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Rheumatoid arthritis (RA) is a common autoimmune disease leading to profound disability and premature death. Although a role for FcγRs and TLRs is accepted, their precise involvement remains to be elucidated. FcγRIIB is an inhibitory FcγR important in the maintenance of tolerance. We hypothesized that the inhibitory FcγRIIB inhibits TLR responses on monocyte-derived dendritic cells (DC) and serves as a counterregulatory mechanism to dampen inflammation, and we surmised that this mechanism might be defective in RA. The expression of the inhibitory FcγRIIB was found to be significantly higher on DCs from RA patients having low RA disease activity in the absence of treatment with antirheumatic drugs. The expression of activating FcγRs was similarly distributed among all RA patients and healthy controls. Intriguingly, only DCs with a high expression of FcγRIIB were able to inhibit TLR4-mediated secretion of proinflammatory cytokines when stimulated with immune complexes. In addition, when these DCs were coincubated with the combination of a TLR4 agonist and immune complexes, a markedly inhibited T cell proliferation was apparent, regulatory T cell development was promoted, and T cells were primed to produce high levels of IL-13 compared with stimulation of the DCs with the TLR4 agonist alone. Blocking FcγRIIB with specific Abs fully abrogated these effects demonstrating the full dependence on the inhibitory FcγRIIB in the induction of these phenomena. This TLR4-FcγRIIB interaction was shown to dependent on the PI3K and Akt pathway.

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conserved pathogen-associated molecular patterns as well as endogenous ligands sparked a revolution of research that constitutes the basis of our current way of thinking regarding the role for TLR in arthritis. First, various research groups have demonstrated that endogenous TLR ligands are abundant in RA patients, both in the circulation as well as in the synovial compartment (18–20). Second, the expression of various TLR subtypes was clearly increased in the synovial compartment of RA patients (3, 4, 21) compared with their healthy counterparts, and TLR ligands induce an augmented inflammatory response by macrophages and DCs from RA patients (3, 22). Finally, studies in mice revealed that the triggering of TLR aggravates arthritis whereas inhibition of the TLR4 pathways either by genetic knockdown (23) or by addition of TLR antagonists drastically reduced the arthritis incidence and severity (24).

The ability of the immune system to distinguish self from non-self is central to its diametrically opposed functions; to protect against invading pathogens and, at the same time, maintain nonresponsiveness to self. Given the ubiquitous nature of endogenous TLR ligands during life, highly regulated counterregulatory responses must be in place to secure an adequate balance between immunity and tolerance (25–27). Led by recent work from our group and others, we postulated that the inhibitory-activation FcγR paradigm might not be the full story in that FcγRIIb might also control TLR4-mediated cell activation (17, 28). This view on TLR4-FcγR cross-talk can be carried over to DCs because these cells have been shown to be under tight control by both receptor systems and to play a decisive role in the regulation of the balance between immunity and tolerance (15, 16, 25, 29). The pathways that underlie cross-talk between TLR4 and FcγR are currently unknown. It has been reported that, at least some of the mediators implicated in FcγR-mediated signaling, are involved in the TLR4 signaling cascade. For example, stimulation of TLR4 results in the recruitment of SHIP to lipid rafts where it is tyrosine phosphorylated, and SHIP appears to be a positive regulator of TLR4 activation by enhancing MAPK phosphorylation and decreasing Akt phosphorylation (30). In turn, PISK reverses the effects of SHIP in both the TLR4 and FcγR pathways, and in both pathways serine/threonine kinase Akt was shown to play a central role.

Here we demonstrate, for the first time, that the inhibitory FcγRIIb directly inhibits TLR4-mediated cell activation and functions as a counterregulatory mechanism designed to dampen TLR-mediated responses. Strikingly, only DCs from RA patients who were able to discontinue their use of disease-modifying antirheumatic drugs (DMARD) without the occurrence of subsequent disease flares expressed remarkably high levels of FcγRIIb, whereas the expression of activating FcγR was unaltered. Exclusively, DCs from those patients who were able to inhibit TLR4-mediated DC activation and subsequent T cell proliferation but also restored the ability to induce T cell-regulatory capacity by DCs. Collectively, here we show a unique counterregulatory pathway for TLR4-mediated immune responses that is aberrant in RA, underscoring the pivotal role for FcγRIIb in RA and opening novel avenues for therapeutic intervention.

