Interleukin 21 Enhances Antibody-Mediated Tumor Rejection

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Abstract
Interleukin-21 (IL-21) is a cytokine with structural and sequence homology to IL-2 and IL-15 that has antitumor activity alone in mouse experimental tumor models and a tolerable safety profile in phase I trials in patients with metastatic melanoma and renal cell carcinoma. Several monoclonal antibodies (mAb) targeted at tumor-associated antigens also have improved antitumor activities in mice when used in combination with IL-21. Recently, we described a rational three antibody-based approach (triple mAb, TrimAb) to eradicating established mouse tumors that required the generation of tumor-reactive CD8+ T cells and IFN-γ. Herein, we show that sequentially combining TrimAb with recombinant IL-21 can significantly improve the antitumor activity of this combination against very advanced disease. These data further support the use of IL-21 in adjuvant settings where strong T cell–mediated immune responses to tumors can be generated.

Introduction
Interleukin-21 (IL-21) binds the IL-21R heterodimer consisting of the IL-21–specific IL-21Rα chain and the common γ chain (γc, CD132), and thus, IL-21 is a member of a family of cytokines defined by the use of γc, which also includes IL-2, IL-4, IL-7, IL-9, and IL-15. IL-21 is produced by activated CD4+ T cells and natural killer (NK) T cells (1, 2), and an initial report described its ability to affect the proliferation and function of NK cells, B cells, and T cells (1). Considering its potential for immune activation, it is not surprising that IL-21 also has antitumor effects. These have been observed in mouse models including melanoma, sarcoma, bladder, and renal cell carcinoma (3–7). The antitumor activity of IL-21 was shown to depend on NK cells or CD8+ T cells or both depending on the tumor model. Tumor rejection typically requires perforin (4, 5), and in some models, rejection occurs via an NKG2D-dependent mechanism (8). Other models have shown that antitumor responses are associated with tumor-infiltrating CD8+ T cells and the induction of long lasting central and effector memory cells (6, 7, 9, 10) or by the activation of NK cells. IL-21 can also enhance endogenous tumor-specific antibody responses (11) and facilitate NK cell differentiation (4) that enhances antibody-dependent cellular cytotoxicity (ADCC; ref. 12). The ability of IL-21 to enhance ADCC preclinically provides the justification for current studies in combination with rituximab in CD20+ B cell non–Hodgkin’s lymphoma (clinicaltrials.gov identifier NCT00347971) or cetuximab in patients with colorectal cancer (Eudract no. 2006-004231-30). Within 4 years of its initial description, human clinical trials of recombinant IL-21 have commenced and anticancer efficacy was shown (13).

Further clinical development is currently under way with IL-21 either as a single agent or in combination with other treatment modalities in multiple cancers. A phase I/II trial combining IL-21 with sorafenib in renal cell carcinoma is now recruiting (clinicaltrials.gov identifier NCT00389285). More novel combinations in mice, such as concurrent treatment with IL-21 and agonistic antibodies against tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) receptors (14), have shown that combinations could be justified on the basis that therapy-induced cell death might enhance access of tumor antigens to antigen-presenting cells (APC), with subsequent improvement of T-cell responses due to concurrent IL-21 administration. In considering this possibility further, we have now undertaken to combine IL-21 with a triple monoclonal antibody (TrimAb) combination that eradicates established tumors in its own right. The TrimAb combination contains three agonistic monoclonal antibodies (anti-DR5/anti-CD40/anti-CD137) that have previously been shown to potently and rapidly eradicate a variety of preestablished tumors (15), compared with dual and single combinations of these antibodies that have minimal or no effects against even smaller tumors. Anti-DR5 mediates apoptosis of TRAIL-sensitive tumor cells and generates a tumor-specific adaptive immune response in its own right (16), whereas in this context, it is believed anti-CD40 and anti-CD137 serve to activate dendritic cells and costimulate T cells, respectively, to further stimulate tumor-specific immunity. TrimAb-mediated antitumor effects were found to critically require CD8+ T cells, IL-12, and IFN-γ in the tumor rejection process (15), and given this mechanism of action, we rationalized that the addition of IL-21 might provide some further benefit to TrimAb-induced antitumor response. In a series of experimental mouse tumor models, we now show that IL-21 can significantly further improve the antitumor activity of an effective combined mAb-based therapy.

