Antibodies to proteinase 3 (PR3-ANCA) prime human oral, lung, and kidney epithelial cells through protease-activated receptor-2 to secrete proinflammatory cytokines upon stimulation with agonists to various Toll-like receptors, NOD1 and NOD2

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Running title: Priming effects of PR3-ANCA in epithelial cells

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Abbreviations: ANCA, anti-neutrophil cytoplasmic antibodies; WG, Wegener’s granulomatosis; TNF-α, tumor necrosis factor-α, IL, interleukin; MCP-1, monocyte chemoattractant protein-1; LPS, lipopolysaccharide; PAR, protease-activated receptor; PAMPs, pathogen-associated molecular patterns; PGNs, peptidoglycans; TLR, Toll-like
receptor; NLR, NOD-like receptor; DAP, diaminopimelic acid; MDP, muramyldipeptide; CDS, non-enzymatic cell dissociation solution; si, short interfering; AP, agonist peptide
ABSTRACT

Anti-neutrophil cytoplasmic antibodies (ANCA) are autoantibodies, the detection of which in sera can be used in the diagnosis of Wegener’s granulomatosis (WG). Proteinase 3 (PR3) is a major target antigen of ANCA in WG patients and the interaction of PR3-ANCA with leukocytes causes a debilitating autoimmune disease. The first signs and symptoms in WG patients are observed in the oral cavity, lungs, and kidneys. Human epithelial cells generally do not secrete proinflammatory cytokines upon stimulation with pathogen-associated molecular patterns (PAMPs). In this study, anti-PR3 Abs and PR3-ANCA sera from WG patients endowed human oral, lung, and kidney epithelial cells with a responsiveness to PAMPs in terms of the production of proinflammatory cytokines such as IL-6, IL-8, MCP-1 and TNF-α. Protease-activated receptor (PAR)-2 agonist peptides mimicked the priming effects of PR3-ANCA against PAMPs. Furthermore, the anti-PR3 Ab-mediated cell activation was significantly abolished by RNA interference targeting PAR-2 and NF-κB. This is the first report of priming effects of anti-PR3 Abs (PR3-ANCA) in epithelial cells. The results suggest that anti-PR3 Abs (PR3-ANCA) prime human epithelial cells to produce cytokines upon stimulation with various PAMPs, and these mechanisms might be involved in severe chronic inflammation in WG.
Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) form a heterogenous group of Abs that target antigens present mostly in azurophilic granules of polymorphonuclear leukocytes. ANCA were first discovered in the 1970s, when cytoplasmic fluorescence was observed during investigations of anti-nuclear Ab using indirect fluorescence in human granulocytes (33). In the 1980s, the spectrum of diseases associated with ANCA became clearer, and vasculitis (7, 14) was identified as a common sign of these diseases. Proteinase 3 (PR3) (21) and myeloperoxidase (MPO) (9) have been identified as two of the main targets of ANCA. PR3-ANCA have been reported to be causally involved in the pathogenesis of Wegener’s granulomatosis (WG); the autoantibody titer correlates with disease activity (27, 32). Anti-PR3 Abs (PR3-ANCA) directly activate a wide variety of inflammatory functions in leukocytes, such as the secretion of cytokines (tumor necrosis factor [TNF]-α and interleukin [IL]-1β, IL-6, IL-8, monocyte chemoattractant protein [MCP]-1), oxygen radicals, proteases, and lipid mediators, once PR3 is expressed on the surface under inflammatory conditions (4, 10, 13, 15, 22, 23, 25, 26). Anti-PR3 Abs provoked a marked release of cytokines in human monocytes with the early appearance of TNF-α and IL-1β and the delayed release of IL-6, IL-8 and thromboxane A₂ (15). In addition, anti-PR3 Abs induced the release of MCP-1 from human mononuclear cells (23). Hattar et al. (16) reported that PR3 was detected in human renal tubular epithelial cells treated with TNF-α, and primed cells responded to anti-PR3 Abs with the activation of a phosphoinositide-related signal transduction pathway. Recently, Bartůňková et al (3) reported that the interaction of PR3-ANCA with TNF-α-primed
mononuclear cells stimulated the release of IL-8 via cross-linking between Fc gamma receptors and PR3 expressed on the monocyte cell surface. Hattar et al. (17) demonstrated a priming effect of PR3-ANCA for the activation of isolated monocytes and neutrophils by bacterial cell-surface components such as lipopolysaccharide (LPS) and lipoteichoic acid. Although the incubation of monocytes and neutrophils with ANCA alone resulted in only a low level of IL-8 release, preincubation with ANCA resulted in a markedly enhanced release of IL-8 upon stimulation with LPS. Recently, we revealed that a murine anti-human PR3 monoclonal Ab primed human monocytic THP-1 cells for enhanced activation upon stimulation with various microbial components (30). These results indicated that PR3-ANCA specifically prime leukocytes and the resulting enhanced responsiveness to bacterial components may contribute to the development and maintenance of inflammatory lesions of WG.

