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Antibody-Opsonized Bacteria Evoke an Inflammatory Dendritic Cell Phenotype and Polyfunctional Th Cells by Cross-Talk between TLRs and FcRs

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During secondary immune responses, Ab-opsonized bacteria are efficiently taken up via FcRs by dendritic cells. We now demonstrate that this process induces cross-talk between FcRs and TLRs, which results in synergistic release of several inflammatory cytokines, as well as altered lipid metabolite profiles. This altered inflammatory profile redirects Th1 polarization toward Th17 cell responses. Interestingly, GM-CSF–producing Th cells were synergistically evoked as well, which suggests the onset of polyfunctional Th17 cells. Synergistic cytokine release was dependent on activation via MyD88 and ITAM signaling pathways through TLRs and FcRs, respectively. Cytokine regulation occurred via transcription-dependent mechanisms for TNF-α and IL-23 and posttranscriptional mechanisms for caspase-1–dependent release of IL-1β. Furthermore, cross-talk between TLRs and FcRs was not restricted to dendritic cells. In conclusion, our results support that bacteria alone initiate fundamentally different immune responses compared with Ab-opsonized bacteria through the combined action of two classes of receptors and, ultimately, may refine new therapies for inflammatory diseases.

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Abbreviations used in this article: AA, arachidonic acid; BSA-B, BSA-coated beads; COX, cyclooxygenase; DC, dendritic cell; iDC, immature DC; IgG-B, IgG-coated beads; inDC, inflammatory DC; LOD, limit of detection; LOX, lipoxygenase; LTB4, leukotriene B4; LXA4, lipoxin A4; PGN-SA, peptidoglycan of S. aureus; PKC, protein kinase C; PMN, polymorphonuclear cell; Poly-L-C, polynonsic acid-polyvalidic acid C; PRR, pathogen recognition receptor; Syk, spleen tyrosine kinase; TXB, thromboxane B.

As such, an additional class of receptors, referred to as FcRs, becomes an important part of the immune response by binding opsonizing Abs (10–12). FcRs are expressed on several immune cell types. For example, DCs express several FcγR subclasses that bind IgG, of which FcγRII (CD32) is the most prominent (11, 12). FcγRII is subdivided into FcγRIIA, which is an activating receptor through ITAM signaling, and FcγRIIB, which contains ITIM and, hence, is an inhibitory receptor. FcR-induced effector functions include phagocytosis, endocytosis, Ab-dependent cellular cytotoxicity, superoxide production, release of cytokines, calcium mobilization, and Ag presentation (10, 13).

Intriguingly, the individual involvement of different types of PRRs or FcRs during bacterial infection has been well established. However, combined activation of these different receptor classes during encounter with Ab-opsonized bacteria has received little attention.

Therefore, we investigated the initiation of immune responses after simultaneous recognition of Ab-opsonized pathogens by PRRs and FcRs on monocyte-derived immature DCs (iDCs), because DCs are the most efficient APCs and secrete a plethora of cytokines and lipid mediators that direct polarization of distinct Th subsets (2).

Th1-mediated responses are induced in the presence of IL-12 (14). Th1 cells, in turn, release IFN-γ, which primarily activates and recruits cytotoxic T lymphocytes, NK cells, and macrophages. Th2 cells that produce IL-4, IL-5, and IL-13 recruit eosinophils and mast cells. Th17 cell differentiation is initiated upon release of IL-1β and sustained via IL-23 (15). Th17 cells stimulate granulopoiesis, because IL-17 induces production of granulocyte-stimulating factor, which favors an increased presence of neutrophils at sites of infection. Recently, it was found that GM-CSF–producing Th cell subsets are involved in the initiation of autoimmune disease (16).

Lipid mediators, such as arachidonic acid (AA)-derived eicosanoids, regulate a variety of processes like cytokine production, Ab formation, Th cell–polarizing ability, and Ag presentation (17–19).

We identified cross-talk between TLRs and FcRs when bacteria were opsonized with Abs. This cross-talk induced synergistic release of inflammatory cytokines and a unique lipid metabolite
profile. The altered inflammatory profile was regulated on both transcriptional and posttranscriptional levels, depending on the distinct cytokine. Importantly, release of this distinct inflammatory mediator profile by DCs redirected DC-induced Th1 responses toward induction of polyfunctional Th cells producing both IL-17 and GM-CSF (20). Finally, combined activation of TLRs and FcRs on iDCs induced functional characteristics that were similar to those described for inflammatory DCs (nDCs). This novel subset was identified recently in synovial fluid of rheumatoid arthritis patients, as well as in inflammatory tumor ascites (21).

In conclusion, this study reveals the distinct impact on cells of the innate immune system, which are simultaneously activated via TLRs and FcRs, as well as the subsequent effects on the initiation of adaptive-immune responses. Thus, our results emphasize the fundamental difference between the development of primary and secondary immune responses.

