Toll-like Receptor 2 Ligands Regulate Monocyte Fcγ Receptor Expression and Function*

Prexy Shah†, Kevin Fatechand‡, Hemal Patel§, Huiqing Fang§, Steven E. Justiniano‡, Xiaokui Mo§, David Jarjoura§, Susheela Tridandapani**‡, and Jonathan P. Butchar***

From the †Department of Internal Medicine, §Center for Biostatistics, **Comprehensive Cancer Center, Ohio State University, Columbus, Ohio 43210

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†To whom correspondence may be addressed: Ohio State University, Department of Internal Medicine, 415 DHRLI, 473 W. 12th Ave., Columbus, OH 43210. Tel.: 614-247-6768; Fax: 614-247-8106; E-mail: butchar.2@osu.edu.

‡To whom correspondence may be addressed: Ohio State University, Department of Internal Medicine, 415 DHRLI, 473 W. 12th Ave., Columbus, OH 43210. Tel.: 614-247-6768; Fax: 614-247-8106; E-mail: tridandapani.2@osu.edu.

The use of therapeutic antibodies is a common form of treatment for cancer, and efforts are ongoing to optimize its effectiveness (1). Here, antibodies bind antigens on tumor cells and effect their destruction through several possible means, including induction of apoptosis, blockage of aberrant intra- or intercellular signaling, and destruction by host immune effector cells (2, 3). By far the most critical mechanism of antibody-mediated tumor cell clearance appears to be through the activity of FcγR3 on host immune effector cells (4). The vast majority of immune cells (with the exception of T cells) express one or more FcγR (5). FcγR bind the Fc portion of antibodies, and their activation leads to effector functions such as phagocytosis, cytokine release, and direct cytotoxicity against the antibody-coated target (5). Among the immune effector cells, monocytes/macrophages play a critical role in that they directly attack opsonized targets and also release a variety of chemokines and cytokines that can attract and activate neighboring cells (6–8). Indeed, in a murine model of CD20 antibody-mediated depletion of B cells, depletion of macrophages abrogated the effect of the antibody (6). Monocytes express FcγR that activate, as well as dampen, downstream events such as phagocytosis and cytokine release. This is mediated through immunoreceptor tyrosine-based activation motifs or immunoreceptor tyrosine-based inhibitory motifs, and FcγR serve to activate or inhibit signaling on the basis of the motif they contain (5).

Toll-like receptors (TLR) are evolutionarily conserved pattern recognition receptors for a wide variety of pathogens and potentially harmful molecules (9). However, utilizing TLR activation as a means of strengthening responses to vaccine treatments or to threats such as viruses and tumors is an area of active research. Purified and/or synthetic TLR agonists such as CpG (for TLR9), imiquimod (for TLR7), flagellin (for TLR5), Pam3CSK4 (for TLR2), and others continue to be evaluated, with many showing promise toward treating asthma, cancer, and viral infections and for strengthening vaccine responses (10).

Fcγ receptor (FcγR) clustering on monocytes/macrophages results in phagocytosis and inflammatory cytokine production, which serve to eliminate antibody-opsonized targets and activate neighboring immune cells. Toll-like receptor 2 (TLR2), which recognizes a range of both bacterial and fungal components, elicits strong proinflammatory responses in these cells when stimulated by ligands, either natural or synthetic. Thus, we explored the possibility that TLR2 agonists could strengthen FcγR activity within the context of antibody therapy. Human peripheral blood monocytes treated with the TLR2 agonist Pam2CSK4 showed significantly enhanced FcγR-mediated cytokine production as well as phagocytic activity. An examination of the molecular mechanism behind this enhancement revealed increased expression of both FcγRIIa and the common γ subunit following Pam2CSK4 treatment. Interestingly however, expression of the inhibitory receptor FcγRIIb was also modestly increased. Further investigation revealed that Pam2CSK4 also dramatically decreased the expression of SHIP, the major mediator of FcγRIIb inhibitory activity. Using a murine Her2/neu solid tumor model of antibody therapy, we found that Pam2CSK4 significantly enhanced the ability of anti-Her2 antibody to reduce the rate of tumor growth. To verify that the FcγR enhancement was not unique to the diacylated Pam2CSK4, we also tested Pam3CSK4, a related triacylated TLR2 agonist. Results showed significant enhancement in FcγR function and expression. Taken together, these findings indicate that TLR2 activation can positively modulate FcγR and suggest that TLR2 agonists should be considered for testing as adjuvants for antitumor antibody therapy.

