FcγRIIa, a novel director of cytokine production in infection and autoimmunity

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IgG opsonization of bacteria promotes Th17 responses via synergy between TLRs and FcγRIIa in human dendritic cells

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Dendritic cells (DCs) are essential in inducing adaptive immune responses against bacteria by expressing cytokines that skew T cell responses towards protective Th17 cells. Although it is widely recognized that induction of these cytokines by DCs involves activation of multiple receptors, it is still incompletely characterized which combination of receptors specifically skews Th17 cell responses. Here we have identified a novel role for Fc gamma receptor IIa (FcγRIIa) in promoting human Th17 cells. Activation of DCs by bacteria opsonized by serum IgG strongly promoted Th17 responses, which was FcγRIIa-dependent and coincided with enhanced production of selected cytokines by DCs, including Th17-promoting IL-1β and IL-23. Notably, FcγRIIa stimulation on DCs did not induce cytokine production when stimulated individually, but selectively amplified cytokine responses through synergy with Toll-like receptor (TLR) 2, 4 or 5. Importantly, this synergy is mediated at two different levels. First, TLR-FcγRIIa co-stimulation strongly increased transcription of pro-IL-1β and IL-23p19. Second, FcγRIIa triggering induced activation of caspase-1, which cleaves pro-IL-1β into its bioactive form and thereby enhanced IL-1β secretion. Taken together, these data identified cross-talk between TLRs and FcγRIIa as a novel mechanism by which DCs promote protective effector Th17 cell responses against bacteria.

INTRODUCTION

Protection against different classes of pathogens requires the activation of antigen-presenting dendritic cells (DCs) to express factors that promote the development of distinct effector T helper cell subsets, which are specialized to combat the class of pathogen involved1. Effective T cell-mediated immunity against extracellular bacteria requires DCs to produce IL-1β, IL-6, and IL-23 that contribute to the development of Th17 cells2-3. The pathogen-induced production of cytokines by DCs is induced upon sensing of pathogens by pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectins, Nod-like receptors and RIG-I-like receptors4-7. Although triggering of individual PRRs is known to induce cytokine production, it is becoming increasingly clear that the ultimate amount and the profile of cytokine production by DCs crucially depends on cross-talk between multiple PRRs8-10. However, our knowledge on these cross-talk mechanisms is likely to be still largely incomplete10.

In this respect, the role of Fc gamma receptors (FcγRs), the family of high- and low-affinity receptors for immunoglobulin G (IgG), in the induction of cytokine production did not receive much attention. IgG is the most prevalent immunoglobulin in the blood and body tissues11,12. Due to the high levels of IgG directed against numerous polyreactive bacterial antigens, invading bacteria are efficiently opsonized as soon as they penetrate the body's barriers, even during primary infection13-17. IgG opsonization can directly lead to pathogen inactivation via complement activation, but can also result in a variety of responses by different effector immune cells such as cell degranulation, production of reactive oxygen species (ROS), or antibody-dependent cellular cytotoxicity (ADCC)18,19.
In addition, binding of opsonized pathogens to low-affinity FcγRs on DCs mediates phagocytosis, degradation, and subsequent presentation of pathogen-derived antigens to T cells\textsuperscript{20}. FcγR stimulation also induces DC maturation\textsuperscript{21-23}. However, the triggering of FcγRs on DCs results in no or only low production of cytokines and has not been demonstrated to play a major role in polarization of human T cell responses in healthy donors\textsuperscript{23,22}.

In the present study we have taken into account that in most conditions DCs will engage bacteria that are IgG opsonized and that such DCs will be simultaneous triggered via FcγRs and bacterial sensors. We here report that the engagement of DCs with opsonized bacteria resulted in strongly up-regulated production of selected cytokines, including IL-1β and IL-23, which favored the development of Th17 cells. This effect was fully dependent on stimulation of the low-affinity IgG receptor FcγRIIa (also known as CD32a), which synergized with TLRs for the amplification of Th17-promoting cytokines by both enhancing cytokine transcription and by activating caspase-1. Taken together, these data identified cross-talk between TLRs and FcγRIIa as a novel mechanism by which DCs promote the development of protective effector T cells in response to bacteria.

RESULTS

Opsonization of bacteria promotes Th17 responses

Upon penetrating the body’s barriers, bacteria are exposed to high concentrations of IgG, which is expressed in both blood and tissue fluids. Here, we set out to determine whether IgG opsonization of bacteria affects DC-mediated T helper cell polarization. First, we determined to what extent human serum indeed contains bacteria-specific IgGs by testing the binding of IgG in pooled human serum to a variety of bacterial species by ELISA. All different bacterial strains we tested were recognized by serum IgG (Fig 1A), indicating that these bacteria are opsonized by IgG upon exposure. The different bacterial strains were recognized by purified IgGs in a similar fashion as by serum IgGs (Fig 1A), thereby confirming IgG-specificity.

To investigate whether opsonization of bacteria affects T helper cell polarization, we incubated opsonized and non-opsonized bacteria with human monocyte-derived DCs and CD4\textsuperscript{+} T cells in serum-free culture medium. To assess Th1 and Th17 skewing we determined the production of IFNγ and IL-17 respectively by intracellular FACS staining upon re-stimulation of the T cells after 12 days. Strikingly, opsonization by human serum IgG strongly enhanced Th17 induction for both Gram-positive \textit{S. aureus} and Gram-negative \textit{K. pneumonia} (Fig 1B). Notably, no consistent differences were observed for Th1 responses (Fig 1B). Taken together, these data indicate that IgG opsonization of bacteria skews adaptive immune responses towards Th17.
Opsonization of bacteria promotes Th17 responses. (A) Bacteria were coated on a 96-wells plate, which were blocked and incubated with a control antibody, human serum or purified IgG, followed by incubation with anti-human IgG-HRP and was subsequently developed. Data shown are mean ± SEM of triplicate measurement from one representative experiment of three. (B) DCs and CD4^+ T cells were incubated with bacteria that were pre-incubated or not in human serum. Intracellular levels of IFNγ and IL-17 were measured at day 12, after 5h of restimulation with PMA, ionomycin, and brefeldin A. FACS plots shown are from one representative of three different donors.

