IL-6 and Maturation Govern TLR2 and TLR4 Induced TLR Agonist Tolerance and Cross-Tolerance in Dendritic Cells1

Julia Geisel,* Frauke Kahl,* Martina Müller,* Hermann Wagner,† Carsten J. Kirschning,† Ingo B. Autenrieth,* and Julia-Stefanie Frick2*

Stimulation of naive mouse dendritic cells (DC) with LPS or Pam3CSK4 (P3C) induces production of TNF-α via TLR4- or TLR2-signaling. Although tolerance in macrophages has been studied in detail, we investigated the role of TLR agonist concentration and IL-6 for tolerance in DC. P3C- or LPS-primed DC were nonresponsive to P3C or LPS restimulation in terms of TNF-α but not IL-6 production. The mechanisms involved in tolerance were dependent on the concentration of the TLR ligand used for DC priming. DC primed with LPS or P3C at high concentrations developed a maturation dependent, IL-6 independent tolerance associated with inhibition of TLR signaling upstream of IkB as indicated by decreased IkB degradation. In contrast, priming of DC with LPS or P3C at low concentrations resulted in IL-6-dependent tolerance, which was abolished in IL-6 deficient DC, and was not accompanied by maturation of DC or by down-regulation of TLR2 or TLR4. In homotolerogenic DC primed with LPS or P3C at high concentrations, degradation of IkB upon restimulation with LPS or P3C was inhibited suggesting tolerance mechanism(s) upstream of IkB; in contrast, cross-tolerance in DC primed with LPS or P3C at low concentrations was not associated with reduced IkB degradation suggesting tolerance mechanisms downstream of IkB. Our data indicate that in naive DC TLR4- and TLR2-stimulation results in homo- and cross-tolerance; the mechanisms involved in tolerance depend on the concentration of the TLR agonist used for DC priming and are governed by IL-6 and maturation. The Journal of Immunology, 2007, 179: 5811–5818.

Dendritic cells (DC)3 are among the first APCs encountering bacteria at mucosal surfaces and play an important role in maintenance of regular homeostasis in the intestine. LPS from the cell wall of Gram-negative bacteria is widely used for DC activation. Activation of DC with TLR agonists leads to activation of NF-κB and MAPK family members (1). Furthermore, cytokines and proinflammatory mediators that affect for instance T cell differentiation are produced rapidly. Stimulation of DC with TLR2 or TLR4 agonists results in different patterns of cytokine secretion. Stimulation with TLR4 promotes induction of IL-12(p70) and the chemokine IFN-γ inducible protein-10, which are both associated with Th1 responses. In contrast, TLR2 stimulation failed to induce IL-12(p70) and inducible protein-10 secretion but resulted in production of IL-12(p40) homodimer and a rapid release of IL-10 (1, 2). Whereas TLR4 transduces enterobacterial LPS signals, TLR2 mediates nonenterobacterial such as Porphyromonas gingivalis (3) and Leptospira interrogans LPS signals (4). TLR1 has been implicated as TLR2 coreceptor and its coexpression increased a TLR4 independent response to Escherichia coli LPS and Neisseria meningitidis lipopoligosaccharide (5).

Following the recognition of LPS by TLR4/MD-2/CD14 complex or P3C (N-palmitoyl-bis(palmitoyloxy)-propyl-cysteinyl-seryl-Lys) by TLR2/TLR1/CD14 complex, the intracellular adaptor protein MyD88 binds to intracellular domains of most TLRs which recruits members of the IL-1 receptor-associated kinase (IRAK) family to the TLR/MyD88 complex (6). IRAK-1 phosphorylates itself and leaves the TLR/MyD88 complex to associate with the TNF receptor-associated factor 6. This leads to downstream activation of signaling pathways involving MAPKs and NF-κB (6). It is known that exposure to LPS both in vitro and in vivo can lead to a desensitization of immune cells to subsequent stimulation with LPS. This finding has been referred to as “endotoxin tolerance.”

