Trastuzumab Triggers Phagocytic Killing of High HER2 Cancer Cells In Vitro and In Vivo by Interaction with Fcγ Receptors on Macrophages

Yun Shi, Xuejun Fan, Hui Deng, Randall J. Brezski, Michael Rycyzyn, Robert E. Jordan, William R. Strohl, Quanming Zou, Ningyan Zhang and Zhiqiang An

*J Immunol* 2015; 194:4379-4386; Prepublished online 20 March 2015;
doi: 10.4049/jimmunol.1402891
http://www.jimmunol.org/content/194/9/4379

References

This article cites 39 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/194/9/4379.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Trastuzumab Triggers Phagocytic Killing of High HER2 Cancer Cells In Vitro and In Vivo by Interaction with Fcγ Receptors on Macrophages

Yun Shi,*† Xuejun Fan,† Hui Deng,* Randall J. Brezski,‡ Michael Rycyzyn,‡ Robert E. Jordan,*§ William R. Stroph,† Quanming Zou,† Ningyan Zhang,* and Zhiqiang An*†

Trastuzumab has been used for the treatment of HER2-overexpressing breast cancer for more than a decade, but the mechanisms of action for the therapy are still being actively investigated. Ab-dependent cell-mediated cytotoxicity mediated by NK cells is well recognized as one of the key mechanisms of action for trastuzumab, but trastuzumab-mediated Ab-dependent cellular phagocytosis (ADCP) has not been established. In this study, we demonstrate that macrophages, by way of phagocytic engulfment, can mediate ADCP and cancer cell killing in the presence of trastuzumab. Increased infiltration of macrophages in the tumor tissue was associated with enhanced efficacy of trastuzumab whereas depletion of macrophages resulted in reduced antitumor efficacy in mouse xenograft tumor models. Among the four mouse FcγRs, FcγRIII exhibits the strongest binding affinity to trastuzumab. Knockdown of FcγRII in mouse macrophages reduced cancer cell killing and ADCP activity triggered by trastuzumab. Consistently, an upregulation of FcγRIII expression by IFN-γ triggered an increased ADCP activity by trastuzumab. In an analogous fashion, IFN-γ priming of human macrophages increased the expression of FcγRIII, the ortholog of murine FcγRIII, and increased trastuzumab-mediated cancer cell killing. Thus, in two independent systems, the results indicated that activation of macrophages in combination with trastuzumab can serve as a therapeutic strategy for treating high HER2 breast cancer by boosting ADCP killing of cancer cells. *The Journal of Immunology, 2015, 194: 4379–4386.

Trastuzumab is a humanized mAb for the treatment of HER2-overexpressing breast cancer (1). After >15 years of successful clinical use, the mechanisms of action for trastuzumab are still being investigated. Among the established modes of action for trastuzumab are inhibition of HER2-mediated cell signaling (2–4) and Ab-dependent cell-mediated cytotoxicity (ADCC) (5). Our group demonstrated recently that engagement of immune cells that express FcγRs with trastuzumab-coated cancer cells mediated HER2 downregulation in the target cells (6).

NK cells play an important role in ADCC, and an increased infiltration of NK cells within breast tumors has been linked to the efficacy of trastuzumab (5). Additionally, macrophages participate in both innate and acquired immunity in cancer. The roles of tumor-associated macrophages (TAMs) are often associated with the promotion of tumor progression and metastasis (7, 8). TAMs still retain Fc-dependent antitumor function despite promoting tumor invasion (8). A study showed that increased TAM infiltration correlated with a positive prognosis in follicular lymphoma patients undergoing rituximab treatment (9). Consistent with this observation is that macrophages are essential for the Ab-dependent depletion of cancer cells mediated by anti-CD20 (10), anti-CD30 (11), and anti-CD40 (12) mAbs in preclinical studies. Macrophages express FcγRs that interact with the Fc portion of IgG Abs (13). Ab-dependent cellular phagocytosis (ADCP) mediated by activated macrophages can kill tumor cells (14). It has been reported that trastuzumab could mediate ADCP against HER2-expressing cancer cells by PBMCs (15) and macrophages (16) in vitro. However, the role of macrophages in response to trastuzumab in antitumor efficacy has not been established in vivo. With this context, a study to clarify the role of macrophages and ADCP in trastuzumab-mediated antitumor efficacy was undertaken.

