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IL-33 Enhances Lipopolysaccharide-Induced Inflammatory Cytokine Production from Mouse Macrophages by Regulating Lipopolysaccharide Receptor Complex

Quentin Espinassous,2,* Elvira Garcia-de-Paco,2,* Ignacio Garcia-Verdugo,* Monique Synguelakis,* Sonja von Aulock,† Jean-Michel Sallenave,§ Andrew N. J. McKenzie,¶ and Jean Kanellopoulos3*

Bacterial LPS triggers monocytes and macrophages to produce several inflammatory cytokines and mediators. However, once exposed to LPS, they become hyporesponsive to a subsequent endotoxin challenge. This phenomenon is defined as LPS desensitization or tolerance. Previous studies have identified some components of the biochemical pathways involved in negative modulation of LPS responses. In particular, it has been shown that the IL-1R-related protein ST2 could be implicated in LPS tolerance. The natural ligand of ST2 was recently identified as IL-33, a new member of the IL-1 family. In this study, we investigated whether IL-33 triggering of ST2 would be able to induce LPS desensitization of mouse macrophages. We found that IL-33 actually enhances the LPS response of macrophages and does not induce LPS desensitization. We demonstrate that this IL-33 enhancing effect of LPS response is mediated by the ST2 receptor because it is not found in ST2 knockout mice. The biochemical consequences of IL-33 pretreatment of mouse macrophages were investigated. Our results show that IL-33 increases the expression of the LPS receptor components MD2 (myeloid differentiation protein 2) and TLR-4, the soluble form of CD14 and the MyD88 adaptor molecule. In addition, IL-33 pretreatment of macrophages enhances the cytokine response to TLR-2 but not to TLR-3 ligands. Thus, IL-33 treatment preferentially affects the MyD88-dependent pathway activated by the TLR. The Journal of Immunology, 2009, 183: 1446–1455.

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2 Abbreviations used in this paper: TRIF, Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF) signaling pathways (6), secretion of anti-inflammatory cytokines, and down-modulation of transcription factors such as NF-κB and AP-1. More recently, Brint et al. (7) demonstrated that macrophages from IL-1R-related protein ST2 knock-out (KO) mice cannot be desensitized to LPS. In addition, in HEK 293R1 cells overexpressing ST2 and MyD88, they showed that MyD88 was able to bind ST2 rather than TLR-4. These authors (8) suggested that this mechanism of sequestration is involved in the phenomenon of LPS desensitization. At that time, the ligand of ST2 was unknown and this receptor was an orphan member of the TLR-IL1-R superfamily.

Recently, Schmitz et al. (8) identified IL-33 as the natural ligand of ST2. IL-33 is one of the newly described members of the IL-1 family that was identified on the basis of its structural similarities with other IL-1 family members (8). These cytokines play a major role in a wide range of infectious, inflammatory, and autoimmune diseases (9). IL-33, like IL-1α, IL-1β, and IL-18, is produced as a result of hyporesponsiveness to an endotoxin challenge due to a prior exposure of hosts to sublethal doses of endotoxins. Macrophages tolerant to LPS produce much lower levels of inflammatory cytokines such as TNF-α, IL-6, and IL-1β than when they are stimulated with endotoxin for the first time. This state of desensitization is not characterized by a general inhibition of macrophage secretion, because IL-10 (1), TGF-β (2), and IL-1R antagonist are up-regulated (3). Thus, Shnyra et al. (4) have suggested that LPS desensitization induces a reprogramming of macrophages that leads to an altered response to endotoxin. Several mechanisms to explain LPS tolerance have been described (reviewed in Ref. 5): down-modulation of TLR-4, modification of various components of the MyD88 or Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF) signaling pathways (6), secretion of anti-inflammatory cytokines, and down-modulation of transcription factors such as NF-κB and AP-1. More recently, Brint et al. (7) demonstrated that macrophages from IL-1R-related protein ST2 knock-out (KO) mice cannot be desensitized to LPS. In addition, in HEK 293R1 cells overexpressing ST2 and MyD88, they showed that MyD88 was able to bind ST2 rather than TLR-4. These authors (8) suggested that this mechanism of sequestration is involved in the phenomenon of LPS desensitization. At that time, the ligand of ST2 was unknown and this receptor was an orphan member of the TLR-IL1-R superfamily.