One of the main characteristics of LPS tolerance in vitro is a change in the pattern of inflammatory gene expression when responses to a single or two sequential LPS exposures are compared (7). Furthermore stimulation with TLR2 agonists leads to desensitization toward a subsequent TLR2 stimulus (8). In macrophages priming with a TLR2 stimulus reduces responsiveness to restimulation with LPS (9). In addition, in human promonocyctic cells priming with LPS leads to cross-tolerance to TLR2 restimulation (10). These phenomena of cross-tolerance both have been reported to be associated with down-regulation of IRAK-1 (10, 11). However, a major effect accompanying tolerance via TLR4 might be induction of p50p50 homodimer formation (11–15). In contrast to p65, the p50 protein does not transactivate, but binds NF-κB recognition elements in gene promoters thus preventing expression of NF-κB driven genes such as TNF-α (16). Additionally, a role for suppressor of cytokine signaling 1 in LPS tolerance has been implicated (17, 18). Furthermore, IRAK-M has been reported to prevent dissociation of...
the IRAK-1/IRAK-4 complex thereby preventing formation of the IRAK-1/ TNF receptor-associated factor 6 complex and subsequent TLR signaling (19).

We have shown recently that treatment of DC with *Bacteroides vulgatus*—an anaerobe Gram-negative rod closely related to *P. gingivalis*—phylogenetically and recognized via TLR2 mainly leads to a reduced responsiveness to restimulation with *E. coli* in bone-marrow-derived DC and differentiates DC toward a semimature status (20).

In this study, we investigated mechanisms underlying TLR2/4 induced DC tolerance toward restimulation with specific TLR agonists. Our main focus was on DC maturation and activation, as well as TLR2/4 surface expression and the role of IL-6 in the modulation of these processes. Our results implicate that TLR2 or TLR4 signals induce homo- and cross-tolerogenic DC. We observed that different mechanisms account for induction of tolerogenic DC; on the one hand, maturation processes and, on the other hand, an IL-6 dependent differentiation to a semimature tolerogenic DC phenotype.

**Materials and Methods**

**Mice**

C57Bl/6 × 129Sv, IL-6−/− (21), and TLR2/4−/− mice were obtained from own breeding. All mice were kept under SPF conditions. Male and female mice were sacrificed at 6–10 wk of age. Animal experiments were reviewed and approved by the responsible institutional review committee.

**Abs and reagents**

P3C was purchased from ECM microcollections and ultra pure LPS *S. enterica* serovar Minnesota from Calbiochem. The following Abs were used for flow cytometry: PE conjugated anti-mouse CD11c, clone HL3 and FITC conjugated anti-mouse CD40, clone HM 40–3, CD80, clone 16–10A1, CD86, clone 16–10A1, and CD86, clone GL1 and MHC class II clone 2G9; isotype control hamster IgG1 and hamster IgM, hamster IgG2a, (BD Biosciences); anti-mouse TLR4, clone MTS 510 and anti-mouse TLR2, clone 6C2; isotype control rat IgG2a and rat IgG2b (eBioscience/NatUCell); anti IκB-α (Cell Signaling Technology) and anti-mouse β-actin (Sigma-Aldrich); and secondary Ab anti-rabbit (Cell Signaling Technology) and rabbit anti-mouse IgG HRP (DakoCytomation) were used. Recombinant mouse IL-6 was purchased from R&D Systems.

**Mouse DC isolation**

Bone marrow cells were isolated and cultured as described previously (22) with minor modifications. Cells were harvested at day 8 and used to evaluate the effects of cellular challenge with LPS and P3C on cytokine release and expression of surface markers as described below.

**Stimulation of isolated DC**

DC were stimulated with LPS or P3C at different concentrations (1 ng/ ml–1000 ng/ml). After 24 h, cell culture supernatant was harvested for analysis of cytokine expression and cells were used for flow cytometry of surface marker expression. For sequential stimulation, cells were challenged with LPS or P3C at day 7 and restimulated with LPS or P3C at day 8. Twenty-four hours later, cell culture supernatants and cells were harvested.

**Cytokine analysis by ELISA**

For analysis of IL-6, IL-10, and TNF-α concentrations of cell culture supernatants commercially available ELISA kits (BD Biosciences) were used according to the manufacturer’s instructions.

