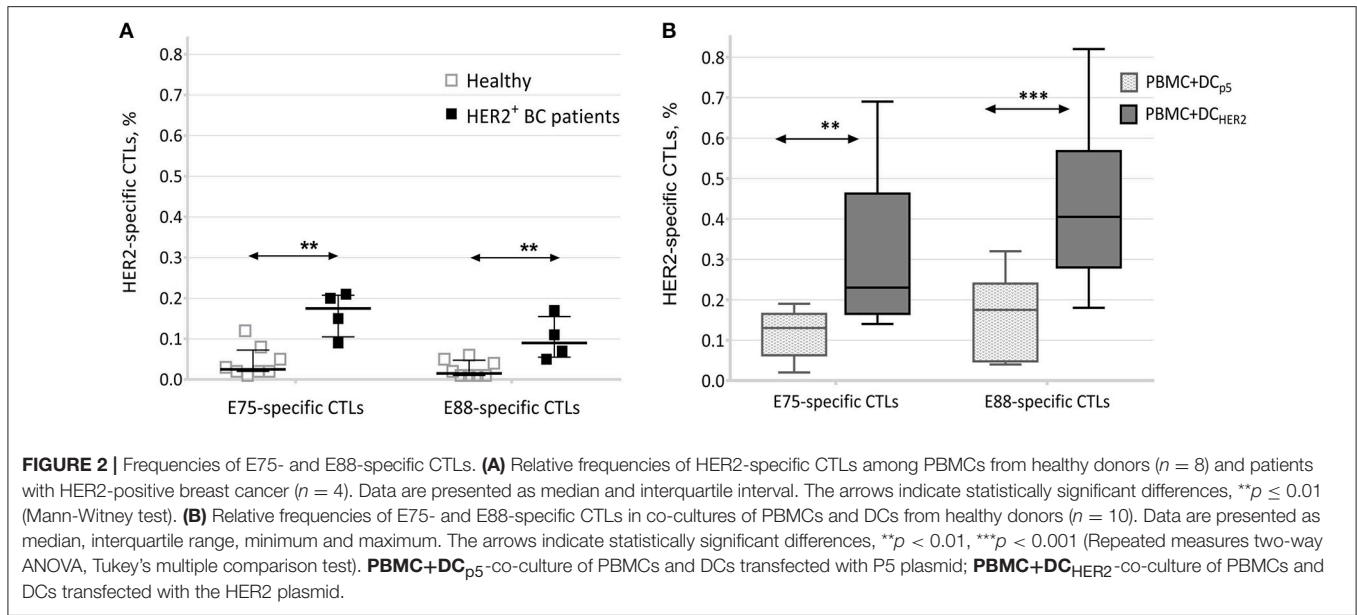
Among E75- and E88-specific CD8<sup>+</sup> T cells (two rightmost columns in Figure 3C), similar patterns were observed in the distribution of T-cell subsets (i.e., no significant differences between E75- and E88-specific CTLs), although both differed from bulk non-stimulated CD8<sup>+</sup> T-cells. E75- and E88-specific CD8<sup>+</sup> T cells contained significantly fewer T<sub>N</sub> cells (4.9% among E75-specific CTLs, and 3.2% among E88-specific CTLs; 35.5% among non-stimulated CD8<sup>+</sup> T cells, 33.3% among DC<sub>p5</sub>-stimulated CD8<sup>+</sup> T cells and 28.8% within DC<sub>HER2</sub>-stimulated CD8<sup>+</sup> T cells) and significantly more T<sub>SCM</sub> cells (41.6% among E75-specific CTLs, and 52.0% among E88-specific CTLs; 7.5% among non-stimulated CD8<sup>+</sup> T cells, 10.2% among DC<sub>p5</sub>-stimulated CD8<sup>+</sup> T cells and 9.1% within DC<sub>HER2</sub>-stimulated CD8<sup>+</sup> T cells).

A comparison of CD27 and CD28 expression within the T<sub>EMRA</sub>, T<sub>EM</sub> and T<sub>CM</sub> subsets is shown in Figure 4.

T<sub>EMRA</sub> cells among the total CD8<sup>+</sup> T cell population are mostly double-negative cells, reflecting a high degree of cell differentiation. However, T<sub>EMRA</sub> cells among HER2-specific CTLs were characterized by a high frequency of CD27<sup>+</sup>CD28<sup>+</sup> cells and a low frequency of CD27<sup>-</sup>CD28<sup>-</sup> cells.

