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Interleukin-21 Enhances NK Cell Activation in Response to Antibody-Coated Targets

Julie M. Roda,* Robin Parihar,† Amy Lehman,‡ Aruna Mani,§ Susheela Tridandapani,¶ and William E. Carson III∥

NK cells express an activating FcR (FcγRIIIa) that mediates Ab-dependent cellular cytotoxicity and the production of immune modulatory cytokines in response to Ab-coated targets. IL-21 has antitumor activity in murine models that depends in part on its ability to promote NK cell cytotoxicity and IFN-γ secretion. We hypothesized that the NK cell response to FcR stimulation would be enhanced by the administration of IL-21. Human NK cells cultured with IL-21 and immobilized IgG or human breast cancer cells coated with a therapeutic mAb (trastuzumab) secreted large amounts of IFN-γ. Increased secretion of TNF-α and the chemokines IL-8, MIP-1α, and RANTES was also observed under these conditions. NK cell IFN-γ production was dependent on distinct signals mediated by the IL-21R and the FcR and was abrogated in STAT1-deficient NK cells. Supernatants derived from NK cells that had been stimulated with IL-21 and mAb-coated breast cancer cells were able to drive the migration of naïve and activated T cells in an in vitro chemotaxis assay. IL-21 also enhanced NK cell lytic activity against Ab-coated tumor cells. Co-administration of IL-21 and Ab-coated tumor cells to immunocompetent mice led to synergistic production of IFN-γ by NK cells. Furthermore, the administration of IL-21 augmented the effects of an anti-HER2/new mAb in a murine tumor model, an effect that required IFN-γ. These findings demonstrate that IL-21 significantly enhances the NK cell response to Ab-coated targets and suggest that IL-21 would be an effective adjuvant to administer in combination with therapeutic mAbs. The Journal of Immunology, 2006, 177: 120–129.

Natural killer cells are large, granular lymphocytes that participate in innate immune responses to viruses, bacteria, and neoplastic cells (1). Although most innate immune cells express both inhibitory and activating FcRs, NK cells are unique in that they constitutively express only a low affinity, activating FcR (FcγRIIIa or CD16), which enables them to interact with Ab-coated targets. In addition to their ability to mediate Ab-dependent cellular cytotoxicity (ADCC), FcγR-activated NK cells have also been shown to secrete cytokines such as IFN-γ, TNF-α, and chemokines that inhibit tumor cell proliferation, enhance Ag presentation, and aid in the chemotaxis of T cells (1). The activity of mAbs directed against tumor Ags has largely been attributed to the direct, antiproliferative, or proapoptotic effects of the Abs on the tumor cells (2). However, in a murine xenograft model, the antitumor effects of trastuzumab (an anti-HER2/new mAb) were at least partially dependent upon the presence of FcR-bearing immune cells, including NK cells (3). The observation that FcR-dependent mechanisms contributed to the effects of antitumor mAbs suggested that their efficacy could be enhanced via the administration of immune modulatory cytokines with the capacity to activate NK cells.

IL-21 is a product of activated CD4+ T cells that stimulates NK and T cells to secrete IFN-γ (4). It has also been shown to enhance the proliferation and cytotoxicity of NK cells and CD8+ T cells (5). In keeping with these activities, IL-21 has shown promising antitumor activity in a variety of murine models (6–9). In the B16 melanoma model, the antitumor effect of IL-21 was found to be dependent on NK cells, but not T cells, as depletion of NK cells prevented tumor regression following IL-21 administration (6, 8). IFN-γ has also been shown to be an important component of the IL-21-mediated antitumor response, as regression of TS/A tumors was inhibited in IFN-γ-deficient mice (9). These studies suggest a vital role for NK cells and their cytokine products in mediating the antitumor activity of IL-21.

Previous reports have demonstrated that activation of human NK cells with immune stimulatory cytokines enhances IFN-γ secretion in response to CD16 cross-linking (10, 11). We have extended these results by showing that NK cell production of IFN-γ and T cell-attracting chemokines in response to Ab-coated tumor cells is synergistically enhanced in the presence of IL-2, IL-12, and reagents that activate TLR 9 (12–14). Given the stimulatory effects of IL-21 on NK cells and its NK cell-dependent antitumor effects in vivo (15), we hypothesized that this cytokine would uniquely augment FcR-mediated NK cell effector functions.