In this study, we investigated the role of macrophages and ADCP for trastuzumab antitumor efficacy using both in vitro coculturing of macrophages with cancer cells as well as in vivo mouse xenograft tumor models. We demonstrated that FcγRIV expressed on mouse macrophages interact with the Fc domains of cell-bound trastuzumab and thereby induced ADCP-mediated cancer cell killing. The results from this study not only validated ADCP as a new mechanism of action for trastuzumab, but also suggested that the activation of macrophages can improve the anticancer efficacy of trastuzumab and other Ab immune therapies by boosting ADCP.
Materials and Methods

Cell lines and reagents

Cell lines BT474 (human breast cancer cell line), SKOV-3 (human ovarian carcinoma cells), RAW264.7 (mouse macrophage cell line), THP-1 (human monocyte cell line), and HEK 293T cells (a human embryonic kidney 293T cell line) were obtained from American Type Culture Collection (Manassas, VA) and cultured in American Tissue Culture Collection-recommended conditions. The L929 cell line (murine anepithelial fibrosarcoma cell line) was from European Collection of Cell Cultures (Sigma-Aldrich, St. Louis, MO). IFN-γ and IL-4 were from ProSpec TechnoGene (Rehovot, Israel). LPS was from Sigma-Aldrich. Cell culture media RPMI 1640 was from Thermo Fisher Scientific (Pittsburgh, PA). FCS was from Invitrogen (Carlsbad, CA).

Generation of mouse bone marrow-derived macrophages and stimulation of different types of macrophages in vitro

Bone marrow–derived macrophages (BMMs) were differentiated from bone marrow macrophages as reported (17). Briefly, the isolated bone marrow cells were cultured in complete RPMI 1640 medium supplemented with 10% FCS and 30% pretested conditioned medium from the L929 cell line as a source of M-CSF for 5 d (17). BMMs were >95% CD11b+ as measured by flow cytometry. The BMMs were further polarized to M1 by IFN-γ (50 ng/ml) and LPS (10 ng/ml), and to type 2 macrophage (M2) by IL-4 (20 ng/ml). Conditioned medium (cm) from 3-d BT474 cancer cell culture was used to polarize BMMs to M1.

Cancer cell killing assay

Cancer cell killing was monitored continuously and noninvasively using the xCELLigence instrument (Roche, Mannheim, Germany) as described previously (18). Briefly, high HER2-expressing SKOV-3 cancer cells were seeded in E-plate 96 (Acea Biosciences, San Diego, CA), followed by the addition of macrophages (BMMs or RAW264.7 cells) as immune effector cells in the presence of trastuzumab or isotype IgG control (5 μg/ml). Cells along with the effector were used as baseline index control. Three wells were used for each treatment group in a 96-well plate assay, and average of the three wells was used as cell growth index of the treatment. Cell growth (measured as cell index) was monitored continuously for 2 d. The E:T ratio was 5:1 or 2:1. The cell index was normalized when the effector cells were added. The normalized cell index recorded after 24 or 48 h of trastuzumab treatment was used to calculate the percentage of cancer cell lysis using the formula: [(cell index of the macrophage plus trastuzumab group – cell index of the macrophage minus trastuzumab group)/cell index of the macrophage group] × 100. The results were expressed as mean ± SD. Experiments were repeated three times and each treatment contained three replicates.