T<sub>CM</sub> cells were mostly represented by CD27<sup>+</sup>CD28<sup>+</sup> cells within all populations investigated. T<sub>EM</sub> cells were represented in comparable parts by CD27<sup>+</sup>CD28<sup>+</sup>, CD27<sup>-</sup>CD28<sup>+</sup>, and CD27<sup>-</sup>CD28<sup>-</sup> cells, and by a minor population of CD27<sup>+</sup>CD28<sup>-</sup> cells; this was true for all three populations of CD8<sup>+</sup> T cells. However, among HER2-specific CTLs there was a trend toward increased frequency of CD27<sup>-</sup>CD28<sup>-</sup> cells compared with bulk CD8<sup>+</sup> T cells.

In summary, the approach used to activate HER2-specific CTLs using DCs loaded with HER2 epitopes led to a decrease in the frequency of CD8<sup>+</sup> T<sub>N</sub> cells. The distribution of T-cell subsets within DC<sub>HER2</sub>-activated E75-specific CTLs was not significantly different from that of DC<sub>HER2</sub>-activated E88-specific CTLs. However, the distribution of subsets within both populations of



HER2-specific T cells revealed a significant reduction of  $T_N$  cells and increase in  $T_{SCM}$  cells compared with bulk  $CD8^+$  T cells.

## Evaluation of the Effector Properties of HER2 Epitope-Specific CTLs

Analysis of the cytotoxic activity of E75- and E88-specific CTLs showed that the intrinsic cytotoxicity of the cells against HER2<sup>+</sup> MCF7 cells was 29.20% for E88-specific cells and 22.20% for E75-specific CTLs. These values significantly exceeded the intrinsic cytotoxicity of control cultures of PBMCs and DCs (5.78%) and of  $CD8^+$  cells (7.25%) (**Figure 5A**).

Thus, HER2 epitope-specific T-lymphocytes had increased cytotoxicity against the MCF-7 cell line.

To evaluate one of the potential cytotoxicity mechanisms, we estimated the concentration of  $IFN-\gamma$  in conditioned media from 2-day effector cell cultures with and without the MCF-7 tumor cell line (see **Figure 5B**).

After 48 h of co-cultivation with target cells, the  $IFN-\gamma$  content in media from culture of E75- and E88-specific CTLs increased by more than 6-fold compared with CTL<sub>0</sub> samples that did not contain target cells (from 4.46 to 29.00 pg/mL for E75-specific CTLs and from 4.10 to 27.47 pg/mL for E88-specific CTLs). The concentration of  $IFN-\gamma$  in the culture media of E75-specific CTLs and E88-specific CTLs significantly exceeded that of PBMC+DC co-cultures (6.36 pg/mL) as well as  $CD8^+$  T cells (16.66 pg/mL).

