IL-21 Enhances Tumor-Specific CTL Induction by Anti-DR5 Antibody Therapy

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Tumor cell apoptosis is the basis of many cancer therapies, and tumor-specific T cells are the principal effectors of successful anti-tumor immunotherapies. In this study, we have examined the ability of IL-21 to suppress tumor metastases and growth when used sequentially in combination with an anti-DR5 mAb directed at TRAIL-sensitive tumors. We have demonstrated in a series of in vivo models that IL-21 promoted tumor-specific CTL activity and enhanced memory responses to tumor rechallenge. This study illustrates the principle that IL-21 may be used to support the anti-tumor activity of many clinically useful mAbs in cancer treatment.

Materials and Methods

Mice

Inbred BALB/c and C57BL/6 (B6) wild-type (WT) and BALB/c SCID mice were purchased from the Walter and Eliza Hall Institute (Parkville, Australia). B6 RAG-1−/−, BALB/c perforin (pfp)-/−, BALB/c IFN-γ−/−, and BALB/c TRAIL−/− mice were bred and maintained at the Peter MacCallum Cancer Centre (Peter Mac). All experiments were performed in accordance with the institutional Animal Ethics Committee of both the Walter and Eliza Hall Institute and Peter MacCallum Cancer Centre. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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accordance with guidelines set out by the Peter Mac animal experimental ethics committee.

Reagents
Recombinant mouse IL-21 was provided by ZymoGenetics. The preparations of IL-21 were diluted in PBS immediately before use. Agonistic anti-mouse DR5 mAb (MD5-1), depleting anti-mouse CD4 mAb (GK1.5), depleting anti-mouse CD8 mAb (53-6.7), and neutralizing anti-CD11b mAb (5C6) were prepared and purified in our laboratory as described previously (12, 30). Previously, the schedules of anti-CD4 and anti-CD8 mAb have been used and shown to effectively deplete these lymphocyte subsets (31). NK cells, but not invariant NKT cells, were specifically depleted in BALB/c mice using 100 μg i.p. rabbit anti-asialoGM1 Ab (WAKO) as described (32, 33).

Tumor cell lines
BALB/c-derived TRAIL-sensitive 4T1 mammary carcinoma (30, 34) and TRAIL-sensitive R331 renal carcinoma (30, 35) were maintained as described previously. FLIP- or mock-transfected R331 were established as described previously (35).

4T1 mammary tumor growth
Groups of five BALB/c WT mice were inoculated in the mammary fat pad with various doses of 4T1 tumor cells on day 0. Groups of mice were then treated i.p. with the following: control Ig (cIg) and PBS, anti-DR5 (MD5-1) and PBS, cIg and IL-21, or a combination of MD5-1 and IL-21 at the doses and times indicated. Tumor size was measured every second day with a caliper as the product of two perpendicular diameters (cm²). Some groups of mice were injected in the mammary fat pad with 4T1 tumor cells, and 28 days later, the primary developing tumor was resected and groups of mice were then treated i.p. with cIg and PBS, anti-DR5 (MD5-1) and PBS, cIg and IL-21, or a combination of MD5-1 and IL-21 at doses and times indicated (34, 36). Survival of the mice was monitored for 150 days. Spleen cells from MD5-1- and MD5-1/IL-21-treated mice that were tumor free after 6 wk were then adoptively transferred (i.v. at the doses indicated) into groups of five BALB/c SCID mice that had received 5 × 10⁵ 4T1 tumor cells in the mammary gland 24 h earlier. SCID mice injected with 4T1 cells but not receiving a spleen T cell transfer were used as controls. Mammary tumor growth was measured every second day, and tumor sizes represent the mean ± SE of five mice in each group. Similar results for A were obtained in two independent experiments.

