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Cancer Therapy: Preclinical

Clinical Cancer Research

Reciprocal Regulation of Activating and Inhibitory Fcγ Receptors by TLR7/8 Activation: Implications for Tumor Immunotherapy

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Abstract

Purpose: Activation of Toll-like receptors (TLR) 7 and 8 by engineered agonists has been shown to aid in combating viruses and tumors. Here, we wished to test the effect of TLR7/8 activation on monocyte Fcγ receptor (FcγR) function, as they are critical mediators of antibody therapy.

Experimental Design: The effect of the TLR7/8 agonist R-848 on cytokine production and antibody-dependent cellular cytotoxicity by human peripheral blood monocytes was tested. Affymetrix microarrays were done to examine genomewide transcriptional responses of monocytes to R-848 and Western blots were done to measure protein levels of FcγR. Murine bone marrow–derived macrophages from WT and knockout mice were examined to determine the downstream pathway involved with regulating FcγR expression. The efficacy of R-848 as an adjuvant for antibody therapy was tested using a CT26-HER2/neu solid tumor model.

Results: Overnight incubation with R-848 increased FcγR-mediated cytokine production and antibody-dependent cellular cytotoxicity in human peripheral blood monocytes. Expression of FcγRI, FcγRIIa, and the common γ-subunit was increased. Surprisingly, expression of the inhibitory FcγRIIb was almost completely abolished. In bone marrow–derived macrophage, this required TLR7 and MyD88, as R-848 did not increase expression of the γ-subunit in $TLR7^{-/-}$ nor $MyD88^{-/-}$ cells. In a mouse solid tumor model, R-848 treatment superadditively enhanced the effects of antitumor antibody.

Conclusions: These results show an as-yet-undiscovered regulatory and functional link between the TLR7/8 and FcγR pathways. This suggests that TLR7/8 agonists may be especially beneficial during antibody therapy. *Clin Cancer Res; 16(7); 2065–75. ©2010 AACR.*

Monocyte Fcγ receptors (FcγR) mediate clearance of IgG-immune complexes and IgG-coated tumor targets. Binding of IgG complexes to FcγR results in receptor clustering, which activates downstream events such as phagocytosis (1), release of reactive oxygen species (2), and cytokine production (3).

The strength of FcγR response is largely determined by the ratio of activating (FcγRI, FcγRIIa, FcγRIII, and the γ-subunit) to inhibitory (FcγRIIb) receptors, as mice genetically deleted for FcγRIIb show markedly enhanced antibody-mediated tumor clearance *in vivo* (4). Conversely, mice lacking the common γ-subunit show very poor antibody-dependent cytotoxicity as mice do not express the γ-subunit–independent FcγRIIa (5). It has also been shown that Toll-like receptor (TLR) activation can enhance FcγR expression and function. For example, the TLR4 ligand lipopolysaccharide has been shown to increase FcγR-mediated phagocytosis (6) and tumor cell lysis (7). Unmethylated DNA (CpG oligonucleotides), which activates TLR9, has also proven effective, enhancing antibody-dependent cellular cytotoxicity (ADCC) against tumors (8).

Agonists of TLR7 and TLR8 have come to light as an effective means of enhancing immune responses. The TLR7 agonist imiquimod has been shown *in vivo* to reduce the growth of MC-26 tumor cells (9), an effect abolished by blocking IFN-α. Both TLR7 and TLR7/8 agonists show antitumor (10) and antiviral (11) activities. Their major mode of action seems to be induction of cytokine production, leading to stronger proinflammatory responses (12).

Here, we have studied the effects of the TLR7/8 agonist R-848 on human monocytes within the context of FcγR expression and function. Results show that R-848 regulates

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Translational Relevance

Antibody therapy against tumors has proven to be a valuable tool in combating cancer but is only partially effective or ineffective for many patients. Because of this, there is a continued attempt to find means of enhancing the efficacy of antibody treatment. Here, we provide both functional and mechanistic evidence that activation of TLR7/8 enhances FcγR expression and activity. Treatment with the TLR7/8 agonist R-848 leads to enhanced destruction of antibody-coated tumor cells by monocytes *in vitro* and to attenuated growth of solid tumors *in vivo*. Hence, TLR7/8 agonists may be an effective adjuvant for antibody therapy.

FcγR transcript and protein, upregulating the activating FcγR and downregulating the inhibitory FcγRIIb. Studies using bone marrow–derived macrophage (BMM) from wild-type (WT) and knockout mice showed that TLR7 and MyD88 are required for the changes in FcγR. Functional assays showed that R-848 treatment synergizes with FcγR function both *in vitro* and in a murine solid tumor model. Hence, TLR7/8 is a novel regulator of FcγR expression and function, suggesting that TLR7/8 agonists may be especially effective as adjuvants for antibody therapy.

Materials and Methods

Antibodies and reagents. R-848 (Resiquimod) was purchased from Alexis Biochemicals and dissolved to 10 mmol/L in DMSO then to 1 mmol/L in RPMI 1640 for working stock. Brefeldin A was purchased from BioLegend and used according to the manufacturer's instructions. PCR primer sets FcγRIa, QT00013475; FcγRIIa, QT01667099; FcγRIIb, QT00086842; γ-subunit, QT00055853; TRAF3, QT00080990; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), QT01192646] were from Qiagen. Trizol was purchased from Invitrogen. Reverse transcriptase, random hexamers, and SYBR Green PCR mix were purchased from Applied Biosystems. $F(ab')_2$ of anti-FcγRI (32.2) and anti-FcγRIIa (IV.3) were obtained from Medarex. The anti–FcR-γ-subunit was from Upstate Cell Signaling. Rabbit polyclonal antibodies specific to hFcγRIIa and hFcγRIIb were generated as previously described (13). Actin, GAPDH, and horseradish peroxidase–conjugated antibodies were from Santa Cruz Biotechnology.