We revealed that proinflammatory cytokines, such as IL-1α, IFN-α, IFN-β and IFN-γ, induced production of PR3 in membrane-bound and secretory forms in human oral epithelial cells, and the addition of anti-PR3 Abs to cytokine-primed oral epithelial cells in culture induced the aggregation of PR3 followed by the activation of protease-activated receptor (PAR)-2, which resulted in remarkable secretion of IL-8 and MCP-1 (31). PAR family members are G protein-coupled receptors characterized by a proteolytic cleavage of the N terminus that exposes tethered ligands and autoactivates the receptor function (6, 8, 24). There are four members of this family. PAR-2 is activated by trypsin and mast cell tryptase as well as coagulation factors VIIa and Xa. Because PARs are expressed on a wide variety
of cell types, including neutrophils, they are believed to play important roles in several pathophysiological processes, including growth, development, inflammation, tissue repair and pain.

The innate immune system recognizes microorganisms through a series of pattern recognition receptors that have been highly conserved during evolution, specific for common motifs found in microorganisms but not in eukaryotes, and designated as pathogen-associated molecular patterns (PAMPs) (1, 18, 20). Representative microbial PAMPs are the lipid A moiety of LPS, lipopeptides, peptidoglycans (PGNs), and viral double-stranded and single-stranded RNA. Akira et al. demonstrated that these PAMPs are recognized specifically by the respective Toll-like receptor (TLR) (2). In addition, some NOD-like receptor (NLR) family members were demonstrated to be intracellular receptors for partial structures of PGN; NOD1 and NOD2 recognize a diaminopimelic acid (DAP)-containing peptide moiety (5, 11, 29) and a muramyldipeptide moiety (12, 19), respectively.

The first signs and symptoms in WG patients are in the oral, lung, and kidney epithelium. Human epithelial cells including those of the oral cavity, lung, and kidney generally do not secrete proinflammatory cytokines upon stimulation with PAMPs (28). In this study, we examined whether anti-PR3 Abs (PR3-ANCA) were capable of priming human oral, lung, and kidney epithelial cells. If they are, the mechanism might be involved in the pathogenesis of ANCA-related inflammatory diseases represented by WG.

MATERIALS AND METHODS
Reagents. Synthetic muramyl dipeptide (MDP; MurNAc-L-Ala-D-isoGln) and an Escherichia coli-type lipid A (LA-15-PP) were purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). Double-stranded Poly I:C was obtained from Sigma-Aldrich (St. Louis, MO). Single-stranded Poly U was purchased from Invivogen (San Diego, CA). A conventional CpG DNA, CpG DNA 1826 (TCCATGACGTTCCTGACGTT [CpG motif is underlined]), was provided by SIGMA Genosys (Tokyo, Japan). A synthetic Mycoplasma-type diacyl lipopeptide FSL-1 (S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-GDPKHPKSF) was purchased from EMC microcollections (Tübingen, Germany). The synthetic desmurmamylpeptides, a PGN fragment containing DAP, FK156 (d-lactoyl-L-Ala-γ-D Glu-meso-DAP-Gly) and its derivative, FK565 (heptanoly-γ-D-Glu-meso-DAP-D-Ala), were supplied by Astellas Pharmaceutical Co. (Tokyo, Japan). Recombinant human IL-1α and TNF-α were provided by Dainippon Pharmaceutical Co. (Osaka, Japan). PAR-2AP (SLIGKV) was synthesized by Takara (Otsu, Japan). Non-enzymatic cell dissociation solution (CDS) was obtained from Sigma-Aldrich. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Serum samples. ANCA sera were obtained from 4 patients with WG at Tohoku University Hospital. The samples were immediately purified by centrifugation, aliquoted, and frozen at –70°C until used. PR3-ANCA and MPO-ANCA titers were re-confirmed by the EIA method by BML Co. (Sendai, Japan). All of the ANCA sera were
PR3-ANCA-positive and MPO-ANCA-negative specimens. Normal serum from a healthy adult donor was used as a control.

