Activation of the FcgammaReceptorIIIa on human natural killer cells leads to increased expression of functional interleukin-21 receptor

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ABSTRACT
Natural killer (NK) cells are innate immune effector cells that play a crucial role in immune surveillance and the destruction of cancer cells. NK cells express a low-affinity receptor for the Fc or constant region of immunoglobulin G (FcγRIIIa) and multiple cytokine receptors that respond to antibody-coated targets and cytokines in the tumor microenvironment. In the present work, microarray gene expression analysis revealed that the IL-21 receptor (IL-21R) was strongly upregulated following FcR stimulation. The IL-21R was found to be upregulated on FcR-stimulated NK cells at the transcript level as determined by reverse transcription polymerase chain reaction (RT-PCR). Immunoblot analysis revealed that protein expression of the IL-21R peaked at 8 h post-stimulation of the FcR. Inhibition of the mitogen-activated protein kinase (MAPK) pathway downstream of the FcR blocked the induction of IL-21R expression. Increased expression of the IL-21R sensitized NK cells to IL-21 stimulation, as treatment of FcR-stimulated NK cells led to significantly increased phosphorylation of STAT1 and STAT3, as measured by intracellular flow cytometry and immunoblot analysis. Following FcR-stimulation, IL-21-activated NK cells were better able to mediate the lysis of trastuzumab-coated human epidermal growth factor receptor 2 (HER2) SK-BR-3 tumor cells as compared to control-treated cells. Likewise, IL-21-induced NK cell secretion of IFN-γ following exposure to antibody-coated tumor cells was enhanced following FcR-stimulation. The analysis of NK cells from patients receiving trastuzumab therapy for HER2 cancer exhibited increased levels of the IL-21R following the administration of antibody suggesting that the presence of monoclonal antibody-coated tumor cells in vivo can stimulate the increased expression of IL-21R on NK cells.

ARTICLE HISTORY
Received 21 February 2017
Revised 21 March 2017
Accepted 23 March 2017

KEYWORDS
Fc receptor; FcgammaRIIIa; IL-21; IL-21 receptor; natural killer cell

Introduction
Natural killer (NK) cells are bone-marrow-derived, large granular lymphocytes that contain abundant cytolytic granules and express numerous adhesion molecules. NK cells are unique in their constitutive expression of receptors for numerous cytokines and an activating receptor for the Fc region of IgG (FcγRIIIa). In addition to their ability to mediate antibody-dependent cellular cytotoxicity (ADCC), FcR-activated NK cells can also secrete factors such as IFN-γ, TNF-α, and chemokines that inhibit tumor cell proliferation, enhance antigen presentation, and stimulate the chemotaxis of T cells, respectively. Properties of NK cells provide NK cells with the ability to directly lyse cellular targets as well as coordinate the developing adaptive immune response.

Interleukin-21 (IL-21) is a pleiotropic cytokine produced primarily by activated CD4+ T cells. IL-21 has been implicated in several disease processes including allergies, autoimmune disorders, viral infections, and cancer. The IL-21 receptor (IL-21R) is a type I cytokine receptor with four conserved cysteine residues and an extracellular WSXWS motif that shares the common receptor gamma-chain with IL-2, −4, −7, −9, and −15. The expression of IL-21R on the surface of immune cells helps mediate a variety of effects including its ability to enhance the proliferation, antigen-induced activation, clonal expansion, IFN-γ production, and cytotoxicity of CD8+ T cells. In addition to its effects on CD8+ T cells, IL-21 has context-dependent effects on B cells, amplifies macrophage activation pathways, and inhibits the activation of myeloid dendritic cells. IL-21 also promotes the maturation of murine NK cells and increases human NK cell expression of the NK activation receptors NKp30 and 2B4. IL-21 is able to mediate the regression of established tumors in a variety of murine models and phase I clinical trials. Its mechanism of action has been variously attributed to NK cell cytolytic activity, perforin-mediated CD8+ T cell cytotoxicity, the differentiation...
of CD4+ T cell subsets, the growth and activity of NKT cells, and the anti-angiogenic actions of IL-21-induced IFNγ.21-27

Here, we show that the upregulation of IL-21R has positive implications for the activation of NK cells against HER2 breast cancer cells and that the induction of this receptor is mediated through the MAP kinase pathway. NK cells that had increased IL-21R expression were better able to lyse antibody-coated tumor cells and produced greater amounts of IFNγ than their resting counterparts. In addition, patients receiving the monoclonal antibody (mAb) trastuzumab and exhibiting a clinical response to treatment into the role played by IL-21R in mediating NK cell activation, patients receiving the monoclonal antibody (mAb) trastuzumab and exhibiting a clinical response to treatment (Applied Biosystems, Foster City, CA). Primers that recognize the human β-Actin sequence were used as the internal control in each reaction well. Real-time PCR data were analyzed using the ABI PRISM® 7900 Sequence Detection system.