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Abbreviations used in this paper: TRIF, Toll/IL-1R domain-containing adapter inducing IFN-β; iNKT, invariant NKT; KO, knockout; LTA, lipoteichoic acid; poly(I:C), polyinosinic-polycytidylic acid; PVDF, polyvinylidene difluoride; qPCR, quantitative real-time PCR; sCD14, soluble CD14; SIGIRR, single Ig IL-1R-related; wt, wild type.
31-kDa precursor protein without a signal peptide. This pro-IL-33 can be cleaved in vitro by caspase 1 to generate a mature 18-kDa protein that has an optimal biological activity (8). Recently, Carrière et al. (10) showed that IL-33 is the same molecule as NF-HEV (11), a nuclear factor endowed with transcriptional repressor properties (10). Thus, IL-33, like IL-1β and HMGB1, may belong to this family of proteins with dual functions that can behave as proinflammatory cytokines and transcription factors (12–14).

Schmitz et al. (8) have shown that the following molecules are involved in the signaling pathway of ST2: the adaptor MyD88, the IL-1R-associated kinases 1 and 4, and the TNF receptor-associated factor 6, which activates the MAPKs and the NF-κB (15). These molecules are shared by other TLR-dependent pathways, notably the CD14/MD2/TLR-4 complex, which is triggered by LPS. Recently, the IL-1R accessory protein IL-1RAcP, which is also involved in IL-1 signaling, was found to heterodimerize with ST2 when IL-33 is bound to ST2 (16, 17). ST2 is expressed on mast cells (18), LPS-activated macrophages (7), and Th2 lymphocytes (19) and exists as transmembrane (ST2L) and soluble (sST2) isoforms generated by alternative splicing (20). IL-33 has been shown to induce the secretion of both proinflammatory and anti-inflammatory cytokines and chemokines from mast cells, eosinophils, and Th2 lymphocytes (16, 21, 22). However, the influence of IL-33 on macrophages has not yet been studied. In addition, the outcome of ST2 stimulation by its natural ligand IL-33 and its potential role in LPS desensitization have not been tested.

In this study, we investigated whether IL-33 induces desensitization of macrophages to endotoxin by stimulating the ST2 receptor. We found that mature IL-33 does not promote LPS desensitization, but instead increases the secretion of TNF-α, IL-6, and IL-1β by mouse macrophages in response to LPS. Therefore, we studied the effect of IL-33 on various components of the TLR-4 pathway and identified some key molecules that are up-regulated by this IL.

**Materials and Methods**

**Antibodies**

The following unconjugated Abs were used for immunoprecipitation, Western blotting, flow cytometric analyses, or inhibition experiments: goat anti-IL-1R4 (ST2) (R&D Systems); rat monoclonal anti-T1/ST2 (MD Biosciences); rabbit polyclonal anti-MD2 (Abcam); rat purified anti-mouse

![FIGURE 1](http://www.jimmunol.org/) Expression of ST2 in peritoneal macrophages. Peritoneal macrophages were collected and plated for 2 h at 37°C. Cells were washed once and incubated overnight in RPMI-SP with 100 ng/ml LPS at 37°C. A. The expression of ST2L was measured at mRNA and protein levels. The mouse thymoma BW5147 was used as a positive control of cell surface expression of ST2 (19). RNA of macrophages and BW5147 cells was extracted as described in Materials and Methods. Two micrograms of total RNA were reverse transcribed, and ST2 expression was determined by RT-PCR. Expression of ST2L in macrophages (mØ), BW5147 cells, non-reverse transcribed RNA from macrophages (RNA mØ), and control (H2O) is shown. B. After biotinylation of the macrophage surface molecules, cells were lysed in 0.5 ml of lysis buffer containing protease inhibitors as described in Materials and Methods. Biotinylated ST2L proteins were specifically immunoprecipitated with rat anti-mouse ST2 mAb. Immunoprecipitated proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and immunoblotted with HRP-streptavidin. C. The areas corresponding to the specific ST2L bands were scanned and analyzed by Quantity One one-dimensional analysis software from Bio-Rad. We represented the value of the intensity/surface. D. Expression of ST2 mRNA of macrophages stimulated or not with LPS for 24 h. Five million peritoneal macrophages were treated with LPS or RPMI 1640 for 24 h and RNA was extracted as described in Materials and Methods. Two micrograms of total RNA were reverse transcribed, and ST2 mRNA expression was determined using real time PCR, normalized to that of β-actin. *** * p < 0.001.
CD16/CD32 (BD Biosciences/Pharmingen) used as Fc block; rabbit polyclonal anti-MyD88 (Abcam); mouse monoclonal anti-/H9252-tubulin (D-10; Santa Cruz Biotechnology); and rabbit polyclonal anti-TLR-2 (Santa Cruz Biotechnology). For cytometry, we used the following conjugated-antibodies: rat monoclonal anti-TLR-4 coupled to PE (Biolegend) and its isotype control, i.e., PE-conjugated rat IgG2a, /H9260 (Biolegend); biotin-conjugated mouse monoclonal anti-R-PE (BD PharMingen); rat monoclonal anti-CD14 coupled to allophycocyanin (eBioscience); and goat F(ab’/H11032)2 anti-rabbit IgG (H and L chain-specific) coupled to biotin (Southern Biotech). Affinity-purified rabbit anti-rat IgG Abs coupled to peroxidase (Sigma-Aldrich) and affinity-purified goat anti-rabbit IgG Abs coupled to peroxidase (Rockland Immunochemicals) were used as secondary Abs for Western-blot analyses.