**Figure 1. Opsonization of bacteria promotes Th17 responses.** (A) Bacteria were coated on a 96-wells plate, which were blocked and incubated with a control antibody, human serum or purified IgG, followed by incubation with anti-human IgG-HRP and was subsequently developed. Data shown are mean ± SEM of triplicate measurement from one representative experiment of three. (B) DCs and CD4^+ T cells were incubated with bacteria that were pre-incubated or not in human serum. Intracellular levels of IFNγ and IL-17 were measured at day 12, after 5h of restimulation with PMA, ionomycin, and brefeldin A. FACS plots shown are from one representative of three different donors.

**Opsonization of bacteria modulates cytokine production by human DCs**

Next, we set out to determine whether the modulated T cell polarization in response to opsonization of bacteria was mediated by an altered production of polarizing cytokines by DCs. DCs were co-cultured with opsonized or non-opsonized *S. aureus* and after 24h supernatants were harvested and subsequently analyzed for presence of cytokines. Compared to non-opsonized bacteria, activation of DCs with bacteria that were pre-incubated in human serum resulted in strongly enhanced levels of TNFα and Th17 promoting cytokines IL-1β, IL-6, and IL-23 (Fig 2A). Notably, production of the classical Th1-associated cytokine IL-12 was not affected (Fig 2A). Opsonization of bacteria with purified IgG led to an up-regulation of cytokine production in a similar manner as for whole human serum, with increased IL-1β, IL-6, IL-23, and TNFα production, but only very little increase in IL-12 (Fig 2B). Interestingly, incubation with opsonized bacteria did not lead to
enhanced cytokine production by monocytes (Fig 2C). Instead, IL-1β and TNFα production was moderately decreased upon activation with opsonized bacteria, while IL-12 and IL-23 production by monocytes was undetectable, both after incubation with opsonized or non-opsonized bacteria. Thus, these results demonstrate that opsonization of bacteria with

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**Figure 2. Opsonization of bacteria modulates cytokine production by human DCs.** (A, B) DCs were stimulated for 24h with *S. aureus* that was pre-incubated or not in human serum (HS) (A) or purified IgG (B). Cytokine levels were determined by ELISA. Data shown are from one representative experiment of six experiments with different donors. (C) Monocytes were stimulated as described under (B), after which cytokine levels in the supernatant were determined by ELISA. Data shown are from one representative experiment of three experiments with different donors. (error bars, SEM; *P<0.05, **P<0.01; ns = not significant; paired Student’s t-test)
Figure 3. Up-regulation of cytokine production is mediated by FcγRIIa. (A) FcγR expression on immature DCs was measured by FACS. Black line indicates expression, grey line indicates control. Data shown are from one representative experiment of three experiments with different donors. (B) DC were pre-incubated for 30 min. with or without a blocking antibody against FcγRIIa, after which cells were stimulated with IgG-opsonized or non-opsonized S. aureus. Cytokine levels were determined in the supernatant by ELISA. Data shown are from one representative experiment of three experiments with different donors. (C) DCs were pre-incubated for 2h with DMSO or 10 mM Cytochalasin D, after which cells were stimulated with S. aureus that was pre-incubated or not in human serum (HS) or purified IgG (IgG). Cytokine levels were determined in the supernatant by ELISA. Data shown are from one representative experiment of three experiments with different donors. (error bars, SEM; *P<0.05, **P<0.01; ns = not significant; paired Student's t-test)
IgG from serum strongly and selectively modulates cytokine responses by DCs, but not by monocytes.

**Up-regulation of cytokine production is mediated by FcyRIIa**

One of the main receptors on DCs for recognition of IgG-opsonized bacteria is the family of FcyRs. To determine which FcyRs are expressed on DCs we assessed expression by flow cytometry. DCs displayed a low to moderate expression of FcyRI (CD64), FcyRIIb (CD32b), and FcyRIII (CD16), but highly expressed FcyRIIa (CD32a) (Fig 3A). To determine whether FcyRs are responsible for the enhanced cytokine production by DCs induced by opsonized bacteria, we blocked different FcyRs with specific antibodies during DC activation and assessed cytokine production. Blocking of FcyRI and FcyRIII did not have any effect on cytokine production induced by opsonized bacteria (data not shown). In contrast, blocking of FcyRIIa inhibited the up-regulation of IL-1β, IL-6, IL-23, and TNFα production induced by opsonized bacteria and reduced it to the level induced by non-opsonized bacteria (Fig 3B). Thus, FcyRIIa is responsible for the up-regulation of cytokine production by DCs activated by IgG opsonized bacteria.

To determine whether internalization of opsonized bacteria is required for the amplification of cytokines, we blocked internalization using actin-polymerization inhibitor cytochalasin. Importantly, blocking internalization did not prevent the enhanced cytokine production induced by IgG opsonization (Fig 3C) indicating that the induction of cytokines by FcyRIIa does not depend on internalization.