In the current study, we show that costimulation of human NK cells with IL-21 and trastuzumab-coated human breast cancer cells resulted in abundant production of IFN-γ, TNF-α, and a number of chemokines with the capacity to recruit both resting and activated T cells in vitro. In addition, the ability of NK cells to lyse K562 cells or mAb-coated cancer cells was significantly enhanced in the presence of IL-21. Immunocompetent mice receiving IL-21 and...
trastuzumab-coated tumor cells exhibited elevated levels of serum IFN-γ as compared with mice receiving either agent alone. IL-21 also enhanced the effect of an anti-HER2/neu mAb in a murine tumor model. Thus, IL-21 enhances the NK cell response to Ab-coated targets and augments the antitumor effects of therapeutic mAbs.

Materials and Methods

Cytokines and Abs

Recombinant human and murine IL-21 was provided by ZymoGenetics. Polyclonal human IgG (hulgG) was purchased from Sigma-Aldrich. Trastuzumab (Herceptin), a humanized anti-HER2 mAb, was provided by Genentech. Rituximab (Rituxan), a humanized anti-CD20 mAb (Genentech), was obtained from J. Byrd (Ohio State University, Columbus, OH). The 4D5 mouse mAb recognizing human HER2, was purchased from the National Cell Culture Center. Rabbit anti-human phospho- (p-)STAT1, p-STAT-2, and p-STAT5 were purchased from Cell Signaling Technology. Mouse anti-human p-STAT3 mAb was purchased from Upstate Cell Signaling. Rabbit anti-human p-STAT4 mAb was purchased from Zymed Laboratories.

Isolation of human and murine NK cells

Human NK cells (>95% CD56+) were isolated directly from fresh peripheral blood leukopacks (American Red Cross) by 30-min incubation with RosetteSep mixture (StemCell Technologies) before Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich). Human NK cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human AB serum (HAB; C-SIX Diagnostics), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (10% HAB medium) (13). Murine NK cells were harvested from whole splenocytes by density gradient centrifugation (Sigma-Aldrich). Human NK cells were analyzed by ELISA.

FcR stimulation assays

For immobilized IgG experiments, wells of a 96-well flat-bottom plate were coated with 100 µg/ml hulgG in cold PBS overnight at 4°C, washed with cold PBS, and then plated with human immune cells (2 × 105 cells/well) and 10 ng/ml IL-21, as previously described (13). At the indicated time points, cell-free culture supernatants were harvested and analyzed for levels of various cytokines and chemokines using commercially available ELISA kits (R&D Systems). For the in vitro coculture assay, wells of a 96-well flat-bottom plate were seeded with either the HER2-overexpressing human breast cancer cell line SKBR3 or the HER2-negative human breast cancer cell line MDA-468 (American Type Culture Collection) at a density of 5 × 105 tumor cells/well. Tumor cells were grown to confluence overnight and then treated with 100 µg/ml trastuzumab for 1 h at 37°C, as previously described (13). After washing off unbound trastuzumab, purified NK cells were then added at 2 × 106 cells/well in 200 µl of 10% HAB medium containing 10 ng/ml IL-21. Control conditions consisted of NK cells incubated with medium alone, trastuzumab-coated tumor alone, or IL-21 alone. Culture supernatants were harvested after 24 h and analyzed by ELISA.

Real-time PCR

Purified human NK cells were used in an immobilized IgG assay with 10 ng/ml IL-21, as described above. After 6 h, total cellular RNA was isolated using the RNeasy Minikit (Qiagen) and cDNA was generated with random hexamers and Moloney murine leukemia virus-reverse transcriptase, according to the manufacturer’s recommendations (Invitrogen Life Technologies). Using the cDNA as template, real-time PCR for human IFN-γ or TNF-α transcript was performed with primer and probe sets specific for the cytokine transcript and a β-actin internal control (PE Applied Biosystems). Data were analyzed according to the comparative threshold method and normalized against the β-actin transcript (13). Results are semiquantitative and represent the fold difference in transcript levels in a particular sample as compared with the levels in unstimulated cells.