Materials and Methods
Mice. Inbred BALB/c wild-type (WT) mice were purchased from the Walter and Eliza Hall Institute of Medical Research. BALB/c perforin-deficient (pfp−/−), IFN-γ-deficient, and double perforin and IFN-γ-deficient (pfp.IFN-γ−/−) mice were bred and maintained at the Peter MacCallum Cancer Center, as previously described (17, 18). Mice of 8 to 12 wk of age were used in all experiments. Experiments were done according to Animal Experimentation Ethics Committee Guidelines.

Antibodies and IL-21. We prepared and purified agonistic mAb to mouse DR5 (MD5-1), agonistic mAb to mouse CD40 (FGR45; kindly provided by Dr. Antonius Rolink), agonistic mAb to mouse CD137 (3H3 kindly provided by Dr. Robert Mittler), mAb to mouse CD4 (GK1.5), mAb to...
mouse CD8 (S3-6.7), and neutralizing mAb to mouse IL-12 (C17.8), as previously described (15, 16, 19). For tumor therapy, we gave i.p. 100 μg mAbs, control hamster IgG, and/or control rat IgG (Sigma-Aldrich) two to four times as indicated. We purchased antibody to asialo-GM1 (ASGM1) from Wako Pure Chemical Industries Ltd. Recombinant mouse IL-21 protein was provided by Zymogenetics, Inc. Working preparations were diluted in PBS, and mice were injected either i.p. with 20 μg in 100 μL volume on the days indicated in each experiment. Control mice received an equal volume of PBS.

Tumor cell lines. We maintained and used BALB/c-derived TRAIL-sensitive 4T1 mammary carcinoma, TRAIL-sensitive R331 renal carcinoma, and TRAIL-sensitive CT26 colon carcinoma, as previously described (15, 16, 20).

Therapy of transplanted tumors. We s.c. inoculated tumor cells in the hind flank of mice. We commenced single, dual, or triple mAb treatments when tumors developed to a size of $3 \times 3 \times 3$ (9 mm$^3$, 7 d after tumor inoculation), or larger. We measured tumor size with a caliper as the product of two perpendicular diameters (mm$^2$) every second day. We did surgical resection and spontaneous 4T1 metastases experiments as previously reported (15) with mAb treatments commencing on the resection of primary tumor (on day 28).

In vivo cell depletion and IL-12 neutralization. We depleted CD8$^+$ T cells, CD4$^+$ T cells, or NK cells and neutralized IL-12 by injection with the appropriate antibodies after the method described (16, 20).

Statistical analysis. We used Mann-Whitney rank sum test for statistical analysis of the tumor growth. We determined significant differences in tumor rejection and survival from metastases at one time point by using a log-rank and Wilcoxon Fisher’s exact test. $P$ values <0.05 were considered as significant.