Therapy of R331 tumor growth
Groups of five BALB/c WT mice were inoculated s.c. with 1 × 10⁵ R331 tumor cells on day 0. Groups of mice were then treated i.p. with cIg on days 0, 3, and 6 and PBS on days 9–11; anti-DR5 (MD5-1) on days 0, 3, and 6 and PBS on days 9–11; cIg (as above) and 20 μg of mouse IL-21 (IL-21) on days 3–5; cIg (as above) and 20 μg mouse IL-21 (IL-21) on days 6–8; cIg (as above) and 20 μg of mouse IL-21 (IL-21) on days 9–11; or combinations of MD5-1 and IL-21 at similar times as indicated. In another experiment, groups of mice received cIg on days 0, 3, and 6 and PBS or IL-21 on days 9–11 (B); MD5–1 on days 0, 3, and 6 and PBS on days 9–11 (C); or MD5–1 on days 0, 3, and 6 and IL-21 on days 9–11 (D), alone or in combination with 100 μg mAbs to deplete CD4⁺ T, CD8⁺ T, NK cells (anti-asGM1), or neutralize CD11b (5C6) as indicated on days 1, 0, 7, and 14. Mammary tumor growth was measured every second day, and tumor sizes represent the mean ± SE of five mice in each group. Similar results for A were obtained in two independent experiments.

FIGURE 1. Enhanced suppression of s.c. 4T1 tumors by early anti-DR5 mAb and IL-21 therapy. Groups of five BALB/c WT mice were inoculated in the mammary fat pad with 5 × 10⁴ 4T1 tumor cells on day 0. A, Groups of mice were then treated i.p. with the following: 100 μg of cIg on days 0, 3, and 6 and PBS on days 9–11; 100 μg of anti-DR5 (MD5-1) on days 0, 3, and 6 and PBS on days 9–11; cIg (as above) and 20 μg of mouse IL-21 (IL-21) on days 3–5; cIg (as above) and 20 μg mouse IL-21 (IL-21) on days 6–8; cIg (as above) and 20 μg of mouse IL-21 (IL-21) on days 9–11; or combinations of MD5-1 and IL-21 at similar times as indicated. In another experiment, groups of mice received cIg on days 0, 3, and 6 and PBS or IL-21 on days 9–11 (B); MD5–1 on days 0, 3, and 6 and PBS on days 9–11 (C); or MD5–1 on days 0, 3, and 6 and IL-21 on days 9–11 (D), alone or in combination with 100 μg mAbs to deplete CD4⁺ T, CD8⁺ T, NK cells (anti-asGM1), or neutralize CD11b (5C6) as indicated on days 1, 0, 7, and 14. Mammary tumor growth was measured every second day, and tumor sizes represent the mean ± SE of five mice in each group. Similar results for A were obtained in two independent experiments.
splenocytes were cocultured with 2 × 10^6 4T1 tumor cells on day 0. When mammary tumors reached a size of ~8 mm in diameter on day 28, the primary tumor was surgically resected. Groups of mice then received i.p. the following: 100 μg of clg on days 28, 31, 34 and 37; 100 μg of anti-DR5 (MD5-1) on days 28, 31, 34, and 37; clg (as above) and 50 μg of mouse IL-21 (IL-21) on days 28–30; clg (as above) and 50 μg of mouse IL-21 (IL-21) on days 40–42; or combinations of MD5-1 and IL-21 at similar times as indicated. Mice were then monitored for tumor-free survival for up to 150 days as described in Materials and Methods. Similar results were obtained in two independent experiments.

size was measured periodically with a caliper as the product of two perpendicular diameters (cm^2).

**Therapy of R331 tumor metastases**

Groups of BALB/c WT mice were inoculated i.v. with the indicated dose of R331, Renca, or R331-FLIP tumor cells on day 0. Groups of mice then received i.p. clg on days 0, 3, and 6 and PBS on days 9–11; anti-DR5 (MD5-1) on days 0, 3, and 6 and PBS on days 9–11; clg and IL-21 (IL-21) on days 9–11; or combination MD5-1 and IL-21 as indicated. All groups of mice were sacrificed at day 14, and lung tumor metastases counted with the aid of a dissecting microscope. Data are recorded as the mean ± SE of triplicate samples of each group.