Materials and Methods

**Bead coating**

CNBr-activated Sepharose beads (GE Healthcare Life Sciences, Uppsala, Sweden) were coupled with 3 μg purified serum IgG (Sigma-Aldrich, St. Louis, MO) or BSA (Roche Diagnostics, Basel, Switzerland), according to the manufacturers’ instructions. IgG purity was tested by SDS electrophoresis and was ≥95%.

**Isolation and culture of monocyte-derived DCs**

PBMCs were isolated from heparinized blood or buffy coats (Sanquin, Amsterdam, the Netherlands) by density gradient centrifugation using LymphoPrep (Axis-Shield, Oslo, Norway). Monocytes were obtained using a CD14 selection step with magnetic anti-CD14 MicroBeads and MACS system (Milteny Biotec, Bergisch Gladbach, Germany). Monocytes were differentiated into iDCs by culturing for 7 d in complete medium (RPMI 1640 medium). Cells were used to determine phagocytosis of bacteria by flow cytometry (FACSCalibur; Becton Dickinson, San Diego, CA) or LPS (purified by gel-filtration chromatography (Acquity; Ultra performance LC, Waters) and negative-mode electrospray ionization tandem mass spectrometry (Xevo Waters), essentially as described (22), with the following modification: sample was extracted by liquid-liquid extraction using ethylacetate-butyl ether. Chromatographic separation was achieved on a Synergi Hydro-RP column (4 μm, 2.0 mm × 250 mm; Phenomenex) using a flow rate of 0.50 ml/min at 40°C in a 30-min gradient using two solvents: H₂O + 0.1% HAC (solvent A) and 90:10 AcN/isopropanol (solvent B). A set of 17 deuterated internal standards was added prior to the extraction step to correct for matrix effects and recovery efficiency. Calibration curves were obtained by plotting the ratio of peak areas of eicosanoids to the internal standards for each compound. Accuracy and the precision of the method were tested and resulted in a coefficient variation < 20% for most of the compounds.

**Lipidomics analysis of inflammatory lipids**

Eicosanoid detection and quantification (∼120 inflammatory lipids) were performed with a multiple reaction-monitoring assay, using ultra-high-performance liquid chromatography-mass spectrometry (Acquity; Ultra performance LC, Waters) and negative-mode electrospray ionization tandem mass spectrometry (Xevo Waters), essentially as described (22), with the following modification: sample was extracted by liquid-liquid extraction using ethylacetate-butyl ether. Chromatographic separation was achieved on a Synergi Hydro-RP column (4 μm, 2.0 mm × 250 mm; Phenomenex) using a flow rate of 0.50 ml/min at 40°C in a 30-min gradient using two solvents: H₂O + 0.1% HAC (solvent A) and 90:10 AcN/isopropanol (solvent B). A set of 17 deuterated internal standards was added prior to the extraction step to correct for matrix effects and recovery efficiency. Calibration curves were obtained by plotting the ratio of peak areas of eicosanoids to the internal standards for each compound. Accuracy and the precision of the method were tested and resulted in a coefficient variation < 20% for most of the compounds.

**In vitro stimulation of iDCs with isolated TLR ligands**

iDCs (5 × 10⁵ iDCs/500 μl) were stimulated with various TLR ligands (synthetic lipopeptide Pam3CSK4 or flagellin (Bacillus subtilis); InvivoGen, San Diego, CA), LPS (purified by gel-filtration chromatography (Acquity; Ultra performance LC, Waters) and negative-mode electrospray ionization tandem mass spectrometry (Xevo Waters), essentially as described (22), with the following modification: sample was extracted by liquid-liquid extraction using ethylacetate-butyl ether. Chromatographic separation was achieved on a Synergi Hydro-RP column (4 μm, 2.0 mm × 250 mm; Phenomenex) using a flow rate of 0.50 ml/min at 40°C in a 30-min gradient using two solvents: H₂O + 0.1% HAC (solvent A) and 90:10 AcN/isopropanol (solvent B). A set of 17 deuterated internal standards was added prior to the extraction step to correct for matrix effects and recovery efficiency. Calibration curves were obtained by plotting the ratio of peak areas of eicosanoids to the internal standards for each compound. Accuracy and the precision of the method were tested and resulted in a coefficient variation < 20% for most of the compounds.