ADCP assay using flow cytometry and confocal fluorescence microscopy

For the two-color flow cytometry ADCP assay, high HER2-expressing BT474 breast cancer cells were labeled with cell proliferation dye eFlour 670 (eBioscience, San Diego, CA) according to the manufacturer’s instructions. The labeled cells were then incubated with macrophages at a 1:1 ratio with 5 μg/ml trastuzumab or isotype IgG control in RPMI 1640/10% FBS. At the end of 1 h of incubation at 37°C, cells were stained with FITC–anti-CD11b and PE–anti-CD163 (BioLegend, San Diego, CA) for 4°C and analyzed by flow cytometry on a guava easyCyte HT instrument (Millipore, Damers, MA). Cells were gated for CD11b+ cells (macrophages), and the CD11b+ cells were then grouped as single-positive macrophages alone (eFlour 670+, CD11b+) and two-color stained phagocytosed macrophages (eFlour 670+, CD11b+). The percentage of phagocytosis was calculated as the population of phagocytized macrophages among the total macrophages. All tests were performed in triplicate and the results were expressed as mean ± SD. For the ADCP assay by fluorescence microscopy, macrophages were cultured on eight-well chamber slides overnight and eFlour 670–labeled BT474 cells were added to the chamber slides in the presence of trastuzumab (5 μg/ml) or isotype IgG. After 1 h of incubation at 37°C, the media were removed and the slides were washed with 1× PBS. The slides were then stained with FITC–anti-CD11b for 30 min at 4°C and examined under a Carl Zeiss fluorescence microscope (Carl Zeiss, Thornwood, NY). The phagocytosed cancer cells in red were localized in the green–stained macrophages.

Mouse xenograft tumor model and macrophage depletion in vivo

Mouse xenograft studies were carried out in accordance with animal care and use guidelines, and the protocol was approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston. Athymic nu/nu mice (Charles River Laboratories, Wilmington, MA) were used for experiment at age of 7–8 wk. BT474 cells (5 × 10⁶) were implanted s.c. Trastuzumab and isotype IgG were administered once weekly at 5 mg/kg i.p. when tumor size reached 100 mm³. For the macrophage depletion assay, macrophages were depleted by i.p. administration of 200 μl clodrosome (5 mg/ml; Encapsula NanoSciences, Brentwood, TN) weekly as reported (19). Tumor size was measured twice weekly using a Vernier scale caliper. For antitumor efficacy study, mice were treated with trastuzumab or control isotype IgG for 4 wk to observe tumor growth or regress. Tumor biopsy was examined by tissue immunofluorescence for ex vivo isolation of tumor-infiltrating immune cells, tumors were harvested 3 d after the second Ab injection. Tumor tissue was digested using a tumor dissociation kit (Miltenyi Biotec, Auburn, CA) and homogenates to single-cell suspensions using the gentleMACS dissociator (Miltenyi Biotec) according to the manufacturer’s protocol.

Measurement of FcγR expression and FcγRs binding assays

Macrophages were stained with rabbit anti-mouse FcγRIV (Creative Biomart, Shirley, NY) and followed by Alexa Fluor 488–anti-rabbit IgG (Life Technologies). After staining, the cells were detected using the guava easyCyte HT instrument. Binding constant (Kd) of trastuzumab to human FcγRIIIn in comparison with murine FcγRIV was determined using the surface plasmon resonance method with a Biacore T-100 instrument as we described previously (20). Binding of mouse FcγRs with trastuzumab was also determined by ELISA adapted from a reported method (18), and FcγR proteins were measured by flow cytometry. Briefly, FcγR proteins (2 μg/ml) were coated on a MaxiSorp plate (eBioscience), and binding of trastuzumab was detected using an F(ab′)2 fragment of goat anti-human F(ab′)2 Ab conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) using the 4-methylumbelliferyl phosphate substrate (Sigma-Aldrich). Fluorescence intensity was read using a plate reader ( Molecular Devices, Sunnyvale, CA). FcγRIV expression in ex vivo xenograft tumor tissues was examined by tissue immunofluorescence staining. Briefly, xenograft tumor tissues were excised fresh and snap-frozen in OCT solution (Sigma-Aldrich) and frozen slides were made for immunofluorescence staining. The tumor tissue was stained with rabbit anti-mouse FcγRIV Ab and followed by Alexa Fluor 488–anti-rabbit IgG. The expression of FcγRIV was examined using a Carl Zeiss fluorescence microscope.