**Cytotoxicity assay**

Cytotoxicity mediated by MD5-1 was tested by a 4-h 51Cr release assay as described previously (12, 37). Susceptibility of tumor cells to TRAIL-mediated cytotoxicity was examined using mouse TRAIL-transfected 2PK-3 (2PK-3-mTRAIL) or mock-transfected 2PK-3 (2PK-3-m) as the effector cells. Susceptibility to MD5-1 cytotoxicity was tested in the presence of FcR-expressing P815 cells or spleen NK cells prepared from PBS or IL-21-treated B6 RAG-1^-^- mice. Mice received PBS or IL-21 (20 μg i.p.) on days −3, −2, and −1 before spleen harvest. In some experiments, the cytotoxicity assay was performed in the presence of 5 μg/ml MD5-1 or clg. CTLs reactive with tumor were induced as described previously (12). Briefly, splenocytes were prepared from either naive or tumor-bearing mice treated with a combination of anti-DR5 or clg and IL-21 or PBS. Following treatment and on day 28 of tumor growth, splenocytes were harvested from three mice of each group, pooled, and 20 million splenocytes were cocultured with 2 × 10^6 of mitomycin C (MMC; Kyowa Hakko)-treated (200 μg/ml, 2 h) 4T1 cells for 7 days. Seven days later, the cytotoxic activities of these cultures were then tested against either 4T1 or R331 tumor targets at the E:T ratio shown in a 4-h 51Cr release assay as described (34). Results were expressed as the mean ± SE of triplicate samples.

**Statistical analysis**

Statistical analysis was performed by a Mann-Whitney U test for the tumor metastases and growth data. Values of p <0.05 were considered as significant.

**Results**

**Synergistic anti-tumor efficacy of combined anti-DR5 mAb/IL-21 therapy**

We have demonstrated previously that an agonistic anti-DR5 mAb exhibits potent anti-tumor effects against TRAIL-sensitive tumors through induction of tumor cell apoptosis, recruiting Fc receptor-bearing APCs, and generating tumor-specific CTL (12). In addition, IL-21 has been reported to induce CTL-mediated anti-tumor effects by costimulating T cells (25). Given the points of action of these agents, we expected that the combination of anti-DR5 and IL-21 might have enhanced efficacy against primary tumors and metastases. Initially, various early treatment schedules of anti-DR5 mAb (MD5-1) and IL-21 alone or in combination were compared for their anti-tumor efficacy against orthotopic growth of the experimental 4T1 mammary tumor (Fig. 1A). If treatment was commenced on day 0, anti-DR5 mAb alone substantially inhibited the growth of tumors, but never caused tumor rejection (Fig. 1A). IL-21 alone was ineffective unless treatment was administered 3–5 days after tumor inoculation, and even at that early time, tumor growth was inhibited only to a minor extent. However, strikingly, combined anti-DR5 and IL-21 treatment completely suppressed tumor growth in a significant proportion of mice, and tumors did not recur >120 days after the treatment ceased (Fig. 1A). Importantly, combined treatment was most effective when IL-21 treatment was commenced 3 days (days 9–11) after the third and final MD5-1 treatment. A dose response was performed with IL-21 from 1 to 50 μg per injection, and 20 μg per injection was found to be optimal in this experimental setting (data not shown). Earlier administration of IL-21 in combination with MD5-1 was less effective, suggesting that IL-21 was simply not significantly enhancing Ab-dependent cellular cytotoxicity (ADCC) triggered by MD5-1. Indeed, MD5-1 has been reported to mediate apoptosis of tumor cells strictly via DR5 and a caspase-8-dependent pathway rather than via classical ADCC (12). When we examined the cytotoxic activity of MD5-1 against tumor targets in vitro, in the presence of effector splenocytes or P815-expressing Fc receptor, IL-21 treatment did not substantially enhance ADCC by the MD5-1 mAb (data not shown). Thus, the improved in vivo efficacy with delayed IL-21 treatment was consistent with a similar improved effect when using α-GalCer and delayed IL-21 in combination (29) and suggested that IL-21 was promoting adaptive tumor immunity.

We next examined which leukocyte subsets were responsible for the primary anti-tumor effect of combined MD5-1 and IL-21 therapy by depleting NK cells, CD4+ T cells, or CD8+ T cells, or neutralizing CD11b function. As shown previously, clg and IL-21 were ineffective in suppressing growth of 4T1 in the mammary gland, and depletion of NK cells, CD4+ T cells, or CD8+ T cells or neutralization of CD11b did not suppress or enhance 4T1 tumor growth (Fig. 1B). We have reported previously that suppression of tumor growth by MD5-1 was mediated in part by FcR-expressing NK cells and CD11b+ cells (12), and our finding in the orthotopic 4T1 tumor model was in concert with this report (Fig. 1C). In vitro studies have shown previously that both NK cells and macrophages enable DR5-triggered apoptosis of tumor cells after FcR-mediated cross-linking of MD5-1 (12). Anti-CD11b mAb (5C6) inhibits both macrophage and neutrophil recruitment to inflammatory sites. Strikingly, the combination MD5-1 and IL-21 therapy critically required CD11b function and, to a lesser extent, NK cell and CD8+ T cell function (Fig. 1D). The dependence on CD8+ T cell function was supported by the relative ineffectiveness of MD5-1 and IL-21 combination against larger tumors in SCID, compared with WT mice (data not shown). CD4+ T cell function
was not required for combined MD5-1 and IL-21 anti-tumor efficacy.

