Hypofractionated EGFR tyrosine kinase inhibitor limits tumor relapse through triggering innate and adaptive immunity

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Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are a first-line therapy for rapidly killing tumors such as those associated with non–small cell lung cancer by blocking oncogenic receptor signaling, but tumor relapse often occurs. Here, we have observed that hypofractionated EGFR TKI treatment (HypoTKI) is more potent than standard hyperfractionated EGFR TKI treatment (HyperTKI), and its antitumor effect associated with preventing tumor relapse depends on T cells. HypoTKI triggers greater innate sensing for type I IFN and CXCL10 production through the Myd88 signaling pathway to enhance tumor-specific T cell infiltration and reactivation. We also demonstrate that timely programmed cell death ligand–1 (PD-L1) blockade can synergize with HypoTKI to control advanced large tumors and effectively limit tumor relapse without severe side effects. Our study provides evidence for exploring the potential of a proper combination of EGFR TKIs and immunotherapy as a first-line treatment for treating EGFR-driven tumors.

INTRODUCTION

Epidermal growth factor receptors (EGFRs) are a large family of receptor tyrosine kinases and consist of four members: EGFR (ErbB1, HER1), ErbB2 (HER2, neu in rodents), ErbB3 (HER3), and ErbB4 (HER4) (1, 2). EGFR and its family members are well-known oncogenic drivers for many types of cancers, particularly non–small cell lung cancer (3–5). The targeted inhibition of these receptors and their signaling pathways has been considered as one of the most successful examples of targeted cancer therapy, including monoclonal antibodies (e.g., trastuzumab and cetuximab) and tyrosine kinase inhibitors (TKIs) (4, 6, 7). Recently, it has been shown that the new generation of irreversible EGFR TKIs shows greater potency than earlier generations of EGFR TKIs, targeted antibody therapies, or existing chemotherapies (8–12). Although EGFR-targeted therapies can cause late-stage EGFR-dependent cancers to initially regress, patients often relapse and develop resistance to EGFR TKIs (13). Therefore, a major challenge for EGFR TKI treatment is developing new inhibitors or combinational therapies that are not as prone to resistance and cause fewer relapses.

Preclinical studies investigating the antitumor effect of EGFR TKIs have been performed in vitro or using xenograft tumor models in immunodeficient mice, and they have shown that prolonged treatment could potently suppress tumor growth (14–16). These results suggested that the therapeutic effect of EGFR TKIs, especially second- and third-generation EGFR TKIs, likely works through the direct blockade of oncogenic signals and inducing tumor cell death. However, recent studies have shown that EGFR TKI treatment might modulate tumor plasticity and enhance tumor recognition or tumor lysis by innate natural killer (NK) cells and antigen–specific T cells (17, 18). Another study revealed that EGFR TKIs could inhibit T cell activation via down-regulation of the c-Raf/ERK (extracellular signal–regulated kinase) cascade and AKT signaling pathways in vitro (19). The mechanisms by which EGFR TKIs influence the host immune system are still poorly defined. Previous studies have reported that different treatment regimens could affect the antitumor effect of radiation. Hypofractionated radiation (the total dose of radiation is divided into large doses, and treatments are given over short periods of time) has more effective antitumor effects than hyperfractionated radiation (the total dose of radiation is divided into small doses, and treatments are given more than once a day) in a T cell–dependent fashion (20, 21). The role of different treatment regimens on antitumor efficacy of EGFR TKI would be a potentially interesting area of future study. Here, we developed EGFR TKI–sensitive syngeneic murine tumor models that exhibit high relapse rates under common clinical regimens and dosing with HyperTKI (hyperfractionated EGFR TKI: low dose with daily treatment) treatment, closely mimicking clinical phenotypes of patients treated with EGFR TKIs. Unexpectedly, HypoTKI (hypofractionated EGFR TKI: high dose with a low frequency treatment) treatment appeared to be more effective in preventing relapse, and its antitumor effects depend on innate and adaptive immunity. Our study further supports the potential use of immunotherapy as a first-line treatment concurrently with EGFR TKI.

RESULTS

Hypofractionated EGFR TKI notably reduces tumor burden and limits tumor relapse

Several murine tumor lines depend on Her2/neu, which shares the same oncogenic signaling pathways with EGFR and can grow in an immunocompetent host. Two examples are TUBO and NOP23, both of which were derived from spontaneous breast cancer in HER2/neu-transgenic (Tg) mice in BALB/c or C57BL/6 background (22, 23). To test whether these two tumor lines were sensitive to EGFR TKIs, we treated them with afatinib, a second-generation EGFR TKI used for both Her2- and EGFR–driven tumors in the clinic. Her2 signaling–dependent