Results

IL-21 enhances TrimAb-mediated rejection of established primary tumors. We have previously shown the efficacy of a combination of anti-DR5, anti-CD40, and anti-CD137 mAbs (TrimAb) against established primary 4T1 mammary carcinomas (15). In prior experiments, four treatments of TrimAb, spaced 3 or 4 days apart, were sufficient to eradicate the majority of small, established primary 4T1 tumors (<10 mm$^2$). Initially, to determine whether IL-21 could potentiate the efficacy of the TrimAb combination, we only gave two sequential doses of TrimAb (on days 7 and 11 after tumor inoculation) and followed 3 days later by three sequential daily treatments (days 14–16) of what had previously been determined to be an optimal dose of recombinant mouse IL-21. This type of schedule had previously been found to be effective when combining anti-DR5 mAb and IL-21 (14). Consistent with the ability of IL-21 to improve the antitumor activity of anti-DR5, it also significantly enhanced the efficacy of TrimAb ($P < 0.05$; Fig. 1A). Administration of two doses of TrimAb was able to induce only rejection of one of five primary 4T1 tumors compared with our previous treatment regimen, which could induce 60% to 80% rejection of primary 4T1 tumors (15). In contrast, the TrimAb/IL-21 combination completely cured four of five mice, demonstrating the ability of IL-21 to enhance the antitumor response induced by TrimAb therapy. Notably, compared with control immunoglobulin (clg)–treated mice, IL-21 alone had little effect on 4T1 tumor growth (Fig. 1A). These data were further supported by experiments where mice received four doses of TrimAb followed by IL-21 (Fig. 1A). Not surprisingly, TrimAb alone was more effective with sustained tumor suppression beyond 30 days with three of five mice becoming tumor free. However, the TrimAb/IL-21 combination afforded complete tumor eradication in all mice. A remarkably similar pattern of 4T1 tumor growth suppression was observed in TrimAb-treated mice receiving four treatments followed by a single injection of plasmid-encoding mouse IL-21 delivered by hydrodynamic injection as previously described (ref. 4; Supplementary Fig. S1).

We next further tested the TrimAb/IL-21 combination against other types of experimental tumors (Fig. 2) and more established subcutaneous 4T1 tumors (Fig. 3) to determine if this combination induced better eradication of established tumors over TrimAb alone. Indeed the combination of TrimAb and IL-21 was found to be more effective than TrimAb alone against established R331 renal carcinoma (Fig. 2A) and CT26 colon adenocarcinoma (Fig. 2B). Treatment of 4T1 with TrimAb or TrimAb/IL-21 was then delayed until day 11 (mean tumor size, >17 mm$^2$) or day 15 (mean tumor size, >35 mm$^2$; Fig. 3). Even when combined treatment was delayed until day 15, all tumors responded to therapy and three of five were completely eradicated by day 32 (Fig. 3). By contrast, TrimAb treatment alone was less effective in each of the different treatments.
impressive result was obtained with only one of five mice treated with TrimAb/IL-21 dying on day 86, and the remaining four mice were completely tumor-free for at least 150 days (P = 0.0079; Fig. 4A). Interestingly, in mice receiving IL-21 in combination with single mAb treatments, IL-21 was not able to significantly extend the survival of mice receiving anti-CD40 (40.0 ± 0.3 days versus 42.6 ± 1.0 days) but did enhance the survival of mice receiving anti-DR5 (44.8 ± 0.8 versus 65.2 ± 2.3, P = 0.0004) or anti-CD137 (48.6 ± 0.7 versus 54.6 ± 1.4, P = 0.0142; Fig. 4B). In addition, each dual treatment containing anti-DR5 (anti-DR5/anti-CD40 and anti-DR5/anti-CD137) was also significantly enhanced by IL-21, with two of five mice receiving anti-DR5/anti-CD137 mAb and IL-21 remaining tumor-free and the other three of five showing enhanced survival.

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**Figure 2.** IL-21 enhances TrimAb-mediated rejection of established primary renal and colon cancer. A, groups of five BALB/c WT mice were inoculated s.c. with 5 x 10^5 R331 renal carcinoma cells (day 0). Groups of mice were then treated i.p. with either 100 μg clg on days 7 and 11 and PBS on days 14, 15, and 16 (open squares, solid line); 100 μg clg on days 7 and 11 and 20 μg IL-21 on days 14, 15, and 16 (open triangles, solid line); 100 μg each of TrimAb on days 7 and 11 and 16 (open circles, solid line); 100 μg each of TrimAb on days 7 and 11 and IL-21 on days 14, 15, and 16 (closed circles, solid line); 100 μg each of TrimAb on days 11 and 15 and PBS on days 18, 19, and 20 (closed squares); and 100 μg each of TrimAb on days 11 and 15 and IL-21 on days 18, 19, and 20 (closed triangles). B, groups of five BALB/c WT mice were inoculated s.c. with 5 x 10^5 CT26 colon adenocarcinoma cells (day 0). Groups of mice were then treated i.p. as in (A). In A and B, tumor growth was measured every second day and tumor sizes represent the mean ± SE of five mice in each group. The proportion of mice surviving tumor is shown in parentheses. Statistical significance at a time point comparing TrimAb-treated and TrimAb/IL-21–treated mice; *P < 0.05.