Measurement of T cell chemotaxis

Activated T cells were generated by sequential culture of peripheral blood-derived T cells from a normal donor for 2 days with 1 µg/ml PHA (Sigma-Aldrich) and then 3 days with 500 µM human IL-2 in RPMI 1640 complete medium supplemented with 10% FBS. Transwell inserts for transwell migration experiments (5-µm pore size) (Corning Glass) were first prepared by coating with 0.01% gelatin at 37°C overnight, followed by 3 µg of human fibronectin (InVitrogen Life Technologies) at 37°C for 3 h to mimic endothelial and extracellular matrix components, as previously described (16). Migration experiments were performed by placing 2 × 105 purified naive or activated T cells in 100 µl of 10% HAB medium in the upper chambers of fibronectin-coated Transwell inserts. Inserts were then placed into the wells of a 24-well plate. Medium, medium supplemented with 1 µg/ml IFN-γ-inducible protein-10 (positive control for T cell chemotaxis) (17), or NK cell culture supernatants (400 µl) were placed in the lower chambers of the wells. The plates were then incubated for 3 h at 37°C, followed by a 10-min incubation at 4°C to loosen any cell adhering to the undersides of the insert membranes (18). The fluid in the lower chambers was then collected separately, and migrated cells were quantitated using trypan blue exclusion. Some of the cells were stained for CD4 and CD8 expression and analyzed by flow cytometry to assess the CD4:CD8 ratio of migrated T cells.

Cytokine transcript was performed with primer and probe sets specific for the

In vivo costimulation assay

A murine colon carcinoma line overexpressing human HER2/neu, CT-26HER2/neu, was obtained from P. Kaumaya (Ohio State University, Columbus, OH). CT-26HER2/neu cells were incubated at 4°C in PBS supplemented with 5% FBS (cell density = 1 × 106 cells/ml) with either trastuzumab or normal hulgG (both at 1 mg/ml) for 45 min. Cells were then washed twice in sterile PBS, and 4 × 105 cells were injected i.p. into BALB/c mice, aged 5–7 wk (The Jackson Laboratory). Mice also received a separate i.p. injection of 10 µg of murine IL-21. Control groups (n = 8) received injections of IgG-treated tumor alone, trastuzumab-coated tumor alone, or IgG-treated tumor and IL-21. Mice were bled 24 h postinjection, and serum was analyzed for IFN-γ content by ELISA (R&D Systems). The parental CT-26 cell line was also used as a control. In some experiments, mice were depleted of NK cells through i.p. injections of an anti-asialo GM1 Ab (Wako BioProducts) three times per week for 1 wk before coadministration of the tumor cells and IL-21 (200 µg/injection). Control mice received injections of PBS. This technique resulted in >93% depletion of NK cells in the peripheral blood and spleen, as determined by flow cytometric analysis with an anti-murine DX5 Ab.

Murine tumor model

Age-matched, female, wild-type, or IFN-γ-deficient BALB/c mice (The Jackson Laboratory) were injected s.c. on the right flank with 2 × 106 CT-26HER2/neu cells in a 200-µl volume. When the tumors had reached a volume of ~200 mm3 (5–7 days), mice were randomly allocated to treatment with PBS, 10 µg mouse IL-21, 1 mg/kg 4D5, or 4D5 and IL-21 (n = 5 mice/group). The dose of IL-21 was chosen on the basis of titration experiments examining the efficacy of IL-21 alone against the CT-26HER2/neu tumors (data not shown). All treatments were administered i.p. IL-21 was administered daily for the first 10 days of treatment and twice weekly thereafter. The 4D5 was administered thrice weekly. Tumor dimensions were measured daily with calipers, and tumor volume was calculated as follows: tumor volume = 0.5 × (large diameter) × (small diameter)2. Treatment was continued until tumors reached a length of 25 mm in any dimension (~3.5 wk), at which point mice were euthanized. All protocols were approved by the Ohio State University Animal Care and Use Committee, and mice were treated in accordance with institutional guidelines for animal care.