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human breast tumor cell BT474 (fig. S1A) and Her2/neu signaling–
independent mouse breast tumor cell 4T1 (fig. S1B) were used as positive and negative controls. Both TUBO and NOP23 cells were sensitive to afatinib treatment (Fig. 1A). To confirm that the antitumor effect of afatinib relies on blocking Her2/neu signaling, we measured the phosphorylation of Her2/neu and the downstream signaling proteins ERK and AKT. Phosphorylation of Her2/neu, ERK, and AKT proteins was substantially decreased by afatinib treatment in a dose-dependent fashion (Fig. 1B). To evaluate whether afatinib can reduce tumor burden in syngeneic mice, both F1 Neu-Tg mice (BALB/c × FVB Neu Tg mice) and Neu Tg mice were initially used. The immunogenicity of the TUBO and NOP23 tumors in the Tg mice is expected to be very low and closely mimics tumors in cancer patients with EGFR or HER2 mutation and a low tumor mutation burden. F1 Neu-Tg mice (BALB/c × FVB Neu Tg mice) and BALB/c mice bearing established TUBO tumors, or Neu Tg mice and C57BL/6 mice bearing established NOP23 tumors, were treated with clinically used standard-of-care regimen (HyperTKI, low dose with daily treatment). Both TUBO (Fig. 1, C and E) and NOP23 (Fig. 1, D and F) tumor models respond to HyperTKI treatment. All tumors relapsed quickly after stopping treatment (fig. S1, C to F), which suggested TUBO and NOP23 tumor models under regimens that closely mimic clinical protocols resemble phenotypes of patients treated with EGFR TKIs. Previous studies have shown that hypofractionated radiation has more effective antitumor effects than hyperfractionated radiation (20, 21). To evaluate whether HypoTKI is more potent than HyperTKI, mice bearing established TUBO tumors were treated with indicated regimens (fig. S1G). HypoTKI (100 and 200 mg/kg, but not 50 mg/kg) is more potent than HyperTKI (12.5 and 25 mg/kg) in reducing tumor burdens. As very high doses may be associated with greater toxicity in patients, we chose a relatively high-dose regimen (100 mg/kg) as a HypoTKI treatment in the TUBO tumor model. Compared with HyperTKI (fig. S1, C to F), HypoTKI (Fig. 2, A to D) not only effectively reduced tumor burden initially but also limited tumor relapse in both TUBO and NOP23 models. In addition, HypoTKI was more efficient than HyperTKI in blocking Her2 downstream AKT signaling (fig. S2A), inducing apoptosis (caspase-3 cleavage; fig. S2, B and C), and suppressing cell proliferation (fig. S2, D and E). To investigate whether this HypoTKI strategy can apply to other EGFR TKIs, we also tested gefitinib (first-generation EGFR TKI), and osimertinib (third-generation EGFR TKI). Similar to afatinib, HypoTKI (Gefi) and HypoTKI (Osi) are much more potent than HyperTKI (Gefi) (Osi) in reducing tumor burden and limiting tumor relapse (Fig. 2, E and F). To further validate these findings, we tested the A431 (EGFR amplification tumor) xenograft tumor model with adoptive cell transfer, and HypoTKI (Afa) was more potent than HyperTKI (Afa) in reducing tumor burden (fig. S3A). To exclude the possibility that HypoTKI may have off-target effects, we used TUBO-P2J, a Her2/Neu lost variant derived from TUBO that is resistant to EGFR TKI. HypoTKI failed to reduce TUBO-P2J burden (fig. S3B). These data suggest that HypoTKI is more effective than HyperTKI in reducing tumor burden and preventing relapse in Her2/EGFR-driven tumors.

Although targeted therapies are thought to be more specific and less toxic than chemotherapies, patients with prolonged EGFR TKI treatment typically suffer from severe side effects, including acneiform-like rash, diarrhea, and ocular toxicity (24, 25). Because the single dose of EGFR TKI was increased in the HypoTKI regimen, we examined side effects associated with HypoTKI. BALB/c tumor-bearing mice were treated with HyperTKI (50 mg/kg, daily for 15 days) or an equal total dose of HypoTKI (250 mg/kg, three times), and total
body weight loss and ocular toxicity were evaluated. We observed that HyperTKI caused severe body weight loss (fig. S3C) and ocular toxicity (fig. S3D). Unexpectedly, no visible body weight loss or ocular toxicity was observed in HypoTKI-treated mice. Collectively, HypoTKI appears to be more potent than HyperTKI in controlling tumor burden and limiting tumor relapse with fewer side effects.

**T cells are essential for HypoTKI to limit tumor relapse**

Efficiently limiting relapse after such short courses of treatment cannot be only explained by direct killing. This raises the possible mechanisms that HypoTKI may trigger the host immune system to control tumor relapse. We characterized the immune cell profile at 72 hours after EGFR TKI treatment (HyperTKI versus HypoTKI) by flow cytometry and measured CD3+ T cells, CD8+ T cells, CD4+ T cells, regulatory T cells, NK cells, B cells, macrophages, myeloid-derived suppressor cells (MDSC), and CD103+ dendritic cells (DCs) (fig. S4, A to J). We observed that HypoTKI, but not HyperTKI, markedly increased CD3+, CD8+, and CD4+ T and B cells in the tumor microenvironment (TME) after treatment. In addition, we observed that both HypoTKI and HyperTKI treatment could decrease MDSC, whereas increased CD103+ DCs in the TME were observed after EGFR TKI treatment. There were no significant changes among other cell populations. These data indicate that HypoTKI is more potent than HyperTKI with respect to inducing an antitumor microenvironment that limits tumor growth and relapse. To determine the role of host immune cells in limiting tumor relapse, we performed experiments in nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice. Although HypoTKI treatment resulted in rapid tumor regression initially, all tumors relapsed immediately after treatment in NOD-SCID mice (Fig. 3A). Similar results were observed in both B and T cell–deficient Rag1−/− mice bearing syngeneic TUBO or NOP23 tumors treated with HypoTKI (Fig. 3B and fig. S5A) or HyperTKI (Fig. 3C). We also observed the consistent phenotype with the A431 xenograft tumor model (fig. S5B). In addition, we found that macrophage and NK cell depletion did not affect the antitumor efficacy of HypoTKI (fig. S5C). These findings indicate that HypoTKI treatment requires the host adaptive immune system to limit relapse.