**Flow cytometry analysis**

A total of 1 × 10⁶ cells were incubated in 150 μl PBS containing fluorochrome conjugated Abs at a concentration of 0.7 ng/μl (anti-CD11c) and 1.7 ng/μl (anti-CD40, CD80, CD86, and MHC class II). TLR4 and TLR2 Abs were used at a concentration of 3.3 ng/μl after incubation in Fc-block (α-FcγRIII/I, CD16/CD32; BD Biosciences) containing 10% FCS. 30,000 cells were analyzed. A total of 7.5 ng propidium iodide (Sigma-Aldrich) was added 1 min before FACS analyses to 150 μl samples.

**IκB-α Western blot**

A total of 2 × 10⁶ DC were treated with P3C or LPS for 24 h and restimulated for another 15 min. After cells were washed in ice cold PBS the pellet was lysed by adding 75 μl SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.01% w/v bromophenol blue). Cells were sonicated for 10–15 s to shear DNA and reduce viscosity. Samples were heated to 95°C for 5 min and microcentrifuged for another 5 min. A 15-μl sample was loaded onto one lane of a 10% PAA gel. Proteins were transferred to nitrocellulose membrane after subjection to SDS-PAGE. The membrane was incubated in blocking buffer (1 × TBS and 0.1% Tween 20 with 5% nonfat dry milk) for 1 h at room temperature. Incubation with primary Ab overnight at 4°C was followed by incubation with HRP-conjugated secondary Ab for 1 h at room temperature. Proteins were detected by adding Lumiglo (Cell Signaling Technology) and exposure to x-ray film.

**Statistical analysis**

Statistical analysis was performed using Student’s *t* test. Values of *p* < 0.05 were considered significant. Error bars represent ± SEM.

**Results**

Pretreatment with LPS or P3C at high concentrations induces maturation dependent but IL-6 independent cross- and homo-tolerogenic DC

To quantify cytokine secretion of naive DC upon stimulation with TLR agonists, wild-type (Fig. 1, A–C) and IL-6−/− (Fig. 1, D–F) DC were stimulated with LPS or P3C at various concentrations and TNF-α, IL-6, and IL-10 production were determined in supernatants after 24 h. Stimulation with LPS or P3C resulted in dose dependent secretion of TNF-α and IL-6 which was enhanced upon LPS as compared with P3C stimulation. IL-10 was only detectable upon stimulation with LPS but not upon stimulation with P3C. Wild-type and IL-6−/− DC revealed similar cytokine secretion pattern (except for IL-6). (Fig. 1, A–F).

We next addressed the question whether LPS or P3C may induce tolerance in DC. For this purpose, naive DC were stimulated with LPS or P3C at high concentrations (1000 ng/ml) for 24 h. Then, the culture medium was changed and primed DC were restimulated with 1000 ng/ml LPS or P3C. After the following 24 h TNF-α, IL-6, and IL-10 concentrations in culture supernatants were determined. Priming of DC with LPS resulted in a significantly decreased production of TNF-α upon restimulation with LPS or P3C. This nonresponsiveness was designated as homo- (LPS restimulation) or cross- (P3C restimulation) tolerance (Fig. 2A); comparable findings were obtained upon priming of DC at high concentrations of P3C (Fig. 2B). These results suggest that high concentrations of TLR2 or TLR4 agonists induce both LPS/P3C homo- and LPS/P3C cross-tolerant DC designated as LPS<sup>high</sup> and P3C<sup>high</sup> tolerance, respectively.

In contrast, IL-6 production was only inhibited in homo-tolerogenic DC primed with LPS but not in DC primed with P3C or cross-tolerogenic DC (Fig. 2, C and D) suggesting that different pathways control TLR2- and TLR4-induced TNF-α and IL-6 production and thus tolerance in terms of TNF-α and IL-6. Secretion of IL-10 from homo- but not from cross-tolerant DC primed at high concentrations of LPS was reduced, whereas secretion of IL-10 from cross- but not from homo-tolerant DC primed at high concentrations of P3C was unaltered (data not shown).