Statistics

Statistical analyses on ELISA cytokine levels were performed using Student’s t test, with a p value of ≤0.05 considered significant. In the murine tumor costimulation model, changes in tumor volume over time were assessed via a longitudinal model. Tumor values were first log transformed, and then a mixed effects model was applied to the data. Estimated slopes (changes in tumor volume over time) were calculated with 95% confidence intervals,
Results

IL-21 enhances IFN-γ production by human NK cells exposed to immobilized IgG

We were interested in determining the ability of IL-21 to enhance NK cell cytokine production in the presence of immobilized hulG. Purified NK cells cultured for 72 h in the presence of IL-21 alone (1–100 ng/ml) produced significant amounts of IFN-γ as compared with NK cells cultured in unsupplemented medium. IFN-γ secretion in response to IL-21 was dose dependent. Co-stimulation of NK cells with IL-21 and immobilized IgG led to synergistic induction of IFN-γ (Fig. 1a). In parallel experiments, the concentration of immobilized IgG was varied, while the IL-21 dose remained constant. Elevated IFN-γ production was observed even in response to low concentrations of immobilized IgG, while the 100 μg/ml dose appeared to induce maximal NK cell stimulation (Fig. 1b). Time course studies revealed that NK cell production of IFN-γ began within 6 h of exposure to IL-21 and immobilized IgG.
and peaked near 24 h (Fig. 1c and data not shown). Although the level of IFN-γ secretion varied between donors, for all donors tested, costimulation of NK cells with IgG and IL-21 resulted in 5-fold secretion of IFN-γ than stimulation with either agent alone. Costimulation of NK cells with immobilized IgG and IL-21 also resulted in synergistic production of TNF-α and the T cell-attracting chemotactic factors IL-8, RANTES, and MIP-1α, but not MIP-1β, macrophage-derived chemokine, MCP-1, or growth-related oncoreg-c-α (Fig. 1d and data not shown). Analysis by real-time PCR revealed a marked induction in IFN-γ and TNF-α transcript in NK cells costimulated with IgG and IL-21, as compared with cells stimulated with either agent alone (Fig. 1e). In addition, it has been reported that costimulation of NK cells with IL-21 and IL-15 or IL-18 is also an effective stimulus for IFN-γ production (4). We therefore compared NK cell IFN-γ production in response to IL-21 and IL-15/IL-18 with that of NK cells in response to IL-21 and IgG. We found that IgG was a more potent costimulus for NK cell IFN-γ production in the presence of IL-21 than either IL-15 or IL-18 (Fig. 1f). Furthermore, the combination of immobilized IgG and IL-21 elicited 3- to 10-fold greater IFN-γ production than the combination of immobilized IgG and IL-2, IL-15, or IL-18 (data not shown).
IL-21 enhances IFN-γ/H9253 production by NK cells cocultured with Ab-coated tumor cells

Based on the above results, it was hypothesized that IL-21 would enhance the response of NK cells to Ab-coated tumor cells. An in vitro assay was used in which purified immune cells were cocultured with Ab-coated human breast cancer cells. SKBR3 (HER2-overexpressing) and MDA-468 (HER2-negative) cells were treated with trastuzumab (an anti-HER2 mAb) and plated with purified NK cells in the presence or absence of IL-21. After 72 h of coculture, supernatants were harvested and assayed for cytokine content. Costimulation of NK cells with IL-21 and trastuzumab-coated SKBR3 cells resulted in synergistic production of IFN-γ/H9253 (Fig. 2a). In contrast, there was minimal production of IFN-γ/H9253 in the presence of HER2-negative cells, regardless of the presence of IL-21. SKBR3 or MDA-468 tumor cells cultured without NK cells did not secrete IFN-γ/H9253 when treated with trastuzumab and IL-21, confirming that the IFN-γ/H9253 was derived from the NK cells and not the tumor cells (data not shown). Experiments using Transwell compartments confirmed that NK cell IFN-γ/H9253 production in response to trastuzumab-coated tumor cells and IL-21 required direct cell contact between tumor cells and NK cells (data not shown). Costimulation of NK cells also resulted in synergistic production of TNF-α and the chemokines MIP-1α, MIP-1β, MCP-1, and IL-8 (Fig. 2b). In addition, IL-21 and Ab-coated tumor cells each induced significant secretion of RANTES, although this secretion was not synergistically enhanced upon costimulation (Fig. 2b).