**Flow cytometry**

FcγR surface expression was determined with anti-CD64, anti-CD16, and anti-CD161 (all from BD Biosciences Pharmingen), anti-CD4 (BD Pharmingen), and anti-FcγRIII–blocking Abs (clone AT10; Abcam), or with 2B6 Ab (anti-FcγRIIB, own hybridoma production ATCC PTA-4591) in 24-well plates (Greiner Bio-One, Alphen aan den Rijn, the Netherlands). iDCs were stimulated with 0.35 ml/min at 40°C in a 30-min gradient using two solvents: H₂O + 0.1% HAC (solvent A) and 90:10 AcN/isopropanol (solvent B). A set of 17 deuterated internal standards was added prior to the extraction step to correct for matrix effects and recovery efficiency. Calibration curves were obtained by plotting the ratio of peak areas of eicosanoids to the internal standards for each compound. Accuracy and the precision of the method were tested and resulted in a coefficient variation < 20% for most of the compounds.

**Imaging stream flow cytometry**

Alternatively, phagocytic capacity was evaluated with imaging flow cytometry (ImageStreamX). Briefly, bright-field and fluorescence (FITC) images were acquired for a minimum of 3000 cells/sample. A compensation matrix was applied to the acquired images, and properly focused single cells were gated based on the area, aspect ratio intensity, and bright-field gradient RMS features using IDEAS v5.0 software (Aminis). A mask depicting only the intracellular space was designed, and the percentage of FITC⁺ cells was calculated. Also, a feature was designed to count the number of bacteria/cell. This feature (spot count) was based on the Peak mask using a spot/background ratio of 5.75.
**Neutrophil isolation**

Polymorphonuclear cells (PMNs; neutrophils) were isolated from heparin anticoagulated peripheral blood drawn from healthy volunteers by standard LymphoPrep isolation (Axis-Shield), according to the manufacturer’s protocol. Erythrocytes were removed by hypotonic lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) (10 min, 4˚C). Purity of PMNs was determined by cytosine preparation and always exceeded 95%. Cell viability of PMNs exceeded 98%, as assessed by trypan blue staining.

**Quantitative real-time PCR**

mRNA was isolated with an mRNA Capture Kit (Roche Diagnostics), and cDNA was synthesized with the Reverse Transcription System (Promega, Madison, WI), according to the manufacturers’ protocols. PCR amplification (ABI ViiA 7 detection system; Applied Biosystems) was performed using target-specific human primers (for housekeeping gene EF1α: forward 5’-AAGCTGGAAGATGGCCTAAA-3’ and reverse 5’-AAAGCGACCAAAAGTTGATG-3’; for pro-IL-1β: forward 5’-TTTGGATGCTGCCCGATTCCC-3’ and reverse 5’-TCAGTTATATCTCTGGCCGC-3’; for TNF-α: forward 5’-GCCAGGGCAGTCAGATCATC-3’ and reverse 5’-TGCGCTACAGGCTGTTACTC-3’; and for IL-23p19: forward 5’-GCTTGGCCAAGGAGGATCCACCA-3’ and reverse 5’-TCCAGTCTACGAGCTCTTCA-3’) with the SYBR Green detection method (iQ SYBR Green SuperMix 2X; Bio-Rad). The reaction protocol was identical for all PCR products: 2 min at 95˚C, followed by 40 cycles of 15 s at 95˚C and 1 min at 60˚C (+1 cycle for dissociation curve). For each condition the normalized amount of target mRNA (Nt) was calculated from obtained Ct values using the unpaired Student t p values. The p values < 0.05 were considered significant.

**Western blotting**

Cells were collected and lysed in RIPA lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate 0.1% SDS, 10 mM EDTA) for 30 min at 4˚C. The lysate was clarified by centrifugation at 15,000 rpm for 10 min at 4˚C, and the supernatant was used for Western blotting. Alternatively, supernatants of cells were harvested and centrifuged at 15,000 rpm for 10 min at 4˚C; these supernatant samples were used for further analysis. Samples were prepared in Laemmli sample buffer and electrophoresed on 4–20% gradient precast gel (Mini-Protean TGX; Bio-Rad, Hercules, CA). Subsequently, the gels were transferred to polyvinylidene difluoride membranes (Immobilon-FL; Millipore, Bedford, MA). The gels were stained with anti–IL-1β (Abcam 2105) and Goat anti-Rabbit IRDye 800CW (red signal; Li-Cor, Lincoln, NE), according to the manufacturers’ protocol, scanned using the Odyssey system (Li-Cor), and adjusted to grayscale images.

**Statistics**

Data are depicted as mean ± SD. Statistical differences were determined using the unpaired Student t test (two groups) or ANOVA (multiple groups). The p values < 0.05 were considered significant.

