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

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

Jantine E. Bakema,*† Cornelis W. Tuk,† Sandra J. van Vliet,† Sven C. Bruijns,† Joost B. Vos,‡ Sophia Letsiou,§ Christien D. Dijkstra,† Yvette van Kooyk,‡ Arjan B. Brenkman,§ and Marjolein van Egmond‡,*

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. The Journal of Immunology, 2015, 194: 000–000.

Pathogen recognition receptors (PRRs), which are expressed on dendritic cells (DCs), trigger antimicrobial defenses during initial encounters with harmful pathogens (primary immune response) (1–5). PRRs include TLRs, scavenger receptors, C-type lectin receptors, nucleotide oligomerization domain–like receptors, or retinoic acid–inducible gene-I–like receptors. They recognize specific pathogen-associated molecular patterns on pathogens, such as lipids, carbohydrates, peptides, and nucleic acid structures (1, 3). Intact microbial pathogens are composed of a variety of these pathogen-associated molecular patterns, which can simultaneously activate multiple PRRs and initiate a plethora of signaling programs that execute a first line of defense against infection (3, 6–9).

However, secondary immune responses (among others) are characterized by the presence of ample amounts of pathogen-specific Abs.

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 FcyRI subclasses that bind IgG, of which FcyRII (CD32) is the most prominent (11, 12). FcyRII is subdivided into FcyRIIA, which is an activating receptor through ITAM signaling, and FcyRIIB, 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).

Interestingly, 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.
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 functional 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 (inDCs). 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 (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were differentiated into iDCs by culturing for 7 d in complete medium (RPMI 1640 + 2 mM L-glutamine [Life Technologies, Paisley, U.K.], 10% heat-inactivated FCS, and antibiotics) supplemented with IL-4 (500 U/ml) and GM-CSF (800 U/ml; both from Biosource, Camarillo, CA). All donors gave informed consent, according to the guidelines of the Medical Ethical Committee of VU University Medical Center and in accordance with the Declaration of Helsinki.

**Escherichia coli phagocytosis**

E. coli strain R3 (kind gift from Dr. B. Appelmelk, VU University Medical Center) was grown overnight in Luria-Bertani medium and labeled with FITC to phagocytosis experiments. Heat-inactivated bacteria (1 h, 70°C) were incubated with 10 μg/ml FITC (Sigma-Aldrich) in carbonate buffer (0.1 M NaHCO3, [pH 9]) for 1 h at 37°C, after which they were washed extensively in PBS. FITC-labeled E. coli (5 × 10⁷) were incubated with 0.05 mg purified human IgG (Sigma-Aldrich) or heat-inactivated serum IgG (Sigma-Aldrich) for 90 min (37°C), with or without pan anti-FcγRII-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 (0.5 × 10⁶/0.5 ml) were added for the indicated times at 37°C in complete RPMI 1640 medium. Cells were used to determine phagocytosis (FACS Calibur; Becton Dickinson, San Diego, CA) and imaging stream flow cytometry (ImageStreamX, Amnis, EMD Merck Millipore, Seattle, WA). The phagocytic index was calculated as geometric mean fluorescence intensity multiplied by the percentage of gated cells. iDCs also were analyzed for surface markers (see Flow cytometry), in coculture with Th cells (see Th cell–differentiation assay) or for relative mRNA levels (see Quantitative real-time PCR). Supernatants were harvested for cytokine measurements (see Lipidomics analysis of inflammatory lipids).

**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 5000 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 (Amnis). 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.

**Flow cytometry**

FcγR surface expression was determined with anti-CD64, anti-CD14, and anti-CD16 (all from biolegend, own hybridoma production ATCC PTA-4591) for 1 h at 4°C. After washing, cells were incubated with rabbit F(ab’2), anti-mouse IgG-PE (DAKO, Glostrup, Denmark) for 30 min at 4°C. Fluorescence was measured by flow cytometry (FACS Calibur; Becton Dickinson), and the gate was set on iDCs. Inflammatory status of iDCs was determined, as described (21). Cell gates were for BDCA1⁺, CD16⁺, and HLA-DR⁺ and subsequently analyzed for CD1a-PE (BD Biosciences Pharmingen), CD14-PE (BioLegend), CD206 (BD Biosciences Pharmingen), CD11b-PE (BD Pharmingen), FcεRI (BD Biosciences Pharmingen), and CD209-FITC (R&D Systems). FcεRI surface expression was determined by staining for anti-CD89 (PE conjugated; BD Biosciences Pharmingen) for 1 h at 4°C. After washing, fluorescence was determined by FACS.