To investigate whether tolerance of primed DC upon restimulation with LPS or P3C is associated with maturation of DC, expression of activation and maturation markers was analyzed. For this purpose, naive DC were stimulated with LPS or P3C at high concentrations and expression of CD40, MHC class II, CD80, and CD86 was determined by flow cytometry 24 h later. The data demonstrate that priming of naive DC with LPS or P3C both led to enhanced expression of CD40 as well as MHC class II, CD80, and
CD86 expression (Fig. 3), indicating maturation and activation of DC. Moreover, these data indicate that mature DC are tolerant to restimulation with LPS or P3C in terms of TNF-α but not in terms of IL-6 or IL-10.

In macrophages, down-regulation of TLR has been described as a mechanism underlying LPS tolerance (23, 24). Therefore, we addressed whether down-regulation of TLR2 or TLR4 expression might also be involved in LPS/P3C tolerance in DC. To this end, naive DC were stimulated with LPS or P3C at low or high concentrations and surface expression of TLR2 and TLR4 was determined by flow cytometry 24 h later. The results demonstrate that stimulation of naive DC with P3C at low or high concentrations did not significantly change expression of TLR4 or TLR2 (Fig. 4). In contrast, stimulation of DC with LPS at high concentrations led to a profound down-regulation of TLR4, whereas expression of TLR2 was increased (Fig. 4). Moreover, stimulation of DC with LPS at low concentrations did not significantly change expression of TLR4 or TLR2 (Fig. 4). As a control to assess Ab specificity, DC from TLR2−/−/− or TLR4−/−/− mice were used and revealed no significant immunostaining. These results suggest that down-regulation of TLRs in DC might be involved in LPShigh tolerance but not in LPSlow or P3C tolerance.

In previous studies, we observed an important role for IL-6 in tolerance of DC (20). Therefore, we investigated the role of IL-6 in tolerance induction by TLR4 and TLR2 stimulation. Naive DC were prepared from IL-6−/−/− mice (21) and stimulated as described above. Priming of DC from IL-6−/−/− mice with LPS at high concentration abolished responsiveness toward restimulation with LPS or P3C (Fig. 5A). Likewise, IL-6−/−/− DC primed with P3C at high concentration displayed unresponsiveness toward restimulation with P3C or LPS (Fig. 5B). To investigate whether LPShigh and P3Chigh tolerance of primed IL-6−/−/− DC is associated with maturation, expression of activation and maturation markers was analyzed by flow cytometry. The data depicted in Fig. 5C demonstrate that priming of naive IL-6−/−/− DC with LPS or P3C both led to enhanced expression of CD40 (Fig. 5C), as well as MHC class II, CD80, and CD86 expression (data not shown). These data indicate that in DC primed with LPS or P3C at high concentrations endogenous IL-6 is not required for LPShigh/P3Chigh homo- or cross-tolerance.

Degradation of IκB leads to subsequent activation and translocation of NF-κB (25). To analyze whether LPS or P3C tolerance in DC is associated with altered IκB degradation indicating NF-κB activation, DC were primed with LPS or P3C at high concentrations for 24 h and then restimulated for 15 min with LPS or P3C. DC were lysed and subjected to Western blot analyses for analysis of IκB expression. Stimulation of naive DC with LPS or P3C led...
to degradation of IκB after 15 min. After 24 h, IκB expression levels reached those in unstimulated cells (data not shown). In DC primed with LPS or P3C at high concentration, we observed a significantly reduced IκB degradation upon restimulation with LPS or P3C reflecting reduced NF-κB activation (Fig. 6). These data argue that LPS\textsuperscript{high} and P3C\textsuperscript{high} cross- and homo-tolerance in DC is governed by mechanisms upstream of IκB.

**Priming with LPS or P3C at low concentrations induces cross- and homo-tolerant DC dependent on IL-6 but independent of maturation**

Because priming with LPS or P3C at high concentrations led to a DC tolerance phenotype associated with maturation, we next investigated whether maturation-independent tolerance mechanisms may exist in DC. For this purpose, DC were primed with LPS or P3C at low concentrations (1 ng/ml) as described above and re-stimulated with LPS or P3C (1000 ng/ml) 24 h later. Priming of DC with LPS or P3C at low concentrations induced cross- as well as homo-tolerant DC as implied from significantly reduced TNF-α

**FIGURE 3.** CD40, CD80, CD86, and MHC class II expression of LPS or P3C treated DC. DC were stimulated with 1 ng/ml or 1000 ng/ml LPS or P3C for 24 h and analyzed by flow cytometry. Black lines represent the surface marker-specific staining, gray lines the isotype control. The results are representative for at least three independent experiments.