To further evaluate the efficacy of the anti-DR5 and IL-21 combination, we assessed suppression of tumor metastases in the 4T1 tumor model when the primary mammary tumor was resected when it reached ~8 mm in diameter on day 28 (Fig. 2). Typically, at day 28, the mice have a high level of 4T1 metastases in their lung and metastases at additional sites such as liver, lymph node, brain, and bone. All mice eventually succumbed to tumor; however, groups of mice receiving a combination of MD5-1 and delayed IL-21 survived significantly longer than those receiving MD5-1 or IL-21 alone or MD5-1 and IL-21 concurrently. Future experiments will be designed to include additional cycles of treatment with the aim of completely suppressing metastatic disease.

**Combination MD5-1/IL-21 suppresses TRAIL-sensitive renal carcinoma metastases**

We next examined the efficacy of combined MD5-1/IL-21 treatment against experimental renal carcinoma metastases. R331 is an extremely TRAIL-sensitive variant of the Renca renal carcinoma (35). Despite the fact that IL-21 treatment commenced as late as day 9, once again the combination was more effective than either MD5-1 or IL-21 alone against TRAIL-sensitive Renca and R331 renal carcinoma cells (Fig. 3, A and B). By contrast, the combination was ineffective against TRAIL-resistant R331-FLIP tumor cells (Fig. 3C). The mechanism of metastases suppression by MD5-1/IL-21 was once again assessed by depleting various subsets of lymphocytes and neutralizing CD11b (Fig. 3D). Consistent with the 4T1 tumor model, the combination MD5-1 and IL-21 therapy critically required CD11b function and, to a lesser extent, NK cell and CD8+ T cell function to suppress R331 lung metastases (Fig. 3D).

**Combination MD5–1/IL-21 enhances memory T cell response to secondary tumor challenge**

We next examined the ability of combined MD5-1/IL-21 treatment to suppress low doses of s.c. R331 renal carcinoma cells. At this low dose of R331, both groups of WT mice receiving MD5-1 or combination MD5-1/IL-21 remained free of their primary R331 tumor (Fig. 4A). By contrast, all mice receiving control Ig and/or IL-21 developed tumors. Tumor-free mice were then rechallenged s.c. in the opposite flank 12 wk after the primary tumor inoculation with increasing doses of R331 tumor cells (Fig. 4, B–D). Mice that had originally rejected R331 following MD5–1/IL-21 treatment were able to resist even the highest dose of secondary challenge with R331 tumor cells (Fig. 4D). By contrast, mice originally receiving MD5-1 alone were unable to reject secondary R331 challenge at doses of 10⁵ cells (Fig. 4C) and above (Fig. 4D). Groups of MD5-1/IL-21-treated mice that rejected primary R331 tumor challenge were unable to resist a secondary 4T1 tumor challenge in

