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Effective antibody therapy induces host protective antitumor immunity that is augmented by TLR4 agonist treatment

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Abstract

Toll-like receptors are potent activators of the innate immune system and generate signals leading to the initiation of the adaptive immune response that can be utilized for therapeutic purposes. We tested the hypothesis that combined treatment with a toll-like receptor agonist and an anti-tumor monoclonal antibody is effective and induces host-protective anti-tumor immunity. C57BL/6 human mutated HER2 (hmHER2) transgenic mice that constitutively express kinase-deficient human HER2 under control of the CMV promoter were established. These mice demonstrate immunological tolerance to D5-HER2, a syngeneic human HER2-expressing melanoma cell line. This human HER2 tolerant model offers the potential to serve as a preclinical model to test both antibody therapy and the immunization potential of human HER2 targeted therapeutics. Here we show that E6020, a toll like receptor-4 (TLR4) agonist effectively boosted the antitumor efficacy of the monoclonal antibody trastuzumab in immunodeficient C57BL/6 SCID mice as well as in C57BL/6 hmHER2 transgenic mice. E6020 and trastuzumab co-treatment resulted in significantly greater inhibition of tumor growth than was observed with either agent individually. Furthermore, mice treated with the combination of trastuzumab and the TLR4 agonist were protected against re-challenge with human HER2 transfected tumor cells in hmHER2 transgenic mouse strains. These findings suggest that combined treatment with trastuzumab and a TLR4 agonist not only promotes direct anti-tumor effects but also induces a host-protective human HER2-directed adaptive immune response indicative of a memory response. These data provide an immunological rationale for testing TLR4 agonists in combination with antibody therapy in patients with cancer.

Keywords

toll like receptor 4 agonist; HER2; antibody therapy; transgenic mouse; antitumor immunity

Introduction

Unconjugated monoclonal antibodies, which provided some of the earliest demonstrations of effective targeted therapy, have emerged as versatile and useful cancer therapeutics for an

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increasing number of human malignancies [1–5]. Immune mechanisms are increasingly appreciated as playing important therapeutic roles for antibody therapy. The interactions between Fc gamma receptors (FcγR) and antibody Fc regions have been well recognized to contribute to antitumor efficacy [6–9]. FcγRs are key links between therapeutic antibodies and the cellular immune system, and enable monoclonal antibodies to induce adaptive immune responses. For example, induction of a tumor antigen-specific humoral response has been documented in patients treated with trastuzumab [10,11]. Antibody therapy can induce T-cell dependent immune responses directed against the targeted tumor antigen in mice [12]. Antibodies have been combined with cytokines and other immunomodulatory agents with the goal of exerting synergistic antitumor effects [13–15]. The synergy between antibodies and other immune activators may be based upon the amplification of FcγR-mediated mechanisms.

Human HER2 was selected as a model antigen for our studies, since it is a well-known breast tumor-associated antigen that has been successfully targeted for immunotherapy of human breast cancer. Trastuzumab, an approved and widely used HER2-targeted antibody that targets HER2-overexpressing breast cancer, efficiently mediates ADCC [16–18]. Absence of appropriate preclinical models has limited the ability to determine the involvement of acquired immune response in the antitumor activity of anti-human HER2 therapies. Most prior *in vivo* treatment experiments have been conducted using xenograft models in immunodeficient mice to avoid the induction of anti-human HER2 antibodies. Therefore, immunocompetent HER2 tolerant mice are needed to determine the antitumor effect attributable to cellular or humoral adaptive immune responses. Several human HER2 transgenic mouse models have been generated to test drug therapies and tumor vaccines for the treatment of breast cancer [19–21]. These transgenic models express the normal human *HER2* gene under the transcriptional regulation of whey acidic protein (WAP) and MMTV promoters to achieve mammary-specific expression of human HER2. While these models are highly relevant for mechanistic studies of functional inactivation of HER2, segregation of the immune-mediated activities of HER2-targeting antibodies from their direct signaling inhibitory effects may be challenging. We therefore developed a transgenic mouse model where a functionally inactive mutant was created by replacing a conserved lysine residue with methionine in the ATP binding pocket of the kinase domain of HER2. This human mutant HER2 (hmHER2) is ubiquitously expressed in normal tissues under the control of cytomegalovirus promoter and is thus a self-antigen in these mice. The resulting kinase-deficient HER2 gene does not produce tumors in mice and offers an opportunity to study HER2-targeted antibody immunotherapy in a human HER2-tolerant mouse preclinical model. In addition, this mouse model has permitted investigation of the ability of trastuzumab to induce T-cell dependent immune responses directed against human HER2 in a human HER2-tolerant setting that immunologically mimics human biology.