**Multiplex protein analysis and cytokine ELISA**

Custom made 9-plex protein assays (Millipore), including IL-12p70, IL-12p40, IL-6, IL-1β, IL-4, IL-10, IFN-γ, TNF-α, and G-CSF, were performed according to the manufacturer’s protocol using LUMINEX technology (LX200; Millipore). IFN-γ, IL-17, GM-CSF, IL-1β, TNF-α, and IL-23 sandwich ELISAs (eBioscience, San Diego, CA) were performed according to the manufacturer’s instructions.

**Th cell–differentiation assay**

PBMCs were isolated from heparinized blood from healthy volunteers by density gradient centrifugation using LymphoPrep (Axsyl-Shift, Oslo, Norway). Memory CD4⁺ Th cells were isolated using a Memory CD4⁺ Th Cell MACS Isolation Kit (Miltenyi Biotec), according to the manufacturer’s protocol. Memory Th cells were used for coculture with stimulated iDCs (5 × 10⁵ iDCs with 5 × 10⁶ Th cells in complete RPMI 1640 medium supplemented with cytokines (100 pg/ml; Sigma-Aldrich)) for 10 days. After 5 d of culture, supernatants were harvested and analyzed with ELISA for IFN-γ, IL-17, or GM-CSF production to determine Th1 or Th17 subset differentiation or GM-CSF Th cell skewing, respectively.

**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 (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 ethyl-tert-butyl ether. Chromatographic separation was achieved on a Synergi Hydro-RP column (4 μm, 2.0 mm × 250; Phenomenex) using a flow rate of 0.3 mL/min. A linear 90:10 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 against known quantities of eicosanoids, leading to a linear regression line (r² > 0.99). The limits of detection (LODs) were defined by a signal-to-noise ratio of 3:1, and they were in the range of 3 pM to 1 nM, depending on the 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) or LPS [purified by gel-filtration chromatography (Acquity, Ultra performance LC, Waters) and checked for impurities (<1% protein)], peptidoglycan of S. aureus (PGN-SA), viral synthetic agonist polynosinic-polycytidylic acid (Poly-I:C, [synthetic lipopeptide Pam3CSK4 or flagellin [Bacillus subtilis]; Invivogen, San Diego, CA] or LPS [purified by gel-filtration chromatography (Acquity, Ultra performance LC, Waters)]. Tocris Bioscience, Ellsvill, MO), or Raf-1 inhibitor GW5074 (Sigma-Aldrich) and compared with DMSO-treated cells as vehicle control.
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 NaCl, 10 mM KHCO₃, and 0.1 mM EDTA) (10 min, 4°C). Purity of PMNs was determined by cytopsin 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 Transcript System (Promega, Madison, WI), according to the manufacturers’ protocols. PCR amplification (ABI ViiA 7 sequence detection system; Applied Biosystems) was performed using target-specific human primers (for housekeeping gene EF1a: forward 5’-AAGCTGGAAGATGGCCCTAAA-3’ and reverse 5’-AAGCCGACCAAGGTAGTGAAT-3’; for pro–IL-1β: forward 5’-TTTGAATCCTGCCAGCTTCCC-3’ and reverse 5’-TCAGTTATATCCTGGCAGGCC-3’; for TNFα: forward 5’-GCCAGGAGCTGACAGTAC-3’ and reverse 5’-TGACTCACGCTGTGACTCT-3’; and for IL-23p19: forward 5’-GCTTTTGCAAAAGGTACCCCA-3’ and reverse 5’-TCCAGTCCTGAGGCTTCTCA-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 (number of PCR cycles for which the fluorescence signal exceeds the detection threshold value) using the equation Nt = 2^(-Ct(Target))-Ct(Target).

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 phagocytic capacity by flow cytometry. (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 (Th1), 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 120 inflammatory eicosanoids between PRRs and FcRs. Costimulation with *E. coli* and IgG, which supported cross-talk between different TLRs and FcRs, was tested by cocculturing 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.