Western blotting and ELISAs. Cells were lysed in TN1 buffer [50 mmol/L Tris (pH 8.0), 10 mmol/L EDTA, 10 mmol/L $Na_4P_2O_7$, 10 mmol/L NaF, 1% Triton X-100, 125 mmol/L NaCl, 10 mmol/L Na_3VO_4, and 10 μg/mL each aprotinin and leupeptin]. Postnuclear protein-matched lysates were boiled in Laemmli sample buffer and separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with the antibody of interest then developed by enhanced chemiluminescence (GE Healthcare). Cell supernatants were collected, centrifuged at full speed to clear cellular debris then assayed for cytokine through sandwich ELISA (R & D Systems) according to manufacturer protocol.

Real-time reverse transcription-PCR. RNA was extracted from peripheral blood monocyte (PBM) using Trizol, reverse transcribed to cDNA then run in triplicate for each donor on an Applied Biosystems Step One Plus system, with automatically calculated thresholds. Relative expression was calculated as $2^{\wedge -\Delta Ct}$, with ΔCt calculated by subtracting the average cycle threshold (Ct) of two housekeeping controls (TRAF3 and GAPDH) from the experimental sample Ct (14).

BMM isolation and culture. L929 cells, generously provided by Dr. Stéphanie Seveau (The Ohio State University, Columbus, OH), were used to generate conditioned media for culturing of murine BMMs (15). L929 were incubated in minimum essential media (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone), nonessential amino acids, sodium pyruvate, and penicillin/streptomycin (Invitrogen). Conditioned media from the L929 cells was collected after 7 d, passed through a 0.22-μm filter, and added to the BMM media. BMM were cultured from femurs of WT, $TLR7^{-/-}$, $MyD88^{-/-}$, $TRIF^{-/-}$, and $Cryopyrin^{-/-}$ C57/Bl6 mice (16) by flushing the marrow from femurs and plating cells on plastic dishes in DMEM (Invitrogen) containing 10% FBS, 0.1% β-mercaptoethanol (Bio-Rad), and 30% conditioned media from L929 cells. After 6 to 7 d, nonadherent cells were washed away using PBS then the remaining BMM was used for experiments.

Peripheral blood monocyte isolation. PBMs were isolated from Red Cross leukopaks through Ficoll centrifugation (Mediatech) followed by CD14-positive selection using MACS (Miltenyi Biotec, Inc.) as previously described (14). PBMs were resuspended in RPMI 1640 containing 10% heat-inactivated FBS (Hyclone), penicillin/streptomycin, and L-glutamate (Invitrogen). The purity of monocytes obtained was >97%, as determined by flow cytometry with CD14 antibody.

Microarray analysis. RNA was extracted from PBM using Trizol (Invitrogen) then labeled and hybridized to Affymetrix hgu133plus2 chips at The Ohio State University Medical Center Microarray-Genetics core facility. Resulting data files were preprocessed and analyzed using R (17) and BioConductor (18), testing for differentially expressed genes using the "limma" package (19).

ADCC assay. ADCC assays were done as previously described (20, 21). Briefly, PBM were incubated overnight with or without 1 μmol/L R-848 or 10 ng/mL IFNγ and were used as the effector cells. MDA-MB-468 breast cancer cells were used as the targets. These cells were incubated at 37°C with 0.3 mCi ^{51}Cr for 30 to 60 min, then incubated with no antibody, 10 μg/mL of Rituximab (negative control antibody), or with cetuximab. Monocytes and MDA cells were then coincubated at a 50:1 ratio in V-bottomed 95-well plates for 18 h. Supernatants were harvested

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and counted for radiolabeled Chromium using a γ counter. Percent cytotoxicity was calculated as [(sample – minimum control)/(maximum control – minimum control) * 100], in which minimum controls were target cells incubated alone and maximum controls were target cells incubated alone and lysed using 10% SDS. To derive values for antibody-dependent cytotoxicity, values from no-antibody controls were subtracted from values from cetuximab-treated targets.

Murine solid tumor model. CT26-HER2/neu colon carcinoma cells (22) were grown in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, and L-glutamate; washed to remove nonadherent cells; then resuspended using enzyme-free cell dissociation buffer (Invitrogen). Cells were centrifuged and resuspended at 10×10^6 per milliliter in RPMI 1640. The murine tumor model was performed in accordance with Penichet et al. (22) and Roda et al. (21). Briefly, 5-wk-old female BALB/cJ mice (The Jackson Laboratory) were injected s.c. with 1×10^6 of syngeneic CT26-HER2/neu cells. Mice were left for 7 d to allow tumors to develop. I.p. injections with treatments were then done thrice per week and tumor measurements were done on each treatment day. Tumor volumes were calculated as [0.5 × (length measurement) * (height measurement)^2], in which length was the longest diameter of the tumor. Treatments consisted of 4D5 anti-HER2 antibody at 1 mg/kg, R-848 at 100 μg/kg, 4D5 plus R-848, or DMSO vehicle control. All *in vivo* experiments were done in strict accordance to guidelines set by the Institutional Animal Care and Use Committee.

Statistical analyses. For all experiments done *in vitro*, Student's *t* tests were used to test for statistically significant differences. Statistics for the murine solid tumor model experiment were done by the Center for Biostatistics at The Ohio State University. Briefly, data were transformed by cube root, then a linear mixed model was applied, followed by an interaction contrast to test for synergy. The SAS (SAS Institute, Inc.) software was used to analyze the *in vivo* experiment.

Results

R-848 enhances FcγR function. The TLR7/8 agonist R-848 has been shown to increase cytokine production (23), so we asked whether it would lead to an additive or synergistic increase in FcγR-mediated cytokine production. To test this, we incubated PBM overnight with 1 μmol/L R-848 then plated them on immobilized IgG to cluster the FcγR. As shown in Fig. 1A, R-848 treatment alone caused PBM to secrete tumor necrosis factor α (TNFα). However, when R-848 treatment and FcγR activation were combined, there was a superadditive level of TNFα secretion. This suggests that the TLR7/8 pathways functionally synergize with FcγR.