**FcyRIIa ligation up-regulates cytokine production via synergy with TLR stimulation**

Our data showed that FcyRIIa ligation by opsonized bacteria strongly up-regulated the production of particular cytokines. However, in previous studies FcyRIIa has been described as a poor inducer of cytokine responses by DCs. To more specifically investigate the role of FcyRIIa in cytokine induction, we stimulated DCs overnight with plate-bound IgG, which is recognized by low-affinity FcyRs such as FcyRIIa. Consistent with previous findings, stimulation with complexed IgG alone did not induce any detectable amount of cytokines, with the exception of little amounts of IL-1β and IL-8 (Fig 4A, 4B, and S1). However, besides FcyRs, opsonized bacteria are recognized through various PRRs, which sense microbial structures that induce cytokine production. The best-studied PRRs belong to the family of TLRs, of which TLR2, 4 and 5 are known to play a major role in recognition of bacteria. As expected, TLR4 stimulation by LPS and TLR2 stimulation by PGN alone induced production of all measured cytokines (Fig 4A, 4B, and S1), while stimulation of TLR5 by flagellin induced very little amounts (Fig S2A).

In order to mimic the condition of stimulation with IgG-opsonized bacteria, we stimulated DCs simultaneously with complexed IgG and TLR ligands. Strikingly, co-stimulation of LPS with IgG strongly amplified TLR4-induced production of IL-1β, IL-23,
Figure 4. FcγRIIa ligation up-regulates Th17-promoting cytokines via synergy with TLR stimulation. (A,B) DCs were stimulated with plate-bound IgG, LPS (A), PGN (B), or combination. After 24h cytokine levels in the supernatant were determined by ELISA. Data shown are from one representative experiment of at least five experiments with different donors. (C) Fold-increase in cytokine production upon LPS or PGN stimulation combined with plate-bound IgG stimulation compared to LPS or PGN stimulation without plate-bound IgG. For every cytokine, every dot represents a different donor tested in an individual experiment. (D) DCs were pre-incubated for 30 min. with or without a blocking antibody against FcγRIIa, after which cells were stimulated with LPS or PGN in combination with plate-bound IgG. Data shown are from one representative experiment of three independent experiments with different donors. (E) DCs were pre-incubated for 30 min. with or without a blocking antibody against FcγRIIa. Subsequently, the cells were stimulated with LPS or PGN in combination with plate-bound IgG and co-cultured with CD4+ T cells. IL-17 levels were measured by ELISA at day 12, after re-stimulation with a CD3-specific and a CD28-specific antibody. Data shown are from one representative of three different donors. (error bars, SEM; *P<0.05, **P<0.01; ns = not significant; paired Student’s t-test)
and TNFα, but not or only moderately affected production of IL-6, IL-8, IL-10, and IL-12 (Fig 4A and S3). Similarly, stimulation with complexed IgG up-regulated PGN-induced IL-1β, IL-23, and TNFα production (Fig 4B). In contrast to LPS stimulation, co-ligation with PGN moderately increased production of IL-6, while IL-12 remained undetectable both with and without IgG co-ligation (Fig 4B). Cytokine production induced by TLR5-ligand flagellin was modulated in a similar manner as PGN (Fig S2A). To compensate for potential donor differences we calculated the fold-increase of the different cytokines upon co-stimulation for multiple donors. In line with the previous results, stimulation with complexed IgG strongly up-regulated LPS- and PGN-induced IL-1β, IL-23, and TNFα, while IL-6 and IL-12 production remained generally unaffected (Fig 4C).

Next, to determine whether the enhanced cytokine production induced by complexed IgG is dependent on FcyRIIa, we blocked the receptor by pre-incubating DCs with an FcyRIIa-specific antibody. IgG-induced up-regulation of IL-1β, IL-23, and TNFα was completely blocked by anti-FcyRIIa (Fig 4D and S2B). To ensure that the observed block did not result from unrecognized effects from using whole anti-FcyRIIa IgGs, we generated Fab-fragments of the anti-FcyRIIa antibody. Anti-FcyRIIa Fabs displayed an almost identical block of IgG-induced cytokine production compared to whole antibody (Fig S3A). In addition, specific block of FcyRIIb, which is closely related to FcyRIIa, did not inhibit IgG-induced cytokine up-regulation (Fig S3B), demonstrating that the effect fully depends on FcyRIIa.

Thus, consistent with previous findings, FcyRIIa is a poor inducer of cytokines when stimulated alone. However, FcyRIIa synergizes with TLR2, 4, and 5 for the selective up-regulation of IL-1β, IL-23, and TNFα, thereby correlating with the up-regulated cytokine profile observed after opsonization of bacteria.

Co-stimulation of TLRs and FcyRIIa promotes Th17 responses
Cytokines IL-1β and IL-23, which are up-regulated by TLR-FcyRIIa co-stimulation, are pivotal for human Th17 development. To determine whether TLR-FcyRIIa co-stimulation, similar to what occurs upon stimulation with opsonized bacteria, indeed induces Th17 responses, we co-cultured stimulated DCs with CD4⁺ T cells and quantitatively determined IL-17 secretion in the supernatant upon re-stimulation after 12 days. As expected from the little amount of cytokine production, stimulation with complexed IgG alone did not induce any IL-17 secretion (Fig 4E). However, stimulation of DCs with complexed IgG strongly enhanced both LPS- and PGN-induced IL-17 production by T cells (Fig 4E). The enhanced IL-17 production was mediated by FcyRIIa, since addition of a blocking antibody attenuated IL-17 production to the level of TLR stimulation alone (Fig 4E). Thus, FcyRIIa cooperates with TLRs on DCs for the promotion of Th17 responses.
FcγRIIa ligation strongly increases sensitivity to TLR ligands and relies on IgG1 and IgG2

To investigate the effect of IgG stimulation on TLR responses in more detail, we stimulated DCs at serial-step dilutions of LPS and PGN and measured cytokine production after co-stimulation with plate-bound IgG. Strikingly, IgG co-stimulation promoted TLR-induced IL-1β, IL-23, and TNFα even at TLR agonist concentrations that were 100 to 1000 fold below optimum, which are concentrations that did not induce any detectable cytokines in the absence of FcγR co-stimulation (Fig 5A). This demonstrates that FcγRIIa stimulation drastically increases the sensitivity of DCs for TLR ligands.