Materials and Methods

Study population

A total of 32 RA patients attending the Department of Rheumatology at the Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands) and 10 healthy controls were included. The patients were selected from our well-documented prospective cohort consisting of >500 RA patients. All patients who were not receiving DMARD therapy for >2 years were selected; this resulted in a total population of 11 patients. By extensive screening outside of this cohort, five additional RA patients not receiving DMARD therapy were found in our outpatient clinic. RA patients receiving DMARD therapy were all selected from our prospective cohort. All patients fulfilled the American College of Rheumatology criteria for RA at the time of disease diagnosis and gave their informed consent (31). Patients using biological agents and/or prednisolone were excluded from the study. Before every vena puncture, to obtain monocytes for DC culture, the disease activity of the RA patients was determined. To quantify the disease activity, the disease activity score (DAS28) was used. The DAS28 incorporates the number of swollen and tender joints determined for 28 joints, the erythrocyte sedimentation rate, and a score on the visual analog scale on well-being. The presence of an erosive disease was scored as positive if on the last x-rays from the feet or hands at least one erosion was present scored by means of the modified Sharp/Van der Heide method (32). The local Medical Ethics Committee approved the study protocol.

Culture of monocyte-derived DCs

PBMCs were isolated from heparinized venous blood by using density gradient centrifugation over Ficoll-Paque (Amersham Bioscience). Monocytes were obtained using CD14 microbeads and mass spectroscopy columns (Miltenyi Biotec). This isolation method results in the isolation of untouched monocytes and circumvents artificial activation by FcγR ligation as occurs during the isolation of monocytes by means of adherence with human serum as used previously (32). DCs were generated by culturing isolated monocytes in RPMI 1640 Dutch modification (Invitrogen Life Technologies) supplemented with 10% FCS and antibiotic-antimycotic (Invitrogen Life Technologies) in the presence of IL-4 (500 U/ml; Schering-Plough) and GM-CSF (800 U/ml; Schering-Plough) for 6 days at a concentration of 10 × 10^6 cells/10 ml of culture medium in 75-cm 2 cell culture flasks (Corning). Fresh culture medium (5 ml) with the same supplements was added at day 3, after which the DCs were harvested at day 6. DCs were resuspended in fresh culture medium at a concentration of 0.5 × 10^7 DCs/ml, transferred to either 24-well (1 ml) or 96-well (0.1 ml) culture plates, and stimulated as described.

Stimulation of PBLs

The PBLs that remained after the extraction of the CD14 + cells, as described in “Culture of monocyte-derived DCs,” were washed with PBS containing 5% FCS and resuspended in culture medium in a concentration of 1 × 10^6 cells/ml. Then, 1 × 10^6 cells were plated per well in a 96-well flat-bottom plate in triplicate and stimulated overnight with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml, Sigma-Aldrich). The supernatants were collected for cytokine measurements.

Phenotypical analysis of monocyte-derived DCs

Using standardized flow cytometry protocols as described previously (33), the phenotypical analysis of monocytes and monocyte-derived DCs was performed. The expression of FcγRs was determined on monocytes and monocyte-derived DCs using the Abs for human FcγRI (CD64, clone 10.1; Dako) and FcγRII (CD16, clone D130c; Dako), the FcγRIIb-specific FITC-labeled labeled Ab 2B6 (Macrogenics) and clone IV.3 which preferentially binds to FcγRIIa (Medarex, a gift from Dr. J. Ronnelid, Ref. 15). Immature DCs were further characterized by staining with mAbs against human CD14 (Dako/cytometry), CD80 (BD Biosciences), CD83 (Beckman Coulter), CD86 (BD Pharmingen), MHC class II (MHC II) DR/DP (clone Q154), ILT3 (R&D Systems), ILT4 (R&D Systems), and DC immune receptor (BD Biosciences). As a secondary Ab, FITC-conjugated goat anti-mouse IgG (Zymed Laboratories) was used. DCs matured for 24 h with LPS in the presence or absence of IC were analyzed for the expression of CD86 and MHC II as classical markers for DC activation. The level of apoptosis of the stimulated DCs was determined by annexin V staining and with propidium iodide. Cells were analyzed with a FACSCalibur (BD Biosciences) for the proportion of positive cells and the mean fluorescence intensity relative to cells stained with the relevant IgG isotypes.