Knockdown of FcγRIV in RAW264.7 cells

The RNAi Consortium lentiviral mouse FcγRIV short hairpin RNA (shRNA) was ordered from Thermo Scientific (Fremont, CA). To package the lentivirus, HEK 293T cells were cotransfected by FcγRIV shRNA plasmid psPAX2 and PMD2.G envelope plasmid using 293fectinfection reagent (Invitrogen) according to the manufacturer’s instructions. At 48 h, the virus was collected to infect the RAW264.7 cells in the presence of Polybrene (4 μg/ml; Sigma-Aldrich). Three days after infection, the cells were selected by puromycin (Sigma-Aldrich). The selected cells were amplified in the presence of puromycin, and FcγRIV expression knockdown was confirmed by flow cytometry.

Statistical analysis

Where appropriate, an unpaired t test between two treatment groups was performed using the GraphPad Prism (version 5) software. A p value < 0.05 between treatment groups was considered significantly different.

Results

Macrophages induced trastuzumab-dependent cancer cell killing

We tested whether trastuzumab can mediate cancer cell killing using BMMs or a murine macrophage cell line (RAW264.7 cells) as effector cells and the high HER2 expression SKOV-3 cells as target cells. Cancer cell lysis mediated by macrophages in the presence and absence of trastuzumab was monitored in real-time and noninvasively using the xCELLigence instrument. The ratio of macrophages (effector cells) and cancer cells (target cells) was 2:1 or 5:1. The cell index was normalized at the point of trastuzumab binding to human FcγRIV.
and macrophage addition (horizontal open arrows in Fig. 1A). As shown in Fig. 1A, the normalized cell index in the presence of trastuzumab was markedly reduced compared with the cells treated with an isotype control IgG shortly after the addition of BMMs; however, the cell growth index with trastuzumab in the absence of BMMs was similar to that of cancer cells alone. In comparison with the cell indices in the isotype control Ab group, the killing of cancer cells by BMMs in the presence of trastuzumab reached a maximum of 40% at the E:T ratio of 2:1, and >60% when the E:T ratio was increased to 5:1 after 24 h of coculture of cancer cells and macrophages (Fig. 1B). The same trend was also observed using the mouse macrophage RAW264.7 cells (Fig. 1C). In comparison with the cell indexes in the isotype control Ab group, the killing of cancer cells by RAW264.7 cells in the presence of trastuzumab was ~20% at the E:T ratio of 2:1, and >30% when the E:T ratio was increased to 5:1 (Fig. 1D).

**Trastuzumab-opsonized high HER2-expressing cancer cells underwent phagocytosis by macrophages**

To determine whether ADCP played key role in cancer cell killing by trastuzumab in the presence of macrophages, we investigated macrophage-mediated phagocytosis in the presence of trastuzumab using both flow cytometry and confocal fluorescence imaging methods. Fluorescent dye eFluor 670–labeled high HER2–expressing BT474 breast cancer cells were used as target cells and incubated with BMMs or RAW264.7 cells as effector cells at an E:T ratio of 1:1 in the presence of trastuzumab or an isotype IgG control. Before detection of phagocytosis of the eFluor 670–labeled BT474 cancer cells, macrophages were stained with CD11b–FITC at 4˚C and cells with dual colors (red for cancer cells and green for macrophages) were detected by flow cytometry or confocal fluorescence imaging. To measure the population of macrophages with cancer cell phagocytosis, we first gated CD11b+ cells and then analyzed the percentage of macrophages with double staining. As shown in Fig. 1A, a significant increase of the CD11b+/eFlour 670+ cell population was observed in the trastuzumab-treated groups when compared with the isotype IgG control group, suggesting that BT474 cancer cells were phagocytized by BMMs or RAW264.7 cells. To confirm that the double-positive cells detected by flow cytometry were a true representation of phagocytized cancer cells in macrophages, we imaged the phagocytized cells using a confocal fluorescence microscope. In the presence of trastuzumab, the red BT474 cells were clearly visible within the green-labeled macrophage cells (Fig. 1B), whereas little phagocytosis of cancer cells by macrophages was observed in the isotype IgG control group. To investigate whether a different activation status of macrophages impacts the phagocytic killing of Ab opsonized cancer cells, we derived macrophages into type 1 (M1) and type 2 (M2) as well as those derived with cancer cell–conditioned medium (Mcm). The M1 macrophages exhibited the most potent phagocytosis of high HER2 cancer cells in the presence of trastuzumab among the tested groups, and both BMMs and M1 macrophages showed significantly more phagocytosis in the presence of trastuzumab than did that of the isotype Ab control (Fig. 1C). However, M2 and Mcm macrophages had no significant increase of ADCP activities in the presence of trastuzumab in comparison with the presence of isotype Ab control (Fig. 1C), suggesting that activation status of macrophages has an impact on ADCP activity.