**Methods**

**NK cells isolation, cell lines, and reagents**

Human natural killer (NK) cells were isolated from fresh peripheral blood leukopacks (American Red Cross, Columbus, OH) by 30-min incubation with RosetteSep cocktail (Stem Cell Technologies) before Ficoll Hypaque (Sigma) density gradient centrifugation and cultured as previously described, with ~97% purity.28 Peripheral blood mononuclear cells (PBMCs) were also procured from patients with HER2-positive breast cancer who were receiving trastuzumab therapy (OSU Protocol No. OSU-09142 and OSU-08153). Polyclonal human IgG (IgG) was purchased from Sigma-Aldrich. Trastuzumab (Herceptin™), an anti-HER2/neu mAb, was provided by Genentech, Inc. Recombinant human interleukin-21 (IL-21) was supplied by ZymoGenetics, Inc. (Seattle, WA). The YT-CD16 cell line was a gift from Dr. Michael Caligiuri (The Ohio State University, Columbus, OH). The following cell lines were purchased from ATCC: SK-BR-3 (HER2-overexpressing breast cancer), Ramos and HeLa.

**FcR-stimulation assays**

For FcR-stimulation assays by immobilized IgG, wells of 96-well flat-bottom plates were coated with 100 μg/mL of IgG in cold PBS overnight at 4°C, washed with cold phosphate buffer saline (PBS), and then plated with human NK cells at 2 × 10⁵ cells/well as previously described.29 NK cells classified as “resting” were plated in 96-well flat-bottom plates in a similar fashion to FcR-stimulated NK cells, minus the addition of IgG.

**Preparation of labeled RNA and microarray hybridization and gene microarray data analysis**

cDNA was prepared from total cellular RNA of NK cells obtained from individual healthy donors and subject to a cleanup protocol as previously described.30 Affymetrix GeneChip expression array U133A was hybridized with each prepared cRNA target in duplicate, according to the manufacturer’s instructions. The raw and processed microarray data have been made publicly available in GEO (GSE63038).30 The gene microarray data were analyzed as previously described.30

**RT-PCR.** The expression values of IL-21R identified via the microarray experiment were validated by real-time PCR. Following RNeasy purification of NK cells from the immobilized IgG assay at various time points, 2 μg of total RNA was reverse transcribed and the resulting cDNA was used as a template to measure IL-21R transcript using pre-designed primer/probe sets according to the manufacturer’s recommendations (Applied Biosystems, Foster City, CA). Primers that recognize the human β-Actin sequence were used as the internal control in each reaction well. Real-time PCR data were analyzed using the ABI PRISM® 7900 Sequence Detection system.

**Immunoblot analysis**

NK cell expression of IL-21R was verified via immunoblot analysis. Lysates were prepared from human NK cells as previously described31, 32 following FcR-stimulation by immobilized IgG and assayed for the expression of IL-21R (MAB991; R&D Systems Inc., Minneapolis, MN) or β-Actin as a loading control (clone AC-74; Sigma Aldrich, St. Louis, MO). Lysates were separated on a 10% SDS-PAGE gels, transferred to a nitrocellulose membrane, and blocked in 5% nonfat milk prior to incubation with primary and secondary antibodies. For pathway analysis, 25 μM of the ERK inhibitor U0126 was incubated with NK cells for 30 min prior to the preparation of lysates (Sigma Aldrich).

**Flow cytometry**

NK cells activated through their FcR by immobilized IgG were subject to flow cytometry to determine cell surface expression of IL-21R. Resting or FcR-stimulated NK cells were incubated on ice for 30 min in flow buffer (5% FBS in PBS) with anti-CD56-PE (clone B159) and anti-IL-21R-APC (clone 17A12) or isotype control antibodies (BD Biosciences, San Jose, CA). Cells were then washed and fixed in 1% formalin. Non-specific staining with an isotype control Ab was employed to determine the percent positive population. To detect intracellular levels of phospho (p)-STAT1 and p-STAT3 induced by IL-21 treatment, unstimulated NK cells or FcR-stimulated NK cells were cultured with or without IL-21 (10 ng/mL) for 30 min, washed, and stained with an anti-p-STAT1-FITC mAb or an anti-p-STAT3-FITC mAb in combination with the NK cell marker CD56 as previously described (BD Biosciences).29 Percentages reported are of dual-positive populations (Q2) as determined by the removal of isotype-control background fluorescence.