The discovery of mammalian toll like receptors (TLRs) and other pattern recognition receptors (PRRs) has provided potential targets for the design of molecules that can be used to manipulate innate immune responses. As potent activators of the innate immune system, TLR agonists can activate most Fc receptor bearing effector cells, and thus may be appropriate adjuvants for antibody therapy. The novel synthetic TLR4 agonist E6020 was developed as a lipid A mimetic that maintains most of the immunostimulatory activity of lipopolysaccharide (LPS). Unlike LPS, E6020 is a simplified, synthetic agonist, with a promising preclinical safety profile [22]. E6020 activates NF-κB signaling and stimulates cytokine production only through TLR4 [23]. In animal models, E6020 has been proven to be a potent, non-toxic vaccine adjuvant that provides protective immune responses when administered with a number of protein antigens, where E6020 enhances Th1 responses characterized through the production of IFN-γ [24–26]. These factors led us to hypothesize that E6020 may serve as a suitable adjuvant for antibody therapy. We therefore evaluated

the ability of promoting antitumor effect through activation of tumor-specific immune response by a TLR4 agonist. We show that treatment with this TLR4 agonist and trastuzumab can effectively enhance the anti-tumor effects of trastuzumab in this model, and that effective therapy induces host-protective, T-cell dependent antitumor immunity.

Materials and Methods

Tumor cell lines

The D5 murine melanoma cell line is a poorly immunogenic subclone of the spontaneously arising B16BL6 melanoma (kindly provided by S. Shu, Cleveland Clinic Foundation, Cleveland, OH). D5-HER2 is a stable clone of D5 transfected with full-length cDNA of the human *HER2* gene. The abundant expression of HER2 on the cell surface has been repeatedly confirmed by flow cytometry using the PE-conjugated anti-HER2 antibody (BD Biosciences Pharmingen) and immunohistochemistry. Tumor cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin. D5-HER2 cell line was maintained in medium containing 0.8 mg/ml G418.

Mice

C57BL/6 mice and C57BL/6 SCID mice were purchased from Jackson Laboratory and housed at the Georgetown University Animal Resources Facility and Fox Chase Cancer Center Laboratory Animal Facilities under specific pathogen-free conditions. All animal work was performed under protocols approved by the Animal Care and Use Committees of Georgetown University Medical Center and Fox Chase Cancer Center.

Generation and identification of human mutated HER2 transgenic mice

Full-length human HER2 transgene was mutated at position 753 to replace a conserved lysine residue with methionine. The cDNA was cloned under the control of the CMV promoter to give rise to a CMV-HER2 cassette. Linear DNA fragments for microinjection were obtained and injected by the Fox Chase Cancer Center Transgenic Mouse Facility. The FVB inbred mouse strain was used. Transgenic animals from the F1 generation were crossed with C57BL/6 mice for 14 generations to establish human HER2 transgenic mice in the C57BL/6 background. RT-PCR analysis was used to determine human *HER2* RNA expression in various tissues derived from transgenic mouse and non-transgenic mice. For routine screening of human mutated HER2 transgenic mice (hmHER2 Tg), tail tissue was used for PCR analysis.

Tumor mouse model

Cohorts of 8–12 week old C57BL/6 SCID mice, and hmHER2 Tg mice were given s.c. injections into the flanks with either D5 or D5-HER2 tumor cells at inocula ranging from 10^3 – 10^4 cells. Following tumor inoculation, mice were monitored twice weekly for the development of palpable tumors at the challenge site with tumor volume calculated as the product of length $\times$ (width)² $\times$ 0.5. Animals were sacrificed before tumors reached 2 cm in maximum diameter, or if there was any sign of distress. Tumor tissue was analyzed for human HER2 expression as described below.

Antibody and TLR4 agonist

Trastuzumab (Herceptin; Genentech, South San Francisco, CA), a humanized IgG1 that recognizes the extracellular domain of the HER2 oncoprotein, was purchased from the Fox Chase Cancer Center pharmacy. E6020, a TLR4 agonist, was supplied by Eisai Research

Institute of Boston (Andover, MA). Hybridomas producing depleting anti-CD4 (GK1.5) and anti-CD8 (2.43) mAb were obtained from the ATCC.

Treatment with trastuzumab and TLR4 agonist

C57BL/6 SCID mice as well as hmHER2 Tg mice were treated on a twice weekly schedule starting on day 1 following tumor inoculation for four weeks. Animals were treated with PBS, or single agent or combined trastuzumab (200 µg) and E6020 (10 µg) intraperitoneally, in a total volume of 1ml PBS.

Immunohistochemical analysis of HER2 expression

Immunohistochemical analysis of HER2 protein expression in hmHER2 Tg mouse organs and tumors was performed on paraffin sections. The sections were subjected to antigen retrieval by steaming for 20 min in citrate buffer (10 mM). After blocking endogenous peroxidase activity by using 0.3% hydrogen peroxide, the sections were incubated overnight with trastuzumab at 4°C in a humidified chamber. Immunostaining was developed using a biotinylated goat anti-human IgG followed by exposure to streptavidin–horseradish peroxidase complex using VIP as substrate (Vector VIP substrate kit, Vector Laboratories, Burlingame, USA). Immunostained sections were counterstained with hematoxylin. The primary antibody was replaced with human IgG as a negative control.