**Background:** Toll-like receptors recognize bacterial components, leading to immune activation.

**Results:** TLR2 ligands alter monocyte/macrophage FcγR and phosphatase expression to improve function both in vitro and in vivo.

**Conclusion:** TLR2 ligands enhance monocyte/macrophage function.

**Significance:** FcγR function is critical for antibody therapy. Deciphering the effect of TLR2 ligands provides a potential means to enhance therapy.
TLR2 Ligands Regulate FcγR Expression and Function

Toll-like receptor 2 (TLR2) responds to a variety of natural stimuli such as Gram-positive bacterial cell wall components and zymogens as well as synthetic compounds such as tri- and diacylated lipopeptides. TLR2 recognizes these components through interacting with other host cell receptors such as TLR1, TLR6, CD14, CD36, and Dectin-1 (10–12). As with most TLR, it involves the downstream adapter MyD88, resulting in cellular responses such as activation of MAPKs and NF-κB (13). The TLR2 agonist Pam3CSK4 is currently being investigated for its ability to induce vaccine responses (14), for protection against ischemia/reperfusion injury (15) and sepsis (16), and for modulating allergic responses (17), as well as for the study of TLR2 activation within the context of other conditions such as rheumatoid arthritis (18) and acute lung injury (19). Hence, such TLR2 agonists may prove to be effective, clinically relevant immunomodulators.

Here we explored the possibility that TLR2 activation could interact with the FcγR pathway, leading to enhancements in FcγR function. We found that the TLR2 agonist Pam2CSK4 enhanced FcγR-mediated cytokine production as well as phagocytic ability. In addition, Pam2CSK4 plus antibody was significantly more effective than antibody alone in slowing the rate of tumor growth in a murine model of antibody therapy. An examination of the underlying mechanisms revealed an up-regulated expression of FcγRIIa, the common γ subunit on monocytes, and, to some small degree, the inhibitory FcγRIIB. However, Pam2CSK4 also up-regulated miR-155 and down-regulated the inhibitory phosphatase SHIP, which is the major mediator of FcγRIIB function. We also found that the related triacylated Pam3CSK4 was equally effective in modulating FcγR expression and in enhancing monocyte-mediated phagocytosis in vitro. These results suggest that TLR2 activation is an effective means of strengthening monocyte FcγR expression and function and that such agonists could be further examined as potential adjuvants for antitumor antibody therapy.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Pam2CSK4 was purchased from Invivogen and dissolved to 1 mg/ml in endotoxin-free water and then to 10 μg/ml in endotoxin-free water for working stock. Brefeldin A was purchased from BioLegend (San Diego, CA) and supplemented with 20 ng/ml M-CSF (R&D Systems) for 7 days before the differentiated cells were used in experiments.

Peripheral Blood Monocyte Isolation—Peripheral blood monocytes (PBMC) were isolated from Red Cross Leukopaks via Ficoll centrifugation (Mediatech, Manassas, VA) followed by CD14-positive selection using MACS (Miltenyi Biotec, Inc., Cambridge, MA) as described previously (21). PBMC were resuspended in RPMI 1640 containing 10% heat-inactivated FBS (Hyclone, Logan, UT), penicillin/streptomycin, and 1-glutamate (Invitrogen). The purity of monocytes obtained was >97% as determined by flow cytometry with CD14 antibody.

Bone Marrow-derived Macrophage Isolation and Culture—Wild-type Balb/c mice were sacrificed according to institution-approved animal care and use protocols. Briefly, bone marrow cells were cultured in RPMI containing 10% fetal bovine serum plus 10 μg/ml polymixin B (Calbiochem, San Diego, CA) and supplemented with 20 ng/ml M-CSF (R&D Systems) for 7 days before the differentiated cells were used in experiments.

Phagocytosis—Phagocytosis assays were performed as described previously (20). Briefly, IgG-coated PKH26-labeled sheep red blood cells (SRBC) were added to the PBMC. Cells were briefly pelleted by slow centrifugation followed by 30 min of incubation at 37°C. Unphagocytosed RBC were subjected to hypotonic lysis with water and PBS wash prior to fixation with 1% paraformaldehyde. The samples were analyzed by fluorescence microscopy in a blinded fashion. The phagocytic index is defined as the total number of SRBCs ingested by 100 phagocytes.