Next, we examined the ability of R-848 to enhance the destruction of antibody-coated tumor cells *in vitro*. We treated human PBM overnight with or without 1 μmol/L R-848 then tested them in an ADCC assay (24, 25) using

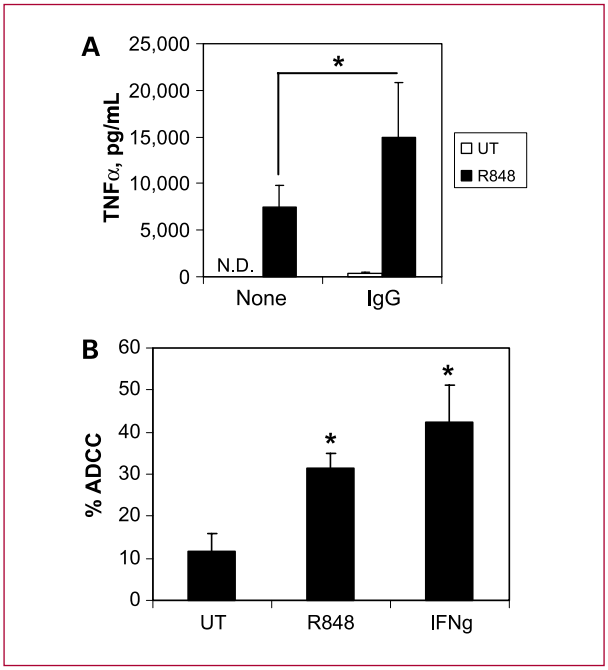

Fig. 1. R-848 enhances FcγR function. A, PBM were treated overnight with 1 μmol/L R-848 (R848) or were left nontreated (UT). Following this, they were incubated in 96-well plates with (IgG) or without (none) immobilized IgG for 24 h. Supernatants were collected and analyzed for TNFα by sandwich ELISA. Columns, mean of three separate donors; bars, SD. *, statistical significance at $P \leq 0.05$. N.D., not detected. B, R-848 enhances monocyte ADCC. Human PBM from four donors were incubated overnight with 1 μmol/L R-848 or 10 ng/mL IFNγ then tested in an ADCC assay (described in Materials and Methods) with cetuximab-coated MDA-MB-468 cells. The percent cytotoxicity after subtraction of no-antibody controls is plotted. Columns, mean; bars, SD. *, statistical significance versus nontreated ($P < 0.01$).

cetuximab-coated MDA-MB-468 cells. Because it has been shown that IFNγ can enhance ADCC in human monocytes (26–28), we also used 10 ng/mL IFNγ as a positive control. Results showed that R-848 treatment significantly increased antibody-dependent cytotoxicity, even to levels approaching those seen after IFNγ treatment (Fig. 1B). Hence, R-848 promotes the destruction of antibody-coated tumor target cells *in vitro*.

Microarray analysis of R-848. We next examined the genomewide transcriptional responses of monocytes to R-848 in an effort to gain insights on how it enhanced monocyte function. To do this, we incubated PBM with 1 μmol/L R-848 for 18 h, extracted RNA, and performed Affymetrix microarray analysis. We then searched for significantly different transcripts within the "immune response" or "inflammatory" ontologies that were upregulated 2-fold or more and with an average $\log_2$ expression of 3 or higher. There were a total of 119 unique transcripts, shown in Table 1. As expected based on previous literature, we found upregulation of cytokines such as interleukin (IL)-6, IL-12 p40, and TNFα. FcγRs are critical for antibody-mediated clearance of tumor cells, such as that seen in Fig. 1B (29). It was possible that part of the R-848–mediated

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Table 1. Microarray analysis of monocytes treated with R-848. PBM from 3 different donors were incubated overnight with or without 1 μmol/L R-848 and then subjected to Affymetrix microarray analysis

Symbol	FC	*P*	Description	Symbol	FC	*P*	Description
ADA	4.317346	1.4E-05	Adenosine deaminase	*IL1RN*	25.6737	4.5E-07	IL-1 receptor antagonist
ADORA2A	40.55086	8.8E-12	Adenosine A2a receptor	*IL2RA*	43.53276	1.6E-07	IL-2 receptor, α
ADRB2	16.73249	8.7E-06	Adrenergic, β-2-, receptor, surface	*IL2RG*	2.428115	0.004	IL-2 receptor, γ
AGER	3.71607	9.8E-05	Adv glycosylation end product–specific receptor	*IL4R*	13.91337	1.3E-06	IL-4 receptor
APOL3	2.290186	0.045	Apolipoprotein L, 3	*IL6*	489.0295	5.4E-09	IL-6 (IFN, β 2)
AQP9	5.560471	1.2E-08	Aquaporin 9	*IL6ST*	3.797162	0.001	IL-6 signal transducer
B4GALT1	7.023606	1.5E-06	β 1,4- Galactosyltransferase, polypeptide 1	*IL7R*	45.28874	1.8E-07	IL-7 receptor
BCL3	2.973849	1.4E-06	B-cell CLL/lymphoma 3	*IL8*	3.7873	0.005	IL-8
BCL6	4.485563	2.8E-11	B-cell CLL/lymphoma 6	*IRAK2*	25.49053	3.2E-08	IL-1 receptor–associated kinase 2
CCL18	8.512269	4.8E-06	Chemokine (C-C motif) ligand 18	*ITCH*	6.845941	0.005	Itchy homologue E3 ubiquitin protein ligase
CCL19	190.8174	4.1E-09	Chemokine (C-C motif) ligand 19	*ITGAL*	3.649082	6.4E-05	Integrin, α L (antigen CD11A (p180)
CCL2	6.25498	0.024	Chemokine (C-C motif) ligand 2	*LAIR1*	7.521857	2.5E-06	Leukocyte-associated Ig-like receptor 1
CCL20	55.74429	3.0E-07	Chemokine (C-C motif) ligand 20	*LCP2*	2.979506	3.8E-04	Lymphocyte cytosolic protein 2
CCL3	20.34984	1.4E-06	Chemokine (C-C motif) ligand 3	*LILRA1*	27.92517	3.2E-12	Leukocyte immunoglobulin-like receptor, A1
CCL4	18.7361	1.6E-06	Chemokine (C-C motif) ligand 4	*LILRA2*	2.971885	0.003	Leukocyte immunoglobulin-like receptor, A2
CCL7	4.251229	0.040	Chemokine (C-C motif) ligand 7	*LILRA3*	35.13229	2.9E-09	Leukocyte immunoglobulin-like receptor, A3
CD163	28.96202	1.2E-06	CD163 molecule	*LILRB1*	6.353475	7.9E-08	Leukocyte immunoglobulin-like receptor, B1
CD274	31.7408	3.6E-09	CD274 molecule	*LILRB2*	7.090295	7.9E-08	Leukocyte immunoglobulin-like receptor, B2
CD40	3.132347	0.028	CD40 molecule	*LILRB3*	3.073823	1.5E-06	Leukocyte immunoglobulin-like receptor, B3
CD55	4.733903	1.3E-06	CD55 molecule	*LILRB4*	2.554202	0.016	Leukocyte immunoglobulin-like receptor, B4
CD59	19.06185	2.9E-09	CD59 molecule	*LOC653879*	3.415538	0.030	Similar to Complement C3 precursor
CD80	18.26656	1.9E-05	CD80 molecule	*MEFV*	14.03661	0.001	Mediterranean fever
CD83	2.445838	0.003	CD83 molecule	*MGLL*	2.640648	0.023	Monoglyceride lipase
CFB	59.44535	1.4E-08	Complement factor B	*MS4A1*	3.285111	0.006	Membrane-spanning 4-domains, A1
CHST2	4.048418	0.001	Carbohydrate sulfotransferase 2	*NFE2L1*	3.47481	1.3E-04	Nuclear factor (erythroid derived 2)–like 1