**Cells and cell culture.** The human oral epithelial cell line HSC-2, human lung epithelial cell line A549, and human kidney epithelial cell line Caki-1 were obtained from the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). These cells were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% heat-inactivated FCS with a change of medium every 3 days. To avoid cell surface markers, we used Sigma’s CDS. CDS contains no protein and allows the dislodging of cells without enzymatic modification or the adsorption of foreign proteins.

**Cytokine measurements.** To investigate the production of inflammatory cytokines by epithelial cells, we collected the supernatant from each culture. The production of cytokines (IL-6, IL-8, MCP-1, and TNF-α) was measured using OptEIA ELISA kits (PharMingen, San Diego, CA). The concentrations of the cytokines in the supernatants were determined using the LS-PLATEmanager 2004 data analysis program (Wako Pure Chemical Industries, Osaka, Japan).

**RNA Interference.** Transfections for targeting endogenous PAR-2, NF-κB p65 and Lamin A/C were carried out with Lipofectamine 2000 (Invitrogen, Carlsbad) and short interfering (si) RNA (final concentration, 200 nM) for 24 h at 37°C, according to the manufacturer’s instructions. The viability of the cells after transfection was more than 95%, as assessed by a 0.2% trypan blue exclusion test, and the cells’ morphological character was un-changed after transfection. siRNAs for PAR-2 and NF-κB p65 were purchased from
Santa Cruz Biotechnology, and the siRNA of Lamin A/C was purchased from B-Bridge International.

**Results**

**Human oral, lung, and kidney epithelial cells do not secrete proinflammatory cytokines upon stimulation with PAMPs.** We previously reported that various human epithelial cells did not secrete proinflammatory cytokines (28). As shown in Fig. 1, human oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells did not secrete IL-8 upon stimulation with synthetic PAMPs; FSL-1 (TLR2 agonist), Poly I:C (TLR3 agonist), lipid A (TLR4 agonist), Poly U (TLR7 agonist), CpG DNA (TLR9 agonist), FK156/565 (NOD1 agonist), and MDP (NOD2 agonist). In contrast, human epithelial cells secreted IL-8 in response to TNF-α and IL-1α as positive controls.

**Treatment with anti-PR3 Abs primed human oral, lung and kidney epithelial cells to secrete IL-6, IL-8, MCP-1 and TNF-α upon stimulation with PAMPs.** We demonstrated that anti-PR3 Abs enhanced TLR- and NOD-agonistic PAMP-induced secretion of proinflammatory cytokines in human monocytic THP-1 cells and human PBMCs (30). We examined the production of inflammatory cytokines upon stimulation with PAMPs after priming with anti-PR3 Abs in human epithelial cells. When human oral, lung, and kidney epithelial cells were preincubated with 1 µg/ml of anti-PR3 Abs for 6 h, and subsequently challenged with the various TLR- and NOD-agonistic PAMPs for a further 18 h, massive production of IL-8 was observed, whereas stimulation with the anti-PR3 Abs by themselves
scarcely had any effect (Fig. 2). Priming effects were also observed for the production of IL-6, MCP-1 and TNF-α (Fig. 3).