## DISCUSSION

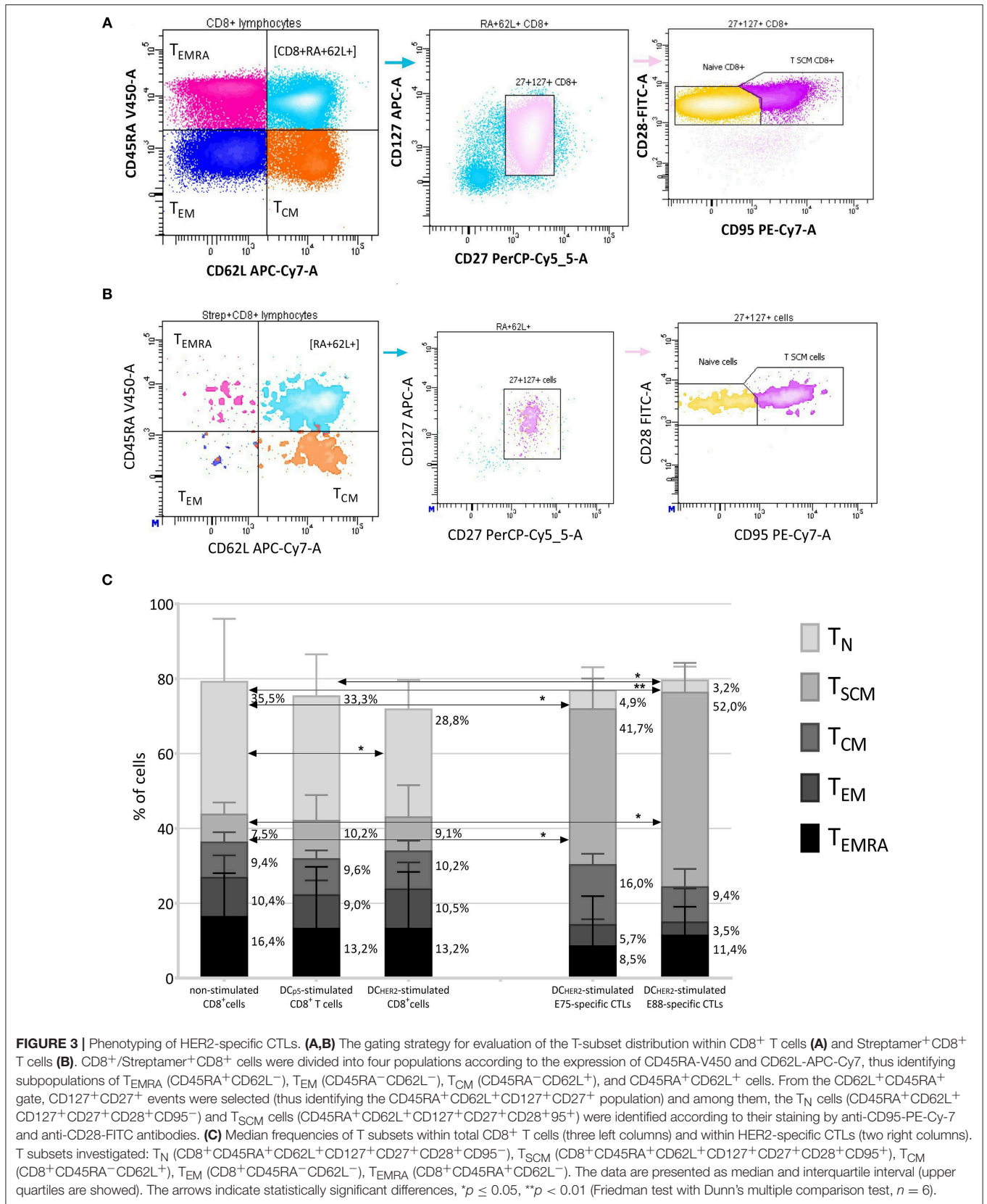
In this study, we selected immunogenic HER2 epitopes with the highest affinities for HLA-A02 alleles, which therefore are most effectively recognized by T-lymphocytes: E75 (HER2 p369–377, KIFGSLAFL) and E88 (HER2 p689–697, RLLQETELV) (13). We chose the HLA-A02 alleles since these are the most common alleles in the human population (29). Moreover, analysis of the distribution of HLA-A alleles among human populations on five