FACS analysis for determination of HER-2 specific antibody levels in mouse

Human HER2 expressing SKOV-3 ovarian cancer cells were incubated with mouse serum samples (1:100 dilution) for 45 min at 4 °C in PBS with 1% BSA. Cells were then washed and further incubated with PE-conjugated goat anti-mouse IgG (BD Biosciences Pharmingen). Cells were analyzed for fluorescence on a FACScan (Becton Dickinson). The results were expressed as mean fluorescence intensity (MFI).

Tumor re-challenge experiments

C57BL/6 hmHER2 Tg mice that had rejected D5-HER2 tumor after treatment were re-challenged 120 days after the first challenge by injecting the opposite flank with 5-fold higher dose than the initial challenge D5 or D5-HER2 tumor cells, without further treatment. Mice were examined twice weekly to detect tumor growth, and the percentage of tumor-free mice was recorded.

In vivo depletion of T lymphocytes, macrophages and NK cells

mAb GK1.5 and 2.43 were used to deplete CD4⁺ and CD8⁺ T cells respectively. Each mouse was injected i.p. on days 5, 2 before tumor re-challenge with 500 µg of mAb GK1.5 or 2.43. Thereafter, depletion was maintained by i.p. injection with GK1.5 or 2.43 every 3 days. Macrophages were depleted by i.p. injection of 200 µl clodronate-containing liposomes (Encapsula NanoSciences, Nashville, TN, USA) the day before tumor cells injection and thereafter every 5 days. For depletion of NK cells, mice were injected i.p. with 25 µg of anti Asialo GM1 antibody (Wako, Richmond, VA, USA) before and after tumor injection every 5 days. Depletion was verified by FACS analysis of splenocytes 6 days after the first injection (data not shown).

Phagocytosis assay

Macrophage antibody-dependent cell-mediated phagocytosis (ADCP) was determined by flow cytometry. Peritoneal macrophages were isolated from hmHER2 Tg mice and were pre-incubated with varying concentrations of E6020 for 20 h. Target D5-HER2 cells were labeled with CellTracker Green CMFDA (Invitrogen, San Diego, CA, USA) and were added at 4:1 effector:target. Antibody 4D5 or control murine IgG antibodies were incubated with

the target cell and effector cell at 37°C for 4 h. Cells were detached from the plate surface with HyQase, stained with APC-anti-CD11b and fixed with 1% paraformaldehyde. Phagocytosis was analyzed using a flow cytometer and percent phagocytosis was calculated as the number of double-positive cells divided by the total number of tumor cells. All tests were performed in triplicate and the results were expressed as mean $\pm$ standard deviation (SD).

Intracellular cytokine staining

Splenocytes were stimulated in 96-well plates ($1-2 \times 10^6$ /well) with HER2 peptide mix (ECD or ICD) (2.6 μ g/mL was used; BD Bioscience, San Jose, CA). After 5 hours incubation, BD GolgiPlug containing brefeldin A (0.5 μ l/well; BD Bioscience) was added and incubated for additional 4 hours at 37°C. Cells were collected and stained for CD4, CD8, CD69 and intracellular IFN- γ using the Cytotfix/Cytoperm kit (BD Bioscience). Total cells were acquired and the number of dual IFN- γ /CD69 positives was determined for the CD4-gated or CD8- gated populations.

ADCC assay

Mouse splenocytes with or without E6020 treatment served as the effector cells for this assay. The target cells were labeled with Na⁵¹CrO₄ (100 μ Ci/ 10^6 targets; PerkinElmer, Waltham, MA) for 1 h at 37°C in RPMI 1640 medium and then washed twice and resuspended at 2×10^5 cells/mL. 50 μ l of labeled target cells were added to individual wells of 96-well plates containing effector cells and/or Abs. Effector cells were added to yield a different E:T ratio in the presence or absence of various concentrations of 4D5 IgG2a. Each well contained a total volume of 200 μ l, and all assays were performed in triplicate. The plates were centrifuged at $300 \times g$ for 3 min, incubated for 4 h in a 5% CO₂ incubator at 37°C, then centrifuged again at $300 \times g$ for 3 min. A total of 100 μ l of supernatant were removed from each well for counting on a gamma-counter (PerkinElmer). Cytotoxicity was estimated by measuring the quantity of label released into culture supernatants using the

$$\text{percentage of lysis} = ((\text{experimental release cpm} - \text{spontaneous release cpm}) / (\text{total added counts cpm} - \text{spontaneous release cpm})) \times 100$$

formula: $\frac{(\text{experimental release cpm} - \text{spontaneous release cpm})}{(\text{total added counts cpm} - \text{spontaneous release cpm})} \times 100$, where the experimental release was defined as cpm released by target cells in wells in the presence of effector cells and/or Ab and the spontaneous release was defined as cpm released by target cells alone.

Statistical analyses

Statistical analysis was performed using Prism Software (GraphPad software, Inc.) and SAS. Survival analysis was performed using the log-rank test. Differences were considered statistically significant at P value of <0.05, using a two-tailed Student's test.