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Symbol	FC	*P*	Description	Symbol	FC	*P*	Description
CLEC4D	5.729237	3.9E-05	C-type lectin domain family 4, member D	*NLRP3*	3.529458	0.026	NLR family, pyrin domain containing 3
CLEC4E	2.414824	0.020	C-type lectin domain family 4, member E	*NR3C1*	4.879714	3.8E-08	Nuclear receptor subfamily 3, C1
CLU	2.289794	0.021	Clusterin	*PAG1*	13.00331	2.6E-08	P-protein assoc with glycosphingolipid microdomains 1
COL4A3BP	2.603515	4.2E-04	Collagen, type IV, α 3 binding protein	*PDCD1LG2*	7.568735	7.5E-06	Programmed cell death 1 ligand 2
CSF3	36.21415	2.0E-07	Colony-stimulating factor 3	*POMP*	2.532659	3.7E-04	Proteasome maturation protein
CXCL1	97.88367	1.2E-12	Chemokine (C-X-C motif) ligand 1	*POU2F2*	2.35123	4.9E-05	POU class 2 homeobox 2
CXCL13	116.9714	2.0E-09	Chemokine (C-X-C motif) ligand 13	*PRELID1*	3.945781	1.3E-05	PRELI domain containing 1
CXCL2	8.593548	1.1E-05	Chemokine (C-X-C motif) ligand 2	*PRG2*	3.749564	4.8E-04	Proteoglycan 2, bone marrow
CXCL3	17.77199	7.6E-07	Chemokine (C-X-C motif) ligand 3	*PRKCA*	3.283269	0.010	Protein kinase C, α
CXCL5	15.40997	6.8E-05	Chemokine (C-X-C motif) ligand 5	*PTGS2*	121.221	2.5E-10	Prostaglandin-endoperoxide synthase 2
CYBB	3.719852	0.002	Cytochrome b-245, β polypeptide	*PTX3*	34.45957	6.8E-14	Pentraxin-related, rapidly induced by IL-1 β
DPP8	3.208713	0.021	Dipeptidyl-peptidase 8	*RAC1*	3.784847	0.005	Ras-related C3 botulinum toxin substrate 1
EBI3	25.25581	9.2E-07	EBV-induced gene 3	*RELA*	11.36527	4.4E-04	V-rel homologue A, p65
EREG	4.980403	3.3E-04	Epiregulin	*RIPK2*	2.190812	0.034	Receptor-interacting serine-threonine kinase 2
ETS1	13.56834	4.3E-04	V-ets E26 homologue 1	*RNF19B*	10.31492	6.0E-05	Ring finger protein 19B
F3	18.19057	5.3E-06	Coagulation factor III	*S100A12*	3.133215	0.014	S100 calcium binding protein A12
FCER1G	2.391636	0.001	Fc fragment of IgE, γ polypeptide	*S100A8*	15.18746	2.1E-04	S100 calcium binding protein A8
FCGR1A	21.46627	1.8E-07	Fc fragment of IgG, high affinity Ia, receptor (CD64)	*S100A9*	3.26703	0.006	S100 calcium binding protein A9
FCGR1B	3.287157	0.042	Fc fragment of IgG, high affinity Ib, receptor (CD64)	*SEMA3C*	22.85418	1.0E-11	Sema, immunoglobulin, short basic, secreted, 3C
FCGR2A	2.545392	0.001	Fc fragment of IgG, low affinity IIa, receptor (CD32)	*SLC11A1*	10.12225	7.2E-08	Solute carrier family 11, member 1
FOXP1	3.44095	3.0E-06	Forkhead box P1	*SNF1LK*	7.138636	0.001	SNF1-like kinase
FPR1	3.575507	3.0E-04	Formyl peptide receptor 1	*SPN*	3.129464	0.027	Sialophorin (leukosialin, CD43)
FPRL1	34.54009	5.9E-09	Formyl peptide receptor-like 1	*TARP*	11.3274	0.011	T-cell receptor γ constant 2
FUS	6.003565	0.016	Fusion (in t(12;16) in malignant liposarcoma)	*TBK1*	2.765583	3.2E-05	TANK-binding kinase 1
HAMP	59.32565	1.4E-12	Hepcidin antimicrobial peptide	*TGM2*	16.02947	4.5E-08	Transglutaminase 2
HDAC7A	2.141959	0.045	Histone deacetylase 7A	*TLR1*	2.055846	0.005	TLR 1
HRH1	10.03894	7.2E-06	Histamine receptor H1	*TLR4*	2.422299	0.033	TLR 4