To understand which cell populations in the adaptive immune system are required to limit tumor relapse after HypoTKI treatment, we treated BALB/c mice bearing established TUBO tumors with HypoTKI, and B or T cells were depleted by intraperitoneal injection of anti-CD20 or anti-CD4/anti-CD8 antibodies, respectively. The limiting tumor relapse effect of HypoTKI was abolished entirely in the absence of T cells, whereas depletion of B cells did not alter efficacy of HypoTKI treatment (Fig. 3D). This suggests that T cells are involved in the mechanism by which HypoTKI treatment limits tumor relapse. A recent study has revealed that the first-generation EGFR TKI–erlotinib can inhibit T cell activation via down-regulation of the c-Raf/ERK cascade and AKT signaling pathways in vitro (19). We found that a relatively high dose of afatinib can suppress proinflammatory cytokines [interferon-γ (IFN-γ) and tumor necrosis factor–α (TNF-α)] production from activated T cells in vitro (19).

To investigate whether HypoTKI can generate more potent antitumor-specific T cell responses in the TME than HyperTKI, we treated TUBO-HA (influenza hemagglutinin antigen overexpressing TUBO cells) tumor–bearing mice with HypoTKI or HyperTKI. To determine whether HypoTKI can generate more potent antitumor-specific T cell responses systemically, we treated TUBO (influenza hemagglutinin antigen overexpressing TUBO cells) tumor-bearing mice with HypoTKI (fig. S4B). We observed that HypoTKI, but not HyperTKI, was associated with a marked increase in CD3+ T cells in the TME 6 days after EGFR TKI treatment (Fig. 3E).

To investigate whether HypoTKI can generate more potent antitumor-specific T cell responses systemically, we treated TUBO-HA (influenza hemagglutinin antigen overexpressing TUBO cells) tumor–bearing mice with HypoTKI or HyperTKI. Results indicated that HypoTKI can increase the number of HA tetramer+ tumor-specific T cells than HyperTKI in tumor tissues (Fig. 3F). To further investigate whether HypoTKI can generate more antitumor–specific T cell responses systemically, we treated TUBO (influenza hemagglutinin antigen overexpressing TUBO cells) tumor–bearing mice with HypoTKI or HyperTKI, and splenocytes from treated mice were restimulated with irradiated TUBO cells. IFN-γ–producing cells were detected via enzyme-linked immune absorbent spot (ELISpot) assay. More IFN-γ–producing cells were detected in the HypoTKI-treated group than the control or HyperTKI group (fig. S5F). A similar ELISpot result was observed in the NOP23 tumor model (fig. S5G). An FTY720
blocking assay was performed to explore the origin of tumor-specific T cells that are required for limiting tumor growth. FTY 720 was injected 5 days before HypoTKI treatment and repeatedly injected every other day during the experiment, and HypoTKI achieved similar tumor control as compared with no FTY720 treatment (Fig. 3G), which suggested that preexisting tumor-specific T cells were sufficient to control tumor growth and limit tumor relapse. To rule out the direct effect of EGFR TKI on antigen-specific T cells, HPV E7 tetramer+ CD8+ T cells were measured in non–tumor-bearing mice vaccinated with HPV E7 vaccine without or without HyperTKI/HypoTKI treatment. We observed that both HyperTKI and HypoTKI did not affect antigen-specific T cell responses (fig. S5H). These data indicate that EGFR TKI did not directly increase antigen-specific T cell responses.

To further determine whether HypoTKI treatment results in prolonged protective antitumor T cell responses, 40 days after complete tumor regression, we rechallenged mice with a higher dose (2.5 × 10^6 cells) of TUBO tumor cells on the opposite flank. Results showed that HypoTKI-cured mice were resistant to rechallenge, whereas naïve mice were not.
showed rapid tumor progression (Fig. 3H). Collectively, these results demonstrate that HypoTKI treatment can enhance the tumor-specific T cell responses to limit tumor relapse.

Tumor-resident CD103+ DCs recently gained attention for their critical role in priming tumor-specific T cell responses during treatment (26–28). We have observed that HypoTKI could markedly increase CD103+ DCs in tumor tissues (fig. S4I). To further explore whether this subset of DCs is essential for the antitumor effect of HypoTKI, we used Batf3−/− mice that are deficient in CD103+ DCs. C57BL/6 and Batf3−/− mice bearing NOP23 tumors were treated with HypoTKI. In contrast to C57BL/6 mice, which can efficiently reduce tumor burden and limit tumor relapse after HypoTKI treatment, CD103+ DC deficiency markedly impaired the antitumor efficacy of HypoTKI (Fig. 3I). This suggests that CD103+ DCs are critical for HypoTKI to bridge innate immunity and adaptive immunity to enhance the tumor-specific T cell responses.