Results

Human mutated HER2 (hmHER2) transgenic mice are tolerant to human HER2

A C57BL/6 strain mouse line that constitutively expresses kinase-deficient human HER2 in several organs was established. In this HER2 transgene, a critical lysine 753 residue that is required for ATP binding by the protein kinase domain was substituted with methionine and cloned into a CMV-promoter vector (Fig. 1a). To determine the transgene expression in mouse organs, RT-PCR analysis was performed. As shown in Fig. 1b, HER2 RNA expression was positive in selected organs of the transgenic mice including kidney, liver, lung and heart. No specific PCR products were observed in any non-transgenic littermates. A high level of RNA expression was detected in kidney, which was confirmed by protein expression as assessed by immunohistochemistry (Fig. 1c).

The C57BL/6 syngeneic D5 melanoma tumor cell line was transfected with the full length human *HER2* CMV-transgene to yield the D5-HER2 cell line. This cell line abundantly expresses human HER2 on the cell surface at a level comparable to the human ovarian cancer cell line SKOV-3 (Fig. S1 and 2c). No HER2 expression was detected on untransfected D5 melanoma cells (Figs. S1, 2c). The tumorigenicity of D5 and D5-HER2 tumor cells was evaluated *in vivo* to determine if hmHER2 Tg mice are tolerant to HER2. C57BL/6 SCID mice, C57BL/6 and C57BL/6 hmHER2 Tg mice were subcutaneously injected with 10^3 – 10^4 D5 or D5-HER2 cells per mouse and monitored for the development of palpable tumors. D5 cells formed rapidly growing tumors in all three strains. In immunodeficient C57BL/6 SCID mice, both D5 and D5-HER2 cells showed similar growth rates. The wild-type C57BL/6 mice rejected D5-HER2 cells or exhibited delayed tumor formation when injected with 10^3 cells per animal. However, a 10-fold higher inoculum was not rejected (Fig. 2a). In contrast, hmHER2 Tg mice demonstrated tolerance to D5-HER2 tumors. The minimum cell dose required for tumor growth in 100% of animals was 10-fold lower for the transgenic mice as compared with wild-type mice. To examine the induction of anti-HER2 antibodies in these mice, sera were collected 1 month after D5-HER2 tumor inoculation. Anti-HER2 antibodies were not detected in SCID or hmHER2 Tg mice, while significant levels of anti-HER2 antibodies were detected in wild-type mice (Fig. 2b).

The expression of HER2 as detected by immunohistochemical staining was uniform throughout the tumor in SCID and Tg mice (Fig. 2c). In wild-type mice, however, the HER2 staining was patchy. The slower growth of D5-HER2 tumors in immunocompetent wild-type C57BL/6 mice was associated with the induction of anti-human HER2 antibodies, intratumoral leukocyte infiltration (not shown) as well as the altered expression of human HER2 in the tumor specimen (Fig. 2c). None of these findings was observed in the human HER2 transgenic strain.

Amplification of the antitumor efficacy of trastuzumab by the TLR4 agonist E6020 in immunodeficient and human HER2-tolerant immunocompetent mice

The antitumor effect of trastuzumab, or in conjunction with E6020 was evaluated in a D5-HER2/C57BL/6 model system. Trastuzumab has been proven to be an effective treatment in animal and human studies. The efficacy of trastuzumab has been shown to be dependent on the presence of Fc γ R [6]. E6020 is an LPS derivative with potent agonistic activity through the TLR4 signaling pathway. TLR4 is predominantly expressed on the cells of the innate immune system, including monocytes/macrophages, dendritic cells and neutrophils, all of which are Fc receptor-bearing cells.

The first experiment was conducted in SCID mice lacking B-cell and T-cell immunity. As shown in Fig. 3a, mice treated with trastuzumab alone survived longer than either PBS or E6020 treated mice. However, when E6020 was combined with trastuzumab, mice survived significantly longer than those receiving trastuzumab alone ($P < 0.001$).

To determine whether similar efficacy would be observed in an immunocompetent, HER2-tolerant mouse model, hmHER2 Tg mice were treated using the same schema as described above. As shown in Fig. 3b, Trastuzumab treatment alone protected 4/12 mice from tumor growth, while the combination of a TLR4 agonist and trastuzumab protected most of the animals (10/12) from tumor growth (Fig. 3b) ($P < 0.05$). Of the few animals in the trastuzumab plus E6020 treatment group that eventually developed tumors, the appearance of these tumors was significantly delayed, resulting in improved survival in this treatment group.

These data demonstrate that the antitumor efficacy of trastuzumab can be amplified by the TLR4 agonist E6020 in immunodeficient and human HER2-tolerant immunocompetent

mice. Hence, innate immunity is sufficient to promote trastuzumab-targeted rejection of primary tumor challenge.