DC stimulation

Day 6 DCs were replated in a concentration of 0.5 × 10^7 DCs/ml and transferred to either 24-well (1 ml) or 96-well (0.1 ml) culture plates. DCs were then put in contact with medium with heat-aggregated human IgG (IC; in all experiments used in a concentration of 50 μg/ml), prepared, and used as previously described (33), with ICs derived from the serum of healthy controls, RA patients, or RA synovial fluid (pegylated-IC; Peg-IC), double-purified LPS, or the combination of IC or Peg-IC and double-purified LPS. The Peg-IC precipitates were purified and washed in a single-step centrifugation procedure as described in Ref. 34; briefly, 1 ml of PBS containing 5% human serum albumin (HSA) and 2.5% PEG 6000 (PBS-HSA-PEG) was added to 1.5-ml autoclaved Eppendorf tubes. Plastic cylinders made from 5-ml autoclaved pipet tips (by cutting off ~1.5 cm of the tips) were...
introduced into the Eppendorf tubes containing PBS-HSA-PEG. Hyaluronic acid (Sigma-Aldrich)-treated synovial fluid or sera precipitated overnight were diluted 1/3 in RPMI 1640 containing 2.5% PEG 6000 and then placed on top of the PBS-HSA-PEG in the pipet tips. An interface was formed with the less dense, red RPMI 1640 solution on top. The tubes were then centrifuged at 2100 × g for 4°C for 20 min, whereby the precipitates in the upper 2.5% PEG-RPMI solution were centrifuged down to the bottom of the Eppendorf tube. The remaining PBS-HSA-PEG solution was removed, and the precipitated pellet was immediately resolubilized in ice-cold sterile PBS to the original serum volume. The precipitates were totally resolved in PBS, leaving no insoluble aggregates. The dissolved PEG precipitates were then placed on ice until used in cell culture experiments.

The used Escherichia coli LPS (100 ng/ml; Sigma-Aldrich) was double-purified at our laboratory using the phenol-water extraction method to remove any endotoxin present by using amion (35). In experiments using intracellular signaling molecule inhibitors, DCs were pretreated with these inhibitors for 1 h at 37°C before adding the stimulants. The following inhibitors were used in the mentioned concentrations: Wortmannin (PI3K inhibitor, 0.1 μM); Akt inhibitor IV (0.1 μM); Akt inhibitor X (0.5 and 5 μM); SB203580 (p38 inhibitor, 20 μM); rottlerin (protein kinase C6 (PKCδ) inhibitor, 10 μM); and LFM-A13 (PI3K inhibitor, 50 μM). All inhibitors were obtained from Calbiochem. Supernatants were collected after 24 h for cytokine measurement.

Monocyte-derived DCs from RA patients able to halt DMARD use express FcyRIIB at high levels