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Symbol	FC	*P*	Description	Symbol	FC	*P*	Description
HSPC111	6.28524	0.008	Hypothetical protein HSPC111	*TNF*	12.9597	3.4E-07	TNF, member 2
IFNG	26.61823	1.4E-04	IFN, γ	*TNFAIP1*	2.2312	1.9E-04	TNF- α–induced protein 1
IL10	39.7384	3.3E-08	IL-10	*TNFAIP6*	14.66536	6.8E-07	TNF-α–induced protein 6
IL12B	3.38804	2.6E-04	IL-12B, p40	*TNFRSF9*	2.658242	0.020	TNF receptor, member 9
IL1A	61.94209	8.4E-08	IL-1, α	*TNFSF9*	3.521049	8.9E-05	TNF, member 9
IL1B	26.07393	7.7E-09	IL-1, β	*TREM1*	6.539373	6.1E-06	Triggering receptor expressed on myeloid cells 1
IL1F9	18.53308	3.7E-05	IL-1 family, member 9	*VAV1*	7.233896	1.8E-07	Vav 1 guanine nucleotide exchange factor
IL1RAP	3.382441	1.2E-04	IL-1, receptor accessory protein				

NOTE: Transcripts with fold increases of ≥2, with average $\log_2$ expression of ≥3, and with ontologies of "immune response" or "inflammatory" were selected. Highlighted in gray are the activating FcγR.
Abbreviation: CLL, chronic lymphocytic leukemia.

enhancement of this clearance was due to the upregulation of these receptors and this was found in the analysis results (Table 1, gray highlights). Unlike this upregulation of activating FcγR, the array data showed a 6-fold downregulation of the inhibitory FcγRIIb (data not shown). These results suggest that R-848 regulates FcγR at the transcriptional level and that this may largely account for the increased FcγR-mediated cytokine production and ADCC.

R-848 alters FcγR protein expression. We next verified that the altered transcription of FcγR led to changes in protein expression and tested for the lowest required dosage. We treated PBM overnight with 0, 0.01, 0.1, or 1.0 μmol/L R-848, or with 0, 1, 10, or 100 μmol/L R-848. Western blots were done to measure expression of FcγRIIa, the common γ-subunit, and FcγRIIb. As shown in Fig. 2A and B, a dosage of 1 μmol/L was sufficient to alter FcγR expression and higher dosages did not lead to greater changes.

To confirm that changes in FcγR also occurred on the cell surface, we treated PBM overnight with 1 μmol/L R-848 and measured surface expression of FcγRIa and FcγRIIa using flow cytometry. Compared with nontreated PBM, there were significant increases after R-848 treatment (Fig. 2C). Overnight treatments (14-18 hours) with R-848 elicited changes in FcγR, so we next tested whether short (1-3 hours) treatment times would be sufficient. Hence, we treated PBM for 1, 3, or 14 hours then measured FcγR by Western blotting. Results showed that increases in FcγRIIa occurred at the late stage (Fig. 2D, top), whereas small increases in the γ-chain appeared at 3 hours but were higher at 14 hours (Fig. 2D, middle). However, decreases in FcγRIIb protein were seen within 1 hour (Fig. 2D, bottom), whereas the transcript for FcγRIIb remained to 4 hours (data not shown). This suggests that R-848 triggered an immediate degradation of the FcγRIIb protein, followed by a later reduction in FcγRIIb transcript.

Secreted factors mediate increases in activating FcγR. Results from the microarray analysis showed that numerous cytokines were upregulated, many of which were known to influence FcγR expression. To test whether secretion of these cytokines may have been responsible for the R-848–mediated changes in FcγR, we pretreated PBM for 30 minutes with Brefeldin A, an inhibitor of secretion. Following this, PBM were treated for 12 hours with R-848 and FcγR expression was measured by both real-time reverse transcription-PCR and Western blotting. Results showed that pretreatment with Brefeldin A prevented the R-848–mediated increases in FcγRI, FcγRIIa, and γ-subunit transcripts, whereas the decrease in FcγRIIb was not affected (Fig. 3A). Similarly, Western blotting showed that Brefeldin A pretreatment inhibited the R-848–mediated increases in FcγRIIa and the γ-subunit but did not prevent the reduction in FcγRIIb (Fig. 3B). These results suggest that R-848 drives production of secreted factors that act in an autocrine/paracrine fashion to increase expression of the activating FcγR, but that the effect of R-848 on FcγRIIb is mediated through a different mechanism.

Mechanisms of R-848–mediated regulation of FcγR. We next wished to test whether a similar effect of R-848 on FcγR expression would occur in mice and to determine the mechanism by which R-848 mediates its effects. R-848 signals through the MyD88 adapter protein (30) and the Nalp3/Cryopyrin cytosolic sensor (31). To determine which were required for regulation of FcγR and to rule out a role for the MyD88-independent TRIF, we isolated BMMs from WT, $TLR7^{-/-}$, $MyD88^{-/-}$, $TRIF^{-/-}$, and $Cryopyrin^{-/-}$ mice. BMMs were treated overnight with

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or without R-848 then expression of the γ-subunit was measured by Western blotting. Of note, FcγRIIa is not expressed in mice (32). As shown in Fig. 4A (top and middle, respectively), neither TLR7$^{-/-}$ nor MyD88$^{-/-}$ cells showed R-848–mediated increases in the γ-subunit. However, R-848 did increase γ-subunit expression in Cyropyrin and TRIF knockout BMM (Fig. 4A, top and bottom, respectively). These results indicate that R-848–mediated effects on FcγR require TLR7 and MyD88. As a functional control, TNFα production was also examined in supernatants from BMM treated with or without R-848. Results were in accordance with those previously reported by Hemmi et al. (30), showing that TLR7$^{-/-}$ and MyD88$^{-/-}$ cells did not produce TNFα in response to R-848 (Fig. 4B). Because R-848 does not seem to activate mouse TLR8 (33), all of its effect would be expected to require TLR7. In humans, however, R-848 can activate both TLR7 and TLR8 (33) and can signal through MyD88-independent pathways (31). Hence, further experiments will be required to rule out the involvement of a MyD88-independent pathway or a possible interaction between TLR7 and TLR8 activation in humans.