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**Figure 3.** Delayed TrimAb/IL-21 combination eradicates established metastatic breast cancer. Groups of five BALB/c WT mice were inoculated s.c. with 2 x 10^5 4T1 tumor cells (day 0). Groups of mice were then treated i.p. with either of the following: A, 100 μg clg on days 7 and 11 and PBS on days 14, 15, and 16 (open squares); 100 μg clg on days 7 and 11 and 20 μg IL-21 on days 1 to 6 (closed triangles); 100 μg clg on days 7 and 11 and 20 μg IL-21 on days 10 to 16 (open triangles); 100 μg each of TrimAb on days 7 and 11 and PBS on days 10 to 16 (open circles); or combinations of TrimAb on days 7 and 11 and IL-21 on days 10 to 16 (closed circles). B, groups of five mice were then treated i.p. with either 100 μg clg on days 11 and 15 and 20 μg IL-21 on days 1, 2, and 3 (closed triangles, solid line); 100 μg clg on days 11 and 15 and 20 μg IL-21 on days 18, 19, and 20 (open circles, solid line); 100 μg each of TrimAb on days 11 and 15 and PBS on days 18, 19, and 20 (open circles, dotted line); a combination of TrimAb on days 11 and 15 and IL-21 on days 18, 19, and 20 (closed circles, solid line); 100 μg clg on days 15 and 19 and 20 μg IL-21 on days 22, 23, and 24 (open triangles, dotted line); 100 μg each of TrimAb on days 15 and 19 and PBS on days 22, 23, and 24 (open circles, dotted line); or a combination of TrimAb on days 15 and 19 and IL-21 on days 22, 23, and 24 (closed circles, dotted line). In both parts (A and B), 4T1 tumor growth was measured every second day and tumor sizes represent the mean ± SE of five mice in each group. The proportion of mice surviving tumor is shown in parentheses.
Figure 4. IL-21 enhances TrimAb-mediated rejection of established metastatic breast cancer. Groups of 5 BALB/c WT mice were inoculated orthotopically into the fourth mammary gland with 5 × 10⁴ 4T1 tumor cells (day 0). On day 28, all mice underwent surgery to remove their primary tumor leaving the mice heavily burdened with metastatic lesions in the lung, lymph nodes, liver, and other organs. Groups of mice were then treated i.p. with either of the following: A, 100 μg clg on days 28 and 31 and PBS on days 34, 35, and 36 (open squares); 100 μg clg on days 28 and 31 and 20 μg IL-21 on days 34, 35, and 36 (open triangles); 100 μg each of TrimAb on days 28 and 31 and PBS on days 34, 35, and 36 (open circles); or combinations of TrimAb and IL-21 at similar times as indicated (closed circles). B, clg (squares), anti-DR5 (triangles), anti-CD40 (circles), or anti-CD137 (circles dotted line; 100 μg each) on days 28 and 31 and either PBS (open symbols) or 20 μg IL-21 (closed symbols) on days 34, 35, and 36. C, dual anti-DR5/anti-CD40 (triangles), anti-DR5/anti-CD137 (triangles dotted line), anti-CD40/anti-CD137 (circles; 100 μg of each antibody on days 28 and 31 and either PBS (open symbols) or 20 μg IL-21 (closed symbols) on days 34, 35, and 36. In parts A to C, percentage of tumor-free survival of mice is plotted against days after 4T1 tumor inoculation. Statistical significance comparing survival of treated mice with those additionally receiving IL-21; **, P < 0.005.

over those mice receiving anti-DR5/anti-CD137 alone (Fig. 4C). Further examination of the efficacy of TrimAb in combination with IL-21 in the same 4T1 surgery/metastases model revealed that this combination was more effective than anti-DR5/anti-CD137 and IL-21, particularly when only one round of mAb treatments were given (Supplementary Fig. S2). Therefore, these data illustrated that, although IL-21 could significantly enhance the efficacy with single mAb combinations (DR5 and CD137), optimal efficacy was seen in combination with TrimAb against primary and metastatic disease, in particular strongly correlating with its effect on anti-DR5 mAb.