RA is a tremendously heterogeneous disease in nature characterized by disease flares and remissions as measured by the DAS28 now generally accepted and used worldwide (37). Next to this, the dependency on DMARDs ranges from those perpetually in need of potent immunosuppressive drugs to a subset of patients who are able to discontinue its use. On the basis of these facts, we divided RA patients into four categories using a well-documented prospective cohort of RA patients (38–40). Accordingly, at the time of study inclusion, RA patients were divided into those having moderate to high disease activity (DAS28 > 3.2) and those having a low disease activity (DAS28 < 3.2) with or without the use of DMARDs. All patients in the current study fulfilled the American College of Radiology criteria for RA at the time of inclusion in the inception cohort and had longstanding RA with a mean disease duration of 13 years (range, 2–33 years) for RA patients not receiving DMARD therapy and 9 years (2–20 years) for those who were receiving DMARD therapy at the start of the current study. No significant differences were observed between both groups regarding rheumatoid factor, disease duration, or the presence of erosions (Table I). We examined the expression of FcγR subtypes in RA patients using the unique Abs recently described to discriminate the FcγRIIa and FcγRIIB isoforms (15, 33). Compared with healthy controls, all patients having a moderate to high DAS28 (regardless of DMARD use) or those having a low DAS28 using DMARDs (DMARD(+) RA) displayed a similar expression profile of both activating and inhibitory FcγRs (Fig. 1, A and B). Strikingly and in sharp contrast, all patients not receiving DMARD therapy and having a low disease activity (DAS28 < 3.2) (further designated as DMARD(−) RA) expressed a significantly higher level of FcγRIIB on their monocyte-derived DCs, whereas the expression of activating FcγR was not notably different (Fig. 1, A and B). With respect to various DC markers (CD14, CD80, CD83, CD25, CD127, CD19, CD56, CD235, CD33, CD34, and CD141) and the T cell purity of 96.29 ± 1.57%, no significant differences were observed between both groups regarding the expression of FoxP3.
CD86, MHC II) as well as the ITIM-bearing molecules ILT3, ILT4, and DC immunoreceptor, which are known to be expressed on tolerogenic DCs (41, 42), no differences were observed (data not shown). In patients treated with DMARD therapy, no correlation between DAS28 and FcγRIIb expression could be observed, whereas this correlation was clearly present in those individuals who did not use antirheumatic drugs (Fig. 1C). These observations substantiate that the lack of DMARD use alone did not explain the high FcγRIIb expression in RA patients with a self-regulated low disease activity but rather indicates that they constitute a different class of RA patients. We also evaluated the expression of the FcγR on monocyte-derived DCs from patients having other immune-related diseases such as systemic lupus erythematosus and psoriatic arthritis. None of these patients DCs expressed FcγRIIb to such high levels as observed in RA patients having quiescent disease (data not shown). To determine whether the differences in FcγRIIb were already present on the progenitor cells of the DCs, monocytes from healthy controls, DMARD(+) RA patients, and DMARD(-) RA patients were evaluated for their expression of FcγRIIb. No significant differences in the expression of FcγRIIb were found (Fig. 1D).

ICs inhibit TLR4-mediated cytokine release on DCs from DMARD(−) RA patients

We surmised that the high expression of FcγRIIb on DCs from DMARD(−) RA patients has clear functional consequences. To