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NK cell IFN-γ production in response to IL-21 is not enhanced in the presence of APCs

To compare the NK cell response to IL-21 plus IgG to that of whole PBMCs, total PBMCs or purified NK cells from the same donor were cultured in the presence or absence of IL-21 on plates that had been precoated with immobilized IgG. IFN-γ/H9253 production by whole PBMCs was consistently ~10–20% of IFN-γ/H9253 production by an equal number of NK cells from the same donor (Fig. 3a). Dual parameter flow cytometry for intracellular IFN-γ/H9253 and specific markers of immune cell subsets demonstrated that the IFN-γ/H9253 was derived from the NK cell compartment (data not shown). CD56+ NK cells, CD3+ T cells, and CD14+ monocytes (all ~96% pure)
were then isolated from a single donor and cultured in the presence or absence of IL-21 on IgG-coated plates. Both purified NK cells and purified T cells secreted IFN-γ in response to IL-21 stimulation, but the T cell IFN-γ response was modest and was not enhanced in the presence of immobilized IgG (Fig. 3b). No IFN-γ production from monocytes was observed under any condition. Based on the potential for interaction between monocytes and NK cells through cytokine signaling and/or direct cell contact (13), depletion experiments were conducted in the context of the co-stimulation assay. The removal of CD14+ monocytes from the PBMC population did not reduce IFN-γ secretion in this system (data not shown). Conversely, addition of monocytes to NK cells at various ratios (NK:monocyte ratios of 1:1, 1:2, and 1:4) did not enhance NK cell IFN-γ secretion in response to IL-21 and immobilized IgG at any ratio examined (data not shown). Collectively, this evidence demonstrated that NK cells are the primary source of IFN-γ produced in response to IL-21, and that this response was not enhanced in the presence of APCs.

**IL-21 enhances NK cell lytic activity**

The effect of overnight treatment of NK cells with IL-21 was examined in an ADCC assay using BT-474 (HER2-positive) tumor cells as targets. Human NK cells were cultured overnight in medium alone or medium supplemented with 10 ng/ml IL-21. Target cells were labeled with 51Cr and treated with either huIgG or tras-tuzumab alone or medium supplemented with 10 ng/ml IL-21. Target cells as targets. Human NK cells were cultured overnight in medium supplemented with 50 ng/ml PMA for 5 min and analyzed for levels of intracellular p-Erk by flow cytometry, to confirm the efficacy of Erk inhibition (data not shown).

**FIGURE 5.** IFN-γ production in response to IL-21 and IgG is dependent on activation of STAT1 and the p44/42 MAPK. A, NK cells were stimulated with medium alone, 10 ng/ml IL-21, huIgG, or huIgG and IL-21 for 15 min and then analyzed for intracellular levels of phosphorylated STAT proteins by flow cytometry, as previously described (19). B, NK cells from STAT1−/− or wild-type mice were isolated from splenocytes by magnetic labeling and plated on wells precoated with murine IgG in the presence of 10 ng/ml murine IL-21. Culture supernatants were harvested at 24 h and analyzed for IFN-γ content by ELISA. C, Human NK cells were pretreated with 10 μM DMSO (vehicle control) or 25 μM U0126 before use in an immobilized IgG assay with 10 ng/ml IL-21. Supernatants were harvested at 24 h and analyzed by ELISA. D, NK cells pretreated with DMSO or U0126 (25 μM) were stimulated with medium or huIgG and IL-21 (10 ng/ml), then stained for intracellular p-STAT1 by flow cytometry. Aliquots of the same cells were stimulated with 50 ng/ml PMA for 5 min and analyzed for levels of intracellular p-Erk by flow cytometry, to confirm the efficacy of Erk inhibition (data not shown).

NK cell IFN-γ secretion in response to IL-21 and IgG is dependent on activation of STAT1 and the p44/42 MAPK To elucidate the mechanism by which trastuzumab and IL-21 synergize to induce NK cell cytokine secretion, both IL-21R and FcγRIIIa signaling pathways were examined. The STATs are important transcription factors that mediate IL-21-induced IFN-γ secretion in NK cells (4). NK cells from normal human donors were stimulated for 15 min with medium alone, IL-21 (10 ng/ml), immobilized IgG, or IgG and IL-21, and then examined for intracellular levels of p-STAT1, p-STAT2, p-STAT3, p-STAT4, and p-STAT5 by flow cytometry (19). STAT2 and STAT4 were not activated in response to IL-21 or FcR stimulation (data not shown). IL-21 treatment induced activation of STAT1, STAT3, and STAT5; however, this effect was not significantly enhanced by the addition of FcR stimulation (Fig. 5a). This suggested that the STAT proteins were specifically activated in response to IL-21. To
further test this hypothesis, splenic NK cells were isolated from wild-type mice and STAT1-deficient (STAT1<sup>−/−</sup>) mice and cultured on plates that had been precoated with murine IgG in the presence of murine IL-21. Whereas wild-type NK cells displayed synergistic IFN-γ production in response to immobilized IgG and IL-21, cells from STAT1<sup>−/−</sup> mice were unable to secrete IFN-γ in response to stimulation either with IL-21 alone or IL-21 and immobilized IgG (Fig. 5b). Importantly, IFN-γ secretion in response to immobilized IgG alone, while low, was not affected in STAT1<sup>−/−</sup> cells, further emphasizing the specificity of STAT1 activation in response to occupation of the IL-21R.