In addition, we assessed the role of different IgG subclasses. Human serum and

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**Figure 5.** FcγRIIa ligation increases sensitivity to TLR ligands and relies on IgG1 and IgG2. (A) DCs were stimulated with plate-bound IgG and different concentrations of LPS or PGN. After 24h cytokine levels in the supernatant were determined by ELISA. Data shown are from one representative experiment of at least five experiments with different donors. (B) DCs were stimulated with LPS in combination with either plate-bound total IgG, IgG1, IgG2, or IgG3. After 24h cytokine levels in the supernatant were determined by ELISA. Data shown are from one representative experiment of at least three experiments with different donors. (error bars, SEM; *P<0.05, **P<0.01; ns = not significant; paired Student’s t-test)
tissue fluids contain a mixture of different IgG subclasses, which may differently stimulate FcγRIIa and consequently give rise to different responses. Therefore, we selectively co-stimulated DCs with either IgG1, IgG2, or IgG3. IgG4 was omitted considering its extremely low affinity to FcγRs. While co-stimulation with IgG1 and IgG2 up-regulated IL-1β, IL-23, and TNFα in a similar manner as total IgG, co-stimulation with IgG3 hardly induced any up-regulation of cytokines (Fig 5B). These data indicate that the subclass of IgG plays an important role in the orchestration of Th17 responses against bacteria. Taken together, FcγRIIa stimulation strongly enhances the sensitivity of DCs for TLR agonists, and FcγRIIa-induced up-regulation of cytokines is mainly induced by IgG1 and IgG2.

**FcγRIIa ligation modulates cytokine production through both enhanced transcription and caspase-1 activation**

Regulation of cytokine production can be organized at several levels. To determine whether the up-regulation of cytokines by TLR and FcγRIIa co-ligation is orchestrated at the level of transcription, we determined mRNA expression over time by quantitative RT-PCR after co-stimulation with TLR ligands and plate-bound IgG. Similar to protein production of the cytokines, stimulation of DCs with LPS or PGN alone induced transcription of IL-1β, IL-12p35, IL-12p40, IL-23p19, and TNFα, while stimulation with complexed IgG merely induced little amounts of IL-1β mRNA, but not other cytokine genes (Fig 6A and S4). However, co-stimulation of TLRs with FcγRIIa strongly enhanced IL-23p19 and TNFα transcription, correlating with the enhanced production at protein level. Remarkably, IL-12p40 mRNA production was strongly reduced after co-stimulation with IgG (Fig S4), indicating that for particular genes FcγRIIa co-stimulation also induces transcriptional down-regulation. Taken together, these data suggest that the synergy between TLRs and FcγRIIa for induction of IL-23, and TNFα is mediated at the level of transcription.

Remarkably, IL-1β production seemed to be substantially less up-regulated on mRNA level than on protein level after TLR-FcγRIIa co-stimulation (Fig 6A and 4C respectively), suggesting that other mechanisms of synergy between these two receptors are involved. The release of biologically active IL-1β is known to be a two-step process: besides transcription and translation, caspase-1 needs to be activated, which processes pro-IL-1β to functional IL-1β. To investigate whether caspase-1 is involved we determined its activation after stimulation using fluorescent caspase-1-binding compound FAM-YVAD-FMK and analyzed results by flow cytometry. Strikingly, stimulation with complexed IgG rapidly activated caspase-1 (Fig 5B), indicating that FcγR stimulation alone is sufficient for inflammasome activation. In addition, co-stimulation with IgG further increased LPS and PGN-induced caspase-1 activation in an additive manner (Fig 5B). Overall, these data indicate that Th17 induction after IgG opsonization of bacteria is mediated by synergy between TLRs and FcγRIIa on DCs, which enhances the production of Th17-polarizing cytokines via both enhanced transcription and caspase-1 activation.
Figure 6. FcγRIIa ligation enhances cytokine transcription and activates caspase-1. (A) Relative mRNA expression was determined in DCs that were not stimulated, stimulated with plate-bound IgG, LPS, PGN, or a combination of LPS or PGN with plate-bound IgG. Lysates were made at the indicated time points, after which mRNA expression of the indicated genes was determined by quantitative RT-PCR. Expression is normalized to GAPDH and set at 1 for unstimulated cells at 0h. Data shown are from one representative experiment of five experiments using different donors. (B) Caspase-1 activation was determined in DCs that were unstimulated, stimulated with plate-bound-IgG, LPS, PGN, or a combination of LPS or PGN with plate-bound IgG. After stimulation at indicated time points, cells were washed and incubated with a fluorescent caspase-1 inhibitor for 1h, after which cells were washed and fluorescence was measured using flow cytometry. Data shown are from one representative experiment of three experiments using different donors.
DISCUSSION