**Results**

Ab-opsonized E. coli evoke distinct iDC effector functions with subsequent altered Th cell polarization

To investigate coactivation of PRRs and FcRs, iDCs were incubated with E. coli in the presence or absence of Ab opsonization. Subsequent iDC cellular responses, as well as Th cell polarization, were analyzed. Ab-dependent uptake of FITC-labeled E. coli was observed in the presence of purified human IgG or human serum (containing specific Abs) (Fig. 1A, Supplemental Fig. 1A) (23). Using imaging stream technology, we visualized and confirmed enhanced phagocytosis and not merely increased binding of opsonized bacteria by iDCs (Fig. 1B). The phagocytic index decreased after 4 h of phagocytosis, which was likely due to acidic pH quenching of the FITC label during intracellular bacterial processing within lysosomes. These differentially stimulated iDCs were analyzed further for maturation markers (data not shown). No major differences in the expression of DC maturation markers CD80, CD83, CD86, and HLA-DR were observed after stimulation with E. coli, IgG, or IgG-opsonized E. coli, which suggests no major modulation of DC maturation compared with individual activation of PRRs (1, 2) and FcRs (24, 25).

**FIGURE 1.** Ab-opsonized E. coli evokes distinct iDC effector functions. (A) iDCs were stimulated with FITC-labeled E. coli, IgG, or IgG-opsonized FITC-labeled E. coli for the indicated times and analyzed for phagocytic capacity by flow cytometry. (B) Uptake of FITC-labeled E. coli by iDCs after 2.5 h was visualized by imaging stream technology (original magnification ×60; Ch01: bright-field; Ch02: FITC). Unstimulated iDCs served as negative control. Bar graphs depict phagocytosis calculated as the percentage of FITC+ cells (upper right panel) and number of internalized E. coli/iDC (lower right panel). (C) iDCs were stimulated as described in (A) for 16 h. Indicated cytokines were measured in supernatants by multiplex protein analysis. IL-23 was measured in a separated sandwich ELISA. (D) Stained iDCs, as indicated, were cocultured with memory Th cells for 5 d, after which supernatants were analyzed for IFN-γ and IL-17 production as markers for Th1 and Th17 differentiation, respectively. Data are mean ± SD (n = 3). *p < 0.05.
Next, we investigated the secretory profile of iDCs that had been incubated with E. coli or IgG-opsonized E. coli. Cytokine release was determined, which included the Th-polarizing cytokines IL-12p40, IL-12p70, IL-6, IL-1β, and IL-23 (Th17 cells), IL-4 (Th2), IL-10 (regulatory T cells), IFN-γ, TNF-α, and G-CSF (Fig. 1C). Minimal production of IL-4 by DCs was observed under all conditions because this cytokine is primarily secreted by Th cells themselves and not by DCs. IgG opsonization of E. coli did not increase secretion of IL-12p40 compared with activation via E. coli alone. IL-12p70, IL-6, IL-10, and IFN-γ were additively released when iDCs were incubated with IgG-opsonized E. coli compared with cytokine release in the presence of either E. coli or IgG. Interestingly, synergistic release of TNF-α, G-CSF, and the Th17-polarizing cytokines IL-1β and IL-23 was observed after costimulation with E. coli and IgG, which supported cross-talk between PRRs and FcRs.

Additionally, we used a novel quantitative mass spectrometry lipidomics approach (22) to profile 12 inflammatory eicoanoids (17–19, 26). iDCs stimulated with IgG-opsonized E. coli demonstrated clear synergistic differences in the secretion of cyclooxygenase (COX)-dependent and lipoxigenase (LOX)-dependent metabolites compared with iDCs stimulated with E. coli (Table I). This included synergistic upregulation of AA metabolites, like PGF2α, PGJ2 (metabolic end product of PGD2), and thromboxane B (TXB)2. Other TXB family members (TXB1 and TXB3), which are linoleic acid–derived metabolites, were upregulated as well (data not shown). Furthermore, increased levels of the chemotactic leukotriene B4 (LTB4) and its counter-regulator, lipoxin A4 (LXA4), were found. In contrast, secretion of PGE2 was reduced (beneath detection level) when iDCs were stimulated with IgG-opsonized bacteria compared with nonopsonized bacteria.

Next, Th cell polarization was tested by coculturing of Th cells and iDCs, which had been stimulated for 24 h with the indicated stimuli (Fig. 1D). Release of IFN-γ and IL-17 was used as a measure of Th1 and Th17 polarization, respectively. No difference in IFN-γ level was found between iDCs cocultured with Th cells and stimulated with IgG-opsonized bacteria or nonopsonized bacteria. However, cocultures of Th cells with iDCs that had been stimulated with IgG-opsonized E. coli evoked IL-17 production, indicative of polarization into Th17 cells; this was not observed in cocultures with unstimulated, IgG-stimulated, or E. coli–stimulated iDCs. Taken together, iDCs stimulated simultaneously with PRRs and FcRs displayed clearly different cellular immune responses compared with single stimulation with PRRs or FcRs, based on the inflammatory cytokine– and lipidomic-profiles, as well as Th cell polarization.