FcγR bearing macrophages but not NK cells are partly responsible for the antitumor effects of combination therapy with trastuzumab and E6020

Since the TLR4 agonist increased the antitumor efficacy of antibody, we asked if this synergistic increase is FcγR-mediated. First we tested the effect of E6020 on the FcγR expression and activation on FcγR-bearing cells, i.e., macrophages, dendritic cells and NK cells. As shown in Fig. 4, *in vivo* therapy with E6020 induced up-regulation of FcγRs and the CD80 costimulatory molecule of macrophages and dendritic cells but not NK cells, indicating activation and maturation of macrophages and dendritic cells by E6020 stimulation.

To further explore which FcγR-bearing cells were responsible for the antitumor effect of combination therapy, we performed cell depletion experiments. Four groups of mice were given subcutaneous injections of 10^3 D5HER2 cells and 200 μl clodronate-containing liposomes or PBS-containing liposomes the day before tumor cell injections, and thereafter every 5 days during therapy with trastuzumab and E6020. As shown in Fig. 5a, treatment with trastuzumab and E6020 in the presence of macrophage-depleting liposomal clodronate markedly decreased the efficacy of trastuzumab plus E6020, suggesting that macrophages are involved in the antitumor effect of combination treatment. An *in vitro* antibody-dependent cellular phagocytosis (ADCP) experiment (Figure 5b) confirmed this finding. Peritoneal macrophages from hmHER2 Tg mice incubated in E6020 exhibited significantly increased FcγR-mediated phagocytosis. In contrast, depletion of NK cells with anti-Asialo GM1 antibody did not attenuate the antitumor effects of combination therapy (Fig. 6a). This finding was consistent with ADCC assay results (Figure 6b), which showed that E6020 only modestly enhanced ADCC.

Protective adaptive antitumor immunity induced by trastuzumab-based treatment can be enhanced by E6020 therapy in HER2 tolerant immunocompetent mice

To further determine whether trastuzumab-based treatment can induce an adaptive immune response, and whether such immunity is sufficient to evoke long-term memory and antitumor protection, we evaluated cured mice for long-term protection against tumor re-challenge. HmHER2 Tg mice surviving tumor-free for more than 120 days after the initial challenge with D5-HER2 cells were re-challenged subcutaneously with 5000 D5-HER2 or D5 tumor cells. All re-challenged mice had received no therapy for a minimum of 90 days, and circulating trastuzumab could not be detected by ELISA at the time of re-challenge (not shown). The mice were monitored for the development of palpable tumors. As shown in Fig. 7a, 16 of 36 (44%) mice treated initially with trastuzumab survived D5-HER2 tumor cell re-challenge without evidence of disease for at least 80 days. In addition, among the tumor-free mice initially treated with trastuzumab plus E6020, 22/30 (73%) survived tumor re-challenge for more than 80 days ($P = 0.005$ versus trastuzumab monotherapy) while none of the eight mice challenged with D5 cells were able to reject D5 re-challenge. Very low levels of murine anti-human HER2 antibodies were detected in mice before and after re-challenge (data not shown), suggesting that the antitumor protection was primarily attributable to T cells. These data strongly supports the idea that anti-HER2 antibody-mediated tumor regression generates long-term immune memory immunity against the human HER2 tumor autoantigen in immunologically tolerant hmHER2 transgenic mice capable of protecting the host from re-challenge. And this antibody induced adaptive immunity can be amplified by E6020 therapy.

To further determine if the protection was T-cell mediated and explore the relative importance of various T cell subsets in the antitumor response, systemic depletion of CD4⁺ and CD8⁺ T cells by antibodies was performed in tumor-free hmHER2 Tg mice prior to and following the tumor re-challenge. T cell depletion was maintained by twice-weekly i.p. injections of anti-CD4 antibody (GK1.5) or anti-CD8 antibody (2.43) and was verified by FACS analysis of splenic T cells isolated from members of each group (not shown). Fig. 7b, 7c demonstrate that depletion of CD4⁺ T cells or CD8⁺ T cells greatly reduced immune protection for both treatments. Depletion of both CD4⁺ and CD8⁺ T cell populations abolished the protection, and all mice re-challenged with D5-HER2 cells developed tumors. Therefore, both CD4⁺ and CD8⁺ T cells were required for D5-HER2 tumor rejection upon tumor re-challenge.

To analyze HER2 specific T cell responses, splenocytes from re-challenged mice from both the trastuzumab-treated group and the combination therapy-treated group were isolated two weeks after tumor re-challenge. IFN- γ producing T cells were detected by intracellular IFN- γ cytokine analysis. As shown in Figure 7d, a significant increase in HER2 ECD or ICD-specific CD4⁺ T cell response was observed in mice treated with combination therapy than non-treated Tg mice. These results support recent findings that anti-HER2 antibody treatment engages the adaptive immune response [12], and further demonstrate that this long-term protective immunity can be amplified by prior therapy with the TLR4 agonist E6020.