To investigate the contribution of FcR-induced signaling to the observed IFN-γ secretion, NK cells were pretreated with a specific chemical inhibitor of the p42/44 MAPK Erk before use in the immobilized IgG assay. Inhibition of Erk signal transduction prevented IFN-γ secretion in response to immobilized IgG, although IFN-γ secretion in response to IL-21 was unaffected. Synergistic induction of IFN-γ following costimulation of NK cells with immobilized IgG and IL-21 was also lost following inhibition of Erk signaling (Fig. 5c). In contrast, chemical inhibition of the MAPK family members p38 and JNK did not diminish IFN-γ secretion by costimulated NK cells (data not shown). In a series of immunoblot analyses, NK cells were activated via FcγRIIIa cross-linking in the presence of IL-21, and whole cell lysates were analyzed for p-STAT1, p-STAT3, pSTAT-5, and p-Erk. Elevated levels of activated STAT1, STAT3, and STAT5 were detected within IL-21-stimulated NK cells, and elevated levels of activated Erk were found within FcR-stimulated cells (data not shown). However, no further activation of these signaling intermediates was observed in response to costimulation. In addition, pretreatment of NK cells with U0126 before costimulation had no effect on activation of STAT1, 3, or 5 (Fig. 5d and data not shown). Taken together, these results suggest that cytokine secretion by NK cells in response to activation of FcR and IL-21R is dependent upon distinct and non-interacting signaling cascades.

** Supernatants from NK cells costimulated with Ab-coated tumor and IL-21 induce chemotaxis of naive and activated T cells **

Having demonstrated that NK cells costimulated with Ab and IL-21 produce large quantities of several T cell-attracting chemokines (see Figs. 1c and 2b), we next wished to determine the ability of these NK cell-derived factors to direct T cell migration. Naive or activated T cells were placed in the upper chambers of Transwell inserts. The lower chambers contained NK cell culture supernatants from the conditions of the immobilized IgG experiments and had been confirmed for chemokine content. Culture supernatants from IL-21-stimulated NK cells induced significant chemotaxis of both naive (Fig. 6a) and activated (Fig. 6b) T cells. Enhanced T cell chemotaxis was observed when culture supernatants from NK cells costimulated with IL-21 and IgG were used as the chemokine source, as compared with chemokine in response to culture supernatants derived from the single-stimulation conditions. This effect was synergistic for the migration of activated T cells. Of note, culture supernatants from IL-21-activated NK cells were much more efficient at inducing chemotaxis of naive T cells than supernatants from IL-2- or IL-12-activated NK cells (data not shown).

** IL-21 enhances in vivo IFN-γ production in response to trastuzumab-coated, HER-positive tumor cells **

An in vivo costimulation experiment was performed in which IL-21 and trastuzumab-coated, human HER2-overexpressing murine tumor cells (CT-26<sup>HER2/neu</sup>) were delivered i.p. to naive, immunocompetent mice. Serum IFN-γ content was analyzed by ELISA (24 h postinjection). Coadministration of IL-21 (10 μg/mouse) and trastuzumab-coated, HER-positive tumor cells resulted in increased serum levels of IFN-γ as compared with mice receiving IL-21 and control Ab-treated tumor cells (Fig. 7a). No IFN-γ was detected in the serum of mice receiving either IgG-treated or trastuzumab-coated tumor cells alone. Of note, increased levels of IFN-γ were not detected when mice received IL-21 in combination with trastuzumab-treated CT-26 cells (the HER2-negative, parental cell line), demonstrating that the IFN-γ induced in response to trastuzumab and IL-21 is dependent on tumor cell expression of the HER2 Ag (data not shown). In addition, IFN-γ secretion was markedly decreased when mice were depleted of NK cells before coadministration of trastuzumab-coated CT-26<sup>HER2/neu</sup> cells and
IL-21 (Fig. 7b). These results demonstrate that in vivo administration of IL-21 can enhance the NK cell cytokine response to Ab-coated tumor cells.