Polarization of T helper cell responses is crucial for pathogen clearance, since it tailors immune responses to the class of pathogen involved. Here we have identified opsonization of bacteria by IgG as a new mechanism by which the immune system promotes Th17 responses, which is essential to efficiently combat extracellular pathogens. IgG opsonization of bacteria selectively up-regulates the production of TNFα and Th17-polarizing cytokines IL-1β, IL-6, and IL-23 by human DCs, which fully depends on low-affinity IgG receptor FcγRIIa. Notably, FcγRIIa stimulation does not directly lead to cytokine production by DCs, but cooperates with TLR stimulation for the amplification of cytokine production. This synergy is regulated at two levels. First, co-ligation increases the transcription of pro-IL-1β, IL-23p19, and TNFα. Second, FcγRIIa activates caspase-1 independent of TLR stimulation, thereby further enhancing the production of IL-1β. Importantly, Th17 induction via this mechanism is likely to occur particularly in tissues that are located just below the epithelial barriers that are most frequently the target of bacterial infections and contain abundant amounts of DCs and interstitial IgGs. Thus, TLR-FcγR cross-talk on DCs may be a universally relevant natural mechanism to counteract bacterial infections in numerous sites in the human body.

Upon infection, pathogens are recognized by multiple microbial sensors on antigen presenting cells. The ultimate immune response against a given pathogen critically depends on the interaction between these different receptors. The main current concept is that this cross-talk is predominantly induced between receptors belonging to the families of PRRs, with an underexposed role for other receptors. Here we show that FcγRIIa also plays an important role in pathogen recognition via cross-talk with TLRs. In contrast to PRRs, FcγRIIa does not recognize PAMPs expressed by microbes, but instead recognizes pathogens upon opsonization with IgG. Most likely as a result of previous pathogen exposure, IgG cross-reactivity and expression of common microbial structures recognized by IgGs, invasion of the body by pathogens such as bacteria rapidly leads to opsonization. Recognition of IgG-opsonized microbes by FcγRs has previously been described to mediate various effects on DCs, such as pathogen uptake, degradation of pathogen-derived structures, antigen presentation of pathogen-derived peptides, and DC maturation. However, the capacity of FcγRs to induce production of polarizing cytokines by human DCs is considered to be limited. Here we show a new function of FcγRs in production of Th17-promoting cytokines by DCs via cross-talk with PRRs. As such, this synergy adds another layer of complexity to recognition of pathogens.

Stimulation of FcγRIIa affected TLR-induced cytokine production in a selective manner. Some cytokines were strongly amplified (IL-1β, IL-23, and TNFα), while others were not, or only moderately, affected (IL-8, IL-10, and IL-12). Notably, the effect on IL-12 production could only be assessed upon TLR4 stimulation, since TLR2-ligand PGN is known to be a poor inducer of IL-12. The observation that IL-12 is not enhanced by co-
ligation of TLRs and FcγRIIa was confirmed when DCs were stimulated with opsonized bacteria, indicating that this effect also holds true for whole pathogens. Modulation of IL-6 production appears to be dependent on the type of TLR involved: IgG co-stimulation does not affect TLR4-induced IL-6 production, but does up-regulate TLR2- and TLR5-induced IL-6 production. IgG opsonization of *S. aureus* enhanced IL-6 production by DCs, which may suggest that the modulation of cytokine production for bacteria mainly results from cross-talk with TLR2. Combined, these data strongly suggest that the observed Th17 induction by opsonization of bacteria is mediated via specific up-regulation of particular polarizing cytokines by DCs: besides up-regulating Th17-promoting IL-1β, IL-6, and IL-23, TLR-FcγRIIa cross-talk does not enhance IL-12 production, which is pivotal for Th1 development and in turn can inhibit Th17 responses. In addition, FcγRIIa co-stimulation enhances TNFα production. Although TNFα produced by plasmacytoid DCs has recently been suggested to play a role in Th22 development, the role of TNFα in T cell polarization is still incompletely characterized.

Whether or how IgG opsonization modulates the polarization of immune responses to other classes of pathogens is not yet clear. For example, viral clearance requires Th1 and cytotoxic lymphocyte responses instead of Th17 and therefore FcγRIIa-mediated Th17 promotion would be counterproductive. However, Th17 induction against viruses by this mechanism may be less likely to occur. For example, IgG opsonization of pathogens requires specific anti-microbial IgGs to be present already at the time of infection. While IgGs directed against numerous bacteria are pre-existent at the time of infection, antibodies against viruses are generally highly specific for viral proteins of a particular (sub)type of virus and therefore not (yet) available during primary infection, suggesting that viral opsonization and subsequent TLR-FcγRIIa cross-talk is less likely to occur. In addition, viruses are recognized through different PRRs than bacteria, and cytokine induction by these viral-sensing PRRs may be differently modulated upon co-stimulation with FcγRIIa. Further research on FcγRIIa cross-talk with different families of PRRs will shed light on how this mechanism is involved in conferring pathogen-class specific immunity to other microorganisms. Taken together, these data indicate that TLR-FcγRIIa cross-talk on DCs during stimulation with bacteria enhances the production of particular polarizing cytokines that promote Th17 responses.