Discussion

Antibody therapy has emerged as an important modality in cancer therapy, but there is still a considerable opportunity to enhance the efficacy of such therapy. Importantly, antibodies against cancer-relevant targets can be viewed as highly specific vehicles to induce tumor-specific immune responses [27] via activation of Fc γ R mediated function and engagement of dendritic cells, macrophages, NK and other innate immune cells. Enhancing the efficacy of antibody therapy by stimulating host-protective cancer antigen-directed immune responses thus offers one promising avenue for accomplishing this goal [6, 28]. Here we demonstrate for the first time that administration of a curative trastuzumab regimen leads to the induction of host-protective immunity that allows mice to reject human HER2-expressing syngeneic tumor re-challenge in immunocompetent, human HER2-tolerant mice. Co-treatment with the TLR4 agonist E6020 amplifies trastuzumab-based protection against tumor re-challenge. Greatly reduced immune protection was observed with CD4⁺ or CD8⁺ T cell depletion (Fig. 7b, 7c). Therefore, T cells comprise the primary cell population mediating D5-HER2 tumor rejection against re-challenge in this experimental system. Additional characterization of this response is under way.

Several previous reports have shown that antibody therapy can induce/enhance tumor-specific cellular immunity when used alone or in combination with chemotherapy [9] or a tumor vaccine [29]. Such data provide evidence that therapeutic antibodies not only function as passive immunotherapy but can also promote tumor-specific active immunity. This results in a long-term protection through augmentation of immunologic memory, and a vaccinal effect [30].

The data presented here confirm and extend the importance of immunomodulation, such as TLR4 activation, as a strategy to improve antibody therapy by demonstrating both immediate effects that are seen in immunodeficient SCID mice, and prolonged immune protection in an immunocompetent, human HER2-tolerant mouse strain. The therapeutic interaction between trastuzumab and E6020 builds upon a prior report by Bevat et al [31], who reported that co-administration of an antitumor antibody and the TLR4 agonist,

monophosphoryl lipid A potentially boosted antitumor effects, with a reduction of pulmonary metastases in a murine melanoma model. The therapeutic benefit of the combination therapy was found to be dependent on the presence of FcγRI, suggesting that the observed phenomenon is due to enhanced ADCC mediated by the innate immune system. However, this report did not examine the effects of combined therapy on the induction of host-protective adaptive immunity. Studies with CpG ODN, a TLR9 agonist, suggested that the enhanced efficacy of combined CpG ODN therapy with antibody therapy is due to both increased expression of the target antigen and enhanced ADCC [32].

Both TLRs and FcγRs were considered to be important regulators of immune responses. A recent report provided evidence for a direct interaction between the TLR4 and FcγR pathways; TLR4 was required for the activation of FcγR and was involved in FcγR III (CD16) signaling [33]. We show here that stimulation with E6020 up-regulated FcγRs expression and activation of macrophages and dendritic cells, thereby amplifying the FcγR mediated phagocytosis. Amplified phagocytosis may facilitate antigen presentation and T cell activation, and thus the vaccinal effects of antibody therapy.

In addition to macrophages and dendritic cells, other innate immune effector cells such as B cells, neutrophils may mediate antibody-induced immunization. Other possible mechanisms may also contribute to enhanced antitumor effects. For example, it has been shown that E6020 stimulates both human and mouse cells in culture and elicits cytokine secretion in a dose-dependent manner when administered to mice [22,23]. These cytokines, including IL-6, IL-10 and TNF-α, may be involved in tumor lysis or have an effect on the development of both humoral and cellular immune responses. Studies are ongoing to determine the precise mechanisms.

The relative contributions of cell signaling effects and immunologic mechanisms to the *in vivo* effects of trastuzumab remain poorly understood. Future studies will determine the degree to which signaling effects are responsible for the improved antitumor activity of trastuzumab in this model system. Furthermore, this human antibody may have an adjuvant effect by inducing xenogeneic immune responses in mice [34]. This can be addressed by employing trastuzumab's fully murine parent, the anti-human HER2 antibody 4D5, in future studies.

In order to rigorously test the capacity of human HER2 directed immunotherapeutics in preclinical models, we developed a human mutated HER2 transgenic mouse model (hmHER2 Tg) syngeneic to the C57BL/6 background in which a HER2 expressing syngeneic murine tumor can be established in an immune tolerant host. We found that the hmHER2 Tg mice were tolerant to human HER2 and permissive of the outgrowth of tumors expressing human HER2 without inducing an anti-HER2 antibody response or intratumoral leukocyte infiltration. Such tumor growth mimicked that seen in immunodeficient SCID mice. In contrast, most of the immunocompetent, non-transgenic mice remained tumor free and mounted robust anti-HER2 antibody responses (Fig. 2b). The CMV promoter used to express the hmHER2 transgene directs widespread expression in a variety of tissues [35], including liver, lung, kidney and heart. The hmHER2 Tg mice developed normally without detectable pathological consequence in organs. These results are comparable to those found in other human HER2 Tg mouse models. In these models, the transgenic mice expressed human HER2 in the brain and mammary gland, and demonstrated tolerance to human HER2 which was partially overcome by DNA vaccination [21]. MMTV.f.huHER2 Tg mice developed metastatic mammary tumors in female founders. Human HER2 expression was detected in mammary glands and other tissues [20]. In the present study, the expression of human mutated HER2 in a C57BL/6 background resulted in a HER2 tolerant mouse strain without spontaneous tumor development. This hmHER2 Tg mouse model permits the

preclinical study of human HER2 targeted therapy as well as vaccination strategies against HER2, though this syngeneic system does not fully replicate the human setting, due to the known differences between the human and murine immune systems, the use of a non-lineage specific tumor, and the non-physiological distribution of human HER2 in the transgenic mice.