Table I. AA-derived metabolites

<table>
<thead>
<tr>
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<th>COX-Dependent AA Metabolites (nM)</th>
<th>LOX-Dependent AA Metabolites (nM)</th>
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<tbody>
<tr>
<td></td>
<td>PGF2α</td>
<td>PGJ2</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E. coli</td>
<td>ND*</td>
<td>0.166</td>
</tr>
<tr>
<td>IgG</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E. coli + IgG</td>
<td>0.489</td>
<td>0.657</td>
</tr>
<tr>
<td>Fold change†</td>
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</table>

†Fold change was calculated between IgG-opsonized E. coli and E. coli–stimulated iDCs.

*LOD of PGF2α = 0.0002 nM.

†LOD of PGF2α = 0.0019 nM.

‡Fold change for E. coli + IgG versus E. coli.

§LOD of 55.6R-LXA4 = 0.0036 nM.

Both FcγRII and MyD88-dependent TLRs are essential for cross-talk

IgG Abs are the main opsonizing Ab isotype present in serum (27). Therefore, we tested the involvement of distinct IgG FcRs (FcγRs) present on iDCs in the cross-talk with PRRs (Fig. 2A). iDCs did not express activating FcγRI or FcγRIII, whereas abundant surface expression of both activating FcγRIIA and inhibiting FcγRIIB was detected. Phagocytosis of IgG-opsonized FITC-labeled E. coli was reduced in the presence of pan anti-FcγRII-blocking Abs (AT10) (Supplemental Fig. 1B, 1C). Similarly, IL-1β and IL-23 release was reduced (Fig. 2B, 2C, respectively), which indicates the absolute requirement of FcγRII to induce an ample amount of Th17-polarizing cytokines. Of note, blocking FcγRIIB with the blocking Ab 2B6 resulted in higher IL-1β release, which was likely due to activation of the FcγRIIA–ITAM–mediated signaling pathway without interference of an inhibitory signal (Fig. 2D) (12, 28). To further delineate cooperation between different TLRs and FcγRs, we next used several isolated TLR ligands and IgG-B (because activation of FcγRs requires cross-linking). The extracellular TLR ligands Pam3CSK4 (TLR1/2), LPS (TLR4), and flagellin (TLR5) were able to induce synergistic IL-1β and IL-23 cytokine release when iDCs were costimulated with IgG-B (Fig. 2E, 2F). PGN-SA (TLR2) already induced high amounts of IL-1β and IL-23, independently of FcγR costimulation. This is likely due to coactivation via cytoplasmic nucleotide oligomerization domain-2 receptors, as described previously (8, 29). The cooperative effect was not restricted to Th17-polarizing cytokines, because costimulation with LPS and IgG-B induced upregulation of IL-6, IL-10, IFN-γ, TNF-α, and G-CSF, as previously observed with IgG-opsonized E. coli (Supplemental Fig. 2A, Fig. 1C, respectively). The intracellular TLR3 ligand Poly-I:C was not able to induce IL-1β or IL-23 (Fig. 2E, 2F). In contrast, stimulation of intracellularly located TLR7/TLR8 with the synthetic agonist R848 and IgG-B resulted in synergistic release of IL-1β (Fig. 2E) and TNF-α (Fig. 2G).

Next, we investigated whether cross-talk is specific for FcγRII on DCs. Prestimulation of iDCs with IFN-γ induced surface expression of FcγRII (30) (Supplemental Fig. 2B, 2II). Coincubation of iDCs with LPS and IgG-B induced augmented synergistic release of IL-1β in FcγRII-expressing IFN-γ-stimulated iDCs (Supplemental Fig. 2C). iDCs expressed FcγRI as well, albeit at low levels (31, 32) (Supplemental Fig. 2BII). Nonetheless, costimulation with LPS and IgA-coated beads induced synergistic release of IL-1β (Supplemental Fig. 2D). In contrast, neutrophils express high levels of FcγRI, which is their most potent FcR (33, 34). We observed synergistic TNF-α release after simultaneous triggering of FcγRI and TLR4 (Supplemental Fig. 2BIV, 2E). Of note, TNF-α was measured because neutrophils are poor IL-1β producers. Thus, cross-talk occurred between TLRs and several types of FcRs and was not constrained to iDCs.

Furthermore, we investigated the effect of differentially stimulated iDCs on Th cell polarization. Th17 differentiation was induced by the extracellular TLR ligands (Pam3CSK4, LPS, or flagellin) combined with IgG-B triggering (Fig. 3A). PGN-SA induced Th17 differentiation independent of FcR activation, because IL-1β and IL-23 release also was produced independent of
FcR. Th17 differentiation after TLR–FcR cross-talk was dependent on the release of IL-1β by iDCs, because costimulation of LPS and IgG-B in the presence of IL-1Ra (an IL-1R antagonist) completely abrogated Th17 differentiation (Fig. 3B).