Role of CD8⁺ T cells, IFN-γ, and IL-12 in rejection of established primary and metastatic breast cancer. We next determined the role of cellular subsets and cytokines in the mechanism of action of a combination of TrimAb/IL-21. TrimAb and IL-21 reduced 4T1 tumor burden and enhanced the survival of mice post surgery, and this effect was completely ablated by the depletion of CD8⁺ T cells (P = 0.004) but not at all by the depletion of CD4⁺ T cells (P = 0.652) and NK cells (P = 0.999; Fig. 5). Consistent with our previous report (15), CD8⁺ T-cell depletion also completely abrogated the antitumor activity of TrimAb (P = 0.004; Fig. 5). In a similar manner, CD8⁺ T cells, but not NK cells or CD4⁺ T cells, were critical for TrimAb/IL-21-mediated suppression of subcutaneous 4T1 tumors when four doses of TrimAb were followed with a single dose of plasmid IL-21 (Supplementary Fig. S3). Using a similar treatment regimen, it was determined that neutralization or deficiency of IL-12 or IFN-γ, respectively, resulted in no therapeutic benefit of TrimAb alone or TrimAb in combination with IL-21 (Supplementary Fig. S4). Perforin deficiency alone resulted in a partial loss of efficacy of TrimAb or TrimAb and IL-21 (Supplementary Fig. S4). These data suggested the mechanism of action of TrimAb alone or in combination with IL-21 were very similar, if not identical, and that IL-21 enhanced the immune response mediated with TrimAb.

IL-21 promotes enhanced tumor-specific memory. Given the enhanced therapeutic efficacy of TrimAb in combination with IL-21, we next determined whether IL-21 was also improving secondary memory responses to tumor rechallenge. Cohorts of mice were cured of their primary subcutaneous (5 × 10⁴ cells) 4T1 tumors with either TrimAb (14 of 15 by day 20) or TrimAb and IL-21 (14 of 15 by day 24; Fig. 6). Approximately, 10 weeks later, mice cured of their primary tumors were rechallenged with 5 × 10⁴, 2 × 10⁵, and 1 × 10⁶ 4T1 cells in the opposite flank. Tumors grew in all naive mice at all of these doses with no mice surviving rechallenge (Fig. 6). Whereas all mice initially cured with TrimAb also resisted tumor rechallenge at 5 × 10⁴ 4T1 cells, three of five mice receiving 2 × 10⁵ 4T1 cells developed tumors and all mice receiving 1 × 10⁶ 4T1 cells developed tumors albeit with a significantly reduced growth rate. By contrast, memory to tumor was greater in mice cured with TrimAb and IL-21 because all these mice resisted tumor rechallenge at 2 × 10⁵ and 5 × 10⁴ 4T1 tumor cells, and one of four mice receiving 1 × 10⁶ 4T1 tumor cells remained tumor-free. These data suggested that IL-21 not only improved primary responses to tumor stimulated by TrimAb, but also enhanced T-cell memory to a secondary challenge.

Discussion
Cancer immunotherapies are becoming increasingly effective as our understanding of tumor immunity advances. Even a relatively short time ago, immunotherapies were effective only in a prophylactic setting (21) or against relatively small metastases (22, 23) or tumors bearing foreign antigens (24–26). Improved approaches led to growth inhibition of more established syngeneic tumors, but eradication of large solid tumors or advanced spontaneously metastatic disease was not widely possible. With further advances
in our understanding of many aspects of immunology including antigen presentation, T-cell costimulation, and memory formation, and as new cytokines and immune system agonists have become available, progress has been made in the immunotherapy of cancer. Recently, complete regression of large syngeneic solid tumors has been shown using combination immunotherapies in a mouse melanoma model (27). It is becoming clear that rationally chosen combinations of immunomodulators offer great potential for effective treatment of established malignant disease.