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**FIGURE 1.** FcγRIIb expression is markedly increased on DCs from RA patients having quiescent disease. *A*, Whereas the expression of activating FcγR subtypes (FcγRI, -IIa, and -IIa) is comparable in all groups (one representative individual from each group is shown), the expression of the inhibitory FcγRIIb is increased only on immature DCs from RA patients having low disease activity who had stopped DMARD use at least 2 years before the experiment. Mean fluorescence intensity (MFI) is presented as measured by flow cytometry. *B*, Expression of activating and inhibitory FcγR on DCs from the different RA subgroups (DMARD(+), DAS28 > 3.2, n = 6; DMARD(+), DAS28 < 3.2, n = 10; DMARD(−), DAS28 > 3.2, n = 5; DMARD(−), DAS28 < 3.2, n = 11) and healthy individuals (n = 10). Mean and SEM are for combined data from 5 to 11 independent experiments. *, p < 0.001, comparing the DMARD(−), DAS28 < 3.2 group with the four other groups. *C*, No correlation is present between FcγRIIb expression on DCs and disease activity in RA patients using DMARDs, whereas there is a clear correlation in patients who halted DMARD use. The disease activity score DAS28 was measured at inclusion of the study. *D*, Expression of FcγRIIb on monocytes from DMARD(+), DAS28 > 3.2 (n = 5); DMARD(−), DAS28 > 3.2 (n = 5) RA patients and healthy individuals (n = 6). Mean and SEM are for combined data from five independent experiments. Pos., positive.
ascertain whether this idea holds true, we measured the production of the proinflammatory mediators TNF-α and IL-12p70 produced by DCs upon coculture with LPS (TLR4 agonist) compared with the combination of LPS and IC. In contrast with DCs from RA patients using DMARDs, which express low FcγRIIb levels (designated as DClow-FcγRIIb), increase TNF-α 2 ± 2% (mean ± SEM) and IL-12p70 19 ± 4%, DCs from DMARD(−) RA patients, which express high FcγRIIb levels (designated as DChigh-FcγRIIb), clearly inhibited the production of TNF-α (−28 ± 2%) and IL-12p70 (−43 ± 4%) upon stimulation with the combination of LPS and IC compared with that seen upon stimulation with LPS alone (Fig. 2A). Importantly, all DChigh-FcγRIIb were derived from DMARD(−) RA patients (untreated RA patients with a low DAS28), and all DCs from DMARD(−) RA patients were DChigh-FcγRIIb, whereas all DClow-FcγRIIb were derived from DMARD(+) RA patients (RA patients receiving DMARD therapy with a low DAS28 score). As expected, like DClow-FcγRIIb, from DMARD(+) RA patients, DCs from healthy controls were unable to suppress TLR4 responses by the ligation of IC (data not shown). Unstimulated or IC-stimulated DChigh-FcγRIIb and DClow-FcγRIIb did not release any detectable levels of TNF-α or IL-12p70 demonstrable of their immature nature. We next determined the inhibitory capacity of FcγRIIb over a range of LPS doses. The inhibitory effect of IC was potent in that it was effective in a wide range of LPS concentrations (10 pg/ml–1 μg/ml; data not shown). The use of heat-aggregated human Igs in our experiments is well controlled but artificial. Therefore, we repeated these experiments to ensure that naturally occurring OCs exert comparable effects with regard to FcγRIIb-mediated TLR inhibition (34). Peg-ICs isolated from the serum from RA patients inhibited TLR4 responses to comparable levels as observed with ICs attesting that circulating ICs can tune TLR-mediated immune responses via FcγRIIb in vivo (Fig. 2B). Peg-ICs isolated from healthy individuals were able to inhibit TLR4-mediated DC activation to the same extent as Peg-ICs from RA patients, again underscoring the importance of FcγRIIb as a checkpoint for tolerance. Peg-ICs isolated from the synovial fluid of RA patients were less able to inhibit TLR4 responses. To exclude the possibility that intracellular retention of TNF-α (43) would explain our results, we compared the release of TNF-α upon stimulation of DCs with the combination of IC and LPS before and after repeated freeze-thaw cycles. The inhibitory effect of IC was still clearly present suggesting that retention of TNF-α was virtually negligible (data not shown). To investigate whether the inhibition of TLR4 by FcγRIIb was prolonged or temporal, we stimulated DChigh-FcγRIIb with LPS in the presence of absence of IC at baseline and measured the amount of TNF-α in the supernatants at several time points. TNF-α production was inhibited to the same extent at all time points (data not shown). Homoaaggregation of FcγRIIb on B cells induces apoptosis. The levels of annexin V and propidium iodide were similar throughout the experiments, which refutes apoptosis as an explanation for our observations (data not shown). Both IC and Peg-IC were found to be negative for LPS contamination using Limulus amebocyte lysate assays (data not shown).

FIGURE 2. Only TLR4-mediated cytokine production by DCs from DMARD(−) RA patients is inhibited by costimulation with IC. A, Immature DCs from RA patients able to successfully discontinue DMARD therapy, all of which expressed high levels of FcγRIIb (further designated as DChigh-FcγRIIb), markedly inhibit TLR4-mediated secretion of TNF-α and IL-12 upon coculture with IC compared with those stimulated with LPS only. In contrast, DCs from RA patients on DMARDs (DClow-FcγRIIb) were unable to inhibit TLR4-mediated cytokine production. The results displayed here originated from 10 independent experiments. B, ICs isolated by PEG precipitation from the serum of healthy controls (n = 11) and RA patients (n = 11) dampen the release of TNF-α to the same extent as do artificial ICs. ICs isolated from synovial fluid (SF) of RA patients (n = 11) are less able to inhibit the production of TNF-α. Values are results from DChigh-FcγRIIb from a representative DMARD(−) RA patient. NH, Normal human; NS, not significant.
FcyRIIb stimulation inhibits TLR4-mediated DC maturation which has clear effects on T cell responses