The main FcγR responsible for the observed effects is FcγRIIa, which is expressed on most hematopoietic cells besides lymphocytes. Notably, FcγRIIa is restrictively expressed in primates, suggesting that the observed effects may be specific for these species. Alternatively, in other species FcγR-TLR cross-talk could be mediated by other receptors. For example, in mice FcγRIIIa is closely related to human FcγRIIa, even though there are differences in intracellular domains and cellular expression between the human and murine orthologue, and FcγRIII in mice has been associated previously with induction of Th2 responses. On most human cell-types including DCs, FcγRIIa is co-expressed with the closely related, but inhibitory, isoform FcγRIIb. While complexed IgG stimulates...
both FcγRIIa and FcγRIIb, the specific up-regulation of cytokines by IgG stimulation was fully dependent on FcγRIIa. Importantly, FcγRIIa is a low-affinity receptor that selectively interacts with IgG in the form of immune complexes, such as expressed on opsonized pathogens, and not with monomeric antibodies\textsuperscript{37}. This feature may be essential for shaping T helper cell responses in an appropriate manner. Monomeric IgGs are present in high concentrations in blood and tissues, but do not induce or amplify cytokine production via triggering of FcγRIIa or other (high or low-affinity) FcγRs. In contrast, only upon pathogen entry and opsonization the complexed IgG on the microbe will stimulate FcγRIIa leading to enhanced cytokine production. Nevertheless, recognition of immune complexes alone, i.e. in absence of TLR stimulation, is also not sufficient for induction of immune responses by DCs. Although FcγRIIa stimulation induces DC maturation, we and others have shown that FcγRIIa stimulation alone induces little production of pro-inflammatory cytokines and hence are unlikely to mediate T cell polarization\textsuperscript{21-23}. Therefore, FcγRIIa activation can only act in concert with a second “danger” signal such as TLR agonists to potentiate immune responses.

Our data show that the mechanism of cross-talk between FcγRIIa and TLRs for the induction of cytokines is orchestrated at at least two different levels. First, FcγRIIa-TLR cross-talk acts at the transcriptional level by selectively amplifying TLR-induced transcription of IL-1β, IL-23p19, and TNFα, while IL-6 transcription is up-regulated depending on the TLR involved (Fig S5). Additionally, IL-12p40, the subunit required for both functional IL-12 and IL-23, was down-regulated after TLR-FcγRIIa co-stimulation. However, since IL-12p40 is usually produced in abundance, IL-23 production most likely mainly depends on enhanced transcription of IL-23p19. As a second mechanism, FcγRIIa up-regulates cytokine production via activation of caspase-1. Production of bioactive IL-1β depends both on gene transcription as well as cleavage of transcribed pro-IL-1β into its functional form by caspase-1\textsuperscript{28,29}. While TLRs are potent inducers of IL-1β transcription, caspase-1 activation by TLR stimulation is limited, especially by LPS\textsuperscript{40,41}. Importantly, here we show that FcγRIIa stimulation, independent of TLR stimulation, activates caspase-1, thereby identifying FcγRs as a new class of receptors that can activate caspase-1. Consequently, TLRs and FcγRIIa collaborate for the induction of IL-1β: TLR stimulation induces IL-1β transcription, while FcγRIIa stimulation enhances IL-1β transcription and activates (LPS) or enhances (PGN) activation of caspase-1, leading to functional IL-1β. Taken together, these two mechanisms enhance the production of particular cytokines by TLRs, while not inducing cytokine production on its own.

Previously, FcγRIIa has been shown to internalize and deliver DNA-containing immune complexes to lysosomes that contain TLR9 in plasmacytoid DCs, and in this manner facilitate cytokine production\textsuperscript{42}. However, blocking internalization of bacteria in DCs did not prevent the enhanced cytokine production induced by IgG opsonization. This indicates that, in contrast to several other FcγR-dependent effects or effects on other cell-types, TLR-FcγRIIa synergy in DCs for induction of Th17 cytokines is not dependent on internalization.
Most likely, FcγRIIa-mediated effects are induced by direct induction of a downstream signaling cascade that modulates TLR responses. It is tempting to speculate on a vital role for spleen tyrosine kinase (Syk), which has previously been described to be involved in several FcγRIIa-mediated effects and is also known to modulate gene transcription and inflammasome activation. In addition, induction of different signaling pathways may explain the distinct effects of different IgG subclasses. While stimulation of FcγRIIa with IgG1 or IgG2 strongly amplified TLR-induced cytokine production, stimulation with IgG3 had little effect. Paradoxically, FcγRIIa has a higher affinity for IgG3 than for IgG1 or IgG2. However, recently it has been shown that for both FcαRI and FcγRIII binding of immunoglobulins with different affinity induces the recruitment of different signaling molecules. Hence, the lower affinity of IgG1 and IgG2 compared to IgG3 for FcγRIIa may induce a different signaling cascade, resulting in distinct effects on TLR-induced cytokine production. Importantly, only minute concentrations of TLR agonists were required for synergy with FcγRIIa. This underlines that FcγRIIa stimulation strongly increases the sensitivity of DCs for TLR ligands, resulting in the promotion of Th17 responses even at very low bacterial presence. Furthermore, this increased sensitivity may be highly relevant for therapeutic purposes such as vaccine development. Remarkably, cross-talk between FcγRIIa and TLRs seems to be specific for DCs. Opsonization of bacteria did not lead to any increase in cytokine production by monocytes, the cell type the in vitro generated DCs are derived from. This difference between cell types does not seem to be dependent on receptor expression, since both TLRs and FcγRIIa are highly expressed on monocytes. Hence, these findings suggest that cell-intrinsic signaling properties are required for TLR-FcγRIIa cross-talk. What these properties are and whether they are present in other cell-types besides DCs is currently under investigation.

Taken together, our data indicate that IgG opsonization of bacteria, besides enhancing uptake and antigen presentation, is a natural mechanism to tailor immune responses to effectively combat extracellular pathogens. From a therapeutic point of view, induction of this TLR-FcγRIIa synergy mechanism on DCs may be a useful tool to skew immune responses to clear pathogenic infections more efficiently.