**The antitumor efficacy of trastuzumab is correlated with the infiltration of macrophages within tumor sites in a mouse xenograft tumor model**

To study the role of macrophages in regard to trastuzumab efficacy in vivo, tumor-infiltrated macrophages were determined following administration of Ab at 5 mg/kg for 2 weekly dosings. The per-

---

**FIGURE 1.** Macrophage-induced killing of high HER2–expressing cancer cells in the presence of trastuzumab. SKOV-3 cancer cells were seeded in E-Plate 96, followed by the addition of BMMs (A and B) or RAW264.7 cells (C and D) as immune effector cells (pointed by the horizontal open arrows) in the presence of trastuzumab or isotype IgG control. Cancer cells plus isotype IgG was used as controls. The cell indexes were monitored in real-time for 80 h to map the cell growth curve. The cell index at 24 h after trastuzumab treatment (indicated by the vertical solid arrows in the kinetic graphs) was used to calculate the percentage of cell lysis as shown in (B) and (D). The percentage of macrophage-mediated cancer cell lysis in response to trastuzumab was calculated using the formula: [(cell index with isotype control — cell index of trastuzumab treatment)/cell index of isotype control] × 100. The ratio of macrophage to cancer cells in the coculture was at 2:1 or 5:1 as indicated in the graphs. The experiments were repeated three times. The error bars in (B) and (D) indicate the SD among three independent experiments.
The percentage of infiltrated macrophages (CD11b+) was found to be significantly higher in tumor tissues in mice treated with trastuzumab (20.88%) compared with the tumors in control IgG-treated mice (11.47%) (Fig. 3A, bar graph). To study whether the increased infiltration of macrophages in the trastuzumab-treated xenograft tumors was a reflection of a general immune amplification in the treated mice, we measured peripheral immune cell populations isolated from spleen tissues of the treated mice. CD11b+ cells in the spleens from mice treated with trastuzumab (13.69%) were significantly higher than in those treated with the control isotype IgG (5.24%) (Fig. 3A). As expected, trastuzumab exhibited strong inhibition of BT474 breast cancer xenograft tumor growth (Fig. 3B). To further investigate the potential role of macrophages for the antitumor efficacy of trastuzumab, we depleted macrophages in vivo using clodrosome nanoparticles. Tumor-bearing mice were administered clodrosome by i.p. injection 1 d before trastuzumab treatment, and the regimen was repeated weekly for 4 wk. Macrophages in the spleens and tumor sites were isolated from fresh tissues obtained 24 h after the last Ab treatments and analyzed by flow cytometry. Macrophages (CD11b+) were minimally detectable in the spleen and tumor tissues in mice treated with clodrosome in either the trastuzumab or isotype IgG treatment groups (Fig. 3A). Strikingly, the inhibition of tumor growth by trastuzumab was significantly impaired in mice treated with clodrosome in comparison with that without the depletion of macrophages (Fig. 3B). Clodrosome depletion of macrophages did not impact the tumor growth in the isotype control IgG treatment group (Fig. 3B), indicating that clodrosome depletion alone had no effect on tumor growth. As expected, trastuzumab showed a partial inhibitory effect on tumor growth in the mouse group treated with clodrosome, when compared with the mice treated with clodrosome and isotype IgG. These results suggest that in addition to macrophages, other mechanisms of action were also involved in the tumor inhibition efficacy of trastuzumab. Collectively, these results suggest that trastuzumab treatment stimulated the overall immune response in mice as reflected by the increased macrophage population in the spleen. More importantly, infiltration of macrophages in tumor sites in mouse xenograft tumors was strongly suggested to be important for the efficacy of trastuzumab. To support this finding, splenocytes were collected from the four treatment groups and an ex vivo assay of cancer cell killing was conducted in the presence of trastuzumab. The results showed that splenocytes from trastuzumab-treated mice exhibited stronger cancer cell killing activity in the presence of trastuzumab than did that of the splenocytes from isotype IgG-treated mice (Fig. 3C). Splenocytes from the clodrosome-treated group exhibited significantly lower cancer cell killing activity in the presence of trastuzumab, suggesting the important role of macrophages in antitumor efficacy of trastuzumab in vivo.
The FcγRIV plays a key role in macrophage-mediated ADCP and killing of Ab-opsonized cancer cells