**FIGURE 3.** Combination MD5–1/IL-21 effectively suppresses TRAIL-sensitive renal carcinoma metastases. Groups of five BALB/c WT mice were inoculated i.v. with the following: 5 × 10⁵ MD5-1-sensitive Renca tumor cells on day 0 (A); 5 × 10⁴ MD5-1-sensitive R331 tumor cells (B); or 5 × 10⁵ MD5–1-resistant R331-FLIP tumor cells (C). Groups of mice then received i.p. the following: 100 µg of cIg on days 0, 3, and 6 and PBS on days 9–11; 100 µg of anti-DR5 (MD5-1) on days 0, 3, and 6 and PBS on days 9–11; clg (as above) and 20 µg of mouse IL-21 (IL-21) on days 9–11; or combination MD5-1 (as above) and 20 µg of mouse IL-21 (IL-21) on days 9–11, alone or in combination with 100 µg of mAbs to deplete CD4+ T, CD8+ T, NK cells (anti-asGM1), or neutralized CD11b (5C6) as indicated on days 1, 0, and 7. All groups of mice were sacrificed at day 14, and lung tumor metastases were counted with the aid of a dissecting microscope. Data were recorded as the mean ± SE of each group. A significant reduction in lung metastases in groups receiving the MD5-1/IL-21 combination, compared with mice receiving MD5-1 alone are shown (∗, p < 0.05). Similar results for A–C were obtained in two independent experiments.
the mammary gland, demonstrating the specificity of the memory response (data not shown). Additional data (Fig. 4) illustrated that primary rejection of R331 conferred protective immunity against both R331 (TRAIL-sensitive) and R331-FLIP (TRAIL-resistant) tumor cells.

In a similar fashion, tumor-specific immunity could be transferred by splenic T cells from MD5-1 or MD5-1/IL-21-treated mice into SCID mice that otherwise could not resist a lethal challenge with 4T1 tumor cells. Both MD5-1 and MD5-1/IL-21 suppressed the outgrowth of primary 4T1 tumor inoculation in BALB/c WT mice, compared with cIg or IL-21 alone (Fig. 5A). Spleen T cells purified from MD5-1- and MD5-1/IL-21-treated mice that were tumor free after 6 wk were then adoptively transferred at two different doses \((5 \times 10^6, 5 \times 10^5)\) into groups of five BALB/c SCID mice that had received \(5 \times 10^3\) 4T1 tumor cells in the mammary gland 24 h earlier. SCID mice injected with 4T1

FIGURE 4. Combination MD5-1/IL-21 enhances memory response to secondary tumor challenge. Groups of five BALB/c WT mice were inoculated s.c. with \(1 \times 10^4\) R331 tumor cells on day 0. A. Groups of mice were then treated i.p. with the following: 100 \(\mu\)g of cIg on days 0, 3, and 6 and PBS on days 9–11; 100 \(\mu\)g of anti-DR5 (MD5-1) on days 0, 3, and 6 and PBS on days 9–11; cIg (as above) and 20 \(\mu\)g of mouse IL-21 (IL-21) on days 9–11; or combinations of MD5-1 and IL-21 at similar times as indicated. Mice treated with either MD5-1 and PBS or MD5-1 and IL-21 that remained tumor-free of their primary R331 tumor were then rechallenged s.c. in the opposite flank 12 wk after the primary tumor inoculation with increasing doses of R331 tumor cells, \(10^4\) cells (B), \(10^5\) cells (C), and \(10^6\) cells (D) as shown. E, Secondary challenge was performed with \(10^5\) R331 or R331-FLIP tumor cells as indicated. Growth after rechallenge was compared with growth at the same doses in naive BALB/c WT mice. In primary and secondary challenge experiments, R331 tumor growth was measured every second day, and tumor sizes represent the mean \pm SE of five mice in each group. Similar results were obtained in two independent experiments for B.
cells, but not receiving a T cell transfer, were used as controls. Clearly SCID mice were well protected from lethal 4T1 tumor challenge when receiving even lower doses of T cells from MD5-1/IL-21-treated WT mice that had rejected primary 4T1 tumor challenge (Fig. 5B). T cells from MD5-1-treated mice also offered some protection at the higher T cell dose transferred. These data illustrated that memory for 4T1 tumor can be more effectively generated and transferred from MD5-1/IL-21-treated mice.