**METHODS**

**BINDING-ELISA WITH BACTERIA**

Sheep blood agarose plates (Biomerieux) were inoculated with *Klebsiella pneumoniae* (clinical isolate), *Escherichia coli* (ATCC 8738), *Salmonella typhimurium* (clinical isolate), *Staphylococcus aureus* (RN4220) or *Staphylococcus epidermidis* (RP62a) and incubated at 37°C overnight. Bacteria were removed from the plate, washed and coated overnight in PBS in 96-well Maxisorp plates (Nunc). Wells were washed three times, blocked with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich) and subsequently incubated with irrelevant human IgG (Humira; Sanquin Blood Supply), human serum (Lonza) or 5 μg/mL purified IgG (Nanogam; Sanquin Blood Supply), washed three times, and incubated
with anti-human IgG-HRP (MH16-1; Sanquin Blood Supply).

**Cells and stimulation**

Monocyte-derived DCs and monocytes were isolated and cultured as described before. Memory CD4 T cells were obtained by isolating human PBMCs from heparinized human peripheral blood by density gradient centrifugation on Lymphoprep (Nycomed), Percoll (Pharmacia), and finally purification using a MACS isolation kit (Miltenyi Biotech) using CD45RO PE (Dakopatts) and anti-PE-beads (Miltenyi Biotech). Bacteria were opsonized by pre-incubation for 2h in human serum or 500 μg/mL purified IgG and were washed three times afterwards in PBS.

Stimulation with *S. aureus* and *K. pneumoniae* was performed in X-VIVO 15 culture medium (Lonza) using 10 bacteria/DC. Stimulation of DCs with with 100 ng/mL LPS (Sigma-Aldrich) or 10 μg/mL peptidoglycan (PGN; Invivogen) was done in Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco) with 2.5% FCS (Lonza). DCs were stimulated with plate-bound IgG by overnight coating of 96-well Maxisorp plates at room temperature with 1 μg/mL of Nanogam, human IgG1, IgG2 (both Sigma-Aldrich), or IgG3 purified from Nanogam (Sanquin Blood Supply) diluted in PBS. Both IgG-coated and control wells were blocked by 2h incubation with PBS with 10% FCS.

FcγRIIa was blocked by pre-incubating DCs with 20 μg/mL IV.3 (Stemcell Technologies) for 30 min at 37°C, after which stimuli and medium was added resulting in a final concentration of 5 μg/mL. Uptake of bacteria was inhibited by pre-incubating DCs for 2h at 37°C in X-VIVO medium containing 10 mM Cytochalasin D (Sigma-Aldrich).

**In vitro differentiation of CD4 T cells**

For T helper cell polarization, bacteria were opsonized and co-cultured with 2,000 DCs and 20,000 allogeneic CD4 T cells per well in Maxisorp plates in X-VIVO 15 culture medium in the presence of 10 pg/mL *Staphylococcus aureus* enterotoxin B (SEB; Sigma-Aldrich). After four days, cells were transferred to 96-wells round bottom plates (Greiner Bio-One) and every two days half of the medium was replaced by IMDM with 2.5% FCS and 10 U/mL IL-2 (Chiron) and wells were splitted if necessary. Resting cells were re-stimulated at day 12 with 0.1 μg/mL phoral myristate acetate (PMA), 1 μg/mL ionomycin and 10 μg/mL brefeldin A (Sigma-Aldrich) for 5h. Cells were washed, fixed with 4% paraformaldehyde (Merck) for 15 minutes, washed again, permeabilized with 0.5% saponin (Calbiochem) in PBS containing 1% BSA and incubated with anti-IL-17-biotin (eBioscience) followed by streptavidin-PE (BD Pharmingen) and anti-IFNy-FITC (BD Biosciences) for 30 minutes at room temperature and analyzed by flow cytometry. For measuring cytokines in supernatant, cells were re-stimulated with 1 μg/mL anti-CD3 (clone 1XE, Sanquin Blood Supply) and anti-CD28 (15E8, Sanquin Blood Supply) for 24h. IL-17 was measured using coated anti-IL-17A (eBio64CAP17, eBioscience) and biotinylated anti-IL-17A (eBio64DEC17, eBioscience), and recombinant IL-17A (R&D Systems) as a standard.
ELISA and Quantitative RT-PCR
DCs (50,000/well) or monocytes (100,000/well) were stimulated in 96-well Maxisorp plates and supernatants were harvested after 24h and stored at -20°C until the levels of IL-1β (Endogen), IL-6 (Biosource), IL-12 (UCytech), IL-23 (eBioscience), and TNFα (eBioscience) were measured by sandwich ELISA, as described previously. mRNA production was determined using quantitative RT-PCR (iCycler iQ Multi-Color Real Time PCR Detection System; Bio-Rad). DCs were lysed at the indicated time points, after which mRNA extraction was done using the NucleoSpin RNA Isolation Kit (Macherey-Nagel) and cDNA synthesis using MBI Fermentas Kit. For each sample, the normalized amount of target mRNA was calculated from the obtained Ct values (defined as the number of PCR cycles where the fluorescence signal exceeds the detection threshold value) for both target and GAPDH mRNA with $N_t = 2^{\Delta \text{Ct(GAPDH)} - \text{Ct(target)}}$. Data were expressed as relative mRNA expression, which represents the mRNA induction compared to unstimulated cells (which was set at 1). For overview of used primers see Table S1.

FcγR expression and FLICA
FcγR expression was determined by staining cells with anti-CD64 (Sanquin Blood Supply), anti-CD32a (IV.3), or anti-CD16 (Sanquin Blood Supply) followed by PE-conjugated goat-anti-mouse (Jackson ImmunoResearch), or directly labeled anti-CD32b-AF488 (2B6; Sanquin Blood Supply), and analysis by flow cytometry. Caspase-1 activation was determined using the FLICA Apoptosis Detection Kit for Caspase-1 (Immunochemistry Technologies) according to the manufacturer’s guidelines. Fluorescence of the cells was assessed using flow cytometry.

