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Intramural Delivery of IL-21 Overcomes Anti-Her2/Neu Resistance through Shifting Tumor-Associated Macrophages from M2 to M1 Phenotype

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Tumor resistance is a major hurdle to anti-Her2/neu Ab–based cancer therapy. Current strategies to overcome tumor resistance focus on tumor cell–intrinsic resistance. However, the extrinsic mechanisms, especially the tumor microenvironment, also play important roles in modulating the therapeutic response and resistance of the Ab. In this study, we demonstrate that tumor progression is highly associated with TAMs with immune-suppressive M2 phenotypes, and deletion of TAMs markedly enhanced the therapeutic effects of anti-Her2/neu Ab in a HER2/neu-dependent breast cancer cell TUBO model. Tumor local delivery of IL-21 can skew TAM polarization away from the M2 phenotype to a tumor-inhibiting M1 phenotype, which rapidly stimulates T cell responses against tumor and dramatically promotes the therapeutic effect of anti-Her2 Ab. Skewing of TAM polarization by IL-21 relies substantially on direct action of IL-21 on TAMs rather than stimulation of T and NK cells. Thus, our findings identify the abundant TAMs as a major extrinsic barrier for anti-Her2/neu Ab therapy and present a novel approach to combat this extrinsic resistance by tumor local delivery of IL-21 to skew TAM polarization. This study offers a therapeutic strategy to modulate the tumor microenvironment to overcome tumor-extrinsic resistance. The Journal of Immunology, 2015, 194: 4997–5006.
Macrophages are highly plastic and can rapidly change their phenotypes in response to their local signals. Exposure to bacterial products and Th1 cytokines (e.g., LPS and IFN-γ) drives macrophages toward a classic (M1) activation state characterized by pro-inflammatory activity, tumoricidal function, and the promotion of T cell responses (21, 22). In response to Th2 cytokines IL-4 and IL-13, macrophages undergo alternative (M2) activation, with the ability to support tumor growth, inhibit antitumor immunity, and promote tissue repair. In malignant tumors (21, 23), TAMS resemble M2-type macrophages and are considered a promising target for tumor therapy (24). Studies of TAM-targeted therapies are primarily done by inhibiting macrophage recruitment, survival, and tumor-promoting activity in tumor, but the most potent antitumor strategy should be skewing tumor-promoting M2 TAMS to tumor-suppressive M1-type macrophages (25, 26).

In this study, we identify the abundant M2 TAMS in Her2/neu breast tumor as a barrier to anti-Her2/neu Ab therapy. Tumor local delivery of IL-21 can skew TAM polarization from the M2 to the M1 phenotype, which reverses immunosuppression and invokes the active antitumor potential that bridges innate and T cell responses for tumor regression. Therefore, our study points to a new strategy to treat established tumor with abundant and unmanageable suppressive macrophages.

Materials and Methods

Mice

BALB/c and C57BL/6 female mice (6–8 wk) were purchased from Vitalriver Experimental Animal (Beijing, China). BALB/c Rag-1 and FVB/N- transgenic (MMTv-neu) mice were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions at the Institute of Biophysics. The study was approved by the Institutional Animal Care and Use Committee of the Institute of Biophysics, and all experiments conformed to the relevant regulatory standards.

Cell lines and reagents

TUBO, cloned from a spontaneous mammary tumor in a BALB/c Neu-transgenic mouse (27), was a gift from Joseph Lustgarten (Mayo Clinic, Scottsdale, AZ). It was cultured in 5% CO2 and maintained in DMEM supplemented with 1% heat-inactivated FBS (HyClone), 2 mmol/l L-glutamine, 0.1 mmol/l MEM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. 3T3/Kb and 3T3/Nkb cells were provided by Dr. Wei-Zen Wei (Wayne State University, Detroit, MI) (28). 3T3/Kb cells expressing the MHC-I molecule H2-Kd and B7.1 were maintained in supplemented DMEM (as above) with the addition of 600 μg/ml G418 and 600 μg/ml Zeocin (both from Invitrogen) and 0.5 mmol/l puromycin (Invitrogen) and 600 μg/ml G418.

Anti-neu Ab 7.16.4, produced in-house (ATCC HB-10493), recognizes the juxtamembranal region of rat neu and competes with 4D5, the precursor of Her2/neu (30). The efficiency of macrophage depletion was assessed by CD11b and F4/80 staining.