***R-848 reduces tumor growth* in vivo.** Results showed that R-848 could regulate FcγR expression and synergize with FcγR function, so we next asked whether R-848 could improve antibody therapy *in vivo*. To test this, we used a solid tumor model using CT26 cells expressing human HER2/neu (21, 22). Here, CT26-HER2/neu colon carcinoma cells were s.c. injected into syngeneic BALB/c J mice. After 7 days to allow tumors to develop, mice were injected i.p. with antibody alone, R-848 alone, R-848 plus antibody, or vehicle alone thrice per week. Tumors were measured on each treatment day. After 13 days, there was a significantly reduced rate of growth in the mice receiving R-848 plus antibody (Fig. 5). Statistical tests showed synergism between 4D5 and R-848 ($P = 0.03$) for reducing the rate of tumor growth. Hence, R-848 plus antitumor antibody leads to synergistic reduction of tumor growth *in vivo*.

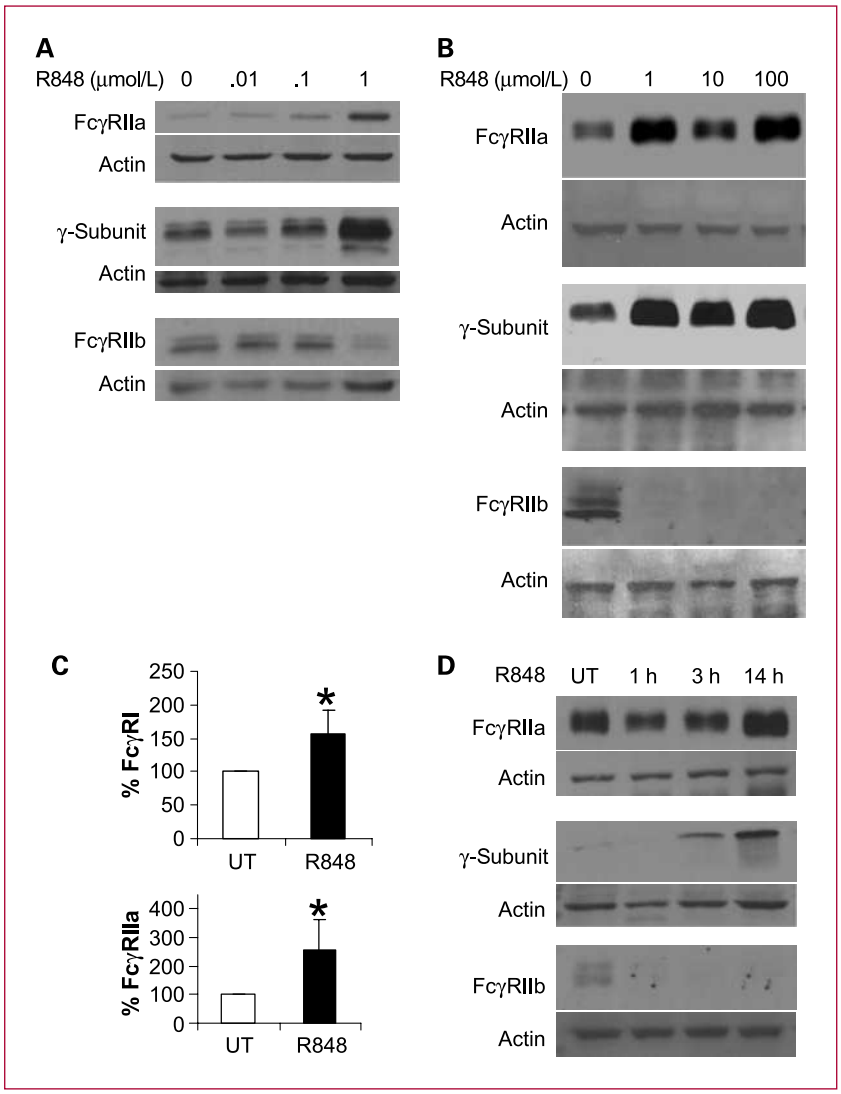

Fig. 2. Dose response to R-848. A and B, PBM were incubated with 0, 0.01, 0.1, or 1.0 μmol/L (A) or 0, 1, 10, or 100 μmol/L (B) R-848, with DMSO concentrations equalized across all treatments. Western blotting was done to measure FcγRIIa (top), the γ-subunit (middle), and FcγRIIb (bottom). Blots represent three independent experiments. C, flow cytometry was done to measure surface expression of FcγRI (top) and FcγRIIa (bottom) using $F(ab')_2$ fragments of 32.2 and IV.3 antibodies, respectively, followed by $F(ab')_2$ goat anti-mouse FITC. The percent increases over nontreated (UT) were plotted as bar graphs. *, statistical significance at $P \leq 0.05$; columns, mean ($n = 3$); bars, SD. D, time course of R-848 influence on FcγR expression. PBM were incubated for 0, 1, 3, or 14 h with 1 μmol/L R-848 then protein lysates were extracted and analyzed by Western blotting for FcγRIIa (top), the γ-subunit (middle), or FcγRIIb (bottom). Blots represent three independent experiments.