To further delineate whether the TLR4-dependent phenotypic maturation of DCs was affected by IC, DChigh-FcyRIIb were stimulated with the combination LPS and IC and compared with those stimulated with LPS alone. Subsequent analysis of CD86 and MHC II demonstrated that the addition of ICs to LPS halted the phenotypic maturation significantly (Fig. 3A). In sharp contrast, IC had no significant effect on DClow-FcyRIIb. Because the expression of such maturation markers exerts direct effects on T cell instruction, we next examined their capacity to induce T cell proliferation of allogeneic maturation markers exerts direct effects on T cell instruction, we next examined their capacity to induce T cell proliferation upon stimulation with the combination of LPS and IC was markedly diminished compared with stimulation with LPS alone (p < 0.05; Fig. 3B). As expected, on DClow-FcyRIIb the addition of IC to LPS had no inhibitory effect. Because the pathogenic role for Th1 cells in RA is evident from a wide range of clinical and experimental observations, we extended our result by analysis of T cell cytokines considered to reflect Th1 (IFN-γ) or Th2 (IL-13) status. Exposure of T cells to DChigh-FcyRIIb, incubated with the combination of LPS and IC strongly increased the potential of T cells to secrete IL-13 compared with DClow-FcyRIIb incubated with LPS alone, whereas this effect was absent on DClow-FcyRIIb (Fig. 4A). IL-4 was also detectable in the MLR supernatants in a pattern similar to that of IL-13, but at much lower concentrations (data not shown). Strikingly, T cells directly isolated from the circulation from RA patients with high FcyRIIb levels produce significantly higher levels of IL-4 and IL-13 than those from patients characterized by DCs with a low FcyRIIb (Fig. 4B). Because T cells able to secrete IL-17, designated Th17, have recently been mentioned as mediators of inflammation in experimental arthritis models, we measured the IL-17 content in MLR with CD3⁺ CD25⁺ CD127⁺ T cells and DChigh-FcyRIIb activated by LPS alone or in combination with IC. No significant differences were observed (Ref. 44 and data not shown). These observations may suggest that FcyRIIb-mediated TLR4 inhibition has clear consequences on the Th1-Th2 axis but does not touch Th17 development. To further explore the modulating effect of FcyRIIb signaling on the ability of DCs to influence T cell differentiation, we next studied the capacity of DChigh-FcyRIIb and DClow-FcyRIIb to induce Tregs. To this end, CD3⁺ CD25⁺ CD127⁺ T cells were stimulated with DChigh-FcyRIIb or DClow-FcyRIIb activated with medium, IC, LPS or the combination of IC and LPS and were analyzed by flow cytometry after 6 days of coculture. Newly induced CD4⁺ CD25⁺ FoxP3⁺ CD127⁺ T cells were present in all the cultures. However, the relative presence of CD4⁺ CD25⁺ FoxP3⁺ CD127⁺ T cells was significantly diminished by the prior activation of the DCs with LPS. The inhibitory effect of TLR4 on the relative presence of Tregs observed by the addition of LPS was completely abolished by the addition of IC to DChigh-FcyRIIb. The addition of IC had no effect with DClow-FcyRIIb (Fig. 4, C and D). Collectively, these data indicate that the FcyRIIb-mediated inhibition of TLR4 responses has clear functional consequences for DC-mediated T cell instruction and might explain the higher number of Th2 cells found in vivo in DMARD(−) RA patients (45).

The effect of IC on TLR4 signaling release is FcyRIIb dependent

There is a remarkably clear correlation (R² = 0.89, p = 0.001) between the level of FcyRIIb expression and the potential to inhibit TNF secretion upon LPS plus IC stimulation by DCs in RA patients (Fig. 5A). This strongly suggests that the level of FcyRIIb expression on DCs determines the level of inhibition of TLR4 responses instrumented by the addition of IC. By using a blocking Ab against FcyRIIb, we confirmed that the inhibitory effect of IC on TLR4 signaling in DChigh-FcyRIIb is fully dependent on FcyRIIb (Fig. 5B and Refs. 15 and 16). In contrast, blocking FcyRIIb had no effect on IC-mediated dampening of TLR4-dependent TNF-α secretion. The expression of costimulatory molecules (data not shown), inhibition of T cell proliferation (Fig. 5C), and increased production of IL-13 by T cells (Fig. 5D) mediated by the addition
of IC to DC<sub>high-FcγRIIb</sub> were fully abrogated by blocking FcγRIIb, implying that the inhibitory FcγRIIb was solely responsible for the inhibitory effect of IC on TLR4 immune responses. The inhibitory effect of FcγRIIb is mediated via the PI3K/Akt pathway. To further dissect the cross-talk between FcγRIIb and TLR, we first explored the effect of inhibiting PI3K and Akt in our system, given that these are intricately involved in FcγR and TLR signaling. Inhibition of the PI3K/Akt pathway, with three separate PI3K or Akt inhibitors, led to an increased release of TNF-α upon LPS stimulation of DCs (Fig. 6A). Extensive dose-response curves were performed to obtain the levels at which the inhibitors had their optimal effect (data not shown). In addition, we found that both the PI3K inhibitor and the Akt inhibitors fully abrogated the inhibitory effect of FcγRIIb on LPS-mediated TNF-α and IL-12p70 production by DCs (Fig. 6, B and C). Inhibition of the MAPK p38, PKCζ, and Btk that also have been advocated to play a part in the
signaling of either TLR or FcγR did not have any effect on the FcγRIIb inhibitory potential (Fig. 6, D and E).