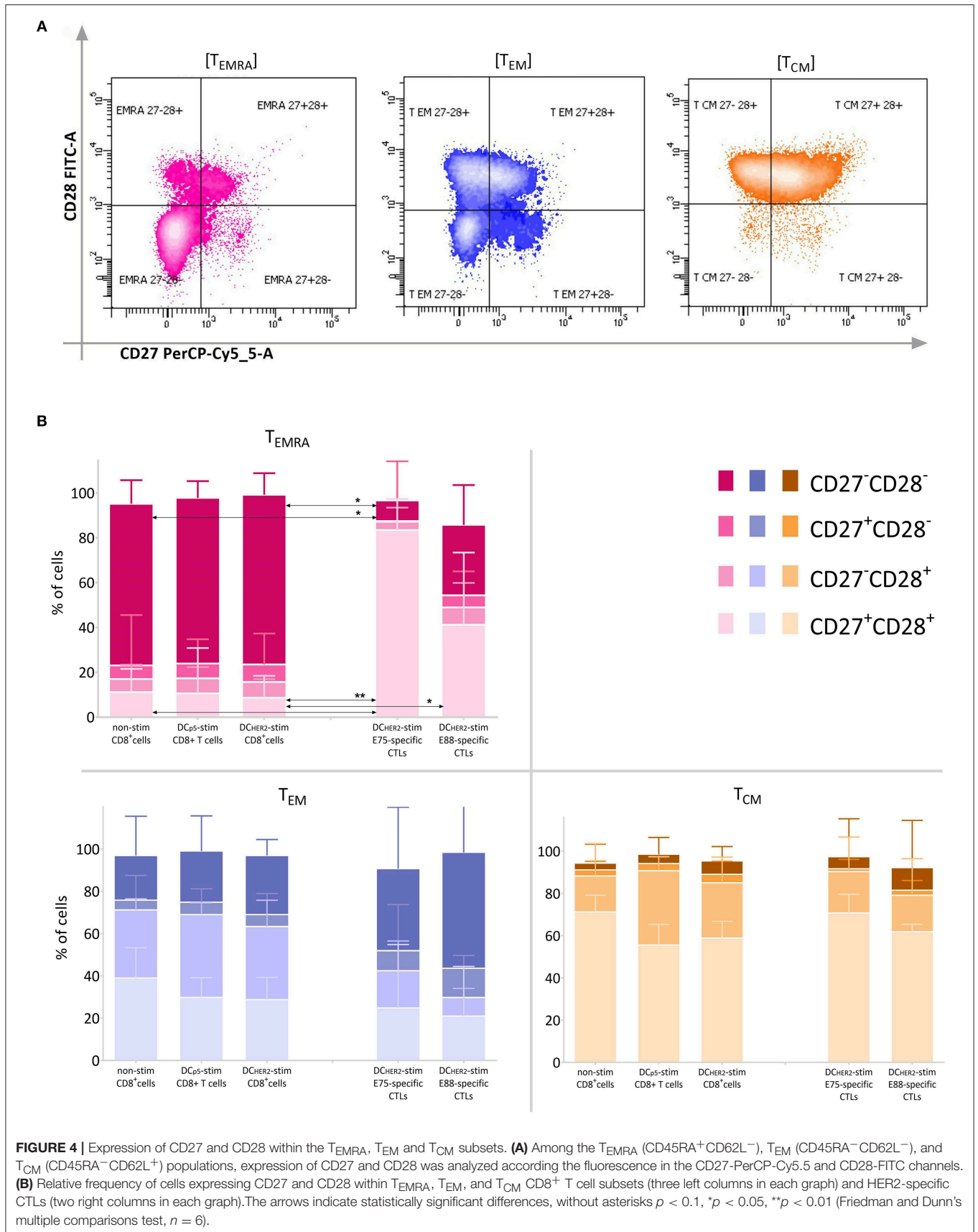
continents showed that A\*02:01 is one of the few HLA class I alleles found in a large proportion of human populations (30).

Among the breast cancer patients initially recruited according to HER2 positive expression status criterium (1+ /2+ /3+), there were both patients in remission and progression, as well as patients after complex treatment and surgery, and previously untreated patients. The final group of 4 patients included two patients after complex treatment and surgery and two patients with primary tumors. All of them were HER2 (3+). It also should be noted that in clinical practice the HER2 expression status is always analyzed before the HER2-targeted therapy.