**Type I IFN signaling is required for HypoTKI to limit tumor relapse**

Recent studies have demonstrated that type I IFN is released from TME as a key innate sensing molecule to bridge T cell responses. Type I IFN responses have been associated with radiotherapy, anti-Her2–targeted therapy, anti-CD47 therapy, and chemotherapy, and have been linked to improved priming and generation of antitumor T cell responses (29–32). To study this molecular mechanism, we explored whether type I IFN signaling was involved in enhancing T cell response mediated by HypoTKI treatment. Mice bearing established TUBO tumors were treated with HypoTKI or HyperTKI, and HypoTKI was associated with substantially increased production of IFN-β and CXCL10 in tumor tissues compared with HyperTKI treatment (Fig. 4, A and B). These results indicate that type I IFN signaling is enhanced during HypoTKI treatment.

To address whether type I IFN signaling is required for limiting tumor relapse in vivo, we treated C57BL/6 and Ifnar1−/− mice bearing NOP23 tumors with HypoTKI. In contrast to C57BL/6 mice, which can efficiently restrict tumor relapse after HypoTKI treatment, loss of type I IFN signaling impaired the effect of limiting tumor relapse mediated by HypoTKI (Fig. 4C). To further determine whether type I IFN signaling is required for HypoTKI to enhance antitumor-specific T cell responses, we treated NOP23 tumor-bearing C57BL/6 or Ifnar1−/− mice with HypoTKI, and splenocytes were isolated for evaluation of antitumor T cell responses 10 days after the initial treatment. Compared with C57BL/6 mice, type I IFN signaling deficiency markedly reduced the effect of HypoTKI in enhancing tumor-specific T cell responses (Fig. 4D). Together, these data suggest that type I IFN signaling plays an integral role in improving T cell responses mediated by HypoTKI to limit tumor relapse.

**Myd88 signaling is required for HypoTKI to limit tumor relapse**

It is well known that innate sensing pathways, such as cGAS-STING, Toll-like receptors (TLRs)–TRIF, and TLRs-Myd88, are essential for type I IFN production (33–35). EGFR TKI–induced tumor cell death might release tumor-derived danger-associated molecular patterns (DAMPs), which could engage innate sensing pathways for type I IFN production. To determine whether any innate sensing pathways were required for limiting tumor relapse and type I IFN production with HypoTKI treatment, we implanted NOP23 tumors on C57BL/6, Stingmutmut, Trif−/−, and Myd88−/− mice. The tumor reduction was comparable among all the mice strains after initial treatment, suggesting that innate sensing pathways are dispensable for the initial antitumor effect of HypoTKI. In contrast to Stingmutmut and Trif−/− mice, which limit tumor relapse similarly to wild-type (WT) C57BL/6 mice (Fig. 5, A and B), all tumors relapsed in Myd88−/− mice (Fig. 5C). These data suggest that Myd88 signaling is critical for HypoTKI to control tumor relapse.

Our previous studies have shown that type I IFN produced by antigen-presenting cells is required for radiation and anti-CD47–mediated tumor-specific T cell activation in a STING-dependent fashion (29, 36). To evaluate whether Myd88 signaling was essential for induction of type I IFN after HypoTKI treatment specifically in antigen-presenting cells, we cocultured BMDCs from C57BL/6 WT and Myd88−/− mice with NOP23 tumor cells with or without EGFR TKI treatment. IFN-β protein was markedly increased in WT BMDC supernatant, whereas Myd88 deficiency completely abolished the IFN-β production with EGFR TKI treatment (Fig. 5D). Similarly, the expression of CXCL10 was also markedly diminished in Myd88−/−...
were treated with afatinib (25 mg/kg, twice) by gavage. Ten days after the initial treatment, lymphocytes from the spleens were
cells irradiated with 40
mean
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4, eaav6473 (2019)     9 August 2019
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T cell responses and programmed cell death ligand–1 (PD-L1)/
advanced tumors and
PD-L1 blockade synergizes with
programmed cell death–1 (PD-1) blockade on rescuing effector T cell
function, we argue that anti–PD-L1/PD-1 therapy should be used as a
first-line treatment to improve the HypoTKI-mediated T cell
cell line A431 in