Because IL-23R signaling and the Th17 transcription factor RORγt drive expression of the cytokine GM-CSF in Th cells (16, 35), we additionally measured GM-CSF production in cocultures of iDCs and Th cells. iDCs costimulated with FcγRs and TLR ligands (Pam3CSK4, LPS, flagellin, or PGN-SA) induced GM-CSF–producing Th cells (Fig. 3C). Of note, GM-CSF was not detected in supernatants of stimulated iDCs without coculture with Th cells (data not shown) (36).

Thus, because only TLR3, which signals via TRIF, did not show cross-talk with FcγR, these results support that MyD88-dependent TLRs act preferentially in concert with FcγRII on iDCs and, thereby, modulate the inflammatory cytokine profile and Th17 cell polarization. Furthermore, these data support that only coactivation of TLRs and FcγRII on iDCs may evoke GM-CSF–producing Th cells.

**Molecular mechanisms of TLR–FcγR cross-talk regulating cytokine release**

Next, we investigated the molecular mechanisms of synergistic cytokine release via cross-talk between TLRs and FcγRs. In contrast to other cytokines, release of active or mature IL-1β is a two-step process. First, pro–IL-1β mRNA and pro–IL-1β protein are induced via TLRs signaling (37–40). A second activation step through so-called "danger signals" (danger-associated mo-
Synergistic release of mature IL-1β after incubation of iDCs with IgG or serum-opsonized E. coli was observed over time (Fig. 4A, Supplemental Fig. 3A, respectively), and it was reduced by preincubation with the FcγRII-blocking Ab AT10. Additionally, activation of iDCs with LPS and IgG-B demonstrated synergy of IL-1β release (Fig. 4B), independent of LPS concentration or incubation times (Supplemental Fig. 3B). As expected, IL-1β mRNA was induced in iDCs by incubation of E. coli (Fig. 4C, Supplemental Fig. 3C). Interestingly, FcγR triggering did not alter mRNA levels of IL-1β, but it was required for IL-1β protein release. This was further strengthened by blocking FcγRII on iDCs, which abrogated the release of IL-1β protein but did not decrease IL-1β mRNA levels (indicated by # in Fig. 4A, 4C, Supplemental Fig. 3A, 3C). Similarly, IgG-B in combination with LPS, Pam3CSK4, or PGN-SA did not alter IL-1β mRNA levels compared with stimulation of individual TLRs (Fig. 4D, LPS; Supplemental Fig. 3D, other TLR ligands), although flagellin–FcR costimulation affected mRNA levels. Poly-I:C did not induce pro–IL-1β mRNA, which correlates with its inability to initiate IL-1β release, even in the presence of FcγR triggering. Furthermore, expression of intracellular pro–IL-1β protein (∼35 kDa) was present after stimulation of LPS (Fig. 4E, upper panel). However, only simultaneous LPS and IgG stimulation resulted in release of mature IL-1β (17 kDa) (Fig. 4E, lower panel). Thus, FcγR triggering did not affect IL-1β mRNA synthesis or pro–IL-1β protein levels, but it was involved in the process of mature IL-1β secretion as the second step of functional IL-1β production (37, 40). This second step requires caspase-1 activity. Therefore, we established caspase-1 dependency for functional TLR4 and FcγR coactivation. Both IL-1β release (Fig. 5A) and subsequent Th17 cell induction (Fig. 5B) after combined TLR4 and FcγR triggering were abrogated in the presence of the specific caspase-1 inhibitor Ac-YVAD-CMK; thus, caspase-1 activity is required for TLR and FcγR coaxitational–induced release of IL-1β production. Of note, IL-1β mRNA levels (Fig. 5C) and pro–IL-1β protein levels (data not shown) were not decreased after inhibition of caspase-1. Next, we investigated how FcR ITAM signaling affects synergistic release of IL-1β. Inhibition of the downstream mediators of ITAM signaling (12), such as Syk, PKC, or Raf-1, abrogated the synergistic release of IL-1β (Fig. 5D, gray bars). However, neither pro–IL-1β protein levels nor changes in caspase-1 activity were observed in the presence of these inhibitors (data not shown).
Together, these data supported the involvement of FcγRII-dependent ITAM signaling in the release of mature and functional IL-1β rather than in the induction and/or processing of pro–IL-1β. This was in contrast to other cytokines that were released by simultaneous activation of TLRs and FcγRs, such as TNF-α and IL-23. Synergistic release of TNF-α demonstrated that both protein and mRNA were increased over time after incubation of iDCs with IgG-opsonized E. coli (Fig. 6A, 6B; indicated with #). Furthermore, blocking FcγRII hampered the release of TNF-α, as well as synthesis of TNF-α mRNA, which was different compared with IL-1β mRNA levels (Fig. 6B versus Fig. 4C, indicated by #). Stimulation of isolated TLR ligands in combination with IgG-B enhanced both TNF-α protein and mRNA levels (Fig. 6C, 6D). Similar results were obtained for IL-23 (Supplemental Fig. 4A). An increase in IL-23 cytokine release by iDCs stimulated with IgG-opsonized E. coli (Fig. 1C) correlated with increased mRNA levels (Supplemental Fig. 4A). Moreover, blocking FcγRII inhibited both cytokine production and mRNA levels, indicative of FcγRII involvement in regulation of the expression level of transcription. TLR stimulation combined with IgG-B also linked synergistic IL-23 cytokine release with upregulation of mRNA levels, with the exception of LPS (Supplemental Fig. 4B). These data suggest that FcγRs can modulate TLR-initiated cytokine release via different molecular mechanisms, which includes at both the transcriptional and posttranscriptional levels, depending on the specific cytokine.