**Cytotoxicity assay**

NK cells activated through their FcR by immobilized IgG were plated overnight in RPMI media supplemented with 10% human AB (HAB) serum media with or without IL-21 (10 ng/mL). Following overnight incubation, 51Cr-labeled K562 tumor cells were incubated with NK cells at various effector:target (E:T) ratios. Following a 4 hr incubation, supernatants were harvested and percent lysis was calculated as previously described.33
**NK cell cytokine secretion**

For in vitro co-culture assays, wells of a 96-well flat-bottom culture plate were seeded with the HER2-overexpressing human breast cancer cell line SK-BR-3 at a density of $5 \times 10^4$ cells/well. Tumor cells were grown to confluence overnight and then treated with $100 \mu g/mL$ trastuzumab for 1 hr at 37°C. After washing off unbound tumor cells, resting or FcR-stimulated NK cells were added at $2 \times 10^5$ cells/well in 200 $\mu L$ in RPMI media supplemented with 10% human AB (HAB) serum media with or without IL-21 (10 ng/mL). Control conditions consisted of resting or 8 hr FcR-stimulated NK cells incubated with tumor alone or IL-21 alone. Cell-free supernatants were collected following a 48 hr incubation and IFN $\gamma$ levels were measured using commercially available ELISA kits (R&D Systems Inc.).

**Analysis of apoptosis via Annexin V/propidium iodide (PI) staining**

Apoptosis-induced phosphatidyl serine exposure was measured in tumor cells by flow cytometric analysis using propidium iodide, V450-anti-annexin V, and APC-anti-CD56 (BD Biosciences) as previously described. Each analysis was performed utilizing at least 10,000 cellular events. The population with values above an isotype control was calculated within each treatment group, gating on APC-anti-CD56-negative cells, for each treatment group.

**Statistics.** IL-21R expression measured by RT-PCR, IFN $\gamma$ release as measured by ELISA and tumor cell apoptosis measured by chromium release and annexin V/PtdIns staining was analyzed by the analysis of variance. Expression of IL-21R from patient samples before and after treatment was compared using the Wilcoxon signed rank test.

**Results**

**Human NK cells increase expression of IL-21R transcript levels in response to FcR stimulation**

NK cells are known for their ability to upregulate the production of effector cytokines and increase their cytotoxic potential in response to external stimuli. The major goal of this study was to gain insight into the function of NK cells upon encounter with antibody-coated targets as it relates to IL-21 sensitivity. To directly address this question, NK cells were isolated from the peripheral blood of healthy donors and global gene-expression profiling was performed on RNA freshly isolated or isolated after a 12 h stimulation of the NK cell FcR by immobilized IgG. The IL-21R was found to be upregulated in response to FcR stimulation as compared to unstimulated NK cells (Fig. 2A). Consistent with the gene expression microarray data, PCR analysis for IL-21R indicated that FcR stimulation upregulates IL-21R expression at the transcript level by approximately 3-fold ($p < 0.01$; Fig. 1B).

**Upregulation of IL-21R via NK cell FcR stimulation occurs in a time-dependent fashion**

RT-PCR, immunoblot analysis, and flow cytometric analysis were used to characterize the upregulation of the IL-21R in NK cells following FcR stimulation. These analyses revealed that the upregulation of the IL-21R occurs in a time-dependent fashion. The expression of IL-21R at the mRNA level peaked at 8 hr post-FcR-stimulation and was upregulated 6.5-fold compared to unstimulated NK cells at this time point ($p < 0.01$; Fig. 2A). Immunoblot analysis for IL-21R expression was conducted using primary human NK cells and the YT cell line modified to express CD16 (YT-CD16). This analysis revealed marked upregulation of IL-21R following FcR stimulation with expression peaking at 8 hr post-stimulation (Fig. 2B). NK cells were also analyzed for IL-21R levels by flow cytometry using anti-CD56 Ab and anti-IL-21R fluorescence-conjugated mAbs. This experiment showed that IL-21R was upregulated on the surface of NK cells in a time-dependent fashion, with 62% of NK cells expressing surface IL-21R at 8 hr post-IgG stimulation as compared to 21.9% at baseline (Fig. 2C).