Combination MD5-1/IL-21 enhances tumor-specific CTL generation

We also examined the ability of mice that initially resisted a low dose of 4T1 mammary tumor after MD5-1/IL-21 treatment to secondarily reject 4T1 inoculated in the opposite mammary gland (Fig. 6). Early MD5-1/IL-21 treatment effectively suppressed the outgrowth of primary 4T1 tumor inoculation in all mice (Fig. 6A). These tumor-free mice were then re-challenged 7 wk after the primary tumor inoculation with 4T1 tumor cells. Groups of these mice were then treated i.p. with the following: 100 μg of cIg on days 0, 3, and 6 and PBS on days 9–11; 100 μg of anti-DR5 (MD5-1) on days 0, 3, and 6 and PBS on days 9–11; cIg (as above) and 20 μg of mouse IL-21 (IL-21) on days 9–11; or a combination of MD5-1 and IL-21 at similar times as indicated. B, Splenic T cells from MD5-1- and MD5-1/IL-21-treated mice that were tumor free after 6 wk were then adoptively transferred (at the doses indicated) into groups of five BALB/c SCID mice that had received 5 × 10⁷ 4T1 tumor cells in the mammary gland 24 h earlier. SCID mice injected with 4T1 cells, but not receiving a T cell transfer, were used as controls. Mammary tumor growth was measured every second day, and tumor sizes represent the mean ± SE of five mice in each group. Similar results were obtained in two independent experiments for A.

**FIGURE 5.** Enhanced tumor-specific T cell function transferred from MD5-1/IL-21-treated mice. Groups of five BALB/c WT mice were inoculated in the mammary fat pad with 5 × 10⁷ 4T1 tumor cells on day 0. A, Groups of mice were then treated i.p. with the following: 100 μg of cIg on days 0, 3, and 6 and PBS on days 9–11; 100 μg of anti-DR5 (MD5-1) on days 0, 3, and 6 and PBS on days 9–11; cIg (as above) and 20 μg of mouse IL-21 (IL-21) on days 9–11; or a combination of MD5-1 and IL-21 at similar times as indicated. B, Splenic T cells from MD5-1- and MD5-1/IL-21-treated mice that were tumor free after 6 wk were then adoptively transferred (at the doses indicated) into groups of five BALB/c SCID mice that had received 5 × 10⁷ 4T1 tumor cells in the mammary gland 24 h earlier. SCID mice injected with 4T1 cells, but not receiving a T cell transfer, were used as controls. Mammary tumor growth was measured every second day, and tumor sizes represent the mean ± SE of five mice in each group. Similar results were obtained in two independent experiments for A.

**FIGURE 6.** Memory response to secondary tumor challenge is mediated by CD8⁺ T cells. BALB/c WT mice were inoculated in the mammary fat pad with 5 × 10⁷ 4T1 tumor cells (day 0). A, Groups of mice were then treated i.p. with the following: 100 μg of cIg on days 0, 3, and 6 and PBS on days 9, 10 and 11 (n = 5); or 100 μg of anti-DR5 (MD5-1) on days 0, 3, and 6 and 20 μg of IL-21 on days 9–11 as indicated (n = 25). Groups of 25 WT mice treated with MD5-1 and IL-21 that remained tumor free of their primary 4T1 tumor were then rechallenged in the opposite fat pad 7 wk after the priming tumor inoculation with 5 × 10⁷ 4T1 tumor cells. Some groups additionally received 100 μg of mAbs to neutralize CD11b or deplete CD4⁺ T, CD8⁺ T, CD4⁺ and CD8⁺ T cells, and NK cells (anti-asGM1) as indicated on days −1, 0, 7, and 14. Growth after rechallenge was compared with growth at the same doses in naive cIg-treated BALB/c WT mice. In primary and secondary challenge experiments, 4T1 tumor growth was measured every second day, and tumor sizes represent the mean ± SE of five mice in each group.
MD5-1/IL-21 treatment and elimination of primary 4T1 tumor inoculation (data not shown).

Given the ability of MD5–1/IL-21-treated mice to resist secondary tumor challenge in a CD8+ T cell-dependent manner, we next assessed whether this treatment protocol enhanced tumor-specific CTL generation in tumor-bearing mice. Splenocytes from treated naive and 4T1 tumor-bearing mice were harvested and cocultured with 4T1 tumor cells in vitro. Seven days later, the cytotoxic activities of these cultures were then tested against either 4T1 (A and B) or R331 (C and D) tumor targets at the E:T ratio shown. Results represent the mean ± SE of triplicate samples.