NK cells were depleted by i.p. injection of 50 μg anti-asialoGM1 (Wako Pure Chemical Industries) 5 d before IL-21 treatment. Cell-depletion efficacy was monitored by staining peripheral blood and tumor mass for DX5.

Flow cytometry and sorting

Tumor tissues were minced and digested in DMEM with 0.1% collagenase (Sigma-Aldrich) and data were analyzed with FlowJo software. For cell sorting, a FACSAria II (BD Biosciences) was used. TAMS were sorted as CD45+ CD11b+ Gr1+ Ly6c+ F4/80+CD11c+ cells. Spleen was digested and stained with CD11b and F4/80 Abs. CD11b+F4/80+ spleen macrophages were sorted as a control for TAMS. Granulocytic myeloid-derived suppressor cells (G-MDSCs) and monocytic myeloid-derived suppressor cells (M-MDSCs) were gated as CD45+CD11b+Gr1+Ly6c+ and CD45+CD11b+Gr1+Ly6c−, respectively.

Immunohistochemistry

Immunohistochemistry was performed on frozen sections (7 μm) of treated tumors using mAb against CD8 (clone 53-6.7), F4/80 (clone BM8), and MHC class II (clone M5/114.15.2; all from eBioScience). Briefly, sections were incubated with primary mAb (1/200) at 4˚C overnight. After washing, sections were incubated with secondary Ab (anti-rat-HRP) for 30 min and positive signals were detected using DAB (both from ZSGB-BIO). Three microscope fields for each sample (n = 6) were counted, and integral OD of positive cells was assessed using Image-Pro Plus 6.0 software.

Gene-expression analysis

All mRNA analyses were performed using quantitative real-time PCR (qPCR). Briefly, total RNA of cultured cells or sorted cells was extracted by an RNeasy Kit (QIAGEN), following the manufacturer’s instructions. First-strand cDNA was synthesized (NEB) and subjected to qPCR (Bio-Rad CFX96) with SYBR Green qPCR SuperMix (TransGen) using the corresponding primers. Gene expression was normalized to β-actin or 18S RNA using the ΔΔCt method.

Primers

The following primers were used: 18S: 5’-CGCTTGGGCTTATCAGATT-3’ and 5’-TGGGTCTTGTGTTGTTAAT-3’; β-actin: 5’-CACCA CACTCTTCTACA-3’ and 5’-TACCAGCAGAGGATACA-3’; Arg2: 5’-CACCTTTACCTAGTATACTG-3’ and 5’-CCAGAAATCGTCAGA TGTGACGCTTGCTGGTG-3’; Ccl2: 5’-TGCTGGCTGTGATTACCTATCA-3’ and 5’-GAGAGCAGAAGGACACGTTGAGGTCG-3’; Ccr7: 5’-TGCTACACTCTAAGGGGAG TGTCC-3’ and 5’-TTCTGAGGAGCAGCGACTTT-3’; Il10: 5’-GGCAG ACTTTATCGGAAATAG-3’ and 5’-ATCATTCTTCTACGGTCCTCA-3’; Il-23p: 5’-ACCAGCACTTCAAGATAC-3’ and 5’-CGCCAGTGCTGCACATTAGCT-3’; Nos: 5’-AATACTGTGCTTGGGTTGACA-3’ and 5’-GAAGGTTGTGGAGTTGCTCTAAG-3’; Mrc1: 5’-GTTCATCCTCGGAG TATGTCCTTC-3’ and 5’-AGGATGCAGATCAGTCACTCA-3’; Tgfβ: 5’-CCAATAATCCTGCGTGTTACCT-3’ and 5’-TGTATTCGCCGTTCCTTGTTCCA-3’; Tnfα: 5’-AGGCTTTCTGTGCTCATGACT-3’ and 5’-ATCGAGTTGGTGTTGGCTCTG-3’; and Vegf: 5’-AGCAAGAGGAGACCCAGAAGA-3’ and 5’-CACAGGACCGTTGAAGATG-3’.

Proliferation assay

Lymph node cells were prepared from BALB/c mice, labeled with CFSE (2 μM in PBS) at 37˚C for 10 min, and washed with complete RPMI 1640 medium. Then, 0.5–1010 labeled lymphocytes were cultured with isolated TAMS or spleen macrophages at a 1:3 ratio in the presence of anti-CD3 (1 μg/ml) and anti-CD28 Abs (0.5 μg/ml) in triplicates in a 96-well round-bottom plate. The proliferation of total cells was tested by CFSE dilution using flow cytometry on day 3, and the cell-proliferation rate was calculated.