**NK cell IL-21R upregulation is dependent on ERK signal transduction**

The ERK signal transduction pathway is activated in NK cells following engagement of the FcR and results in the
generation of phosphorylated ERK protein, which mediates downstream gene regulation. The ERK inhibitor U0126 was used in an effort to determine the contribution of the ERK signaling pathway to the upregulation of IL-21R on the surface of NK cells following FcR stimulation. As expected, the ERK inhibitor was effective in decreasing levels of activated (phosphorylated) ERK in NK cells following PMA stimulation (Fig. 3A). When NK cells were activated through the FcR for 8 h, an upregulation of IL-21R protein level was detected, but when these FcR-activated NK cells were treated with an ERK inhibitor, expression of the IL-21R was diminished (Fig. 3B).

**Figure 2.** The IL-21R is upregulated on NK cells following FcR stimulation in a time-dependent fashion. NK cells stimulated via the FcR by immobilized IgG were analyzed at varying time points for expression of IL-21R transcript by (A) RT-PCR and IL-21R protein by (B) immunoblot analysis, and (C) flow cytometry. (A) RT-PCR for IL-21R transcript in untreated NK cells and NK cells cultured in the presence of immobilized-IgG at the time points indicated. Data represent the mean fold increase in IL-21R expression in three donors ± SD. The asterisk (*) denotes \( p < 0.01 \) vs. all time points shown. (B) IL-21R expression at the protein level was confirmed by immunoblot analysis in primary NK cells and YT-CD16 cells at the time points indicated. The Ramos tumor cell line served as a positive control. The membranes were re-probed for \( \beta\)-actin to confirm equal loading. (C) IL-21R expression was measured by flow cytometry in resting NK cells and NK cells stimulated with immobilized-IgG. Cells were stained with anti-CD56-PE and anti-IL-21R-APC Abs at the time points indicated and fluorescence was compared to that obtained with an isotype control antibody. HeLa and Ramos acted as negative and positive controls, respectively. Percentages are reflective of dual positive populations (Q2). Each plot depicts the results from one representative donor. Results are representative of three normal donors tested.

**Upregulation of IL-21R on the surface of NK cells leads to enhanced NK cell signal transduction**

The IL-21R signals via the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling
IL-21 is known to activate JAK1 and JAK3, and this activation leads directly to the phosphorylation of STAT1 and STAT3. In order to determine if FcR-mediated upregulation of IL-21R on the surface of NK cells led to increased sensitivity to exogenous IL-21, intracellular levels of p-STAT1 and p-STAT3 post-IL-21 exposure were measured by flow cytometry and immunoblot analysis (Fig. 4). NK cells stimulated through their FcR for 8 h and then exposed to IL-21 for 45 min showed an increase in the generation of p-STAT1 as compared to unstimulated NK cells via flow cytometry. Immunoblot analysis confirmed that exposure of FcR-stimulated NK cells to IL-21 led to increased levels of p-STAT1 in comparison to unstimulated NK cells (Fig. 4A). Similar results were obtained for the generation of p-STAT3 in response to IL-21 (Fig. 4B). Interestingly, total STAT1 and STAT3 levels increased as NK cells were stimulated through their FcR. The upregulation of total STAT1 and STAT3 levels may have contributed to the increased p-STAT1 and pSTAT3 signal observed.

**Upregulation of IL-21R on the surface of NK cells leads to enhanced cytotoxicity, IFNγ production, and NK cell-mediated tumor cell apoptosis**

In order to characterize the consequences of upregulation of the IL-21R on the surface of NK cells following FcR engagement, NK cell cytotoxicity, production of IFNγ, and NK cell-induced tumor cell apoptosis were measured through chromium release, ELISA and annexin V/PI flow cytometry, respectively (Fig. 5). It was hypothesized that NK cell effector functions in response to IL-21 would be enhanced following FcR stimulation. As predicted, FcR-stimulated NK cells were better able to lyse K562 tumor cells in an NK cell cytotoxicity assay following the addition of IL-21, as compared to unstimulated control NK cells ($p < 0.05$; Fig. 5A). Furthermore, exposure of FcR-stimulated NK cells to IL-21 resulted in increased production of IFNγ in response to trastuzumab-coated SK-BR-3 tumor cells as compared to unstimulated control NK cells ($p < 0.01$; Fig. 5B). In addition, FcR-stimulated NK cells mediated greater apoptosis of trastuzumab-coated SK-BR-3 tumor cells in response to IL-21 ($p < 0.05$; Fig. 5C).