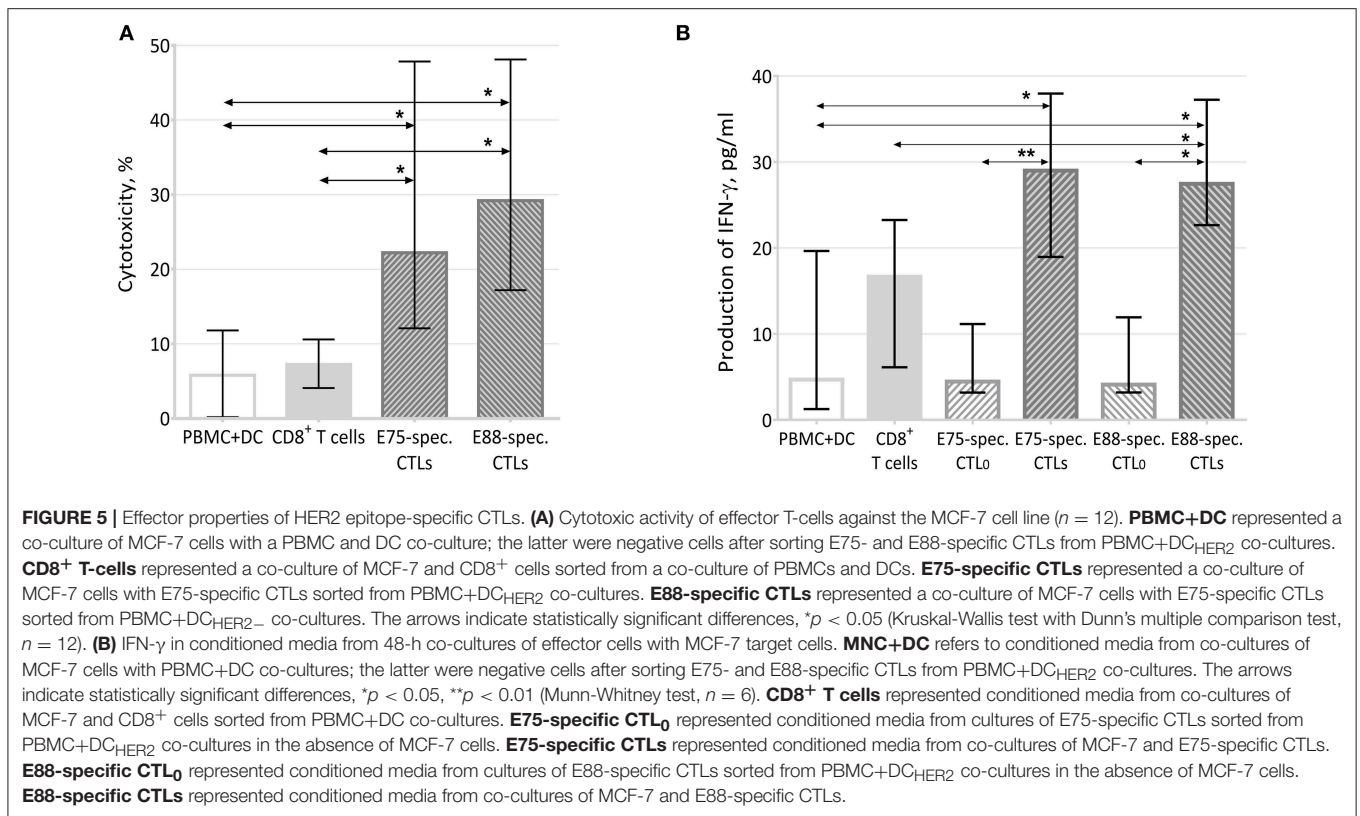
Our evaluation of HER2-specific CTL frequency in the peripheral blood of healthy donors and patients with HER2-overexpressing breast cancer confirmed the formation of a specific immune responses against HER2/neu during the malignant process, despite the fact that this antigen is an epidermal growth factor receptor and is normally present on healthy epithelial cells of a number of organs (17). In 2004, Woll and co-authors showed that the frequency of  $CD8^+$  T cells specific for the HER2/neu E75 epitope was about 0.2–0.3% of bulk peripheral blood lymphocytes in patients with HER2-positive breast cancer (31). These data are consistent with our results. However, we were also interested in the question of whether the ~0.2% of HER2-specific CTLs in patient blood resulted from the development of a specific cellular response due to the HER2/neu overexpression, or, on the contrary, was similar to that of healthy individuals. Our comparison of patients and healthy donors made it possible to clarify that an increase in CTL clones specific to HER2/neu epitopes does occur in patients with breast cancer.

Correct antigen presentation plays a crucial role in immune response generation. The simplest approach for *in vitro* loading of APCs with antigen is the addition of an antigenic peptide to a cell culture, ensuring the capture and processing of the antigen by the MHC II pathway. The major advantage of this approach is









the simplicity of the loading protocol. The use of nucleic acids as a source of antigen is also a common method of APC loading. If tumor material is sufficient, mRNA can be isolated directly from tumor cells and used for transfection of APCs. DNA, however, is more stable and is easier to work with it than with RNA (32–34). A number of studies using DCs transfected with non-viral DNA vectors have shown that loaded DCs can activate an effective antitumor immune response (7, 23, 33, 35). An advantage of nucleofection over other non-viral DNA delivery strategies is the fact that nucleofection allows delivery of molecules not only to the cytoplasm, as for other non-viral transfection methods, but also directly into the cell nucleus (36, 37).