**IL-21 enhances the efficacy of an antitumor mAb in a murine solid tumor model**

A murine tumor model was used to determine whether IL-21 could enhance the effect of an antitumor mAb in vivo. Mice bearing solid tumors overexpressing human HER2 were treated with PBS, murine IL-21 (10 μg/mouse), 4D5 (a murine mAb recognizing human HER2), or the combination of 4D5 and IL-21. Based on a longitudinal model using log-transformed values, no significant differences in tumor volume were found between the four groups at baseline. However, at day 26 of treatment, the average tumor volumes of mice receiving either IL-21 or 4D5 alone were significantly smaller than those of the PBS-treated mice ($p < 0.0001$). Furthermore, the average tumor volumes for mice receiving the combination of 4D5 and IL-21 were significantly smaller than mice receiving IL-21 alone ($p = 0.0007$) or 4D5 alone ($p < 0.0001$). To determine whether the therapeutic efficacy of 4D5/IL-21 is dependent on IFN-γ, wild-type or IFN-γ-deficient mice bearing s.c. CT-26HER2new tumors were treated with PBS or the combination of 4D5 and IL-21 (Fig. 8b). By day 23, wild-type mice receiving 4D5 and IL-21 exhibited a ~50% reduction in tumor volume as compared with wild-type mice receiving PBS, consistent with previous experiments ($p = 0.0005$). In contrast, tumor growth in the IFN-γ-deficient mice receiving 4D5 and IL-21 was not significantly different from tumor growth in the IFN-γ-deficient mice receiving PBS ($p = 0.6172$). Of note, the CT-26HER2new cells grew more rapidly in the IFN-γ-deficient mice, indicating a possible role for IFN-γ in natural immune surveillance against this cell line. These data demonstrate that IL-21 can enhance the effect of a therapeutic mAb in a murine solid tumor model, and that this effect is dependent on IFN-γ.

**Discussion**

In the current study, we have demonstrated that NK cell IFN-γ secretion in response to IL-21 is greatly enhanced in the presence of an FcR stimulus. Costimulation of NK cells with IL-21 and trastuzumab-coated human breast cancer cells caused NK cells to secrete 5- to 10-fold higher levels of IFN-γ as compared with stimulation with either agent alone. In addition, costimulation of NK cells in this fashion also induced the secretion of a number of chemokines with the ability to direct the chemotaxis of both resting and activated T cells. IL-21 enhanced NK cell lytic activity against trastuzumab-coated human breast cancer cells and rituximab-coated human B cell lymphomas. Coadministration of IL-21...
and trastuzumab-coated tumor cells to immunocompetent mice resulted in enhanced circulating levels of NK cell-derived IFN-γ. In addition, in a breast tumor model, IL-21 enhanced the effect of an anti-HER2 mAb in an IFN-γ-dependent fashion. These data demonstrate the ability of IL-21 to enhance NK cell activation in response to mAb-coated targets and suggest a pathway by which NK cells can have an effect on the generation of T cell immunity.

IFN-γ has an important role in the regulation of innate and adaptive immune responses. IFN-γ enhances Ag presentation, activates macrophages and T cells, and promotes the differentiation of CD4⁺ T cells into Th1 cells (20). In addition to its role in regulating immune cell function, IFN-γ also exerts antiproliferative and proapoptotic effects on tumor cells (20–22). These properties have suggested to investigators the potential value of therapeutic strategies designed to enhance the endogenous production of IFN-γ. In the context of Ab therapy, the role of IFN-γ in the clearance of tumor cells was established in a preclinical model of HER2-overexpressing carcinoma (23). Mice were inoculated with a HER2 vaccine to induce circulating anti-HER2 Abs and were then rechallenged with HER2-positive tumor cells. Mice deficient in IFN-γ were unable to clear the subsequent tumor challenge. In the current study, the ability of IL-21 to enhance the effect of an antitumor mAb was dependent on IFN-γ production. Of note, our group has observed elevated circulating levels of NK cell-derived IFN-γ within a subset of patients experiencing a clinical benefit on a phase I trial of trastuzumab and IL-12 (24) and on a follow-up trial of IL-12 plus trastuzumab and paclitaxel (25). These observations suggest that administration of immune modulatory cytokines such as IL-21 in combination with antitumor mAbs may be an effective strategy for inducing NK cell IFN-γ production.