The D5-HER2 syngeneic mouse model system allows us to determine the function of adaptive immunity in the antitumor activity of anti-human HER2 therapies. However, the HER2 expressing D5 melanoma cells (a subclone of B16F10) which is highly aggressive requires early initiation of therapy. No therapy was effective with larger tumor inocula or initiation of therapy at day 9. This limited the clinically relevant therapy against established tumors. Establishing other clinically relevant HER2-expressing cell line is under way.

In summary, we show for the first time that therapy with an anti-human HER2 antibody can protect mice against primary tumor challenges and subsequent tumor re-challenges in an immunocompetent preclinical model characterized by immunological tolerance to human HER2. We further demonstrate that both primary treatment efficacy and subsequent host-protective adaptive immunity can be augmented by concomitant TLR4 agonist therapy. This long-term T cell mediated protection results in a breakage of immune tolerance and enhanced antitumor activity. These observations have potentially important implications for the use of therapeutic anti-tumor monoclonal antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

hmHER2 Tg	human mutated HER2 transgenic mouse
TLR	toll like receptor
ADCC	antibody-dependent cellular cytotoxicity

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Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1. Construction of a human mutated *HER2* transgene and identification of C57BL/6 human mutated *HER2* (hm*HER2*) transgenic mice

a Map of the 7734bp CMV regulated human mutated *HER2* transgene.

b Detection of human *HER2* RNA in different tissues of hm*HER2* transgenic mice. Total RNA was extracted and analyzed for relative levels of *HER2* RNA by RT-PCR. (1. Kidney 2. Live 3. Lung 4. Heart 5. DNA ladder)

c Immunohistochemical analysis of human *HER2* expression by kidney from hm*HER2* transgenic (left panel) and wild-type (right panel) mice. Original magnification: X200.

Fig. 2a

Fig. 2b

Fig. 2c

Fig. 2. Establishment of a murine syngeneic mouse model system

a Kinetics of D5 and D5-HER2 syngeneic melanoma cell growth in mice. C57BL/6 SCID, C57BL/6 and C57BL/6 hmHER2 Tg mice. 10³ or 10⁴ D5 (◆) and D5-HER2 cells (■) were inoculated and tumor growth was monitored.

b Anti-human HER2 antibody induction associated with D5-HER2 tumor growth was measured. Sera from untreated C57BL/6 SCID, C57BL/6 and C57BL/6 hmHER2 Tg tumor bearing mice were collected 21 days after tumor inoculation and used to stain D5-HER2 cells. Bound antibody was detected by flow cytometry, as described in *Materials and Methods*. The results are expressed as the mean fluorescence of individual samples.

c Immunohistochemical detection of human HER2 expression by D5-HER2 tumors and D5 tumors from tumor-bearing C57BL/6 SCID, C57BL/6 wild-type and C57BL/6 hmHER2 Tg mice. Original magnification: X400.

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Fig. 3a

Fig. 3b

Fig. 3. E6020 enhances the efficacy of therapy with trastuzumab in C57BL/6 SCID mice and hmHER2 transgenic mice

Cohorts of 10–12 C57BL/6 SCID mice (Fig. 3a) and hmHER2 mice (Fig. 3b) were challenged with 10^3 D5-HER2 tumor cells s.c. on day 0, and treated starting on day 1 twice weekly by intraperitoneal injections of either PBS, 10 μ g E6020 alone, 200 μ g trastuzumab alone or combined E6020 and trastuzumab. The percentage of tumor-free mice was recorded. The data are presented as Kaplan-Meier survival curves indicating the percentage of tumor-free mice. Similar results were found in three independent experiments. ** $P < 0.001$, * $P < 0.05$ when combined therapy was compared to trastuzumab, E6020 or PBS.

Fig. 4. TLR4 agonist exposure activates FcγR-dependent functions on macrophages and dendritic cells but not NK cells

In vivo therapy with E6020 induces up-regulation of FcγRs and CD80 costimulatory molecules. Cohorts of HmHER2 Tg mice received 10μg E6020, CD11b⁺ peritoneal macrophages, splenic NK1.1⁺ cells or CD11c⁺ dendritic cells were analyzed for murine FcγR expression. Flow cytometry profiles of CD11b⁺, NK1.1⁺ or CD11c⁺ cells gated for FcγR I, FcγR II/III and CD80 expression analysis demonstrate upregulation of FcγRs and the activation of macrophage from E6020 injected hmHER2 Tg mouse. FcγR I but not FcγR II/III is upregulated on CD11c⁺ dendritic cells by E6020 treatment. E6020 shows no effect on FcγR expression in NK cells.