**NK cells from patients receiving monoclonal antibody-therapy demonstrate increased IL-21R expression following treatment**

PBMCs from HER2-positive breast cancer patients receiving trastuzumab therapy were analyzed for expression of IL-21R on CD56-positive NK cells by flow cytometry. The first analysis was conducted with five patients receiving neoadjuvant chemotherapy, four of whom had either a partial response or a complete response. The pre-surgical treatment regimen of these patients with operable, non-metastatic invasive ductal carcinoma consisted of doxorubicin/cyclophosphamide administered every 3 weeks × 4 cycles followed by weekly trastuzumab/paclitaxel. IL-21R expression on patient NK cells increased significantly at the time of trastuzumab administration compared to pre-therapy levels. The percentage of CD56+ NK cells expressing the IL-21R went from an average of 11.8% pre-therapy to 22.4% at 20 weeks ($p = 0.0302$; Fig. 6A). In contrast, a second group of patients with metastatic disease receiving trastuzumab in combination with CpG oligodeoxynucleotides weekly for 12 weeks, followed by weekly trastuzumab monotherapy exhibited no clinical responses and there was only a 1.15% increase in IL-21R expression on NK cells at 20 weeks of therapy compared to pre-therapy levels (ns; Fig. 6B).

**Discussion**

The major goal of this study was to gain insight into the expression of IL-21R on NK cells upon encountering Ab-coated targets. An oligonucleotide microarray experiment revealed increased expression of the IL-21R on NK cells following FcR activation. Immunoblot analysis and flow cytometry confirmed that levels of IL-21R on the surface of NK cells are increased following FcR-stimulation. Upregulation of IL-21R rendered NK cells more sensitive to IL-21 at the level of signal transduction, lytic activity, and IFNγ production. Finally, it was shown that expression of IL-21R on NK cells was upregulated in patients who successfully received trastuzumab-based neoadjuvant chemotherapy.

It has been suggested that IL-21 acts *in vivo* to accelerate the transition between innate and adaptive immunity and that the effects of IL-21 on NK cells may vary depending on the timing and magnitude of the T cell response and subsequent concentrations of IL-21. Due to its immune stimulatory properties on both innate and adaptive immunity, IL-21 administration or IL-21 gene transfer has been widely exploited in preclinical models of cancer immunotherapy either alone or in combination with antibodies, other cytokines or immune checkpoint...
blockers. Early studies in vivo using the systemic expression of IL-21 by plasmid-mediated delivery revealed that IL-21 can inhibit the growth of large melanomas and fibrosarcomas.\textsuperscript{22} The depletion of select populations of immune cells showed that most of the antitumor activity of IL-21 is mediated by NK cells, with a smaller contribution from CD8\textsuperscript{+} T cells.\textsuperscript{21} In syngeneic mouse models of melanoma and renal cell carcinoma, IL-21 administered intratumorally was able to strongly inhibit tumor growth and increase the frequency of tumor-infiltrating CD8\textsuperscript{+} T cells.\textsuperscript{42} In addition, delivery of IL-21 showed low toxicity and did not induce vascular-leak syndrome, a dose-limiting toxicity associated with other clinically utilized cytokines, such as IL-2.\textsuperscript{43} IL-21 antitumor activities have been exploited in association with several other molecules, which showed either additive or synergistic effects.\textsuperscript{44} The use of an anti-CD25 mAb in combination with an IL-21-secreting mammary carcinoma cell vaccine cured 70% of syngeneic mice from lung micrometastases through the induction of antigen-specific CD8\textsuperscript{+} T cell responses and production of IFN\gamma.\textsuperscript{26} In immunodeficient mice bearing human B cell lymphoma xenografts, the addition of IL-21 to rituximab treatment significantly increased survival relative to either agent alone.\textsuperscript{65} These findings illustrate the therapeutic potential for IL-21 administration and suggest that FcR-activated NK cells would be sensitive to this cytokine and able to aid in the eradication of tumor. Indeed, the stimulation of the FcR on the surface of NK cells led to an upregulation of IL-21R and increased NK cell ADCC and IFN\gamma production in response to trastuzumab-coated tumor cells.