### 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 FcRs, we used several isolated TLR ligands and IgG-B (because activation of FcγRs requires cross-linking). The intracellular TLR3 ligands Pam3CSK4 (TLR3/2), 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, 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 FcγRI (30) (Supplemental Fig. 2B1 versus 2BII). Coincubation of iDCs with LPS and IgG-B induced an 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

### Table I. AA-derived metabolites

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>COX-Dependent AA Metabolites (nM)</th>
<th>LOX-Dependent AA Metabolites (nM)</th>
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</thead>
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<tr>
<td></td>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>PG&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>E. coli</em> + IgG</td>
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<td>16.087</td>
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<tr>
<td><em>E. coli</em> + IgG</td>
<td>ND</td>
<td>4.353</td>
</tr>
<tr>
<td>Fold change&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2445</td>
<td>4.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>LOD of PGF<sub>2α</sub> = 0.0002 nM.

<sup>b</sup>LOD of PGE<sub>2</sub> = 0.0019 nM.

<sup>c</sup>Fold change for *E. coli* + IgG versus *E. coli*.

<sup>d</sup>LOD of 5S,6R-LXA<sub>4</sub> = 0.0036 nM.
FcRs. 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 FcγR 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 TLR signaling (37–40). A second activation step through so-called "danger signals" (danger-associated mo-
lecular patterns) is required for cleavage of pro–IL-1β protein into mature IL-1β.

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, 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-YV AD-CMK; thus, caspase-1 activity is required for TLR and FcγR coactivational-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 LPS, with or without IgG-B, correlated with increased mRNA levels (Supplemental Fig. 4A). 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).

**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 FcγRs 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 FcγRII 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-1β 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 FcγRII on DCs, as previously suggested (41). Both FcγRI 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), assembly 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 activation either with blocking Abs or inhibitors of FcγR–ITAM signaling (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 conclusion, this favors a primary role for FcRs in finalizing mature IL-1β secretion, rather than inducing pro–IL-1β or caspase-1 activity. 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 inflammatory PGs, such as PGF_2α, 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). PGF_2α 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. In
contrast, PGE₂ can act as a negative regulator of IL-17 production and RORγt mRNA levels in Th17 cells (45). Thus, the high levels of PGF₂α and low levels of PGE₂ that we observed may contribute to skewing toward Th17 polarization. For the LOX-dependent class of eicosanoids, we observed increased levels of LTB4. LTB4 is a potent inflammatory mediator and neutrophil chemoattractant and is an inducer of RORγt mRNA levels favoring skewing into polyfunctional Th17 cells (46). The LTB4 anti-inflammatory counter-regulator LXA₄ also was increased. Because potent proinflammatory responses are kept under tight control by this class of anti-inflammatory eicosanoids, it is likely that the LXA₄-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-1β 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, Pam₃CSK₄ (as inflammatory stimuli) activation of blood DCs did not induce an infDC subset, because no IL-1β or IL-23 was produced (21). Based on our findings, we postulate that cross-talk of TLRs with FcRs on iDCs induces infDCs, because we demonstrated that this coactivation induced the release of IL-1β and IL-23 with subsequent IL-17–producing Th17 cells. When analyzing infDC surface markers (CD1a⁺, CD14⁺, CD206⁺, CD11b⁺, FcεRI⁺, 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 FcεRI 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-1β and IL-23 release and induction of Th17 polarization) requires coactivation with FcRs, however.

It is likely that coactivation of TLRs and FcγRs 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-γ, TNF-α, G-CSF, and LTB4 (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 FcRs may play a detrimental role in disorders, such as rheumatoid arthritis, and in inflammatory bowel disease because of excess Ab–pathogen complexes.
present in the gut (50). The continuous activation of both receptor classes can result in unmitigating neutrophil recruitment, which contributes to severe tissue damage. More recently, a pathogenic role for (IL-23-driven) GM-CSF was described in chronic inflammatory diseases, because GM-CSF production triggered extramedullary hematopoiisis of granulocyte-monocyte progenitors, resulting in colitis (20). Furthermore, IL-23-dependent secretion of GM-CSF by Th cells was shown to evoke experimental autoimmune encephalomyelitis and sustain neuroinflammation (16). Recent data suggest that a Th17 subset is involved in (anti)-tumor immunity, although the exact nature of this relationship is not clear (35). In patients with head and neck squamous cell carcinoma, Th17 cells represented an important fraction of the tumor-infiltrating lymphocytes in tumor tissue and tumor-draining lymph nodes, which correlated with impaired proliferation and angiogenesis of head and neck squamous cell carcinoma (51). Thus, inducing cross-talk during (therapeutic) antitumor immune responses may boost tumor elimination.