**FIGURE 4.** TLR2 and TLR4 surface expression in DC treated with LPS or P3C. Expression of TLR4 (A) and TLR2 (B) in WT DC treated with different concentrations (1 ng/ml or 1000 ng/ml) LPS or P3C for 24 h. As control TLR2/4 \textsuperscript{−/−} DC or isotype control (IT) were used. The results are representative for at least three independent experiments.
secretion upon restimulation with LPS or P3C (Fig. 2, \( A \) and \( B \)). In contrast, the secretion of IL-6 upon restimulation with LPS or P3C was not altered in LPS\( _{\text{low}} \)- or P3C\( _{\text{low}} \)-tolerant DC (Fig. 2, \( C \) and \( D \)). However, the secretion of IL-10 was significantly impaired in DC primed with low concentrations of LPS or P3C (data not shown). These data demonstrate that LPS\( _{\text{low}} \) and P3C\( _{\text{low}} \) tolerance in DC is associated with reduced production of both TNF-\( \alpha \) and IL-10. However, LPS\( _{\text{low}} \) and P3C\( _{\text{low}} \) tolerance only partially (\( \sim 50-80\% \)) attenuated TNF-\( \alpha \) production as compared with total attenuation of TNF-\( \alpha \) in LPS\( _{\text{high}} \)/P3C\( _{\text{high}} \) tolerance.

Moreover, stimulation of naive DC with LPS or P3C at low concentrations did not induce significant DC maturation but induced a phenotype of semimature DC (20, 26) as indicated by only moderately increased expression of CD40, CD80, CD86, and lack of up-regulation of MHC class II (Fig. 3). Likewise, surface TLR2 and TLR4 expression was unaltered upon stimulation of naive wild-type DC (Fig. 4, \( A \) and \( B \)) or IL-6\( ^{+/+} \) DC (data not shown) with LPS or P3C at low concentrations. These data indicate that LPS\( _{\text{low}} \)/P3C\( _{\text{low}} \) tolerance in DC occurs independent of maturation processes and TLR2/TLR4 down-regulation.

Next, we addressed the role of IL-6 in homo- or cross-tolerance in DC primed with LPS or P3C at low concentrations. To this end, naive DC from IL-6\( ^{-/} \) mice were primed with LPS or P3C and after 24 h restimulated with LPS or P3C as described above. Interestingly, treatment of IL-6\( ^{+/} \) DC with P3C or LPS at low concentrations induced neither homo- nor cross-tolerance as indicated by an unaltered strong TNF-\( \alpha \) response upon restimulation with LPS or P3C (Fig. 5, \( A \) and \( B \)). These data demonstrate that IL-6 is required for LPS\( _{\text{low}} \)/P3C\( _{\text{low}} \) homo- or cross-tolerance in DC.

To investigate whether this effect can be mimicked by priming of DC with IL-6, DC were incubated with rIL-6 for 24 h and then stimulated with LPS or P3C (1000 ng/ml) for another 24 h. We observed a significant reduction (\( \sim 40-50\% \)) of LPS (Fig. 7A) or P3C (Fig. 7B) induced TNF-\( \alpha \) secretion from DC primed with IL-6 as compared with nonprimed DC. Priming of DC with rIL-10 did not change TNF-\( \alpha \) secretion upon restimulation with LPS or P3C (data not shown). These data suggest that LPS\( _{\text{low}} \)/P3C\( _{\text{low}} \) tolerance...
of DC can be mimicked by IL-6 and suggest that this type of tolerance is mediated by an auto or paracrine IL-6 loop.