**Combination MD5-1/IL-21 suppresses established experimental tumor burden**

To examine the efficacy of combined MD5-1/IL-21 treatment against more established tumors, the commencement of treatment was delayed until 12 (2–3 mm in diameter) or 18 (4–5 mm in diameter) days after tumor inoculation. Neither IL-21 nor MD5-1 therapy alone had any discernible effect on R331 tumor growth (Fig. 8). By contrast, the combination of MD5-1 and IL-21 reduced s.c. R331 tumor growth by >50% when commenced from day 12. These data in this instance demonstrate the synergistic activity of anti-DR5 and IL-21 in an advanced tumor model. In this setting, the mechanism of tumor suppression likely involves CTL generation, but it may additionally involve effects on tumor angiogenesis, suppressor macrophages, or suppressor T cell populations, and these possibilities remain to be investigated.
In summary, it is becoming clear that IL-21 is capable of enhancing CD8\(^+\) T cell expansion and function and is necessary for an optimal CD8\(^+\) T cell response to Ag. Many immunotherapies that induce tumor-specific CTL may be enhanced further by combination with IL-21. Pending safety trials of IL-21 in patients with advanced cancer, it should be possible to combine this cytokine with many new CTL-based vaccines.

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Disclosures

P. Sivakumar is an employee of ZymoGenetics, which is currently developing IL-21 for clonal oncology. M. J. Smyth is a consultant for Novo-Nordisk.

References

1. Waldmann, T. A. 2003. Immunotherapy: past, present and future. Nat. Med. 9: 269–277.

2. Nagata, S. 1997. Apoptosis by death factor. Cell 88: 355–365.

3. Ashkenazi, A., and V. M. Dixit. 1998. Death receptors: signaling and modulation. Science 281: 1305–1308.

4. Ashkenazi, A. 2002. Targeting death and decoy receptors of the tumor-necrosis factor superfamily. Nat. Rev. Cancer 2: 420–430.

5. Wiley, S. R., K. Schooley, M. Smolak, W. S. Din, C. P. Huang, J. K. Nicholl, G. H. Rotherland, T. D. Smith, C. A. Smith, et al. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673–682.

6. Pitti, R. M., S. A. Marsters, S. Ruppert, C. J. Donahue, A. Moore, and A. Ashkenazi. 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. 271: 12687–12690.

7. Walczak, H., R. E. Miller, K. Ariail, B. Glijniak, T. S. Griffith, M. Kubin, W. Chin, J. Jones, A. Woodward, T. Le, et al. 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat. Med. 5: 157–163.

8. Ashkenazi, A., and V. M. Dixit. 1999. Apoptosis control by death and decoy receptors. Curr. Opin. Cell Biol. 11: 255–260.

9. Chinnaiyan, A. M., U. Prasad, S. Shankar, D. A. Hamstra, M. Shanaiah, T. L. Chenervet, B. D. Ross, and A. Rehemtulla. 2000. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc. Natl. Acad. Sci. USA 97: 1754–1759.

10. Chantharapai, A., K. Dodge, K. Grimmer, K. Schroeder, S. A. Marsters, H. Koeppen, A. Ashkenazi, and J. K. Kim. 2001. Isotype-dependent inhibition of tumor growth in vivo by monoclonal antibodies to death receptor 4. J. Immunol. 166: 4891–4898.

11. Ichikawa, K., W. Liu, L. Zhao, Z. Wang, D. Liu, T. Ohtsuka, H. Zhang, J. D. Moutz, W. J. Koopman, R. P. Kimberly, and T. Zhou. 2001. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. Nat. Med. 7: 954–960.

12. Takeda, K., N. Yamaguchi, H. Akiba, K. Yojima, Y. Hayakawa, J. E. Tanner, T. J. Sayers, N. Seki, K. Okumura, H. Yagita, and M. J. Smyth. 2004. Induction of tumor-specific T cell immunity by anti-DR5 antibody therapy. J. Exp. Med. 199: 437–448.

13. Selenko, N., O. Maidic, S. Draxier, A. Berer, U. Jager, W. Knapp, and J. Stockl. 2001. CD20 antibody (CB28)-induced apoptosis of lymphoma cells promotes phagocytosis by dendritic cells and cross-priming of CD8\(^+\) cytotoxic T cells. Leukemia 15: 1619–1626.

14. zum Buschenfelde, C. M., C. Hermann, B. Schmidt, C. Peschel, and H. Bernhard. 2002. Antihuman epidermal growth factor receptor 2 (HER2) monoclonal antibody trastuzumab enhances cytolytic activity of class I-restricted HER2-specific T lymphocytes against HER2-overexpressing tumor cells. Cancer Res. 62: 2244–2247.