In summary, we provide data that efficient pathogenic elimination 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 knowledge could be helpful in developing and refining new therapies for inflammatory (autoimmune) diseases, as well as for Ab-mediated treatment of cancer.

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Disclosures

The authors have no financial conflicts of interest.

References


Figure S1: Uptake of serum opsonised E. coli and inflammatory marker profile of iDCs.

iDCs were stimulated with FITC labelled E. coli, heat inactivated human serum (HI) or HI-opsonised FITC labelled E. coli for indicated time points and analysed for (A) phagocytic capacity with flow cytometry. (B, C) iDCs were stimulated for 2.5H with FITC labelled E. coli, IgG or IgG-opsonised FITC labelled E. coli in the presence or absence of pan FcγRI (AT10) blocking antibody. Cells were analysed for phagocytic capacity with (B) flow cytometry or (C) image stream technology (Ch01: bright field, Ch02: FITC channel). Graphs depict internalisation calculated as % FITC positive cells (left graph) and number (#) of internalized E. coli per iDC (right graph). (D) DC were, after 48H of stimulation as indicated, gated for CD10/BDC1a+, CD16- and HLA-DR+ and subsequent analysed for the markers CD1a, CD14, CD206, CD11b, FcγRI, and CD209 (thin line). Isotype control is depicted as filled histograms.
Figure S2: Different cell types stimulated with LPS and IgG-coated beads and analysed for cytokine release. (A) Supernatants of iDCs, which were stimulated (24H) with LPS in combination with IgG-B or BSA-B, were analysed by multiplex protein assay for IL-6, IL-10, IFNγ, TNFα, and G-CSF. (B) Panel I and II: iDCs were primed (24H) with IFNγ (300U/ml) to induce FcγR1 surface expression, which was confirmed by flow cytometry. Panel I represents FcγR1 expression on unstimulated iDCs, panel II depicts FcγR1 expression on IFNγ-stimulated iDCs. Panel III and IV depict FcγR1 surface expression on iDCs and granulocytes (PMNs), respectively. All panels: thin line represents isotype control, filled histograms depict expression of indicated FcγR. (C) iDCs with or without FcγR1 expression (grey versus black bars, respectively) were stimulated with LPS in the presence or absence of IgG-B or BSA-B. Supernatants were harvested after 24H and analysed for IL-1β production. (D) iDCs were stimulated with LPS in the presence or absence of IgG-B (targeting FcγR1) or BSA-B as control. Supernatants (24H) were analysed for IL-1β release. (E) PMNs were stimulated with LPS in the presence or absence of IgG-B (targeting FcγR1) or BSA-B as control. Supernatants (24H) were analysed for TNFα release (n=2).

Data are depicted as mean ± SD.
Supplemental figure 3

(A) iDCs were stimulated for the indicated time points with E. coli, heat inactivated human serum (Hi) or Hi-opsinised E. coli in the presence or absence of pan FcyRII (AT10) blocking antibody as indicated. Supernatants were analysed for IL-1β production. (B) iDCs were stimulated for 4H (left panel) or 24H (right panel) with 10, 100, 500 or 1000 ng/ml LPS (represented by black bars colouring to light grey bars) in combination with IgG-B or BSA-B as control. Supernatants were analysed for IL-1β production. C) iDCs were stimulated as described in (A) and analysed for relative IL-1β mRNA levels expression which was normalized to EF1α. (D) iDCs were stimulated as described with Pam3CysK4 (Pam), Flagellin (Flag), PGN of S. aureus (PGN-SA) or Poly-I:C in the presence or absence of IgG-B or BSA-B as control. Cell lysates were analysed for relative IL-1β mRNA levels and expression was normalized to EF1α. Data are depicted as means ± SD. (n=3).
Figure S4. Molecular mechanisms of TLR-FcγR induced IL-23 release.

(A) IL-23p19 mRNA was measured after stimulation (4H) of IDCs with E. coli in the presence or absence of IgG. When indicated the pan FcγRII blocking antibody (AT10) was added. B) IDCs were stimulated for 4 hours with indicated TLR ligands in presence or absence of IgG-B or BSA-B, after which relative IL-23p19 mRNA levels in cell lysates were determined. Expression was normalized to EF1a (n=3).