Discussion

It is generally thought that clearance of pathogens during recurrent infection is faster as the result of rapid expansion of lymphocytes and more efficient phagocytosis because of the presence of opsonizing Abs (5). We now demonstrate that Ab-opsonized bacteria initiate fundamentally different immune responses compared with non-opsonized bacteria. We identified synergistic activity of TLRs and FcRs leading to release of the Th17 polarizing cytokines IL-1β and IL-23, as well as TNF-α, G-CSF, and a distinct metabolite profile (both COX- and LOX-dependent eicosanoids). Notably, maturation of DCs was not altered during FcγR and TLR cross-talk because both independent stimuli already were capable of inducing a mature DC phenotype (data not shown). Furthermore, in addition to IL-17–producing Th cells, GM-CSF–producing Th cells were evoked, which were recently described as a pathogenic subset involved in autoimmune diseases (16). This suggests that TLR and FcR cross-talk induces polarization into polyfunctional GM-CSF–producing Th17 cells. It was described that RORγt-mediated GM-CSF production was induced by IL-23R signaling (20).

Both surface-expressed TLRs (TLR1/2, 4, 5) and intracellular TLR 7/8 were able to cross-talk with FcγRs. TLRs and FcγR subfamily members are structurally different, which makes a
physical interaction less likely. Therefore, we hypothesized that
the interacting link relied on the signaling pathways downstream
of both receptors. TLR1/2, 4, 5 and TLR 7/8 signal via MyD88,
whereas TLR3, the only TLR unable to synergistically induce
cytokine release, is dependent on TRIF. Moreover, we showed that
FcR-dependent ITAM signaling pathways were essential for IL-1
release. Thus, both MyD88-dependent TLR signaling and FcR-
dependent ITAM signaling are required for functional cross-talk.
Furthermore, cross-talk was not confined to Fc
RII on DCs, as
previously suggested (41). Both FcRI and FcαRI were able to
synergize with TLR4 on DCs and neutrophils. Although infection
is linked with uptake of Ab-opsonized pathogens, we conclude that
amplified secretion of cytokines is already induced by extracellular
ligation of FcRs. This is based on our findings that stimulating cells
with isolated TLR ligands in combination with IgG-coated beads
(average size 90 μm), which were not phagocytosed by iDCs,
resulted in the release of similar cytokine profiles.

TLRs are potent inducers of pro–IL-1β transcription through
activation of MyD88, with subsequent NF-κB activation, but they
are limited in their ability to induce the second step of IL-1β
processing (4, 38, 40). After exposure to a second “so-called”
danger signal (e.g., ATP that is induced via cellular stress), as-
ssembly of the caspase-1–dependent inflammasome is initiated,
which cleaves pro–IL-1β protein into mature IL-1β cytokine.
Finally, an increase in calcium levels in the cell, which is induced
by activating FcRs (12), may provide a mechanism by which
mature IL-1β is released from the cell (38, 40). We demonstrate in
this study that TLR activation is essential for the induction of pro–
IL-1β, and coactivation with FcγRs results in the secretion of
functional and mature IL-1β. Moreover, blocking FcγRII activa-
tion either with blocking Abs or inhibitors of FcγR–ITAM sig-
naling (Syk, PKC, and Raf) resulted in abrogated IL-1β cytokine
release, despite the presence of ample amounts of intracellular
pro–IL-1β protein and the presence of active caspase-1. In con-
clusion, this favors a primary role for FcRs in finalizing mature
IL-1β secretion, rather than inducing pro–IL-1β or caspase-1 ac-
tivity. In contrast, FcγRII was involved in modulation of TNF-
α and IL-23 transcription, because enhanced mRNA levels were observed
when FcγRII and TLRs were activated simultaneously. Moreover,
blocking FcγRII reduced mRNA levels. Thus, FcγR can modulate
specific TLR-initiated cytokine production via different molecular
mechanisms and, in this way, alter the Th cell phenotype.