In the current study described above, we have used IL-21 in combination with antibodies inducing tumor cell apoptosis and immune cell activation and showed complete regression of advanced solid breast tumors. Furthermore, the efficacy of this combination therapy and its reliance on IL-21 was shown for other advanced tumors including renal cancer and colon cancer. In addition, we were able to completely eradicate advanced widespread spontaneously metastatic breast cancer in the majority of mice treated with this regimen. Inclusion of IL-21 in treatment regimens was found to be necessary for optimal effect in all three tumor models.

The TrimAb component of the therapy is thought to operate through the coordination of three molecular and cellular pathways, each stimulated by individual antibodies comprising the TrimAb formulation (15). Early tumor cell apoptosis is mediated by anti-DR5, and as determined by TUNEL analysis, this is not enhanced.

Figure 5. Role of CD8+ T cells in the rejection of established primary and metastatic breast cancer. Groups of five BALB/c WT mice were inoculated orthotopically into the fourth mammary gland with \(5 \times 10^4\) 4T1 tumor cells (day 0). On day 28, all mice underwent surgery to resect the primary tumor. Groups of mice were then treated i.p. with either of the following: A, 100 \(\mu\)g clg on days 28 and 31 and PBS on days 34, 35, and 36 (open squares); 100 \(\mu\)g each of TrimAb on days 28 and 31 and PBS on days 34, 35, and 36 (open circles); the same combination of TrimAb depleted of CD8+ cells (closed circles), or the same combination of TrimAb depleted of NK cells (closed squares). A group of clg-treated mice also received 100 \(\mu\)g of all three depleting mAbs over the same time course (open triangles). B, 100 \(\mu\)g clg on days 28 and 31 and 20 \(\mu\)g IL-21 on days 34, 35, and 36 (open squares); 100 \(\mu\)g each of TrimAb on days 28 and 31 and IL-21 on days 34, 35, and 36 (open circles); the same combination of TrimAb and IL-21 depleted of CD8+ cells (closed circles), the same combination of TrimAb and IL-21 depleted of CD4+ cells (closed circles), or the same combination of TrimAb and IL-21 depleted of NK cells (closed circles). Depletion of NK cells, CD8+ T cells, or CD4+ T cells used 100 \(\mu\)g of mAb as described on day 27, 28, 35, 42, 49, and 56. In all cases, percentage of tumor-free survival of mice is plotted against days after 4T1 tumor inoculation. Statistical significance comparing survival of treated mice with those additionally depleted of CD8+ T cells, CD4+ T cells, or NK cells; *, \(P < 0.05\).

Figure 6. IL-21 promotes enhanced tumor-specific memory. Groups of 5 to 15 BALB/c WT mice were inoculated into the mammary fat pad with \(5 \times 10^4\) 4T1 tumor cells (day 0). Groups of mice were then treated i.p. with either 100 \(\mu\)g clg on days 7 and 11 and PBS on days 14, 15, and 16; 100 \(\mu\)g each of TrimAb on days 7 and 11 and PBS on days 14, 15, and 16; or combinations of TrimAb and IL-21 at similar times as indicated. Mice treated with either TrimAb and PBS (open circles) or TrimAb and IL-21 (closed circles) that remained tumor-free of their primary 4T1 tumor were then rechallenged s.c. in the opposite flank 10 wk after the primary tumor inoculation with increasing doses of 4T1 tumor cells: A, \(1 \times 10^6\); B, \(2 \times 10^5\); and C, \(5 \times 10^4\) cells, as shown. Growth after rechallenge was compared with growth at the same doses in naïve BALB/c WT mice (open squares). In primary and secondary challenge experiments, 4T1 tumor growth was measured every second day and tumor sizes represent the mean ± SE of four to five mice in each group. The proportion of mice surviving tumor is shown in parentheses.
further by the TrimAb combination (data not shown). Furthermore, tumor suppression by anti-DR5 or TrimAb is inhibited by tumor FLIP overexpression (15). Thus, anti-DR5 mAb induces apoptosis of a proportion of tumor cells, thereby releasing potential tumor antigens. These antigens are then presented to T cells by APCs that are activated and matured by anti-CD40 mAb. T cells in turn are activated by licensed APCs and further costimulated by the agonist anti-CD137 mAb, resulting in a robust adaptive immune response against tumor. When IL-21 was used in this setting, a further enhanced antitumor response was observed.