Multiple FcγRs (FcγRI, II, III, and IV) are expressed on murine macrophages (21). With the exception of FcγRII, three of the four FcγRs are activating receptors for immune effector functions such as ADCP upon engagement of macrophage with the Ab Fc. To determine which FcγRs played a dominant role in macrophage-mediated ADCP and killing of Ab-opsonized cancer cells, we first measured the in vitro binding affinities of trastuzumab to the four mouse FcγRs by ELISA. As shown in Fig. 4A, trastuzumab showed concentration-dependent binding to all four mouse FcγRs, and trastuzumab binding to FcγRII was the highest among the four murine FcγRs with an EC₅₀ at 9.8 μg/ml. FcγRII expression was then assessed in tumor-infiltrated immune cells by immunofluorescence staining. As shown in Fig. 4B, trastuzumab-treated tumor tissues showed higher FcγRII expression than that in IgG isotype-treated tumor tissues (Fig. 4B), indicating that more FcγRII-expressing immune cells are recruited to the tumor sites. Flow cytometry analysis showed that M1 macrophages expressed the highest level of FcγRII (mean fluorescence intensity [MFI] = 323), which was approximately twice the level of FcγRI expression on BMMs (MFI = 165), M2 macrophages (MFI = 144), and Mcm macrophages (MFI = 192) (Fig. 4C). The high level of FcγRII expression on M1 macrophages was consistent with the finding that M1 macrophages exhibited more potent killing of high HER2 cancer cells in the presence of trastuzumab than did BMMs, M2 macrophages, and Mcm macrophages (Fig. 2C).

To determine the function of FcγRII expression on macrophages in trastuzumab-mediated ADCP activity, FcγRII expression on the murine macrophage RAW264.7 cells was either knocked down (KD) by the shRNA lentivector system or upregulated by the addition of IFN-γ. As determined by flow cytometry, FcγRII expression in the FcγRII KD RAW264.7 cells was reduced to minimum levels (MFI = 4) in comparison with that in the wild-type (WT) cells (MFI = 25), whereas FcγRII expression in response to IFN-γ stimulation was significantly upregulated (MFI = 75) (Fig. 5A). Cancer cell killing by FcγRII KD RAW264.7 cells with was significantly reduced (16%) when compared with that by the WT RAW 264.7 cells (24%) (Fig. 5B). In contrast, increased FcγRII expression resulting from IFN-γ stimulation showed stronger cancer cell killing (43%) in the presence of trastuzumab (Fig. 5B). We also compared the ADCP activity between FcγRII KD, IFN-γ–stimulated, and WT RAW 264.7 cells. The results showed that FcγRII KD cells had reduced ADCP activity in comparison with WT RAW 264.7 cells, whereas increased FcγRII expression by IFN-γ stimulation resulted in stronger ADCP mediated by trastuzumab (Fig. 5C). Similar to the results from the RAW264.7 cells, BMMs showed increased FcγRII expression by flow analysis when treated with IFN-γ (Fig. 5D), and IFN-γ-stimulated BMMs had stronger killing of high HER2 cancer cells and ADCP activity than that of the control BMMs in the presence of trastuzumab (Fig. 5E, 5F). Collectively, these results suggest that FcγRII expression level plays a key role in macrophage-mediated, trastuzumab-dependent killing of cancer cells.