Murine Solid Tumor Model—CT26-HER2/neu colon carcinoma cells (22) were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin/streptomycin, and 1-glutamate; washed to remove non-adherent cells; and then...
resuspended using enzyme-free cell dissociation buffer (Invitrogen). Cells were centrifuged and resuspended at 10 × 10⁶ cells/ml in RPMI 1640. The murine tumor model (22) was used as described previously (23,24). Briefly, 5-week-old female Balb/c mice (The Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously with 1 × 10⁶ of syngeneic CT26-HER2/neu cells. Mice were left for 7 days to allow tumors to develop. Intraperitoneal injections with treatments (PBS vehicle, 4D5 at 1 mg/kg, Pam2CSK4 at 5 µg/kg, or 4D5 plus Pam2CSK4) were then performed three times per week, with tumor measurements recorded on each treatment day. Tumor volumes were calculated as [0.5 × (length measurement) × (height measurement)]³ (23), where length was the longest diameter of the tumor. Treatments consisted of 4D5 anti-HER2 antibody at 3 mg/kg, Pam2CSK4 at 100 ng/g, 4D5 plus Pam2CSK4, or PBS control. All in vivo experiments were performed in strict accordance with guidelines set by the Institutional Animal Care and Use Committee.

Statistical Analyses—For all experiments performed in vitro, Student’s t tests were used to test for statistically significant differences. Statistical analyses for the murine solid tumor model experiment were done at the Center for Biostatistics, Ohio State University. Briefly, data were transformed by cube root, and then a linear mixed model was applied to identify significant differences between treatment groups using SAS (SAS Institute, Inc., Cary, NC) analysis software.

RESULTS

Pam2CSK4 Enhances FcyR Function—We first determined whether TLR2 activation could strengthen monocyte FcyR activity with regard to both cytokine production and phagocytosis. We first treated PBM overnight with or without 100 ng/ml of the TLR2 agonist Pam2CSK4 and then incubated them for 30 min with fluoresceinated, IgG-opsonized sheep red blood cells SRBC. The number of ingested SRBC was counted using fluorescence microscopy. Results showed that agonist-treated PBM ingested significantly more SRBC than vehicle-treated PBM (Fig. 1A). Next, we treated PBM overnight with or without 100 ng/ml of Pam2CSK4 and then incubated them with or without immobilized IgG. After 24 h, cleared supernatants were collected, and TNFα was measured by ELISA. Results (Fig. 1B) showed that Pam2CSK4 elicited synergistic increases in IgG-mediated TNFα production with very little background TNFα from Pam2CSK4 treatment alone. Collectively, these results show that treatment with the TLR2 agonist Pam2CSK4 can significantly enhance monocyte FcyR function.

Pam2CSK4 Modulates FcyR Expression—To examine the mechanism by which TLR2 activation leads to increased FcyR function, we next tested whether the agonist treatment influenced FcyR expression itself. We treated PBM with vehicle or Pam2CSK4 overnight and subsequently measured FcyR expression by Western blotting. As shown in Fig. 2A, agonist treatment increased the expression of FcyRIIa and the common γ-chain that associates with FcyRI and FcyRIII (left and center panels). Expression of the inhibitory FcyRIIb was largely unchanged, although it did show increases in some donors tested (right panel, showing an increase). The increase in FcyRIIa was verified in subsequent experiments (see Fig. 5). We then examined transcript levels of Fcy-γ following overnight treatment with Pam2CSK4. The results (Fig. 2B) showed increases in FcyRIIa (left panel), the γ-chain (center panel), and FcyRIIb (right panel). These results suggest that TLR2 activation can up-regulate expression of FcyR.

TLR2 Modulates miR-155 and SHIP—Results showed that Pam2CSK4 significantly enhanced FcyR function despite also leading to up-regulation of the inhibitory FcyRIIb. This suggests that FcyRIIb activity is suboptimal, perhaps because of alteration of downstream mediators. Hence, we examined the expression of SHIP, the inositol phosphatase responsible for the majority of FcyRIIb-mediated FcyR inhibition (25–27). Following overnight treatment of PBM with Pam2CSK4, the transcript for SHIP was significantly reduced (Fig. 3A). Because recent studies implicated the microRNA miR-155 as a regulator of SHIP expression (28–30), we also examined the response of miR-155 to Pam2CSK4. As shown in Fig. 3B, Pam2CSK4 significantly up-regulated miR-155. These results suggest that, although this TLR2 agonist may increase expression of FcyRIIb, it also down-regulates the major downstream mediator of this inhibitory FcyR.