BMDCs (Fig. 5E). Given the role of DC activation in enhancing tumor-
specific T cells, we investigated whether Myd88 signaling was required
for DC maturation after EGFR TKI treatment. BMDCs from WT mice
displayed a significantly higher level of CD86 when cocultured with
EGFR TKI–treated NOP23 tumor cells, whereas Myd88 deficiency
markedly impaired CD86 up-regulation (Fig. 5F). These results
demonstrate that HypoTKI induces stress on tumor cells that can
trigger Myd88 signaling in DCs. We have found that EGFR TKI can
increase the release of Myd88 stimulators’ double-stranded DNA
(dsDNA) and RNA from tumor cells in a dose-dependent fashion
(fig. S6, A to F). Accordingly, we also found that EGFR TKIs could
increase lactate dehydrogenase (LDH) release from treated tumor cells
in a dose-dependent manner (fig. S6, G and H), which indicates that
high doses of EGFR TKIs may induce cell membrane disruption
and DNA/RNA release. Together, these data suggest that HypoTKI
can induce more dsDNA and RNA release than HyperTKI in vivo
and trigger Myd88–type I IFN innate sensing pathways. Furthermore,
we observed that Myd88 signaling is essential for HypoTKI treatment–
enhanced antitumor-specific T cell responses (Fig. 5G). All these
data reveal that HypoTKI is more potent than HyperTKI in triggering
a Myd88-dependent type I IFN signaling pathway and enhancing
antitumor-specific T cell responses.

PD-L1 blockade synergizes with HypoTKI to control
advanced tumors and limit tumor relapse
Given the prominent effect of HypoTKI on enhancing tumor-specific
T cell responses and programmed cell death ligand–1 (PD-L1)/
T cell function (43, 44). These data raise the possibility that PD-L1/PD-1 blockade would enhance the T cell responses mediated by HypoTKI treatment and may achieve better control of tumor relapse.

Clinical results have already shown that response rates to anti–PD-1/PD-L1 therapy are very low (about 10%) in cancer patients with EGFR mutations (45, 46). Given the potency of HypoTKI in reducing tumor burden and enhancing T cell responses, we hypothesized that proper use of HypoTKI might overcome the resistance of anti–PD-1/PD-L1 therapy in cancer patients with EGFR mutations. Multiple clinical studies have been initiated to evaluate the combination of standard-of-care HyperTKI and PD-L1/PD-1 blockade. Several trials have been halted prematurely because of severe side effects. Therefore, we examined optimization strategies that combine EGFR TKI and PD-L1 blockade. We treated F1 Neu-Tg mice bearing advanced large tumors with HypoTKI, and anti–PD-L1 was administered at different time points (Fig. 6C). Tumors were completely resistant to anti–PD-L1 monotherapy. HypoTKI markedly reduced tumor burden initially, but all tumors (eight of eight) relapsed eventually. The maximum synergistic effect of combinational therapy depends on the timing of anti–PD-L1 administration. Early administration (anti–PD-L1 treatment started at day 0 or day 3 after the first afatinib treatment) showed total tumor regression and only two of eight tumors relapsed. Almost no synergistic effect was observed when anti–PD-L1 was administered 7 days after the first afatinib treatment, at which time tumors had already relapsed and started to grow (Fig. 6, D to I). Similar results were seen with BALB/c mice. Concurrent treatment of HypoTKI and anti–PD-L1 showed total tumor regression and no relapse. Sequential treatment resulted in similar tumor control at the beginning and partial tumor relapse later but a lower recurrence rate than HypoTKI alone. Again, almost no synergistic effect was observed when anti–PD-L1 was provided 2 weeks after HypoTKI treatment, at which time tumors had already relapsed and started to grow (Fig. S7, A to G). These data suggest that proper timing of anti–PD-L1 and HypoTKI combination shows the maximum synergistic antitumor effect in EGFR/Her2-driven tumors.
Because severe side effects of HyperTKI plus anti–PD-L1/PD-1 have been reported clinically (47), side effects of HypoTKI plus anti–PD-L1 were evaluated here. Mice treated with HyperTKI plus anti–PD-L1 exhibited more severe body weight loss and more apparent periorbital inflammation and edema, with the hematoxylin and eosin (H&E)–stained section of eyelid lesions showing reactive epidermal changes and dense subdermal chronic inflammation with lymphoid aggregates. Encouragingly, no marked body weight loss or eyelid inflammation was observed in HypoTKI plus anti–PD-L1–treated mice (fig. S8, A and B). These data suggest that anti–PD-L1/PD-1 blockade should be combined with HypoTKI concurrently to achieve maximum effect in reducing tumor burden and limiting tumor relapse without severe side effects.

**DISCUSSION**

TKIs that target EGFR family members are considered the most successful example of targeted cancer therapies (2, 4, 5). Although a promising antitumor effect has been shown initially under the standard-of-care HyperTKI regimen, almost all patients eventually face tumor relapse. Achieving both high response rates and low tumor relapse rates remains a big challenge for EGFR TKI treatment. In this study, we demonstrate that the HypoTKI regimen is much more potent than HyperTKI in reducing tumor burdens and limiting tumor relapse in a host T cell–dependent manner and reveal that type I IFN and Myd88 signaling pathways are critical for HypoTKI to enhance tumor-specific T cell responses (fig. S9). Additional carefully timed combination of PD-L1 blockade can safely synergize with HypoTKI to control advanced large tumors and limit tumor relapse. Thus, our study suggests that immunotherapy could be considered as first-line treatment concurrently with TKI.