Measurement of IFN-γ-secreting T cells by ELISPOT assay

Splenocytes were added at 1×106 well to a 96-well MSIPS45 plate (Millipore), which was precoated with rat anti-mouse IFN-γ (U-CyTech). 3T3/Nkb or TUBO cells were added as APCs over the spleen cells, and 3T3/Kb or CT26 cells were used as control. The ratio of responder cells to APC was 10:1.

Tumor-infiltrating CD8+ T cells were enriched from tumor cell suspension by double Percoll density gradients (36 and 63%). The enriched cell suspensions were incubated with PE-conjugated CD8+ mAb (clone 12-

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of the M1-type genes Il12 and Tnf-α. The expression of these genes in splenic macrophages was comparable between tumor-bearing mice and naïve mice. (Fig. 2A). In addition, the sorted TAMs dramatically inhibited T cell proliferation stimulated with anti-CD3 and anti-CD28 mAbs, whereas the splenic macrophages from the same tumor-bearing mice had no suppressive effects (Fig. 2B). These results show that the macrophages were skewed toward the typical M2 phenotype in the tumor microenvironment, which manifested strong immunosuppressive effects.

To determine whether TAMs contribute to extrinsic resistance to anti-Her2/neu therapy, TUBO-bearing mice were treated i.p. with the well-characterized anti-Her2/neu (rat homolog of human Her2) mAb 7.16.4 (13), followed by continuous i.t. injection of cidofovir liposomes (CELs) to delete TAMs (using vehicle liposome as control). Even for larger tumors in which anti-Her2/neu treatment followed by i.t. injection of vehicle liposome only slightly delayed their growth, TAM deletion following anti-Her2/neu treatment resulted in the regression of all tumors (Fig. 2C). The tumors continued to shrink even after the cessation of CEL injections, suggesting that tumor regression is mediated by an antitumor immune response. To assess whether removal of TAMs increased the tumor-specific CD8+ T cell responses, the sorted tumor-infiltrating CD8+ T cells or splenocytes were stimulated with 3T3/NKb cells expressing Her2/neu and H-2Kb (3T3/Kb as negative control) (28) or TUBO tumor cells (CT26 as negative control) 8 d after completion of CEL injections, and IFN-γ-secreting cells were evaluated by ELISPOT. Both tumor-infiltrating CD8+ T cells and splenocytes from the TAM-deleted group had 3-fold more IFN-γ-producing cells than those from the vehicle liposome–treated group (Fig. 2D), indicating the increased responses of local and systemic tumor-specific T cells. Overall, our data suggest that TAMs confer an immune-suppressive environment that inhibits antitumor T cell responses and results in the emergence of further extrinsic resistance to anti-Her2/neu therapy.

Intratumoral treatment of IL-21 dramatically increases the therapeutic effect of anti-Her2/neu Ab

Next, we tried a series of combination treatments to manipulate the immune responses, including anti-CD137 agonist mAb, anti–programmed cell death protein 1 (PD-1)–blocking mAb, and rHL-15/IL-15Rα fusion protein to improve the therapeutic effect of anti-Her2/neu Ab. Impressively, i.t. injection of IL-21 resulted in the most notable therapeutic effects combined with anti-Her2/neu treatment. Injection of low-dose IL-21 (5 μg) alone had no effect on tumor growth, and anti-Her2/neu treatment alone delayed tumor growth compared with control treatment; however, the combination of these two treatments rapidly eradicated the tumor masses in all mice (Fig. 3A). Neu-transgenic mice are tolerant to Rat-neu and are highly resistant to various immunotherapies or anti-Neu Ab. The same therapeutic regimen even rapidly eradicated the established TUBO tumors in all tolerant F1 neu-transgenic mice (BALB/c × FVB/N MMTV-neu) (Fig. 3B). The cured mice rejected the rechallenged tumors, suggesting that long-term immune memory was generated (Fig. 3C). However, the i.v. injection of 5-fold more IL-21 had no synergistic antitumor effect with anti-Her2/neu Ab, suggesting the local effect of IL-21 on tumors (Fig. 3D). These results demonstrate that tumor local delivery of IL-21 could overcome the extrinsic resistance of tumors to anti-Her2/neu therapy and markedly improve the antitumor effects of anti-Her2/neu Ab.