Statistics
Data were analyzed for statistical significance using the paired Student’s t-test with GraphPad Prism 5.0 software (GraphPad Software).

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Author Contributions
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**SUPPLEMENTAL METHODS**

**Cells and stimulation**

Fab fragments of IV.3 and anti-FcγRIIb (2B6; Macrogenics) were used for block in a similar manner as block with IV.3. Since the anti-FcγRIIb antibody may also block the closely related FcγRIIc, inhibition of FcγRIIb was performed on DCs from donors that do not express functional FcγRIIc because of a single nucleotide polymorphism, as determined by quantitative RT-PCR on mRNA samples (for primers see table S1). Fab fragments of IV.3 were generated by papain digestion. 80 μg of mAb was digested with 0.8 μg of pre-activated papain (2.1 mg/mL, 1 mM DTT, 30 min, 37°C) for 100 minutes at 37°C in 100 μL PBS pH 7.4, 3 mM EDTA. DTT was removed from pre-activated papain by repeated dilution/concentration using Amicon Centriprep centrifugal filter devices (Millipore). Fab fragments were separated from Fc fragments by protein A affinity (Protein A 4 fast flow, GE Healthcare) chromatography. Purity was confirmed by SDS-PAGE (NuPage)/silver stain (SilverQuest Silver Staining Kit, Life Technologies). DCs were stimulated with 1 μg/mL flagellin (Invivogen) in Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco) with 2.5% FCS (Lonza).

**ELISA**

DCs (50,000/well) were stimulated in 96-well Maxisorp plates and supernatants were harvested after 24h and stored at -20°C until the levels of IL-8 (Biosource) or IL-10 (BD Pharmingen) were measured by sandwich ELISA.

**Table S1. Primers**

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>TTTGAGTCTGCCAGTTCCC</td>
<td>TCAGTTATATCCTGAGCCGCGC</td>
</tr>
<tr>
<td>IL-6</td>
<td>TGACAAACAAATTCGGTACATCCT</td>
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<td>IL-12p35</td>
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<td>IL-12p40</td>
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<tr>
<td>IL-23p19</td>
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<td>TTTGCAAGCAAGACTGACTGTTG</td>
</tr>
<tr>
<td>TNFα</td>
<td>GGCTCCAGGGCGGTCTCTTGG</td>
<td>CAGATAGATGGGCTCATACCA</td>
</tr>
<tr>
<td>FcγRIIc</td>
<td>GCTATTCCCTGGCTCTGTTG</td>
<td>TCACAGGATCAGTGGAAATTG</td>
</tr>
</tbody>
</table>
**SUPPLEMENTAL DATA**

Figure S1. FcγRIIa co-ligation does not affect IL-8 and only moderately affects IL-10 production. DCs were stimulated with complexed IgG, LPS, or combination. After 24h cytokine levels were determined in the supernatant by ELISA. Data shown are from one representative experiment of three with different donors. (error bars, SEM; *P<0.05, **P<0.01; ns = not significant; Student’s t-test)

Figure S2. FcγRIIa ligation also upregulates cytokines via synergy with TLR5 stimulation. (A) DCs were stimulated with complexed IgG, flagellin, or combination. After 24h cytokine levels were determined in the supernatant by ELISA. Data shown are from one representative experiment of three with different donors. (B) DCs were pre-incubated for 30 min with or without a blocking antibody against FcγRIIa, after which cells were stimulated with flagellin in combination with complexed IgG. Data shown are from one representative experiment of three independent experiments with different donors. (error bars, SEM; *P<0.05, **P<0.01; ns = not significant; Student’s t-test)
Figure S3. IgG-induced cytokine production is blocked by anti-FcγRIIa Fabs, but not by anti-FcγRIIb. (A) DCs were pre-incubated for 30 min. with or without a whole blocking antibody against FcγRIIa or Fab fragments, after which cells were stimulated with complexed IgG, LPS, or combination. After 24h cytokine levels were determined in the supernatant by ELISA. Data shown are from one representative experiment of three with different donors. (B) DC were pre-incubated for 30 min with or without a blocking antibody against FcγRIIa or FcγRIIb, after which cells were stimulated with LPS in combination with complexed IgG. Data shown are from one representative experiment of three independent experiments with different donors. (error bars, SEM; *P<0.05, **P<0.01; ns = not significant; Student’s t-test)
Figure S4. FcγRIIa ligation differently affects transcription of LPS-induced IL-12 subunits. Relative mRNA expression was determined in DCs that were not stimulated, stimulated with complexed IgG, LPS, or a combination. Lysates were made at the indicated time points, after which IL-12p35 and IL-12p40 mRNA expression was determined by quantitative RT-PCR. Expression is normalized to GAPDH and set at 1 for unstimulated cells at 0h. Data shown are from one representative experiment of three using different donors.

Figure S5. FcγRIIa ligation differently modulates LPS- and PGN-induced IL-6 transcription. Relative mRNA expression was determined in DCs that were not stimulated, stimulated with complexed IgG, LPS, PGN, or a combination of LPS or PGN with complexed IgG. Lysates were made at the indicated time points, after which IL-6 mRNA expression was determined by quantitative RT-PCR. Expression is normalized to GAPDH and set at 1 for unstimulated cells at 0h. Data shown are from one representative experiment of three using different donors.

SUPPLEMENTAL REFERENCE