Although the HyperTKI regimen is commonly used in clinical practice, a high dose of EGFR TKI treatment strategies has also been reported. A phase 1 clinical trial (NCT01647711) has demonstrated that the maximal tolerating doses (MTDs) of afatinib could be increased to 150 to 200 mg daily (about fivefold higher than the MTDs of afatinib daily) with the high-dose regimen (once daily for 3 days, repeated every 14 days in a 28-day cycle). The MTD of afatinib would be even higher than 200 mg daily according to our HypoTKI regimen (twice per week). Therefore, the dose of EGFR inhibitors in HypoTKI should be clinically feasible, and toxicity should be manageable. Several studies show that an optimized high dose of EGFR TKI could effectively kill tumor cells and delay the development of drug resistance owing to their direct blocking of oncogenic signaling (48–50). However, our current study shows that HypoTKI is much more potent than HyperTKI in reducing tumor burden and limiting tumor relapse in an adaptive immunity-dependent manner. One potential reason for the different findings between our study and previous ones may be the different preclinical animal models that were used. Most studies have been performed on xenograft tumor models in immunodeficient mice, which could only investigate the direct effect of EGFR TKI on tumor cells but not host immune responses. Using syngeneic murine tumor models, we could investigate the interaction between tumor cells and host immune cells during EGFR TKI treatment. Several studies, including ours, have shown that type I IFN signaling is essential for radiotherapy, CD47 blockade, or chemotherapeutics-enhanced tumor-specific T cell responses (29, 31, 32, 36, 51). However, the mechanism of induction of IFN might depend on distinct pathways. Here, we also reveal that type I IFN production mediated by the Myd88 signaling pathway is essential for HypoTKI to enhance T cell responses. We propose that HypoTKI can rapidly increase cellular stress and induce apoptosis, and then DAMPs, especially DNA or RNA, released from tumor cells will promote innate sensing for type I IFN production. However, which kinds of DAMPs and TLRs are upstream of the Myd88 signaling pathway remain to be determined. Myd88 also works as an adaptor protein for inflammatory signaling pathways downstream of the interleukin-1 (IL-1) receptor families (52, 53). Future studies are needed to determine whether the IL-1α/β/IL-1R/Myd88 axis is also involved in enhancing tumor-specific T cell responses and limiting tumor relapse in mice under HypoTKI treatment.

PD-L1 blockade can only reduce the immunosuppressed status but cannot effectively trigger T cell recruitment and reactivation. HypoTKI not only can reduce tumor burden but also can trigger IFN-dependent tumor-specific T cell responses. IFN could up-regulate PD-L1 expression in the TME. Therefore, HypoTKI and PD-L1 blockade can synergize with each other to achieve the maximum therapeutic effect and reduce relapse. It is noteworthy that recent clinical results from ongoing trials have shown that checkpoint blockade combined with prolonged treatment of EGFR TKIs can achieve a higher response rate, but side effects were also markedly increased, leading to the premature termination of several trials (47). Therefore, careful development of the combination of EGFR TKI and immunotherapy might minimize side effects. In our current study, HypoTKI combined with the PD-L1 blockade effectively controlled advanced large tumors, increased overall survival, and reduced tumor relapse. Compared with HyperTKI plus anti–PD-L1, HypoTKI plus anti–PD-L1 has fewer side effects. Anti–PD-L1 blockade is often used as a second- or third-line treatment in clinics after tumor relapse from first-line therapy. Here, we have observed that the therapeutic effect of concurrent HypoTKI and anti–PD-L1 may be better than anti–PD-L1 administration at a later time point when tumors start to relapse. Our study suggests that anti–PD-L1/PD-1 blockade should be combined with HypoTKI in a carefully timed manner as the first-line therapy for cancer patients with the Her2/EGFR mutation.

Patients with prolonged EGFR TKI treatment eventually develop drug resistance due to various mechanisms, including secondary mutations of EGFR (L858R and T790M), aberrant activation of the bypass pathways (c-Met, HGF, and AXL), and aberrant downstream pathways (K-RAS mutations and loss of PTEN) (8, 13, 54, 55). Several approaches have been developed to overcome drug resistance, such as next-generation EGFR TKIs that target EGFR mutations, and the combination of EGFR TKI with chemotherapeutics or targeted agents for the aberrant pathways (9). Here, we have demonstrated that HypoTKI is more potent than HyperTKI in limiting tumor relapse in a host immune response–dependent manner, and the combination of PD-L1 blockade further enhances the antitumor efficacy of HypoTKI in advanced large tumors. Further investigation is needed to determine whether HypoTKI or combined immunotherapy would overcome drug resistance and improve the overall survival of patients with EGFR/Her2-driven tumors. Our current study focuses on EGFR TKIs and how this hypofractionated regimen can be applied to other tumor-associated protein kinase inhibitors, such as ALK (anaplastic lymphoma kinase), BRAF (serine/threonine-protein kinase B-Raf), or VEGF (vascular endothelial growth factor) inhibitors, which will be an area of future interest.
MATERIALS AND METHODS

Study design
The goal of this study was to explore whether and how optimizing the treatment regimen of EGFR TKIs could enhance antitumor efficacy. We compared treatment regimens between standard-of-care HyperTKI and HypoTKI using murine syngeneic EGFR TKI–sensitive tumor models. The tumor-bearing mice were assigned to different groups by tumor size (the average tumor size is similar among different groups). The sample size is specified in each figure legend, and samples were not blinded or randomized during experiments or analysis.