It was previously shown that CD8+ T cells and the cytokines, IFN-γ and IL-12, were required for the antitumor activity of TrimAb. In an earlier study, treatment efficacy correlated with tumor-specific CD8+ T cells producing IFN-γ (data not shown). It was also interesting that a similar number of mice were cured after treatment with two doses of TrimAb/IL-21 and four doses of TrimAb alone. This suggested that IL-21 amplified at least part of the same mechanism used by TrimAb therapy. Notably IL-21 can enhance perforin expression in CD8+ T cells (28) and NK cells (4), but there seemed no greater dependence on this effector pathway than observed in mice receiving TrimAb alone. Most likely, the effect of IL-21 occurred at the level of activation or expansion of CD8+ T cells, because IL-21 has been shown to promote proliferation of antigen-specific CD8+ T cells in vitro and in vivo (refs. 10, 29; reviewed in ref. 30). In addition, IL-21 has been shown to promote antitumor CTL activity in mice (31) and, in synergy with IL-15, can enhance the expansion of naive and memory CD8+ T cells (32). The effectiveness of the therapeutic regimen against tumors of various histologic origins suggests that immunity can be generated and enhanced against a variety of tumor antigens using the TrimAb and IL-21 combination. One of the aims of cancer immunotherapy is the ability to induce lasting tumor-specific immunity. In our studies, we also showed enhanced tumor-specific memory of mice after treatment with the TrimAb/IL-21 combination compared with TrimAb alone.

IL-21 can also induce maturation and activation of NK cells, thereby increasing their cytolytic potential (4), with induction of various genes involved in the activation of innate immune responses. IL-21 also enhances ADCC by NK cells and can lead to secretion of IFN-γ, tumor necrosis factor-α, IL-8, macrophage inflammatory protein-1α, and RANTES and an enhanced anti-tumor effect (12). This mechanism may well be responsible for the observed modest activity of IL-21 alone against tumor in the absence of additional immune stimulation. However, it seems unlikely that this mechanism plays a major role in augmenting TrimAb activity because depletion of NK cells did not affect significantly antitumor activity. IL-21 has been shown to play a role in CD4+ T-helper cell differentiation, with reports that Th1 and Th2 responses can be elicited in human and mouse systems (33, 34). In addition, IL-21 plays a key role in the differentiation of naive CD4+ T cells into Th17 cells (35, 36) that promote inflammatory responses and plays a pivotal role in autoimmunity. It is likely that the end result of IL-21 signaling on T-helper cell differentiation is dependent upon the context in which it operates. However, in the therapy setting described in the current report, there seems little contribution from T-helper cells because depletion of CD4+ T cells did not affect the increased therapeutic effect afforded by IL-21. Nevertheless, the mechanistic studies described above are not sufficient to exclude a role for IL-21 in the inhibition of CD4+ regulatory T cells (Treg). Indeed, IL-21 has been previously shown to inhibit transforming growth factor–β–driven differentiation of naive T-helper cells into Foxp3+ Treg cells (35, 36), and IL-21–mediated Foxp3 suppression leads to enhanced CTL generation (37). IL-21 can also enable CD4+ T-helper cells to become resistant to the suppressive effects of Treg without directly reversing the function of CD4+CD25+ Treg (38).

In summary, we show that sequentially combining TrimAb with recombinant IL-21 can significantly improve the antitumor activity of this combination against very advanced disease in a number of experimental tumor models. Whereas clinically it will be important to try to simplify combination therapy strategies by minimizing the number of agents used, these data further support the use of IL-21 in adjuvant settings where improved cellular immune responses to tumors are desired and achievable.

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