In addition to enhancing NK cell IFN-γ production, costimulation of NK cells with IL-21 and mAb-coated tumor resulted in the secretion of a number of chemokines that could direct the migration of both resting and activated T cells. In previous studies, we found that patients exhibiting a clinical response on a phase I trial of trastuzumab and IL-12 exhibited increased circulating levels of NK cell-derived chemokines (26). These findings suggested a model in which NK cell-derived chemokines could drive the migration of naïve and activated T cells, promoting the development of an Ag-specific immune response. In the current study, we have shown that costimulation of NK cells with trastuzumab and IL-21 induced a similar chemokine response. Interestingly, supernatants derived from NK cells costimulated with trastuzumab-coated tumor and IL-21 induced much higher levels of chemotaxis by naïve T cells than supernatants derived from NK cells costimulated with trastuzumab and IL-2 or IL-12 (our unpublished data). Other groups have reported that IL-21 can enhance the proliferation of naïve T cells and can enhance the clonal expansion and survival of tumor-specific CD8⁺ T cells (27–29). Taken together, these findings suggest that chemokines produced in response to IL-21 could enhance the transition between the innate and adaptive phase of the immune response (29).

The signaling component of the IL-21R complex is the common γ-chain, which is also a functional component of the IL-2R, IL-4R, and IL-15R (30). Binding of IL-21 to the IL-21R results in the activation of the STAT proteins, which translocate to the nucleus and initiate the transcription of IL-21-responsive genes. IL-21-induced expression of IFN-γ in NK cells and T cells has been shown to be dependent upon the activation of STATs (4). Strengell et al. (4) have reported that IL-21 induced the activation of STAT1, STAT3, and STAT4, but not STAT5, in human NK cells and the NK cell line NK-92. In contrast, we found elevated levels of p-STAT1, p-STAT3, and p-STAT5, but not p-STAT4 or p-STAT2, in NK cells stimulated with IL-21. There was no difference in levels of p-STAT1, p-STAT3, or p-STAT5 in cells that had been costimulated with IL-21 and immobilized IgG as compared with cells that had been stimulated with IL-21 alone. These findings suggested that activation of the STAT proteins in response to IL-21 is not influenced by FcR signal transduction. In addition, while it has been reported that STAT3 is the primary STAT activated by IL-21 (4), we found that NK cells from STAT1 knockout mice were unable to respond to costimulation with IL-21 and murine IgG, suggesting a vital role for STAT1 in mediating NK cell IFN-γ secretion in response to IL-21. However, because STAT3-deficient mice die in early embryogenesis and STAT5-deficient mice lack NK cells, we are unable to confirm the roles of these factors in mediating the NK cell response to IL-21.

FcγRⅢA is known to signal through the Ras-MAPK pathway (31, 32), and IL-21 has been shown to activate the p44/42 MAPK Erk in a promyelocytic cell line (33). Studies in which NK cells were pretreated with chemical inhibitors of specific signal transduction pathways indicated that NK cell IFN-γ secretion in response to IL-21 and immobilized IgG is dependent on activated Erk. However, Erk was not activated in response to IL-21 alone in NK cells, and while chemical inhibition of Erk was able to abrogate NK cell secretion of IFN-γ in response to dual stimulation, this treatment had no effect on the NK cell response to IL-21 alone. In addition, while activation of Erk has been shown to be required for STAT phosphorylation in some systems (34, 35), this did not appear to be the case in the present model, as chemical inhibition of Erk did not diminish phosphorylation of STAT1, STAT3, or STAT5 in response to immobilized IgG and IL-21. These data suggest that stimulation of NK cells via the IL-21R and FcγRⅢA results in the activation of distinct signaling cascades, each of which culminate in the activation of unique transcription factors with the potential to act on the IFN-γ promoter.

IL-21 enhances the NK cell response to mAb-coated targets and serves as an effective adjuvant to therapy with an anti-HER2/neu mAb in a murine breast cancer model. These results suggest that the administration of exogenous IL-21 could be an effective means of enhancing the immune response to antitumor mAbs.

Disclosures

The authors have no financial conflict of interest.

References