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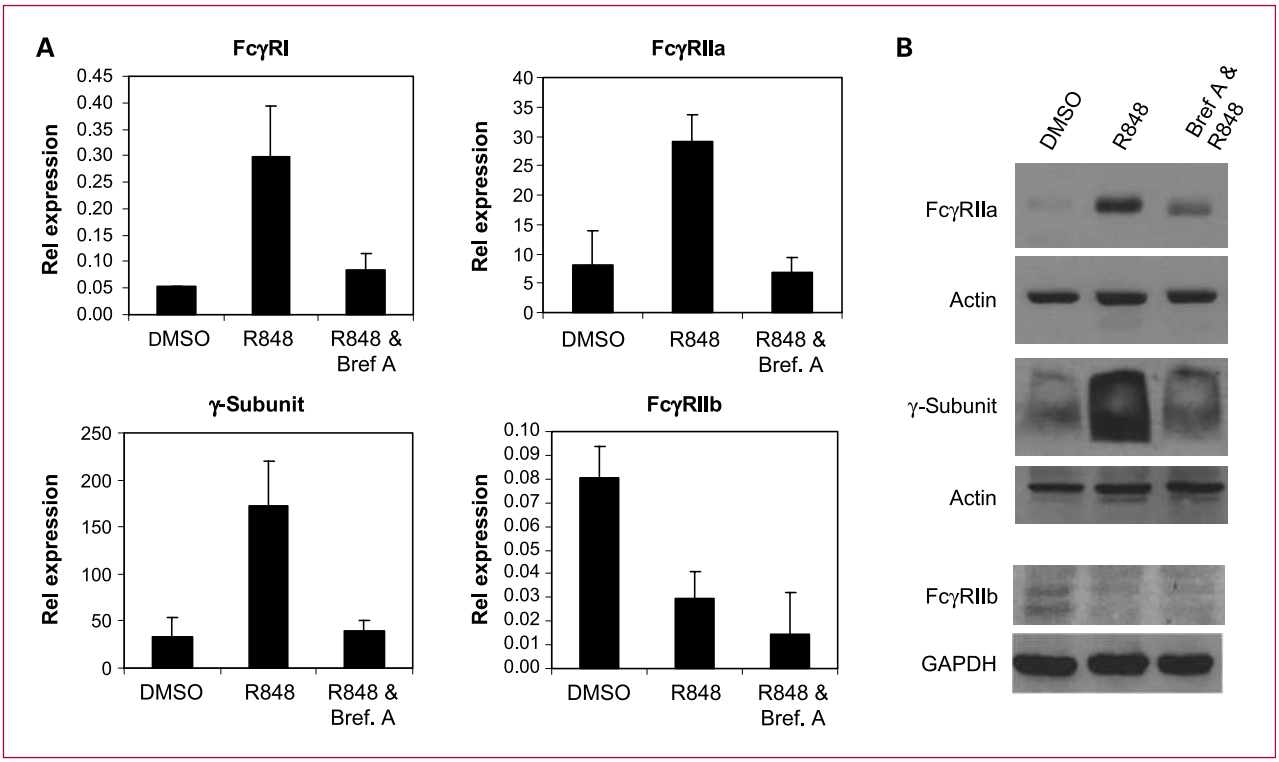

Fig. 3. Requirement of secreted factors for R-848–mediated changes in FcγR expression. A, PBM were pretreated with or without Brefeldin A for 30 min and treated for 12 h with or without 1 μmol/L R848. RNA was extracted and FcγR expression measured by real-time reverse transcription-PCR. DMSO (vehicle control), R-848 alone, and R-848 plus Brefeldin A pretreatment were compared for FcγRI (top left), FcγRIIa (top right), the γ-subunit (bottom left), and FcγRIIb (bottom right). Graphs are representative of three independent experiments. Columns, mean; bars, SD. B, PBM (n = 3) were pretreated with or without Brefeldin A for 30 min then treated for 12 h with or without 1 μmol/L R848. Protein lysates were collected and Western blots done to detect FcγRIIa (top), the γ-subunit (middle), and FcγRIIb (bottom). DMSO (vehicle control), R-848 alone, and R-848 plus Brefeldin A pretreatment were compared. Actin or GAPDH reprobes were done for each blot to verify equivalent loading.

Discussion

Here, we have shown a link between the TLR7/8 and the Fcγ receptor pathways, in which TLR7/8 activation regulates FcγR expression. There is also functional synergism between these two pathways, leading to enhanced FcγR-mediated cytokine release and to a decreased rate of tumor growth. As such, TLR7/8 agonists may be of special benefit in conjunction with antibody therapy against tumors.

Several possible mechanisms may account for the functional synergism between TLR7/8 activation and FcγR activity. First, as shown in Fig. 2 and Table 1, there was increased expression of the activating FcγR and a marked reduction in the inhibitory FcγRIIb. This would promote superadditive responses, as the FcγR would respond more strongly to any given stimulus. With regard to the synergism seen in the murine solid tumor model (Fig. 5), other factors may also contribute. T cells, B cells, dendritic cells, and monocytes/macrophages can all respond to TLR7/8 ligands (34), although it has also been reported that T cells may respond only indirectly (35). Natural killer cells are themselves unaffected by R-848 treatment but respond to monocyte-derived cytokines following R-848 treatment (data not shown; ref. 36). In mice, it has been shown that R-848 elicits cytokine production (23) as well as driving leukocytes from circulation to peripheral organs (35). Together, these two effects might work with the increased FcγR to enhance the antitumor response. First, greater cytokine production would lead to more activation of the immune cells. Second, because migration of leukocytes into peripheral organs is stimulated, presumably there would be more leukocytes migrating to the tumor site as well (35). In addition, R-848 may have shifted the macrophages toward an M1 phenotype and this would have significantly enhanced their ability to combat the tumors. Tumor-associated macrophages possess an M2 phenotype and promote invasion (see refs. 37, 38 for review). However, it has been shown that treatment of squamous cell carcinoma with imiquimod, a TLR7 ligand approved for clinical use, leads to an M1 and Th1 phenotype (39). Collectively, these factors may have contributed to the synergism we observed between TLR7/8 and FcγR. Studies are ongoing to determine the precise mechanisms.