Effect of human FcγRIII, the mouse FcγRIV ortholog, on the phagocytic activity of human macrophages

Human FcγRIII (CD16) shares 60% similarity in amino acid sequence with mouse FcγRIV/CD16-2 (22, 23). To investigate...
whether FcγRIII expressed on human macrophages have a similar effect on trastuzumab-mediated antitumor efficacy, we compared binding affinity ($K_d$) of trastuzumab to human FcγRIII and murine FcγRIV by the surface plasmon resonance method. The $K_d$ to the murine FcγRIV (1.6 $\times 10^{-8}$ M) was 15-fold higher than that to the human FcγRIII (2.7 $\times 10^{-7}$ M). To determine the functional similarity between human FcγRIII and murine FcγRIV, we treated human monocyte cells (THP-1 cells) with human IFN-γ and determined FcγRIII expression by flow cytometry. Similar to the increased expression of FcγRIV in response to IFN-γ stimulation of mouse macrophages, FcγRIII expression on human THP-1 monocyte cells was also increased after treatment with IFN-γ (MFI = 307) when compared with the nonstimulated control THP-1 cells (MFI = 191) (Fig. 6A). ADCP activity of THP-1 monocyte cells in the presence of trastuzumab also increased when cells were stimulated with IFN-γ (Fig. 6B), suggesting that the increased expression of FcγRIII on human macrophages has a similar effect on trastuzumab-mediated antitumor efficacy.

**FIGURE 4.** FcγRIV expression is associated with trastuzumab anticancer efficacy. (A) FcγRIV shows stronger binding affinity to trastuzumab in comparison with other mouse FcγRs. The x-axis is at log scale and data points are average of three replicates by ELISA. Error bars indicate the SDs. (B) Higher FcγRIV expression was detected in tumor-infiltrated immune cells treated with trastuzumab than that of the isotype control by immunofluorescence. OCT frozen tumor tissues were stained with a rabbit anti-FcγRIV Ab and detected with an Alexa Fluor 488-anti-rabbit IgG Ab. A representative image is shown. Original magnification $\times 40$. (C) FcγRIV expression on different types of macrophages by flow cytometry using the same Ab set as in (B). MFI is shown in the histogram.
increased FcγRIII expression in human macrophage cells can promote the trastuzumab-triggered phagocytosis of cancer cells.

Discussion

Immunotherapeutic Abs targeting surface Ags on tumor cells can simultaneously recruit immune effector cells to specifically destroy the malignant cells, an outcome that is mediated by interactions of the IgG Fc region with FcRs on immune cells. Studies have shown that higher affinity interactions between Ab and FcγRs promote more robust ADCC and ADCP activities and are associated with better therapeutic efficacy of the anti-CD20 Ab rituximab (24, 25). Although it had been established that ADCC, mediated by NK cells, contributes to the efficacy of trastuzumab (26–28), a contribution of macrophage-mediated ADCP to anti-tumor efficacy of trastuzumab in vivo has not been reported. In this study, we demonstrated that activated macrophages readily phagocytosed trastuzumab-coated cancer cells (ADCP) and mediated Ab-dependent cancer cell killing in coculture conditions in vitro, and the results are consistent with the study reported previously (16). More importantly, our results demonstrated that monocytes/macrophages contributed to the efficacy of trastuzumab against HER2-expressing tumors by ADCP in vivo. In the high HER2-expressing BT474 mouse xenograft tumor model, the infiltration of macrophages into the tumor was increased in response to the trastuzumab treatment, suggesting that the trastuzumab Ab can recruit and engage macrophages at the tumor site. We also showed that a depletion of macrophages by clodrosome stimulation was analyzed by flow cytometry. MFI is shown in the histogram.