Dose Response to Pam2CSK4—To determine the lowest effective concentration of Pam2CSK4 and to see whether treatment with greater than 100 ng/ml (used above) would lead to still further FcyR enhancements, we treated PBM overnight...
with Pam2CSK4 at concentrations ranging from 0–200 ng/ml. PBM were then tested for phagocytic ability using fluorescein-ated, opsonized SRBC. Results showed that as little as 1 ng/ml Pam2CSK4 was sufficient to drive increases in phagocytic ability (Fig. 4A). We also treated with dosages from 0–200 ng/ml followed by incubation for 24 h with or without immobilized IgG. TNFα levels in cleared supernatants were measured and compared between dosages. Results, in accordance with those from the phagocytosis assay, showed that as little as 1 ng/ml could significantly enhance IgG-mediated cytokine production (Fig. 4B). We also measured FcγR expression by Western blotting, and as shown in Fig. 5A, levels as low as 0.1 ng/ml led to greater expression of activating FcγRIIa (top panel) and the γ-chain (center panel) as well as modestly up-regulating FcγRIIb (bottom panel). As in Fig. 2, there was some donor variability with regard to changes in FcγRIIb expression. We tested dosages as high as 200 ng/ml and found that changes in FcγRI were minimal above the 1–5 ng/ml range (Fig. 5B). Hence, we conclude that as little as 0.1 ng/ml of Pam2CSK4 is sufficient to significantly modulate expression of monocyte FcγR. Dosages higher than this led to minimal further enhancements and, possibly, to small declines.

**FIGURE 2.** Pam2CSK4 influences FcγR expression. PBM were treated with or without Pam2CSK4 (100 ng/ml) for 24 h at 37 °C. A, protein-matched cell lysates were analyzed by Western blotting (IB) for Fcγ receptors. All membranes were reprobed with actin antibody to ensure equivalent loading of protein in all lanes. The line graphs show the densitometric ratio of FcγR to actin for three donors. B, RNA was extracted from treated versus untreated PBM, and FcγR was analyzed using real-time RT-PCR for FcγRIIa, the common γ subunit, and FcγRIIb. Both Cap-1 and GAPDH were measured for all samples as a base line. The graphs include data obtained from three independent donors. Data were analyzed by Student’s t test. *, p < 0.05. UT, untreated; PC, Pam2CSK4.

**FIGURE 3.** Pam2CSK4 modulates SHIP and miR-155. PBM were treated overnight with or without 100 ng/ml Pam2CSK4 at 37 °C. Then, RNA was extracted and analyzed for SHIP (A) and miR-155 (B) transcripts using real-time RT-PCR. Relative copy number (RCN), plotted on the y axes, was calculated against housekeeping controls as described under “Experimental Procedures.” The graphs represent data obtained from three independent donors. Data were analyzed by Student’s t test. *, p < 0.05. UT, untreated.
Murine Macrophages Respond to Pam2CSK4—We then sought to determine whether the murine system would provide a good model in which to test the effect of Pam2CSK4 on FcγR and antibody therapy. To do this, it was first necessary to examine the effects of this agonist on murine FcγR function. To do this, we treated mouse BMM overnight with or without 100 ng/ml of Pam2CSK4 and then tested the BMM in a phagocytosis assay. As anticipated, agonist treatment significantly enhanced the phagocytic ability of BMM (Fig. 6A). We also treated overnight with or without Pam2CSK4 and then incubated the BMM for 24 h with or without immobilized IgG. Supernatants were collected and analyzed for TNFα levels by ELISA. Results showed that Pam2CSK4 increased IgG-mediated TNFα production in these murine cells (Fig. 6B). These experiments suggest that murine cells respond similar to human cells when treated with TLR2 agonist and that the murine system would be a suitable model to test the effect of this agonist on antibody therapy in vivo.

Pam2CSK4 Enhances Antibody Therapy in Vivo—We next tested whether Pam2CSK4 could enhance antibody-mediated antitumor responses using a murine model of antibody therapy (22–24). Here, syngeneic CT26 colon adenocarcinoma cells engineered to express human Her2/neu were injected into the flanks of Balb/cJ mice. Following tumor development, intraperitoneal injections of the 4D5 anti-Her2 antibody with or without Pam2CSK4 were done, and the rate of tumor growth was measured for ~18 days. Using this model, we found that cotreatment with Pam2CSK4 and 4D5 antibody was significantly better than antibody alone at slowing the rate of tumor growth (Fig. 7). These results suggest that Pam2CSK4 could potentially serve as an effective adjuvant for antitumor antibody therapy.