The cytokine response itself is likely responsible for the increase in activating FcγR and might have been responsible for changes in FcγRIIb as well. Upregulation of FcγR by TLR4 has been previously shown in a murine model of arthritis and was found to be largely mediated by

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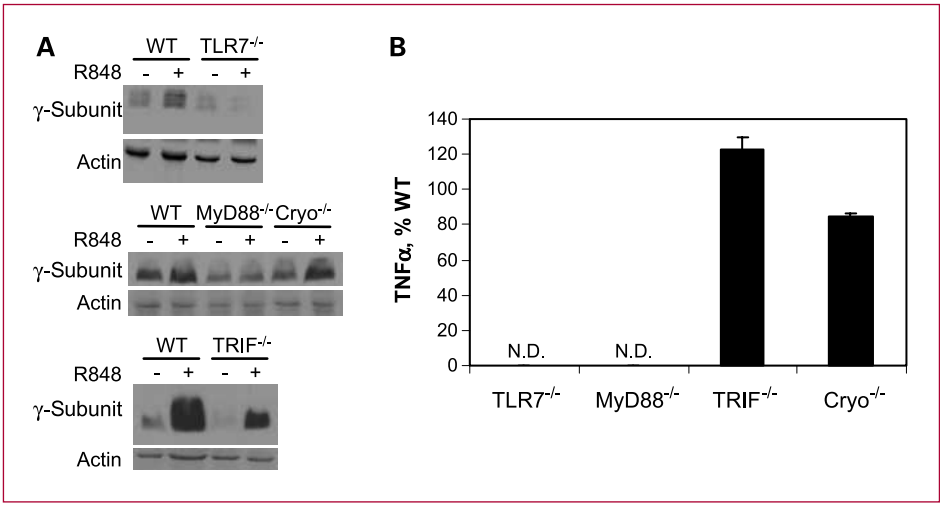

Fig. 4. R-848 requires TLR7 and MyD88 to regulate FcγR expression. A, murine BMMs were isolated, treated overnight with or without 1 μmol/L R-848, then protein lysates were extracted and analyzed by Western blotting for expression of the γ-subunit. Top, BMM from WT versus TLR7 knockout (TLR7$^{-/-}$) were compared. Middle, BMM from WT versus MyD88 (MyD88$^{-/-}$) and Cryopyrin (Cryo$^{-/-}$) knockouts were compared. Bottom, BMM from WT versus TRIF knockouts (TRIF$^{-/-}$) were compared. All blots represent three independent experiments. B, cytokine response of BMM to R-848. BMM from WT, TLR7$^{-/-}$, MyD88$^{-/-}$, TRIF$^{-/-}$, and Cryopyrin$^{-/-}$ mice were treated overnight with 1 μmol/L R-848 or left nontreated (UT). Supernatants were analyzed by sandwich ELISA for TNFα. Graph is representative of at least three different experiments per genotype. N.D., not detected.

IL-10 production (40). IL-10 can lead to increases in activating FcγR (41–43) as well as that of the inhibitory FcγRIIb (43, 44). In contrast to this general upregulation, however, we found a striking decrease in FcγRIIb after TLR7/8 activation. Previous work has shown that IL-4 works with IL-10 to promote expression of FcγRIIb (13, 44), but our microarray analysis found an ~40-fold increase in IL-10 with no increase in IL-4 after R-848 treatment. Concurrently, TNFα and IFNγ, both known to decrease FcγRIIb expression (44, 45), were strongly upregulated.

Hence, it seemed likely that the specific cytokine milieu elicited by R-848 was responsible for the changes in FcγR expression. Indeed, this was likely the case for the upregulation of activating FcγR, as blocking secretion with Brefeldin A abolished the R848-mediated changes. However, Brefeldin A did not prevent the R848-mediated decrease in FcγRIIb (Fig. 3B). Further, FcγRIIb protein was decreased within 1 hour (Fig. 2D), whereas FcγRIIb transcript remained to 4 hours (data not shown). These results strongly suggest a differential regulation of activating versus inhibitory receptors by R-848, in which autocrine/paracrine factors drive the upregulation of activating receptors and different mechanisms—perhaps ubiquitination and proteasomal degradation—cause the almost-immediate decrease in FcγRIIb.

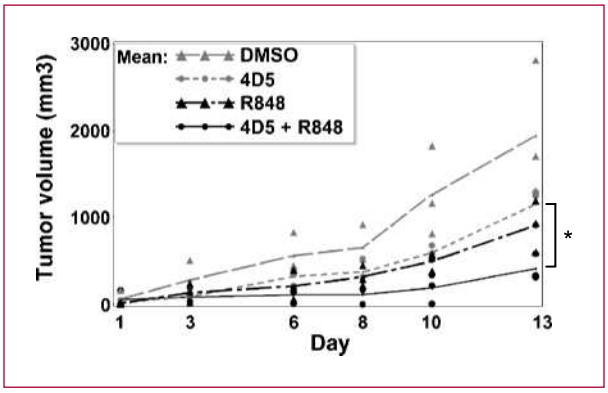

Fig. 5. R-848 enhances antibody therapy *in vivo*. BALB/cJ mice (n = 3 per group) were injected s.c. with 1 × 10^6 CT26-Her2/neu cells and left for 7 d for tumors to develop. Mice were then injected i.p. on every other day with DMSO vehicle, anti-Her2 (4D5) plus DMSO, R-848, or 4D5 plus R-848. Tumor sizes were measured (see Materials and Methods) every other day for 2 wk. *, statistical significance ($P \leq 0.05$).

There is a chance that the culture conditions (10% FBS rather than autologous sera) influenced the maturation and responses of the monocytes. For example, the quantities of many growth, survival, or apoptosis factors that monocytes would normally be exposed to within circulation would have been different with the FBS culture. However, the negative controls within the experiments suggest that R-848 itself drove at least the majority of the FcγR and cytokine responses.

Results from the ADCC (Fig. 1B) and the murine solid tumor (Fig. 5) experiments suggest that although imidazoquinolines have previously been shown to be effective by themselves against certain tumors, they may prove especially useful as adjuvants to antibody therapy. In fact, it is plausible that part of the antitumor effect of R-848 is mediated by autoantibodies against the tumor. Such autoantibodies have been well documented in humans (46). We found that cotreatment with antibody and R-848 led to the greatest effects (Figs. 4 and 5), but it is likely that more effective antibodies may elicit an even stronger TLR7/8-FcγR synergism. Many antibodies have been engineered for better FcγR binding (47, 48) and these may

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prove especially powerful when combined with TLR7/8 agonists.

In summary, we have identified a unique regulatory link between TLR7/8 and FcγR. This not only has implications for the clinical setting, but also uncovers a novel biological regulatory pathway of Fcγ receptors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Reciprocal Regulation of Activating and Inhibitory Fcγ Receptors by TLR7/8 Activation: Implications for Tumor Immunotherapy

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