In addition to the choice of the APC antigen loading strategy, there is also a choice of the cell type, hopefully with the most potent antitumor effects, which should be used for adoptive T-cell immunotherapy. Several studies have used one cell type as effector cells—either CD4<sup>+</sup> T cells or CD8<sup>+</sup> lymphocytes (16, 38, 39)—while others have used mixed cultures of T-helper and T-killer populations or bulk PBMCs (4, 40, 41). Many studies have emphasized that subpopulations of T cells play a synergistic role in orchestrating the immune response. In this regard, interest is growing in the role of T helper cells in the context of antitumor immunotherapy. Wolf and co-authors suggested the forthcoming appearance of cellular therapies that specifically combine CD8<sup>+</sup> with CD4<sup>+</sup> T cells and their testing in phase III clinical trials (5).

At the same time, adoptive transfer of pure populations of antigen-specific circulating CTLs or tumor-infiltrating T-cells has not lost its relevance (6, 16, 23). Despite the generation of an

effective antitumor immune response, the cytotoxic properties of CTLs can be reduced due to the presence of other, non-effector cell types in the cell preparation (3). Our results also confirmed the increased effectiveness of specific antitumor immune responses when using pure populations of antigen-specific T-cells. Cultures of E75- and E88-specific cells are more than 90% composed of CD8<sup>+</sup> T cells of a given antigen specificity (23). These populations have a more pronounced cytotoxic effect against HER2-expressing MCF-7 cells, produce more IFN- $\gamma$  in response to target cells, and in addition, and have increased frequencies of memory cells compared to bulk CD8<sup>+</sup> cells (both unstimulated *in vitro* and stimulated with DC<sub>p5</sub> cells or DC<sub>HER2</sub> cells).

The primary method for assessing peripheral blood T cell heterogeneity is flow cytometry. The main phenotypic characteristic of memory T cells is considered to be the appearance of the CD45RO<sup>+</sup> isoform instead of the CD45RA<sup>+</sup> isoform. In combination with these markers, the CD62L and CCR7 molecules, mediating homing into lymphoid organs, are used to identify memory subsets within CD8<sup>+</sup> T cells (26, 27). It should be noted that there are currently no commonly accepted phenotypic criteria for dividing memory cells into subsets.

CD8<sup>+</sup> T<sub>CM</sub> cells, producing a large amount of IL-2 but low levels of effector cytokines, have the CD45RA<sup>-</sup>CD45RO<sup>+</sup>CCR7<sup>+</sup>CD62L<sup>+</sup> phenotype. These cells differ from T<sub>N</sub> cells only by the CD45 isoform, and are capable of homing to lymphoid organs. CD8<sup>+</sup> T<sub>EM</sub> cells, which produce high levels of IFN- $\gamma$  and contain perforin and granzyme

granules, differ from  $T_{CM}$  cells in their lack of expression of CCR7 and CD62L.

Adding two more important markers to the panel (the CD27 and CD28 molecules responsible for T-cell costimulation) allows assessment of T-cell differentiation level. As an example, some researchers consider cells with the phenotype  $CD45RO^+CCR7^-CD62L^-CD27^-CD28^-$  as effector memory cells and  $CD45RO^+CCR7^-CD62L^-CD27^-CD28^+$  cells as “transitional” memory T-cells; the latter are more differentiated than  $T_{CM}$  cells but less differentiated than  $T_{EM}$  cells according to a number of phenotypic characteristics (42, 43) and based on their proliferation in response to IL-15 *in vivo* (44, 45). Terminally-differentiated  $CD45RA^+CCR7^-CD62L^-$  T-cells negative for CD27 and CD28 are described as short-lived CTLs with pronounced effector properties that can be activated under the influence of non-professional APCs, but die immediately after executing their cytotoxic function (46).

$T_{SCM}$  cells were first discovered in 2005 in the peripheral blood of mice in a study of experimental graft-vs.-host disease (47) and were later identified in human peripheral blood as well (48, 49). The principal difference in these cells from  $T_N$  cells was their high expression of CD95 and CD122; in common with  $T_N$  cells, they expressed CCR7, CD45RA, CD62L, CD27, CD28, and CD127 (IL-7Ra) but were completely negative for CD45RO (49).