**PR3-ANCA serum primed human oral, lung and kidney epithelial cells to secrete IL-8 upon stimulation with PAMPs.** To further demonstrate the possible immunopathological properties of ANCA from WG patients, we examined whether the ANCA sera are capable of priming human epithelial cells similarly to murine anti-PR3 Abs. We obtained sera from patients with WG at Tohoku University Hospital, and carried out experiments. The titers of PR3-ANCA and MPO-ANCA in serum were determined. All sera from 4 patients were PR3-ANCA positive (> 3.0 titer) whereas none was MPO-ANCA positive (<1.3 titer). In this study, PR3-ANCA serum from WG patients (S9-32) was compared with normal serum. We examined the production of IL-8 upon stimulation with TLR- and NOD-agonistic PAMPs. IL-8 production was significantly induced when human oral, lung, and kidney epithelial cells were incubated with the patients’ serum (closed bar) as compared with normal serum (open bar) (Fig. 4). These results clearly indicated that PR3-ANCA in the serum from WG patients exerted a priming effect similar to murine monoclonal anti-human PR3 Abs in vitro.

**Treatment with PAR-2 agonist peptide (AP) primed human oral, lung and kidney epithelial cells to secrete IL-8 upon stimulation with PAMPs.** As we found that anti-PR3 Abs primed human monocytic cells via PAR-2 (30), we examined whether priming effects of PAR-2AP also occurred in human epithelial cells. Clear priming effects by PAR-2AP on epithelial cells in response to TLR- and NOD-agonistic PAMPs were observed
concerning IL-8 production (Fig. 5).

**Priming effect of anti-PR3 Abs occurred in a PAR-2- and NF-κB-dependent manner.** To clarify the signaling pathway of the priming effects of anti-PR3 Abs upon stimulation with TLR- and NOD-agonistic PAMPs, we utilized RNA interference assays targeting PAR-2 and NF-κB p65. PAR-2 and NF-κB p65 protein levels determined by flow cytometry were suppressed by ca. 80% using specific siRNA in the cells up to 72 h (28, 30). As shown in Fig 6, the priming effects induced by anti-PR3 Abs were almost completely inhibited in PAR-2- and NF-κB-silenced cells, but not in Lamin siRNA-silenced cells. These results demonstrated that the priming effect of anti-PR3 Abs occurred in a PAR-2- and NF-κB-dependent manner.

**DISCUSSION**

Among ANCA, those targeting PR3 (PR3-ANCA) have a strong and specific association with WG (27, 32). Besides their significance as seromarkers, a pathogenic role has been proposed for these autoantibodies in relation to their capacity to activate leukocytes in vitro (4, 10, 13, 15, 22, 23, 25, 26). We previously reported that incubation with anti-PR3 Abs significantly up-regulated the production of proinflammatory cytokines upon stimulation with various PAMPs (30). The first signs and symptoms in WG patients are in the oral cavity, lungs, and kidneys. In the present study, an alternative approach was chosen to define the priming effect of ANCA against PR3 (PR3-ANCA) from WG patients on human oral, lung, and kidney epithelial cells; the epithelial cells were preincubated with substimulatory
concentrations of anti-PR3 Abs or PR3-ANCA and their possible activation by various TLR- and NOD-agonistic PAMPs was examined. The epithelial cells generally did not secrete proinflammatory cytokines (Fig. 1). Surprisingly, preincubation with anti-PR3 Abs primed them to secrete proinflammatory cytokines (Figs. 2-3). In addition, PR3-ANCA serum from WG patients, but not control serum, primed the cells to secrete proinflammatory cytokines similar to anti-PR3 Abs (Fig. 4). These results indicated that PR3-ANCA in the serum from WG patients exerted a clear priming effect similar to anti-PR3 Abs in vitro and strengthen our conclusion and the relevance of our study to the human situation.