Intratumoral IL-21 directly skews TAM polarization

Although the antitumor effects of IL-21 are considered to be mediated by NK and/or CD8+ T cells (31–33), their local effects on tumor tissues in the combination therapy with anti-Her2/neu Ab promoted us to pursue their effects on TAMs. Tumor-bearing
mice were injected i.t. with rIL-21 or PBS on days 15 and 17 after tumor inoculation, and the expression of the M1/M2-type genes in TAMs sorted from tumor masses was detected by real-time PCR 3 d after the last injection of IL-21. TAMs from the IL-21–treated group expressed reduced levels of M2-type genes Tgf-β, Il10, Ccl17, Vegf, and Arg2 compared with TAMs from the control group. In contrast, the M1-type genes Cxcl9 and iNos tended to be upregulated in TAMs from the IL-21–treated group (Fig. 4A).

Instead of inhibiting T cell proliferation, TAMs from IL-21–treated tumors enhanced T cell proliferation (Fig. 4B). Immuno-histochemistry analysis showed that IL-21 treatment significantly increased the expression of CD86 on TAMs, whereas it tended to reduce the tumor infiltration of macrophages and enhance MHC class II expression on TAMs (Supplemental Fig. 1).

To test the antitumor effect of IL-21 through TAM polarization in other tumor models, we combined tumor local treatment of IL-21 with the chemotherapeutic doxorubicin in an MCA205 tumor model. Doxorubicin treatment alone had minimal effect on tumor growth, whereas combination with IL-21 treatment dramatically improved its therapeutic effect (Supplemental Fig. 2A). These results demonstrate that tumor local treatment of IL-21 skewed TAM polarization away from the immune-suppressive M2 phenotype toward the immune-stimulatory M1 phenotype.

TAM polarization induced by intratumoral delivery of IL-21 might be explained by the stimulation of tumor-infiltrating CD8+ T cells and NK cells to produce IFN-γ, a strong stimulator of M1 polarization of macrophages. To exclude this possibility, tumor-bearing Rag2−/− mice were injected i.p. with anti-asialo GM1 Ab 5 d before the first i.t. injection of IL-21. TAMs from IL-21–treated mice lacking T, B, and NK cells still upregulated the expression of the M1-type gene iNos and downregulated the expression of the M2-type genes Il10, Ccl17, Vegf, and Tgf-β to a greater extent than did TAMs from IL-21–treated wild-type mice (Fig. 4C, compared with Fig. 4A). These results suggest that TAM polarization skewed by IL-21 treatment is independent of T, B, or NK cells.

To assess the direct effect of IL-21 on TAM polarization, the freshly sorted TAMs were cultured with IL-21 overnight. They expressed reduced levels of the M2-type genes Vegf and Tgf-β, but increased levels of the M1-type gene Cc17, compared with TAMs from the control group (Fig. 4D). In addition, the in vitro treatment of IL-21

**FIGURE 1.** Correlation of macrophage infiltration with tumor progression. BALB/c mice (n = 3–5) were injected s.c. with 8–10 × 105 TUBO cells and sacrificed at the indicated time points. Tumor-infiltrating myeloid-derived cells and lymphocytes were evaluated by flow cytometry. (A) Percentages of tumor-infiltrating CD45+ cells. (B) Gating strategy for analysis of TAMs (CD45+CD11b+Gr1−Ly6C−/−F4/80+CD11c+). (C) Percentage and absolute number of TAMs. (D) Absolute numbers of tumor-infiltrating CD8+, CD4+, and NK cells. (E) The ratio of macrophages/CD8+ T cells or macrophages/NK cell in tumors. Data are mean ± SEM and are representative of five independent experiments. *p < 0.05, **p < 0.01. n.s, not significant.
also decreased the expression of the M2-type genes \( \text{Vegf} \) and \( \text{Il10} \) in bone marrow–derived macrophages (Supplemental Fig. 3A) and \( \text{Vegf} \), \( \text{Tgf-\beta} \), \( \text{Il10} \), and \( \text{Ccl17} \) in peritoneal macrophages, and it markedly increased the expression of the M1-type genes \( \text{Tnf-\alpha} \) and \( \text{Il6} \) in peritoneal macrophages induced by a low dose of LPS (Supplemental Fig. 3B); this suggested that the effect of IL-21 on macrophage polarization was not specific to TAMs. These results indicate that IL-21 could directly skew macrophages away from the M2 phenotype.