Discussion

This study establishes a novel role of the inhibitory FcγRIIb, providing a general point of our immune system tuning TLR4-mediated immune activation. The ability of the immune system to distinguish self from nonself is seminal to the ability to protect the host from the detrimental effects of invading pathogens. The mechanisms that orchestrate these properties operate at discrete checkpoints involving central and peripheral tolerance. Given the complexity of these processes, it is not surprising that central tolerance is frequently incomplete. Therefore, inhibitory signaling has emerged as a critical feature of peripheral tolerance. The role of TLR in the initiation of immunity is extensively shown. However, the counterregulatory mechanisms that control this inflammation and thus should prohibit chronic inflammation and uphold tolerance are not yet identified. The FcγRIIb-mediated inhibitory capacity on TLR signaling that we show here provides a novel mechanism on how the immune system exploits FcγRIIb as a counterregulatory mechanism to limit inflammation to prohibit exaggerated damage to host tissues.

In arthritis, it has been irrefutably shown that the balance between activating and inhibitory FcγR is seminal in controlling both susceptibility to and the severity of the disease (5, 10, 11, 17, 34, 46–49). Here we demonstrate that the expression of FcγRIIb was insufficient to inhibit TLR4-mediated immune activation in all but RA patients able to suppress disease activity in the absence of antirheumatic drugs, suggesting that the high FcγRIIb expression observed in these latter patients might underlie their state of disease remission. Further evidence for the existence of such a repressed FcγRIIb system in RA comes from the use of i.v. Igs (IVIG) that have been applied successfully to treat a variety of immune-related diseases such as immune thrombocytopenic purpura, systemic lupus erythematosus, Kawasaki disease, and Guillain-Barré syndrome. Whereas the mechanisms that exert the effects of IVIG remain enigmatic, binding to and up-regulation of the inhibitory FcγRIIb is advocated (Ref. 50; reviewed in Ref. 51). In this line, it is remarkable that the administration of IVIG to RA patients has been disappointing (52, 53). Recently, Kaneko et al. (54) provided evidence for the role of sialylation of IC as another mechanism underlying the distinct effect of IC observed during inflammation and health. Our data underscore the existence of such a mechanism, given that Peg-IC obtained from the synovial fluid,
thus originating from a more inflammatory environment, exerted significant less inhibitory capacity compared with Peg-IC obtained from the peripheral blood of RA patients. However, the fact that these former ICs are still able to inhibit TLR4 responses indicates that the inhibitory capacity of the FcγRIIB system is superior over the level of sialylation.