To analyze whether priming of DC with LPS or P3C at low concentrations has a different impact on IκB degradation in wild-type and IL-6−/− DC, we performed Western blot analyzes as described above. Although IκB was not degraded in LPSlow primed DC restimulated with LPS (reflects LPSlow homo-tolerance), IκB was degraded in LPSlow primed IL-6<sup>−/−</sup> DC restimulated with LPS (no LPSlow homo-tolerance), suggesting that LPS<sup>low</sup> homo-tolerance in DC is mediated by IL-6 and occurs upstream of IκB (Fig. 6A). Similar results were obtained in P3C<sup>low</sup> homo-tolerance (Fig. 6B). Thus, we observed an inhibition of IκB degradation in homo-tolerogenic DC but not in cross-tolerogenic DC. Furthermore, inhibition of IκB degradation in homo-tolerogenic DC proved to be IL-6 dependent because it was not observed in IL-6<sup>−/−</sup> DC.

Discussion

We examined the effect of priming DC with a bacterial cell wall component or a lipopeptide analog that are both recognized by distinct TLRs on their state of tolerance toward subsequent restimulation. We used P3C and LPS as agonists for TLR2 and TLR4, respectively (27, 28). Stimulation of naïve DC with LPS or P3C resulted in a dose-dependent increase of TNF-α and IL-6 secretion and expression of activation surface markers such as CD40, CD80, CD86, and MHC class II (29–32). As reported previously (33), stimulation via TLR4 was more efficient in inducing TNF-α secretion and expression of activation surface markers such as CD40, CD80, CD86, and MHC class II. Thus, we observed an inhibition of IκB degradation in homo-tolerogenic DC but not in cross-tolerogenic DC. Furthermore, inhibition of IκB degradation in homo-tolerogenic DC proved to be IL-6 dependent because it was not observed in IL-6<sup>−/−</sup> DC.

| Concentration 1<sup>st</sup> stimulus | Homo-tolerance | Cross-tolerance |
|--------------------------------------|----------------|-----------------|
| Maturation                           | high           | high            |
| TLR downregulation                   | yes (TLR4)     | yes (TLR4)      |
| IL-6 dependency                      | yes            | yes             |
| IκB degradation                      | no             | yes             |
| LPS<sup>low</sup>                     | no             | yes             |

FIGURE 8. The chart illustrates the synopsis of the mechanism described within this study, that might contribute to tolerance induction in DC.

Previously, we reported on a major role of IL-6 in induction of tolerogenic DC upon stimulation with commensal bacteria (20). Stimulation of DC with E. coli induced TNF-α, IL-12 and IL-6 secretion and expression of activation-markers, whereas stimulation with B. vulgatus led only to secretion of IL-6 and DC were driven to a semimature state with low expression of activation-markers. Those semimature DC were nonresponsive to stimulation by E. coli in terms of maturation, TNF-α but not IL-6 production. The nonresponsiveness of B. vulgatus stimulated DC was abrogated by addition of anti-IL-6-mAb or mimicked with rIL-6 (20). In this study, we observed that treatment with P3C or LPS at low concentrations induced tolerogenic DC which depended on IL-6 but not on maturation or reduced expression of TLR. We assume consequently that by an autocrine as well as a paracrine loop IL-6...
induces inhibition of TLR signaling because the effect was not observed in IL-6−/− DC. Vice versa, priming of DC with rIL-6 mimicked priming with LPS/P3C at low concentrations. Homotolerance was associated to inhibition of IL-6 signaling by LPS or P3C at low concentrations. Cross-tolerance involved a different and currently unknown mechanism. IL-6 dependent cross-tolerance induced by LPS<sub>low</sub> or P3C<sub>low</sub> priming of DC did not inhibit degradation of IL-6 and suggested p50 homodimerization or induction of ReILB as candidate-inhibitors of TNF-α secretion as reported in LPS tolerance in macrophages or human promonocyte cells respectively (11, 38). Moreover in keeping with our previous data obtained by stimulation of DC with B. vulgatus or E. coli and our present data, there is evidence that B. vulgatus, which is phylogenetically closely related to Porphyromonas gingivalis (8) primarily signals via TLR2 whereas E. coli signals via both, TLR2 and TLR4.