Mice
Female C57BL/6j and BALB/c mice were purchased from University of Texas (UT) Southwestern Medical Center Breeding Core or the Jackson Laboratory. Rag1−/− mice in both C57BL/6 and BALB/c background, Sting−/−/−, Myd88−/−/−, Trif−/−/−, Batf3−/−/−, FVB/N-Tg (MMTVneu) and C57BL/6-Tg[TcraTcrrb]1100Mjb/J (OT-I CD8+ T cell receptor–Tg mice) were purchased from the Jackson Laboratory. NOD-SCID mice were obtained from the UT Southwestern Medical Center Breeding Core. Ifnar1−/−/− mice were provided by A. Chong at the University of Chicago. NeuO1/Tg-1/Tg mouse and provided by H. Nelson at Trev & Joyce Deely Research Centre, British Columbia, Canada. We obtained F1 Neu-Tg mice from crossing FVB/N-Tg (MMTVneu) and BALB/c mice. More information can be found in table S1. All mice were maintained under specific pathogen–free conditions, and all animal procedures were performed in accordance with the experimental animal guidelines set by the Institutional Animal Care and Use Committee of the UT Southwestern Medical Center.

Cell lines and reagents
TUBO was derived from a spontaneous mammary tumor in a BALB/c Neu-Tg mouse (22). NOP23 was cloned from a spontaneous mammary tumor in a B6 NeuOT-I/OT-II Tg mouse and provided by B. H. Nelson at Trev & Joyce Deely Research Centre, Canada. BT474, 411, and A431 cells were purchased from the American Type Culture Collection. Cells were cultured in 5% CO2 and maintained in vitro in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), penicillin (100 U/ml), and streptomycin (100 µg/ml). The second generation of EGFR TKI–afatinib and FTY720 were purchased from Selleckchem. Gefitinib and osimertinib were purchased from LC Laboratories. (100 U/ml), and streptomycin (100 µg/ml). The second generation of EGFR TKI–afatinib and FTY720 were purchased from Selleckchem. Gefitinib and osimertinib were purchased from LC Laboratories. Anti-CD8 (YTS 169.4), anti-CD4 (GK1.5), and anti-CD20 (5D2) antibody were purchased from Bio X Cell. Anti-CD8 T cell depletion experiments, anti-CD8 and anti-CD4 antibodies (200 µg of each antibody on the same day of the first treatment and then 100 µg of each antibody every 3 days for a total of four times) were injected intraperitoneally during the EGFR TKI treatment. For B cell depletion experiments, 200 µg of anti-CD20 antibody was injected intraperitoneally twice a week during the EGFR TKI treatment. For the PD-L1 blockade experiments, 200 µg of anti–PD-L1 (clone 10F.9G2) was administered intraperitoneally to mice every 2 days for a total of three times.

Cytotoxicity assays
Cytotoxicity of afatinib on tested cell lines was performed using Cell Counting Kit-8 (CCK-8) according to the manufacturer’s instructions (Dojindo Inc.). Briefly, 5000 to 8000 cells were seeded into 96-well plates and cultured overnight. The following day, medium containing various concentrations of afatinib was added. Forty-eight hours later, a volume of 20 µl of CCK-8 reagent was added to the wells and incubated for 1 to 2 hours at 37°C. The absorbance of the samples at 450 nm was measured with SPECTROstar Nano (Biotec Solutions Inc.).

Immunoblotting
Protein sample preparation and immunoblot procedures were performed as previously described (36). Antibodies to p-HER2/Neu (6G7), HER2/Neu (D8F12), p-ERK1/2 (Thr202/Tyr 204), ERK1/2 (137F5), p-akt (D9E), AKT (pan) (C67E7), caspase-3, activated caspase-3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin were purchased from Santa Cruz Biotechnology or Cell Signaling Technology. Detailed information can be found in table S1. Briefly, cells were seeded into 12-well culture plates overnight and treated with different doses of afatinib. Four hours later, cells were lysed and run on SDS-PAGE (polyacrylamide gel electrophoresis) gels for immunoblotting. Tumor tissues were lysed with radiolabeled antibodies followed by horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (Abcam) secondary antibody. Color was developed with diaminobenzidine substrate kit (Abcam). The images were taken with NanoZoomer (Hamamatsu Photonics K.K.).

Histological and immunohistochemical staining
Skin and tumor tissues were fixed with 10% formalin and embedded in paraffin. Sections from skin around the eyelid were stained with H&E as previously described (57). For immunohistochemical staining, sections of tumor tissues were stained with anti-mouse cleaved caspase-3 (Asp175) (Cell Signaling Technology) or anti-mouse Ki-67 (Abcam) primary antibody followed by horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (Abcam) secondary antibody. Color was developed with diaminobenzidine substrate kit (Abcam). The images were taken with NanoZoomer (Hamamatsu Photonics K.K.).