Several potential mechanisms of action have been proposed for the antitumor effects of mAbs in vivo, including cross-linking-mediated activation of signaling cascades that lead to tumor cell apoptosis, blockade of ligands required for tumor cell survival, recruitment of the complement cascade, and recruitment of cytotoxic FcR-positive effector cells.\textsuperscript{46} NK cells are able to directly kill transformed cells due to the interaction of the FcγRIIIa with bound mAbs.\textsuperscript{47, 48} The FcγRIIIa on NK cells initiates cellular activation through an intracellular immunoreceptor tyrosine-based activation motif.\textsuperscript{59, 60} We have also shown
that CD56dimCD16+ NK cells co-stimulated via the FcR and IL-12R increase their expression of genes encoding cytotoxicity receptors, apoptotic proteins, intracellular signaling molecules, and cytokines that could mediate enhanced cytotoxicity and interactions with other immune cells within inflammatory tissues. Upregulation of the IL-21 cytokine receptor has positive implications for both the proliferation and maturation of NK cell populations from the bone marrow, as IL-21 has been shown to synergistically enhance IL-15- and Flt3L-mediated NK cell generation from CD34+ haematopoietic progenitor cells. Based on this data, the use of IL-21 in combination with therapeutic mAbs is thought to exert a positive antitumor effect. The fact that NK cell IL-21R expression was correlated with favorable clinical outcomes in patients receiving trastuzumab alone supports this contention. It should be noted that studies in patients receiving trastuzumab are limited and results are preliminary. The results of correlative studies of NK cells in patients with HER2-positive cancers that received trastuzumab are interesting but are preliminary in nature and must be confirmed in larger prospective studies. Furthermore, it is very possible that the surface expression of other receptors would also increase following mAb therapy, including CD25. Functional studies with NK cells isolated from patients receiving trastuzumab therapy could add to this manuscript. However, our group has observed low NK cell activity from patient PBMC that were cryopreserved and then thawed. This limitation reduced our enthusiasm to conduct functional assays using total PBMC in an ex vivo cytotoxicity assay.

In the present study, the IL-21R was shown to be upregulated at the transcriptional and protein levels in NK cells following FcR-stimulation. Upregulation of the IL-21R allowed NK cells to be more sensitive to IL-21 in terms of signal transduction and effector functions. It was also shown that IL-21R expression was upregulated on the surface of NK cells from patients who had a clinical response to antibody-based chemotherapy. These results support further investigation into the use of IL-21 in patients receiving mAb therapy.
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reported are of dual positive populations (Q2).

Disease. Percentages of patients achieving complete response, partial response, or stable disease (CR, PR, or SD) were determined by a gating strategy utilizing an isotype control antibody. The asterisk denotes p = 0.0302 vs. pre-therapy. (B) IL-2R expression in NK cells of patients with metastatic breast cancer who successfully received doxorubicin/plus cyclophosphamide every 3 weeks × 4 cycles followed by weekly trastuzumab plus paclitaxel and exhibited clinical benefit (complete response, partial response, or stable disease = CR, PR, or SD). Time points are pre-therapy and at the 20 week time point at which time patients were receiving trastuzumab. Each data point is reflective of double positive populations determined by a gating strategy utilizing an isotype control antibody. The asterisk (*) denotes p = 0.0302 vs. pre-therapy. (A) IL-2R expression in NK cells of patients with operable breast cancer who successfully received trastuzumab-based therapy were analyzed for IL-21R expression. PBMC were stained with Abs specific for CD56 and IL-2R and analyzed by flow cytometry. (A) IL-2R expression in NK cells of patients with operable breast cancer who successfully received doxorubicin/plus cyclophosphamide every 3 weeks × 4 cycles followed by weekly trastuzumab plus paclitaxel and exhibited clinical benefit (complete response, partial response, or stable disease = CR, PR, or SD). Time points are pre-therapy and at the 20 week time point at which time patients were receiving trastuzumab. Each data point is reflective of double positive populations determined by a gating strategy utilizing an isotype control antibody. The asterisk (*) denotes p = 0.0302 vs. pre-therapy. (B) IL-2R expression in NK cells of patients with metastatic breast cancer treated with trastuzumab and CpG oligodeoxynucleotides. Each data point is reflective of double positive populations determined by a gating strategy utilizing an isotype control antibody. Time points are pre-therapy and the 20 week mark at which time patients were receiving trastuzumab therapy. All patients eventually experienced progression of disease. Percentages reported are of dual positive populations (Q2).

Disclosure of potential conflicts of interest

The authors have no conflicts of interest to disclose.

Funding

This work was supported by NIH Grants P01 CA095426 (M. Caligiuri), P30 CA16058 (M. Caligiuri), CA84402, K24 CA93670 (W.E. Carson, III), T32 GM068412 (ACJ-R), and T32 CA009338.

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