**IL-21 amplifies the tumor-specific CD8\(^+\) T cell response through polarizing TAMs**

Our previous study showed that anti-Her2/neu Ab treatment increased tumor infiltration of CD8\(^+\) T and NK cells (13). Flow cytometry and immunohistochemistry analysis showed that the combination treatment of IL-21 and anti-Her2/neu Ab further increased tumor-infiltrating CD8\(^+\) T cells by 2-fold, which reached 20% of tumor-infiltrating CD4\(^+\) T cells and NK cells in tumors compared with anti-Her2/neu Ab alone (Fig. 5A, 5B). Strikingly, the percentage of IFN-\(\gamma\)-producing cells among tumor-infiltrating CD8\(^+\) T cells from the combined treatment group was also 2-fold more than that of the anti-Her2/neu group (Fig. 5C). Thus, tumor local delivery of IL-21 increased the quantity and enhanced the function of CD8\(^+\) T cells in tumors treated with anti-Her2/neu Ab.

To underscore the role of TAM polarization skewed by IL-21 treatment in our combination therapy, we compared the effects
of TAM deletion on therapy with anti-Her2/neu Ab alone and combination therapy. Anti-Her2/neu treatment alone only delayed the tumor growth, whereas TAM deletion following Ab treatment markedly enhanced the therapeutic effects of anti-Her2/neu Ab, which resulted in tumor regression in four of six mice, indicating that TAMs were predominantly of tumor-promoting M2 phenotype in Ab-treated tumors. Conversely, combination therapy of anti-Her2/neu and IL-21 resulted in rapid elimination of all detectable tumors, whereas the TAM deletion prevented the tumor regression induced by the combination treatment and caused rapid grow-up of tumors in two of six mice, suggesting that TAMs are tumor-inhibitory M1 phenotype in tumors with the combination treatment (Fig. 5D). Concomitant with the contrary polarization of TAMs with anti-Her2/neu Ab alone and combination therapy, TAM deletion increased IFN-γ–producing CD8+ T cells in tumors treated with anti-Her2/neu Ab alone while dramatically reducing IFN-γ–producing CD8+ T cells in tumors treated with combination therapy to the same level as that seen with anti-Her2/neu mono-therapy (Fig. 5E). Collectively, these results show that the combination treatment of IL-21 and anti-Her2/neu Ab can greatly amplify the tumor-specific CD8+ T cell response in the tumor microenvironment triggered by anti-Her2/neu therapy through reprogramming TAMs from the M2 phenotype to the M1 phenotype. Moreover, the reprogrammed M1 TAMs are required for the tumor regression mediated by the combination therapy of anti-Her2/neu and IL-21.

**Discussion**

In this study, we found that TAMs with the M2 phenotype are gradually enriched within growing tumors and confer tumor resistance to anti-Her2/neu therapy as a major extrinsic limitation. Next, we demonstrated an effective strategy to prevent anti-Her2/neu–resistant breast cancer: combination therapy using anti-Her2/neu Ab and tumor local IL-21. The mechanism underlying its superior effect is that IL-21 has the potential to skew TAMs away from the immune-suppressive M2 phenotype and, therefore, break the immune tolerance of the tumor microenvironment.