Recently it has been reported that DC primed with LPS or P3C and rested for 48 h showed an enhanced production of IL-10 and reduced IL-12p40 production upon TLR4 restimulation (34). In the primed DC highly activated ERK1/2 and TRAF3 inducing selective production of IL-10 appeared to compensate for reduced p38 MAPK phosphorylation, resulting in enhanced and reduced production of IL-10 and IL-12p40, respectively (34). However, in our model LPS<sub>low</sub>/P3C<sub>low</sub> stimulation of naive DC did not induce significant IL-10 production arguing against an impact of IL-10 in LPS<sub>low</sub>/P3C<sub>low</sub> tolerance. Although naive DC stimulated with LPS at high concentrations produced high quantities of IL-10 which thus might contribute to the nonresponsiveness, priming of DC with rIL-10, however, did not result in a reduced responsiveness toward subsequent restimulation (data not shown).

Although BMDC closely resemble myeloid DC occurring in vivo, we cannot exclude that tolerance mechanisms in vivo may be different from what we observed for BMDC in vitro. Nevertheless, given that comparable mechanisms may operate in DC in vivo we would like to speculate that the mechanisms reported herein might contribute to the maintenance of intestinal homeostasis even though this hypothesis is highly speculative. In such a model scenario we propose that under physiologic conditions DC might be exposed to only a low stimulation of TLR. In fact, the physiologic gastrointestinal microbiota is separated from the intestinal DC by a thick mucus layer (39). This might lead to the induction of tolerogenic DC in dependency of IL-6, thereby, contributing to the perpetuation of intestinal homeostasis and tolerance of occasional stimulation via TLR2 or TLR4. In contrast, in a state of chronic disease the microbiota might overcome a reduced mucus layer (39), leading to activation and maturation of DC, which provide a proinflammatory cytokine response, trigger T cell proliferation and an inflammatory host reaction before shading off into a maturation dependent, IL-6 independent state of tolerance.

IL-6 is known to influence cell growth, differentiation and migration during immune response, hematopoiesis, and inflammation (40). IL-6 also affects the differentiation of myeloid lineages, including macrophages and DC (41, 42). In the presence of IL-6 the number of resting/immature DC in lymph nodes and spleen is increased, whereas the number of activated mature DC is decreased upon stimulation with LPS in vivo (43). IL-6 plays an important role in T cell differentiation (42) and promotes of Th2 polarized T cell differentiation (44). Second, IL-6 up-regulates the expression of suppressor of cytokine signaling (SOCS1) in CD4<sup>+</sup> cells which inhibits IFN-γ signaling and thus Th1 differentiation (45). The presence of IL-6 may shift the Th1/Th2 balance toward Th2 (44). In keeping with these findings we hypothesize that in our model IL-6 triggered by low dose TLR priming induces semimature DC which inhibit the induction of proinflammatory Th1 responses.

Therefore, it is tempting to speculate that IL-6, in the absence of TNF-α and IL-12(p70), can be considered as a modulatory rather than simply a proinflammatory cytokine. In contrast, IL-6 secreted from splenic DC upon TLR stimulation was an important factor of T cell activation by overcoming T mediated suppression of T cell proliferation (46). Production of IL-6 by DC in response to TLR ligation during stimulation appears to be critical for T cell activation, because it allows pathogen specific T cells to overcome the suppressive effect of CD4<sup>+</sup>CD25<sup>+</sup> T cells (46). However, as splenic DCs include different subsets of DC we cannot exclude that our findings are limited to a system working with only myeloid DC. Furthermore, studies describe that in macrophages in absence of SOCS3 IL-6 induces an anti-inflammatory response, indicating that SOCS3 selectively blocks signaling by IL-6, thereby preventing its ability to inhibit LPS signaling (47). The important role of SOCS3 in tolerance is supported by another study reporting on the silencing of SOCS3 in DC by RNA interference. Silencing of SOCS3 led to induction of IDO and onset of tolerogenic properties in DC upon CD28-ig treatment. The authors suggest that these effects appear to result from a combination of unopposed IFN-γ signaling and the occurrence of IFN-γ like actions by IL-6 (48). Together, the data provide evidence that IL-6 might play an ambiguous role in inflammatory diseases. Depending on the host status IL-6 may act as proinflammatory or anti-inflammatory cytokine promoting or inhibiting T cell priming and Th1 responses.

However, future studies will have to elucidate the mechanism contributing to IL-6 dependent DC tolerance and the relevance of IL-6 dependent tolerogenic DC for intestinal immune homeostasis and on disease in vivo.

**Disclosures**

The authors have no financial conflict of interest.

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