Furthermore, we found that several COX-dependent inflam-
matory PGs, such as PGF$_2$α, PGJ$_2$ (as PGD$_2$ stable end product),
and thromboxanes (TXA$_2$), were upregulated (42) when IgG-
opsonized E. coli were used as stimulus for iDCs (Table I). It is
noteworthy that IL-1β and TNF-α are regulators of COX enzymes
(18, 43). PGE$_2$ is known to be crucial for polarization toward the
Th17 cell type (44) by binding its receptor on CD4+ Th cells; in
this way, it upregulates levels of RORγt transcription factor.
contrast, PGE$_2$ can act as a negative regulator of IL-17 production and ROR$\gamma$ mRNA levels in Th17 cells (45). Thus, the high levels of PGF$_2\alpha$ and low levels of PGE$_2$ that we observed may contribute to skewing toward Th17 polarization. For the LOX-dependent class of eicosanoids, we observed increased levels of LTB$_4$. LTB$_4$ is a potent inflammatory mediator and neutrophil chemoattractant and is an inducer of ROR$\gamma$ mRNA levels favoring skewing into polyfunctional Th17 cells (46). The LTB$_4$ anti-inflammatory counter-regulator LXA$_4$ also was increased. Because potent proinflammatory responses are kept under tight control by this class of anti-inflammatory eicosanoids, it is likely that the LXA$_4$-feedback mechanism was already activated after 16 h. Interestingly, the overall secretory profile of iDCs and subsequent Th cell polarization after TLR–FcR cross-talk resemble those of the recently identified infDCs (21). This DC subset was found in synovial fluid of arthritic joints and tumor ascites and was designated as a novel DC subset. infDCs are particularly efficient at inducing Th17 polarization by secreting the polarizing cytokines IL-1$\beta$ and IL-23. It is clear that onset of these infDCs resides in inflammatory microenvironments. However, the trigger for DCs to develop into this inflammatory phenotype remains unknown and was not dependent on ICOS/ICOS-L interaction, which is important for Th17 polarization (21, 47). Moreover, Pam$_3$CSK$_4$ (as inflammatory stimuli) activation of blood DCs did not induce an infDC subset, because no IL-1$\beta$ or IL-23 was produced (21). Based on our findings, we postulate that cross-talk of TLRs with FeRs on iDCs induces infDCs, because we demonstrated that this coactivation induced the release of IL-1$\beta$ and IL-23 with subsequent IL-17–producing Th17 cells. When analyzing infDC surface markers (CD1a$^+$, CD14$^+$, CD206$^+$, CD11b$^+$, FcR$\gamma$I$^+$, and CD209$^+$ [gated for BDCA$^+$, HLA-DR$^+$, and CD16$^+$ DCs]), we observed that iDCs activated with IgG-opsonized E. coli exhibited some expression of inflammatory markers (higher CD14 and CD206 expression, but CD1a, CD14, CD206, and CD11b already were present on E. coli–stimulated DCs; levels of FcR$\gamma$ and CD209 on infDCs could not be confirmed in our experiments) (Supplemental Fig. 1D). This suggests that the infDC phenotype (based on markers) is induced by inflammatory stimuli (like TLR stimulation). We hypothesize that functionality of infDCs (IL-1$\beta$ and IL-23 release and induction of Th17 polarization) requires coactivation with FeRs, however. It is likely that coactivation of TLRs and FcR$\gamma$s favors recruitment, activation, and prolonged survival of neutrophils at inflammatory sites, which will facilitate clearance of bacterial infections, because the observed secretory profile includes IFN-\gamma, TNF-\alpha, G-CSF, and LTB$_4$ (48). Additionally, Th17 cells stimulate production of G-CSF and granulopoiesis, thereby orchestrating and amplifying neutrophil function (49), whereas GM-CSF is a neutrophil growth factor as well. However, cross-talk between PRRs and FeRs may play a detrimental role in disorders, such as rheumatoid arthritis, and in inflammatory bowel disease because of excess Ab–pathogen complexes.
hematopoiesis of granulocyte-macrophage progenitors, resulting in
induction of an essentially different immune response compared to
MyD88-dependent TLRs and activating FcRs, with subsequent
antitumor immune responses may boost tumor elimination.

In summary, we provide data that efficient pathogenic elimina-
tion during secondary infections induces cross-talk between
MyD88-dependent TLRs and activating FcRs, with subsequent
induction of an essentially different immune response compared with
non-Ab-mediated primary immune responses. This knowl-
edge could be helpful in developing and refining new therapies for
autoimmune diseases, as well as for Ab-mediated treatment of cancer.

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Disclosures

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