Fig. 5a

Fig. 5b

Fig. 5. Macrophages mediate the antitumor effect of combination therapy with trastuzumab plus E6020

a Depletion of macrophages impairs the enhanced efficacy of combination therapy with trastuzumab plus E6020. Cohorts of 10–12 C57BL/6 hmHER2 mice were challenged with 10^3 D5-HER2 tumor cells s.c. on day 0, and treated starting on day 1 twice weekly by intraperitoneal injections of either PBS, or combination therapy with trastuzumab plus E6020. Macrophages were depleted by i.p. injection of 200 μ l clodronate-containing liposomes the day prior to tumor cell inoculation and thereafter every 5 days. The percentage of tumor-free mice was recorded. * $P < 0.01$ when combination therapy with macrophages depletion was compared to that without macrophage depletion.

b E6020 enhances macrophage phagocytosis *in vitro*. Peritoneal macrophages were isolated from hmHER2 Tg mice and were pre-incubated with 0, 5, 10 μ g/ml E6020 for 20 h then tested in an ADCP assay with 4D5 coated D5-HER2 cells. D5-HER2 cells were labeled with CMFDA-green for tracking purposes, and treated with non-binding control IgG or 4D5 MAb at 0, 10 μ g/ml and mixed with macrophages. Macrophages were stained with APC-conjugated anti-CD11b. Flow cytometry was used to calculate the percentage of tumor cell engulfment by macrophages. The effector: target ratio used was 4:1. Percent phagocytosis

was determined as the number of double-positive cells divided by the total number of CMFDA-positive cells.

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Fig. 6a

Fig. 6b

Fig. 6. NK cells are not responsible for the antitumor effect of combination therapy with trastuzumab plus E6020

a Cohorts of 10–12 C57BL/6 hmHER2 mice were challenged with 10^3 D5-HER2 tumor cells s.c. on day 0, and treated starting on day 1 twice weekly by intraperitoneal injections of either PBS, or combined E6020 and trastuzumab. NK cells were depleted by i.p. injection of anti-Asialo GM1 antibody the day prior to tumor cell inoculation and thereafter every 5 days. The percentage of tumor-free mice was recorded.

b Splenocytes were isolated from hmHER2 Tg mice that received 10μg E6020. The ADCC assay was measured using a standard ^{51}Cr release assay as described in *Materials and Methods*. A representative assay and the mean $\pm$ SD of triplicate samples are shown; each assay was repeated at least twice.

Fig. 7a

Fig. 7b

Fig. 7c

Fig. 7d

Fig. 7. Combination therapy with trastuzumab plus E6020 enhances host-protective HER2-directed adaptive immune responses

a C57BL/6 hmHER2 mice treated with either the combination of trastuzumab and E6020 or trastuzumab alone were protected from D5-HER2 but not D5 re-challenge. C57BL/6 hmHER2 mice were s.c. inoculated with 1×10^3 D5-HER2 tumor cells on day 0. Treatment with trastuzumab plus E6020 or trastuzumab was initiated on day 1 and continued for 30 days, as described in *Materials and Methods*. One hundred and twenty days following the initial challenge, tumor-free mice that previously received combined treatment with 200 μ g trastuzumab and 10 μ g E6020 or treatment with 200 μ g trastuzumab alone were inoculated with 5×10^3 D5-HER2 cells or D5 cells (i.e., a 5-fold higher challenge than was initially administered) in a site opposite that of the first s.c. injection. Tumor recurrence was

monitored by palpation for a minimum of two months. The data are presented as Kaplan-Meier survival curves indicating the percentage of tumor-free mice. $*P = 0.005$ when initial combined therapy was compared to trastuzumab. The data are combined from at least two replicate experiments.

b, c Both $CD4^+$ and $CD8^+$ T cells are required for D5-HER2 tumor rejection upon tumor re-challenge. C57BL/6 hmHER Tg mice that rejected D5-HER2 tumors after initial treatment with trastuzumab plus E6020 (Fig. 7b) or trastuzumab alone (Fig. 7c) were depleted of $CD4^+$, $CD8^+$ T cells or both as described in *Materials and Methods*. Animals were then re-challenged in the opposite flank with D5-HER2 cells (5-fold higher dose than the initial challenge). The mice were monitored for the development of palpable tumors and the percentage of tumor-free mice was recorded. The data from two separate experiments with similar outcomes were combined.

d Antigen specificity was detected by intracellular IFN γ cytokine analysis. Tumor-free Tg mice from both the trastuzumab-treated group and the combination-treated group were re-challenged as described above. Two weeks after tumor re-challenge, mice were sacrificed and splenocytes were isolated. Splenocytes were re-stimulated with HER2 peptide mix (ECD or ICD peptide mix) and assayed for IFN- γ production using an intracellular cytokine staining. The percentage of $CD4^+$ /IFN- γ^+ T cells is shown.