The panel of antibodies we selected for eight-color flow cytometry included antibodies against human CD8, CD45RA, CD62L, CD27, CD28, CD95, and CD127 as well as fluorochrome-conjugated antigen-specific reagents (Streptamers, Iba, Germany). This panel allowed us to identify all major circulating  $CD8^+$  T cell subsets among the E75- and E88-specific CTL populations.

A significant decrease in the frequency of  $T_N$  cells among E75- and E88-specific CTLs, as well as among bulk  $DC_{HER2}$ -activated  $CD8^+$  cells, indicated that DCs transfected with the HER2 plasmid could effectively activate specific T-cell cytotoxic immune responses. It is also noteworthy that the largest component of total HER2-specific cells was  $T_{SCM}$  subset. This population is characterized by pronounced effector properties and long-term self-maintenance, which has attracted interest in these cells for cancer immunotherapy (27, 49, 50).  $T_{SCM}$  cells are known to be the least differentiated of all memory subsets, but at the same time are able to secrete IFN- $\gamma$ , IL-2, and TNF- $\alpha$  in response to  $\alpha$ -CD3/ $\alpha$ -CD2/ $\alpha$ -CD28 stimulation (49). These properties make  $T_{SCM}$  cells promising for further use in adoptive T-cell therapy. We attribute the increase in  $T_{SCM}$  frequency to the method for antigen-specific T-cell isolation we used: the isolated HER2-specific T-lymphocytes were cultured in the presence of IL-2, IL-7, and IL-15 (50 ng/mL each) (23). IL-2 is necessary to maintain the viability of T-lymphocytes, and IL-7 and IL-15 play a key role in triggering *in vitro* proliferation, in addition to signals from the T-cell receptor and costimulatory molecules. As mentioned earlier,  $T_{SCM}$  cells are characterized by high expression of CD122 (total  $\beta$ -chain of the receptor for IL-2 and IL-15) and CD127 (IL-7R $\alpha$ ).

We demonstrated a six-fold increase in IFN- $\gamma$  production by  $DC_{HER2}$ -activated E75- and E88-specific CTLs in response to target cells, which is consistent with a reduction in

the number of  $T_N$  cells and an increase in  $T_{SCM}$  content within these populations. In addition, IFN- $\gamma$  production by HER2-specific CTLs was almost 2-fold higher than that of activated bulk  $CD8^+$  cells of all other specificities. Thus, some level of IFN- $\gamma$  production occurs in antigen non-specific T-cell immune responses, but IFN- $\gamma$  production is significantly higher in antigen-specific T cells that executing their cytotoxic function.

## CONCLUSION

In this study, we demonstrated the presence of HER2-specific T-lymphocytes in the blood of healthy donors and a significant increase in their frequency in the blood of patients with HER2-overexpressing breast cancer. We showed the effectiveness of a technique to isolate CTL populations specific for HER2/neu epitopes. The resulting E75- and E88-specific CTLs consisted of more than 40% of  $T_{SCM}$  cells. These cells exhibited pronounced cytotoxicity and a higher level of IFN- $\gamma$  production against the HER2-expressing tumor line MCF-7 cells compared with activated PBMCs. We expect that this approach will be effective for obtaining antigen-specific T-cells for adoptive T-cell transfer. Specifically, we assume that the cells obtained in this way can be effective for the elimination of HER2-expressing tumor cells in patients with HER2-overexpressing tumors after resection of the primary tumor, which may help in preventing of relapses and metastasis.

## ETHICS STATEMENT

This study was carried out in accordance with the Declaration of Helsinki with written informed consent from all subjects. The protocol was approved by the local ethics committee of Research Institute of Fundamental and Clinical Immunology (RIFCI).

## AUTHOR CONTRIBUTIONS

MK contributed to the design, experimental work, and optimization of each experimental stage, analysis, and interpretation of data, drafting of the manuscript, and infographics. JL contributed to the conception, design, and contribution to data interpretation. JS performed experimental work (DC transfection) and contributed to data interpretation. AS performed experimental work (optimization of adherent PBMCs isolation protocol) and revision of the manuscript. AM performed experimental work (preparation of DNA constructs). SS contributed to the conception, design, revision, and final approval of the manuscript. All authors read and approved the final version of this manuscript.

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