The precise pathways underlying FcγRIIB-mediated inhibition of ITAM are extremely complex and not completely elucidated let alone the mechanisms by which it might inhibit other immune receptors such as TLR4. Prime suspects are PI3K and its counteracting opponent SHIP. Whereas PI3K is known to increase phosphatidylinositol (3,4,5)-trisphosphate levels and
thereby the level of phosphorylated Akt, SHIP degrades phosphatidylinositol (3,4,5)-trisphosphate and reduces the level of Akt phosphorylation. Inhibiting Akt or PI3K led to an increase in TLR4 induced cytokine production by DCs in our experimental set-up. On the basis of our observations, we propose a mechanism centered on the PI3K/Akt-SHIP balance by which FcγRIIb might inhibit TLR4 signaling. In our conceptual framework, FcγRIIb activation leads to the recruitment and phosphorylation of SHIP to the cell membrane, decreasing the level of SHIP available for enhancement of the TLR4 pathway. Increased levels of phosphorylated Akt might thus arise due to a shifted balance toward PI3K, instead of SHIP, closely associated with the TLR4 signaling cascade, leading to decreased LPS-mediated DC activation. The likely massive presence of phosphorylated SHIP near FcγRIIb in DC<sub>high-FcγRIIb</sub> activated by IC thus potentially increases the PI3K/Akt inhibitory signal leading to a decrease in TLR4-mediated cytokine production. Most likely, however, this proposition is still an oversimplified scheme of the signaling events taking place. SHIP for example has been found to bind to adaptor molecules belonging to the family of Dok proteins which were first identified as substrates for the p21<sup>(ras/raf)</sup> oncoprotein and are implicated in inhibitory signaling (55–57). The function of the Dok proteins, which are phosphorylated by Lyn, has been linked to the facilitation or sustainment of the activation of SHIP and the inhibition of the ras pathway (58, 59). In addition, ligation of TLR4 by LPS has been shown to rapidly induce the phosphorylation and adaptor function of Dok proteins and the absence of Dok proteins resulted in the elevated activation of Erk and hyperproduction of TNF-α (60). Whether this inhibitory effect of the Dok proteins on TLR4 signaling is linked to their role as adaptor molecules needs further investigation. It does, however, demonstrate that the events taking place intracellularly in our experiments are far more intricate than we have tried to contemplate. The changes in intracellular TLR4 signaling induced by FcγRIIb leads to an increased release of the proinflammatory cytokines TNF-α, IL-6, and IL-12p70 as well as an increased capability of the DCs to induce Tregs and Th2 cells. These changes are seen as important events in the resolution of Th1-mediated inflammation. Intriguingly, the absence of SHIP in mice results in the increased presence of alternatively activated macrophages as well as a spontaneous allergic inflammation in the lungs characterized by elevated levels of the Th2 cytokines IL-13 and IL-4 (61). These findings make it even more tempting to speculate that FcγRIIb signaling is designed to induce alternatively activated DCs capable of dampening inflammation.

DCs from patients having quiescent disease display a more tolerogenic phenotype as than those from RA patients having active disease, which is witnessed by the lower level of cytokine secretion, higher levels of Foxp3-expressing T cells, and higher production of Th2 cytokines by T cells primed by these DCs. These observations are in keeping with that of Dhodapkar et al. (16, 62) demonstrating that FcγRIIb is crucial to keep DCs in an immature state in steady-state conditions. Because we show that healthy individuals had circulating IC, which exerted an inhibitory capacity comparable with that seen by RA IC, these findings suggest that circulating ICs orchestrate the DC<sub>high-FcγRIIb</sub> tolerogenic phenotype by a constant down-tuning of TLR-mediated immune activation. As anticipated, healthy individuals did not express FcγRIIb at high levels potentially mirroring their steady-state situation. These data further substantiate that FcγRIIb is designed to tune immune responses, restoring steady state conditions. Presumably, at some stage of immune activation, pathways must be turned on that lead to a clear shift in FcγR expression toward the inhibitory subtype. The pathways that regulate the expression of FcγR remain obscure. Although IL-13/IL-4 with and without the combination of IL-10 has been demonstrated to skew the FcγR balance toward the inhibitory subtype, whereas IFN-γ strongly shifts this balance toward activating FcγR. Although we have previously shown that the regulatory capacity of IL-13 is lost in RA patients, the high levels of FcγRIIb observed in DMARD(−) RA patients could not be explained by IL-13 or IL-10 because blocking these mediators during culture of DCs from RA patients with quiescent disease did not show any effect (Ref. 63 and data not shown). In addition, the expression of FcγRIIb seen in these patients reached such extraordinarily high levels that they could not be reached by the addition of these mediators to DCs from healthy controls. Thus, these data indicate that other mechanisms must be responsible for the regulation of FcγRIIb expression. The identification of such pathways is likely to result in the delineation of the processes underlying the deranged up-regulation of FcγRIIb that was found to be specific for RA patients. This, in turn, would significantly contribute to the development of therapeutic targets that are specifically designed to act on the defective pathway that underlies the chronic course of RA, a current ummet need in the treatment of this condition.

Here we show for the first time that, taken together, most RA patients have a deranged expression of the inhibitory FcγRIIb, rendering them incapable of controlling TLR-mediated immune activation. Our data strongly suggest that the up-regulation of FcγRIIb expression opens novel therapeutic avenues for the treatment of RA and other autoimmune conditions where TLR signaling is implicated.

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References