**FIGURE 3.** Combination therapy of anti-Her2/neu Ab with tumor local IL-21. (A) TUBO-bearing mice (n = 5) were treated i.p. with 100 μg anti-Her2/neu and/or i.t. with 5 μg IL-21 (PBS as control) on days 14 and 17, as indicated by arrows. (B) TUBO-bearing Neu-transgenic F1 mice (n = 5–7) were treated i.p. with 150 μg anti-Her2/neu on day 20 and injected i.t. with 5 μg IL-21 (PBS as control) on days 20 and 23, as indicated by the arrows. (C) The cured mice or naive mice (n = 5) were rechallenged s.c. with 5 × 10^6 TUBO cells on the opposite side from the primary tumors ≥1 mo after complete rejection of primary tumors. (D) Tumor-bearing mice (n = 5) were treated i.p. with 100 μg anti-Her2/neu on day 15 and injected i.v. with 25 μg or injected i.t. with 5 μg IL-21 on days 15 and 17. Data are representative of three (A and B) or two (C and D) independent experiments. *p < 0.05, **p < 0.001, i.v. versus i.t., two-way ANOVA.
FIGURE 4. IL-21 skews TAMs away from the M2 phenotype and toward the M1 phenotype. (A and B) Tumor-bearing mice (n = 3–5) were injected i.t. with 5 μg IL-21 on days 15 and 17, and the tumor was excised on day 20. (A) qPCR was performed on sorted TAMs. Relative copy number was calculated based on β-actin. (B) CFSE-labeled lymph node cells were cocultured with sorted TAMs at a 3:1 ratio in the presence of anti-CD3 and anti-CD28 Abs for 3 d. T cell proliferation was measured by CFSE dilution. The percentages of CFSEdim cells (at least five divisions) were calculated. (C) TUBO-bearing Rag-1−/− mice (n = 3) were administered 50 μg anti-asialo GM1 Ab i.p. every 4 d, starting on day 13. IL-21 was injected i.p. on days 18, 20, and 21, and tumors were excised on day 22. qPCR was performed on sorted TAMs. Relative copy number was calculated based on β-actin. (D) TAMs were sorted from established TUBO tumors and exposed to IL-21 (100 ng/ml) for 16 h. Relative copy numbers of M2- and M1-type cytokines to β-actin were detected by qPCR. Data are mean ± SEM and are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. n.s, not significant.
Recent work showed convincingly that Ab-mediated tumor control depends on both innate and adaptive immune responses (13–15). However, multiple immune-suppressive mechanisms in the cancer microenvironment impair antitumor immunity and promote tumor recurrence. Targeting intrinsic signaling of tumor cells, as well as the extrinsic immune-suppressive microenvironment, will enhance the ongoing immune response against tumor, resulting in sustained tumor remission (34, 35). One of the most successful strategies is blockade of the B7-H1(PD-L1)/PD-1 pathway. Recent clinical trials demonstrated that ∼40% of patients treated with human anti-PD1 Ab had objective responses, with a durable response observed in only 20% of patients (36). Although this is an exciting strategy because this treatment has a much higher success rate than conventional treatment, the majority of patients still fail to respond to anti-PD1 treatment. Other strategies need to be considered.

The tumor microenvironment contains various cellular and molecular immune-suppressive mechanisms. Different tumors might rely on different key dominant immune-resistant mechanisms. In this TUBO breast cancer model, PD-1 is highly expressed on tumor-infiltrating CD8+ T cells. Blockade of PD-1 signaling by anti–PD-1 Ab improves the therapeutic activity of anti-Her2/neu therapy (15). However, this therapeutic strategy is not the most optimal combinatorial treatment with regard to anti-Her2/neu Ab, especially for larger or advanced tumors (data not shown); this suggests that, in addition to the PD-1 signaling pathway, other immune-suppressive mechanisms are responsible for resistance to anti-Her2/neu therapy. In this study, we characterized the abundant M2 TAMs in HER2/neu+ breast tumor as a dominant extrinsic limitation to confer tumor resistance to anti-Her2/neu Ab therapy. This is consistent with a recent clinical study demonstrating that stromal infiltration of CD68+ macrophages correlated...
inversely with the presence of CD8+ T cells in human breast cancer tissues and that the CD68/CD8 immune signature is an independent prognostic indicator for survival (20, 37). Thus, a similar phenotype of TAMs might exist and mediate resistance to anti-cancer therapy in human breast cancer. TAMs are recognized as a promising target for tumor therapy, and reduction of their tumor-promoting activities has become a hot topic. TAM-targeted anti-tumor approaches principally include inhibition of macrophage recruitment and survival in tumor and blockade of the tumor-promoting activity of TAMs. Macrophages are highly plastic and can adopt a different phenotype depending on the changing milieu. TAMs can be re-educated to become tumoricidal or tumor-suppressive M1-like phenotype. Nonetheless, only a few molecules have been identified to orchestrate this process (25, 38, 39).