15. Pardoll, D., and J. Allison. 2004. Cancer immunotherapy: breaking the barriers to harvest the crop. Nat. Med. 10: 887–892.

16. Lake, R. A., and B. W. Robinson. 2005. Immunotherapy and chemotherapy: a practical partnership. Nat. Rev. Cancer 5: 397–405.

17. Sivakumar, P. V., D. C. Foster, and C. H. Clegg. 2004. Interleukin-21 is a T-helper cytokine that regulates humoral immunity and cell-mediated anti-tumor responses. Immunology 112: 177–182.

18. Mehta, D. S., A. L. Warster, and M. J. Grusby. 2004. Biology of IL-21 and the IL-21 receptor. Immunol. Rev. 202: 84–95.

19. McKelvey, S. L., J. Brady, Y. Hayakawa, and M. J. Smyth. 2004. Interleukin-21: a key player in lymphocyte maturation. Crit. Rev. Immunol. 24: 239–250.

20. Collins, M., M. J. Whitters, and D. A. Young. 2003. IL-21 and IL-21 receptor: a new cytokine pathway modulates innate and adaptive immunity. Immunol. Rev. 208: 131–140.

21. Parrish-Novak, J., S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Muddon, W. Xu, J. West, et al. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature 408: 57–63.

22. Di Carlo, E., A. Comes, A. M. Orenjo, O. Rosso, R. Meazza, P. Musiani, M. P. Colombo, and S. Ferrini. 2004. IL-21 induces tumor rejection by specific
CTL and IFN-γ-dependent CXC chemokines in syngeneic mice. J. Immunol. 172: 1540–1547.

23. Ma, H. L., M. J. Whitters, R. F. Konz, M. Senices, D. A. Young, M. J. Grusby, M. Collins, and K. Dunussi-Joannopoulos. 2003. IL-21 activates both innate and adaptive immunity to generate potent antitumor responses that require perforin but are independent of IFN-γ. J. Immunol. 171: 608–615.

24. Brady, J., Y. Hayakawa, M. J. Smyth, and S. L. Nutt. 2004. IL-21 induces the functional maturation of murine NK cells. J. Immunol. 172: 2048–2058.

25. Moroz, A., C. Eppolito, Q. Li, J. Tao, C. H. Clegg, and P. A. Shrikant. 2004. IL-21 enhances and sustains CD8+ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21. J. Immunol. 173: 900–909.

26. Kasaian, M. T., M. J. Whitters, L. L. Carter, L. D. Lowe, J. M. Jussif, B. Deng, K. A. Johnson, J. S. Witek, M. Senices, R. F. Konz, et al. 2002. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. Immunity 16: 559–569.

27. Takaki, R., Y. Hayakawa, A. Nelson, P. Y. Sivakumar, S. Hughes, M. J. Smyth, and L. L. Lanier. 2005. IL-21 enhances tumor rejection through a NKG2D-dependent mechanism. J. Immunol. 175: 2167–2173.

28. Zeng, R., R. Spolski, S. E. Finkelson, S. Oh, P. E. Kovanan, C. S. Hinrichs, C. A. Pise-Masison, M. F. Radonovich, J. N. Brady, N. P. Restifo, et al. 2005. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. J. Immunol. 172: 139–148.

29. Smyth, M. J., M. E. Wallace, S. L. Nutt, H. Yagita, D. I. Godfrey, and Y. Hayakawa. 2005. Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer. J. Exp. Med. 201: 1973–1985.

30. Takeda, K., M. J. Smyth, E. Cretney, Y. Hayakawa, N. Kayagaki, H. Yagita, and K. Okumura. 2002. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. J. Exp. Med. 195: 161–169.

31. Smyth, M. J., K. Y. Thia, S. E. Street, D. Macgregor, D. I. Godfrey, and J. A. Trapani. 2000. Perforin-mediated cytolysis is critical for surveillance of spontaneous lymphoma. J. Exp. Med. 192: 755–780.

32. Smyth, M. J., K. Y. Thia, E. Cretney, J. M. Kelly, M. B. Snook, C. A. Forbes, and A. A. Scalzo. 1999. Perforin is a major contributor to NK cell control of tumor metastasis. J. Immunol. 162: 6658–6662.