Flow cytometry analysis
Single-cell suspensions of cells were incubated with anti-CD16/32 (anti-FcγRII/III receptor, clone 2.4G2) for 10 min to block nonspecific binding and then subsequently stained with antibodies. All fluorescently labeled antibodies were purchased from BioLegend or eBioscience.
and the detailed information of antibodies is listed in table S1. Fixable Viability Dye eFlour 506 (eBioscience) was used to exclude dead cells. Data were collected on CytoFLEX (Beckman Coulter Inc.) and analyzed by CytExpert (Beckman Coulter Inc.) or FlowJo (Tree Star Inc., Ashland, OR) software.

Measurement of IFN-γ-secreting T cells by ELISPOT
Spleens from afatinib- or vehicle-treated mice were processed to single-cell suspensions and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). A total of 2 × 10^5 or 4 × 10^5 spleen cells were used for the assay. Irradiated TUBO or NOP23 cells were used to restimulate the tumor-specific T cells. The ratio of tumor cells and spleen cells was 1:4. The IFN-γ production was determined 48 hours after incubation with an IFN-γ ELISPOT assay kit according to the manufacturer’s protocol (BD Biosciences). The visualized spots were enumerated with the CTL-Immunospot S6 Analyzer (Cellular Technology Limited).

Generation of bone marrow–derived DCs
Bone marrow–derived DCs (BMDCs) are generated as described previously (58). Briefly, bone marrow (BM) cells were collected from tibias and femurs of female C57BL/6 and 58. Briefly, bone marrow (BM) cells were collected from tibias and femurs of female C57BL/6 and Myd88−/− mice. The BM cells were plated and cultured in a 24-well plate with complete RPMI 1640 medium containing recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/ml, BioLegend). Fresh media with recombinant mouse GM-CSF were added into the culture on day 3. The immature BMDCs were collected and ready to use on day 7.

T cell isolation
CD8+ T cells were isolated from lymph nodes and spleens of OT-1 T cell receptor–Tg mice with a negative CD8 isolation kit (STEMCELL Technologies) following the manufacturer’s instructions.

ELISA and multiplex
Tumor tissues were excised on day 3 after EGFR TKI treatment and homogenized in the Cell Lysis Kit (Bio-Rad Laboratories) with the FastPrep-24 5G Homogenizer. Cell culture supernatants were obtained 24 hours after tumor and BMDC coculture. The concentration of IFN-β was measured with VeriKine-HS Mouse Interferon Beta Serum enzyme-linked immunosorbent assay (ELISA) Kit (PBL Assay Science) in accordance with the manufacturer’s instructions. The concentration of CXCL10 was measured with the Bio-Rad multiplex (mouse chemokines) kit in accordance with the manufacturer’s instructions.

Statistical analysis
All analyses were performed using GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA). Two-way analysis of variance (ANOVA) was used to analyze tumor growth and body weight loss. All the other data were analyzed using unpaired two-tailed t tests. A value of P < 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS
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Fig. S1. HyperTKI could not prevent tumor relapse.
Fig. S2. HypoTKI is more potent in suppressing Her2 downstream Akt signaling, inducing apoptosis, and suppressing tumor cell proliferation than HyperTKI in vivo.
Fig. S3. HyperTKI is more potent than HyperTKI in controlling tumor growth with fewer side effects.

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Acknowledgments: We thank the UT Southwestern Flow Cytometry Facility, Animal Resources Center, and Pathology Core Facility. Y.-X.F. holds the Mary Nell and Ralph B. Rogers Professorship in Immunology. Funding: This work was in part supported by Texas CPRIT grant RP180725 and RR150072 (CPRIT scholar in Cancer Research) to Y.-X.F. Author contributions: Z.L., C.H., and Y.-X.F. designed the experiments and analyzed the data; Z.L. and C.H. did the statistical analysis; Z.L., C.H., A.S., E.H., Z.R., and Y.P. conducted the experiments; J.Q., C.L., L.L., M.C., A.Z., and Y.W. contributed to reagents/materials and provided helpful advice; Y.-X.F. supervised the experiments; Z.L. and Y.-X.F. wrote the manuscript; Z.L., C.H., E.H., C.T., and Y.-X.F. revised the manuscript. Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. The materials that support the findings of this study are available from the corresponding author on reasonable request.

Submitted 6 October 2018
Resubmitted 5 March 2019
Accepted 10 July 2019
Published 9 August 2019
10.1126/sciimmunol.aav6473

Hypofractionated EGFR tyrosine kinase inhibitor limits tumor relapse through triggering innate and adaptive immunity

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Sci. Immunol. 4, eaav6473. DOI: 10.1126/sciimmunol.aav6473

Enhanced inhibitor

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) block oncogenic receptor signaling and are used as a first-line treatment for EGFR-mutated non–small cell lung cancer. Resistance to EGFR TKIs, including the standard hyperfractionated EGFR TKI (HyperTKI) treatment, is a problem that has driven the development of next-generation inhibitors. Here, Liu et al. describe the improved efficacy of hypofractionated EGFR TKI (HypoTKI) relative to HyperTKI in triggering antitumor T cell responses and preventing relapse in a TKI-sensitive syngeneic murine tumor model through a mechanism involving the type I IFN and MyD88 signaling pathways. Coadministration of HypoTKI with an anti–PD-L1 antibody further improved antitumor responses and reduced tumor relapse, thus suggesting that this combined therapy may be a potential alternative to existing treatment regimens.