Pam3CSK4 Also Enhances FcγR Function—We have performed these studies using the diacylated Pam2CSK4, but the related triacylated TLR2 agonist Pam3CSK4 is also being investigated as a putative immunomodulatory agent. Hence, we wished to determine whether this agonist, similar to Pam2CSK4 in structure but leading to TLR2/TLR1 heterodimerization instead of TLR2/TLR6, could lead to similar changes in FcγR expression and function. To begin, we tested function by treating BMM overnight with or without 100 ng/ml Pam3CSK4 and measuring phagocytic ability. Results showed that Pam3CSK4 significantly enhanced the number of targets ingested (Fig. 8A). We then treated BMM overnight with 0, 5, 10, or 100 ng/ml Pam3CSK4 and further incubated cells with or without immobilized IgG for an additional 24 h. ELISAs of cleared supernatants showed that the lowest dose of 5 ng/ml could strongly increase IgG-mediated cytokine production (Fig. 8B). We also measured levels of SHIP and miR-155 after overnight treatment of BMM with 5 ng/ml Pam3CSK4. Pam3CSK4 led to a trend toward decreased SHIP, with 60–65% decreases in two donors and a 10% increase in one donor (p = 0.11, Fig. 8C). However, there was a significant increase in miR-155 in the same three donors (Fig. 8D).

As with Pam2CSK4, we tested the effects of increasing Pam3CSK4 dosages on FcγR expression. Results showed that levels as low as 0.1 ng/ml could increase expression of FcγRIIa (Fig. 8A, top panel), and dosages between 1 and 5 ng/ml increased γ-chain expression (Fig. 9A, bottom panel). As with
Pam2CSK4, dosages above 5 ng/ml did not lead to further increases (Fig. 9B). These results suggest that TLR2 activation itself, whether through heterodimerization of TLR2 with TLR1 (Pam3CSK4) or, as with Pam2CSK4, through TLR6- (31) or non-TLR6-containing complexes (32), can drive positive changes in FcγRI expression and function.

**DISCUSSION**

Immunomodulatory agents have been extensively studied for their potential to enhance antitumor responses. Here we have shown that the TLR2 agonist Pam2CSK4 and the related triacylated Pam3CSK4 can modulate both the expression and function of FcγR. Both compounds led to up-regulation of all FcγR including, to some degree, the inhibitory FcγRIIb. Pam2CSK4 increased monocyte phagocytic ability in vitro and significantly enhanced the effect of antitumor antibody treatment in vivo.

Although the TLR2 agonists up-regulated the activating FcγR, they also led to increases in the inhibitory FcγRIIb. These higher levels of FcγRIIb should have resulted in at least partially attenuated FcγR activity. However, although expression of FcγRIIb was elevated, we simultaneously observed consistent decreases in the inositol phosphatase SHIP (Fig. 3A), which mediates the inhibitory function of FcγRIIb. Hence, FcγRIIb function was likely compromised despite modestly increased expression. In this sense, the up-regulated FcγRIIb would have likely functioned primarily to enhance binding to opsonized target, as its downstream inhibitory functions were compromised.

Up-regulation of miR-155 appears to be a characteristic of TLR activation, as agonists for TLR2, TLR3, TLR4, and TLR9 (33), as well as TLR7/8 (34), induce miR-155 expression. Within the context of FcγR, the concomitant down-regulation of SHIP (28) following miR-155 induction would permit stronger signaling events, leading to more effective responses such as cytokine release and phagocytosis. Although clearly not the only effect of TLR2 activation, this miR-155-SHIP response is likely one of the major mechanisms of enhanced FcγR response. Indeed, the importance of SHIP as a negative regulator within the monocyte FcγR pathway has been well documented (35–37).

The response of FcγRIIb is also interesting in that it is in stark contrast to results seen with the TLR7/8 agonist R-848. (24) R-848 led to rapid decreases in FcγRIIb protein, followed by a decrease in transcript. Pam2CSK4, conversely, elicited modest increases in FcγRIIb protein as well as marked increases in tran-
script. Results were similar when testing Pam3CSK4, suggesting that the difference is more related to TLR2 itself than to which TLR2-binding partner was employed. This is in agreement with Farhat et al. (13), who found that TLR2/1 and TLR2/6 activation led to similar downstream signaling activities. Regarding TLR2 versus TLR7/8, perhaps a kinomics approach comparing the intracellular signaling events downstream of the different TLR would shed light on why activation of one but not the other TLR would down-regulate FcγRIIb.

In summary, we have found that TLR2 agonists are effective agents for enhancing FcγR expression and function. These agonists are currently being examined as putative agents to aid with vaccine efficacy (14), ischemia/reperfusion injuries (15), sepsis (16), and allergies (17). Results from this study suggest that they are powerful modulators of FcγR and should, therefore, also be tested as candidate adjuvants for antitumor antibody therapy.

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