FIGURE 6. Upregulation of human FcγRIII expression by IFN-γ stimulation in human monocyes increases ADCP activity in the presence of trastuzumab. (A) FcγRIII expression in THP-1 cells with or without IFN-γ stimulation was analyzed by flow cytometry. MFI is shown in the histogram. (B) Phagocytosis activity by THP-1 cells treated with or without IFN-γ in the presence of trastuzumab or isotype IgG. The results are shown as mean ± SD. n = 3. *p < 0.05.

Studies have demonstrated that different IgG subclasses selectively interact with certain activating FcγRs on immune cells. Studies have demonstrated that different FcγRs: FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIV (CD16-2). FcγRIV is a new member of the family and maps in the 75-kb genomic interval between FcγRII and FcγRIII; its expression is restricted to myeloid lineage cells (34). Studies have demonstrated that different IgG subclasses selectively interact with certain activating FcγRs in vitro and in vivo (35). FcγRIV binds to mouse IgG2a and IgG2b with intermediate affinity, but it has no detectable binding to mouse IgG1 or IgG3 (22). It has been shown that FcγRIV is important in mediating the function of antitumor Ab (IgG2a) in the model of lung and liver metastases using murine melanoma cells, and in an anti-CD20 Ab-dependent B cell depletion model (24, 36, 37). Interestingly, we found that human IgG1 exhibited the strongest binding to murine FcγRIV in comparison with the binding affinity to the other murine FcγRs, including the generally considered high-affinity FcγRI. This finding has significant implications for our understanding of immune cell engagement by therapeutic Abs of the human IgG1 isotype in mouse xenograft tumor models, because human or humanized Abs in preclinical studies are often evaluated in mouse xenograft tumor models where mouse FcγRs interact with the human IgG1 such as trastuzumab. We showed that FcγRIV-expressing immune cells in tumor tissue were increased in response to trastuzumab treatment in the high HER2 breast cancer xenograft tumor model and demonstrated that FcγRIV expression on the macrophage is associated with ADCP and cancer cell killing. Knockdown of FcγRIV in macrophages significantly reduced trastuzumab-mediated ADCP and cancer cell killing, whereas increased FcγRIV expression on macrophages by IFN-γ stimulation enhanced ADCP and killing of cancer cells in the presence of trastuzumab. Taken together, these results demonstrated that FcγRIV played an important role in immune cell engagement by human IgG1 Abs such as trastuzumab.

Human immune cells have a corresponding family of FcγRs, including FcγRI (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), and FcγRIII (CD16A/B) (35). On the basis of protein sequence and function, human FcγRIII is often considered the ortholog of mouse FcγRI (CD64). Similar to the function of mouse FcγRIV, we showed that FcγRIII expression on human monocyes and macrophages was also increased in response to IFN-γ stimulation, and also resulted in increased ADCP activity and cancer cell killing triggered by trastuzumab. It is well established that immune effector functions depend on the balance of both activating and inhibiting FcγRs. These results indicate that FcγRIII may play an important role in human macrophage-mediated ADCP activity. Clinical studies have implicated the important role of FcγRIII in trastuzumab efficacy (38), even though function of FcγRIII on NK cells is considered mainly to contribute to cancer cell killing through ADC (39). Future studies are warranted to establish the roles of ADCP mediated by macrophages in trastuzumab anticancer efficacy in the clinic. Taken together, this study demonstrated that trastuzumab can trigger ADCP and that increased expression of mouse FcγRIV/human FcγRIII promotes the ADCP activity of macrophages triggered by the Ab. Our results suggest that modulation of macrophages by stimulation of the activating FcγRIII levels can serve as a strategy for enhancing...
Ab-mediated ADCP and ADCC for improved efficacy of anti-cancer Ab immunotherapies.

Acknowledgments
We thank Dr. Qingchun Tong for providing mouse tissue for bone marrow cell isolation. We also thank Dr. Amy Lauren Hazen for expert assistance in flow analysis.

Disclosures
R.J.B., M.R., R.E.J., and W.R.S. were employees of Janssen Research & Development, LLC at the time the studies were undertaken. The remaining authors have no financial conflicts of interest.

References