Concerning signaling pathways, anti-PR3 Abs activated human cells via PAR-2 and NF-κB in a TLR- and NOD-dependent manner (30, 31). PAR-2AP had a similarly potent priming effect to anti-PR3 Abs (Fig. 5) and the effects by anti-PR3 Abs also occurred through PAR-2 and NF-κB in oral, lung, and kidney epithelial cells (Fig. 6). We solely used chemically synthesized PAMPs, because natural microbial components are inevitably contaminated with minor bioactive components that might have affected the results. Therefore, these results clearly indicated that anti-PR3 Abs primed human epithelial cells for TLR- and NOD-dependent cell activation.

It is conceivable that microbial components (PAMPs) exhibit powerful immuno-adjuvant activities against various antigens, including autoantigens, through TLR and NOD pathways, which in turn might induce severe autoimmune diseases. In the important roles of PR3-ANCA in the regulation of inflammatory leukocyte functions, PR3-ANCA, being only weak direct activators of monocytes and neutrophils to release
cytokines per se, exert a definite priming effect on these leukocytes, enhancing their responsiveness to secondary stimulation with PAMPs (30). In this study, we first reported that oral, lung, and kidney epithelial cells were primed to secrete proinflammatory cytokines by anti-PR3 Abs upon stimulation with PAMPs, whereas these epithelial cells normally did not produce proinflammatory cytokines in response to PAMPs. The first signs and symptoms in WG patients are severe inflammation in the oral cavity, lungs and kidneys. Such cooperation between PR3-ANCA and PAMPs may well trigger exacerbations of WG during infections and contribute to the persistence of inflammatory lesions, which might be a novel model for the pathogenesis of WG.
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REFERENCES


express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. Mol Immunol 44:3100-3111.


Figure Legends

FIG. 1. Human oral, lung, and kidney epithelial cells did not secrete IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were stimulated with FSL-1 (1 nM), Poly I:C (10 µg/ml), lipid A (10 ng/ml), Poly U (10 µg/ml), CpG DNA (10 nM), FK156 (100 µg/ml), FK565 (100 µg/ml), MDP (100 µg/ml), TNF-α (10 ng/ml), or IL-1α (10 ng/ml) for 24 h in triplicate. Human TNF-α and IL-1α were used as positive controls. The levels of IL-8 in the culture supernatants were determined using ELISA. Data are expressed as mean values ± SD. *P < 0.01 vs. medium alone. The results are representative of three different experiments demonstrating similar results.

Fig. 2. Human oral, lung, and kidney epithelial cells preincubated with anti-PR3 Abs secreted IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were preincubated for 6 h with anti-PR3 Abs (1 µg/ml; closed bar), with equal amounts of an isotype-matched IgG (open bar). Subsequently, the cells were stimulated with FSL-1 (1 nM), Poly I:C (10 µg/ml), lipid A (10 ng/ml), Poly U (10 µg/ml), CpG DNA (10 nM), FK156 (100 µg/ml), FK565 (100 µg/ml), or MDP (100 µg/ml) for 24 h in triplicate. The levels of IL-8 in the culture supernatants were determined using ELISA. Data are expressed as mean values ± SD. *Significantly different from control IgG-incubated cells and from respective cultures (P < 0.01). The results are representative
of three different experiments demonstrating similar results.

 Fig. 3. Human oral, lung, and kidney epithelial cells preincubated with anti-PR3 Abs secreted IL-6, MCP-1, and TNF-α in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were preincubated for 6 h with anti-PR3 Abs (1 µg/ml; closed bar), with equal amounts of an isotype-matched IgG (open bar). Subsequently, the cells were stimulated with FSL-1 (1 nM), Poly I:C (10 µg/ml), lipid A (10 ng/ml), Poly U (10 µg/ml), CpG DNA (10 nM), FK156 (100 µg/ml), FK565 (100 µg/ml), or MDP (100 µg/ml) for 24 h in triplicate. The levels of IL-6, MCP-1, and TNF-α in the culture supernatants were determined using ELISA. Data are expressed as mean values ± SD. *Significantly different from control IgG-incubated cells and from respective cultures (P < 0.01). The results are representative of three different experiments demonstrating similar results.