IL-21, a pleiotropic common γ-chain (yc) cytokine, is primarily produced by activated CD4+ T cells and NK cells, but it acts on multiple lineages, including T cells, B cells, NK cells, NK cells, and dendritic cells. It should be noted that the role of IL-21 is well established in T and B cells, but it is less well studied in innate immunity (40, 41). Although several studies demonstrated the complex effects of IL-21 on dendritic cells (42, 43), its role in other myeloid cells is poorly documented. It was reported that monocyes/macrophages express IL-21R, and stimulation of IL-21 in vitro enhances phagocytosis and protease activity of monocytes/macrophages (44–47). Our present study reveals an unprecedented role for IL-21 in re-educating TAMs. We found that tumor local delivery of IL-21 can shift TAM polarization from the immune-suppressive M2 phenotype to the tumor-inhibiting M1 phenotype, which dramatically promotes anti-Her-2/neu Ab therapy. The polarization is a direct action of IL-21 on TAMs, but it is not mediated by IFN-γ produced by IL-21-activated CD8+ T cells and/or NK cells, because in vitro stimulation of IL-21 can rapidly skew the polarization of sorted TAMs; elimination of T, B, and NK cells enhances, instead of diminishes, the tumor local IL-21-induced polarization of TAMs to the M1 phenotype; and depletion of TAMs decreases the progression of anti-Her2/neu-treated tumors while increasing the growth of tumors treated with anti-Her2/neu and IL-21, implying that TAMs acquired a tumor-suppressive M1-like phenotype when exposed to local IL-21. Treatment with IL-21 alone also decreased the expression of the M2-type genes Vegf and Il10 in M-CSF-induced bone marrow-derived macrophages and Vegf, Tgf-b, Il10, and Ccl17 in peritoneal macrophages. These results were contrary to the study by Pesce et al. (48), in which pretreatment of bone marrow–derived macrophages with IL-21 increased the expression of the M2-type genes Arg-1 and FIZZI when subsequently stimulated with IL-4 and IL-13. This difference may suggest that macrophage programming by IL-21 depends on the environment of the macrophages. Thus, more research is required to define the effects of IL-21 on macrophages in various conditions.

IL-21 had significant antitumor activity against a variety of tumors in preclinical studies, and it is in a phase II clinical trial for metastatic melanoma (49–52). Depending on the tumor type and the methods of IL-21 application, the antitumor effect of IL-21 was mediated by CD8+ T cells, NK cells, or both. Most of these studies were done by deleting CD8+ T cells or NK cells with Abs. However, it is well known that antitumor CD8+ T cells or/and NK cells in the tumor microenvironment are firmly harnessed by a plethora of immune-suppressive mechanisms, so that they cannot be fully activated if the immune-suppressive mechanisms are not blocked or ameliorated. Therefore, our studies suggest that, in well-established tumors, such as larger or advanced tumors, skewing TAM polarization to the M1-like phenotype is the prerequisite for IL-21 to augment CD8+ T cell and/or NK cell cytotoxicity to exert its antitumor effects. Otherwise, IL-21 should not be able to directly activate CD8+ T cells and NK cells to eliminate the tumors because of the immune-suppressive mechanisms dominated by TAMs. IL-21–induced polarization of TAMs eliminates the immune suppression of TAMs to unleash the tumor-specific CD8+ T cell response, as well as reverses the immune suppression of TAMs to simulate antitumor immune responses. Based on our data, the potential clinical applicability of IL-2–mediated programming of macrophages allows for a more broad and attractive candidate for combination with Ab, as well as with immunogenic chemotherapy, irradiation, and novel immunotherapeutic agents, such as adoptive transfer of TCR-engineered T cells or treatment with anti-PD1 or anti-CTLA4. Because TAM abundance, distribution, and phenotypes varied between mouse models and human patients, even within different types of human cancers, future work should aim to define the response of TAMs to IL-21 stimulation in different types and subtypes of human cancers. To overcome the shortcoming of local injection of IL-21, fusion of IL-21 with the anti-Her2/neu Ab will be a strategy for tumor-targeting the delivery of IL-21 while maintaining the antitumor effects of the Ab.

In conclusion, our study demonstrates that the M2-type of TAM plays a dominant role in extrinsic resistance to Ab therapy, which is a promising anticancer strategy. We combined Ab therapy with targeted delivery of IL-21 to efficiently break the tumor resistance when the M2 type of TAMs becomes dominant. Our findings unveil a role for IL-21 in reprogramming M2 TAMs and highlight the potential of using re-educated TAMs to restore the antitumor Ab response. The study provides an opportunity to develop next-generation biologics by combining anti-Her2/neu Ab with IL-21.

Disclosures
The authors have no financial conflicts of interest.

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