 Fig. 4. Human oral, lung, and kidney epithelial cells preincubated with PR3-ANCA serum secreted IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were preincubated for 6 h with 1:100 dilutions of PR3-ANCA serum from a WG patient (closed bar) or with equal amounts of normal serum (open bar). Subsequently, the cells were stimulated with FSL-1 (1 nM), Poly I:C (10 µg/ml), lipid A (10 ng/ml), Poly U (10 µg/ml), CpG DNA (10 nM), FK156 (100 µg/ml), FK565 (100 µg/ml), or MDP (100 µg/ml) for 24 h in triplicate. The levels of IL-8 in the culture supernatants were determined using ELISA. Data are expressed as mean values ± SD. *Significantly different from control IgG-incubated cells and from respective cultures (P < 0.01). The results are representative of three different experiments demonstrating similar results.
supernatants were determined using ELISA. Data are expressed as mean values ± SD.

*Significantly different from normal serum-incubated cells and from respective cultures (*P* < 0.01). The results are representative of three different experiments demonstrating similar results.

Fig. 5. Human oral, lung, and kidney epithelial cells preincubated with PAR-2AP secreted IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were preincubated for 6 h with PAR-2AP (10 nM; closed bar) or with equal amounts of control peptides (open bar). Subsequently, the cells were stimulated with FSL-1 (1 nM), Poly I:C (10 µg/ml), lipid A (10 ng/ml), Poly U (10 µg/ml), CpG DNA (10 nM), FK156 (100 µg/ml), FK565 (100 µg/ml), or MDP (100 µg/ml) for 24 h in triplicate. The levels of IL-8 in the culture supernatants were determined using ELISA. Data are expressed as mean values ± SD. *Significantly different from control peptide-incubated cells and from respective cultures (*P* < 0.01). The results are representative of three different experiments demonstrating similar results.

Fig. 6. The priming effect of anti-PR3 Abs occurred in a PAR-2- and NF-κB p65-dependent manner. Oral epithelial HSC-2, lung A549, and kidney Caki-1 cells were transfected with PAR-2- (gray bar), NF-κB p65- (black bar), or Lamin A/C (white bar)-specific-siRNA. After 24 h, the transfected cells were preincubated for 6 h with anti-PR3 Abs (1 µg/ml). Subsequently, the cells were stimulated with FSL-1 (1 nM), Poly I:C (10 µg/ml), lipid A (10
ng/ml), Poly U (10 µg/ml), CpG DNA (10 nM), FK156 (100 µg/ml), FK565 (100 µg/ml), or MDP (100 µg/ml) for 24 h in triplicate. The levels of IL-8 in the culture supernatants were determined using ELISA. Data are expressed as mean values ± SD. *,# Values differed significantly from those obtained with medium alone or respective cultures stimulated with the indicated ligands, respectively (P < 0.01). The results presented are representative of three different experiments demonstrating similar results.
Figure 1

HSC-2 cells
A549 cells
Caki-1 cells

* none
F
SL-1
poly I:C
lipid A
poly U
CpG DNA
FK156
FK565
MDP
TNF-α
IL-1α

0 0.5 1 1.5 2
IL-8 (ng/ml)

2.5

Downloaded from http://cvi.asm.org/
Figure 2

[Bar chart showing IL-8 levels in different cell lines (Caki-1, A549, HSC-2) treated with various compounds (MDP, FK565, FK156, poly I:C, lipid A, poly U, CpG DNA, FK156, FK565, poly I:C, FSL-1) and treatment conditions (none, Control Ab, α-PR3 Ab). Significant differences are indicated by asterisks (*) and double asterisks (**) for different conditions.]
Figure 3

(a) HSC-2 cells
(b) A549 cells
(c) Caki-1 cells

Control Ab □, α-PR3 Ab ■
Figure 4
Figure 5

Graphs showing the IL-8 (ng/ml) levels in HSC-2, A549, and Caki-1 cells treated with various control peptides and PAR-2AP. The treatments include FSL-1, poly I:C, lipid A, poly U, CpG DNA, FK156, FK565, and MDP. The asterisks (*) indicate statistically significant differences.
Figure 6