Mice
Eight- to 12-wk-old BALB/c and C57BL/6 mice were purchased from Charles River Laboratories. Female and male mice from C57BL/10 (ScSn) and TLR-4 KO mice (C57BL/10 (ScCr)) (23) were bred in our animal facility and sacrificed at 8 to 20 wk of age. ST2-deficient mice (24) were backcrossed to the BALB/c background for seven generations and compared with wild-type controls backcrossed to BALB/c for six generations. These mice were maintained in the Small Animal Barrier Unit and Central Biomedical Services, Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.

Thioglycolate-elicited peritoneal macrophages
Mice were injected i.p. with 1.5 ml of 4% thioglycolate broth with resazurine (Fluka). ST2-deficient and control mice received 0.4 ml of 12% thioglycolate i.p. Five days later, cells were harvested by peritoneal lavage with RPMI 1640 plus GlutaMAX (Invitrogen) containing 100 U/ml streptomycin (Invitrogen) and 100 /H9262 g/ml penicillin (Invitrogen) (RPMI-SP). The cells were washed twice and 106 peritoneal cells were then added to each well of 24-well plates (Costar). The cells were incubated at 37°C for 2 h in a humidified 5% CO2 incubator to allow macrophages to adhere to the plates. The plates were then washed once with 0.5 ml of RPMI 1640 plus GlutaMAX at room temperature to remove nonadherent cells.
Stimulation of mouse peritoneal macrophages

Salmonella minnesota LPS Re 595, polyinosinic:polycytidylic acid (poly(I:C)), and ATP were purchased from Sigma-Aldrich, and rIL-33 (rIL-33) was from Alexis Biochemicals. Lipoteichoic acid (LTA) was purified as described by Morath et al. (25). This highly purified preparation does not stimulate macrophages from TLR-2 KO mice. To establish the optimum time of incubation with rIL-33, the cells were incubated at 37°C in a humidified 5% CO2 incubator for different times with or without rIL-33 at 5 or 50 ng/ml (the optimal dose of IL-33 varied with the commercial batches). After this preincubation, macrophages were stimulated with 100 ng/ml LPS for 24 h. Supernatants were collected and TNF-α production was determined by ELISA (the values correspond to the mean obtained with macrophages of two mice tested individually ± SD). The results shown in A are representative of three experiments performed on different days. B, Macrophages (10⁶) from WT or ST2 KO mice were treated with IL-33 (50 ng/ml) for 6 h. Medium was eliminated and cells were incubated with LPS (100 ng/ml) for 24 h. Supernatants were collected and TNF-α was measured by ELISA. The values shown in B represent the mean and SD of TNF-α levels secreted by macrophages of one wt and one ST2 KO mouse. Six wt mice and five ST2 KO animals were tested individually. *, p < 0.05; ***, p < 0.001.

Inhibition of IL-33 effect

Macrophages (10⁶) were incubated with different concentrations of anti-ST2 Ab (R&D Systems and MD Biosciences) for 1 h, then 50 ng/ml IL-33 (Alexis Biochemicals) was added for 6 h. Supernatants were eliminated and macrophages were incubated for 24 h with 100 ng/ml LPS. TNF-α levels were measured by ELISA.

Immunoprecipitation

After 24 h of culture in RPMI-SP with or without LPS, 10⁶ macrophages were surface biotinylated. Cells were washed twice with 2 ml PBS and incubated for 30 min with 1.5 ml of EZ-Link sulfo-NHS-LC-biotin (Pierce) (1 mg/ml) in PBS at 4°C with gentle agitation. Cells were washed twice in RPMI 1640 containing 10% FCS and 50 mM glycine and once with PBS at 4°C. Cells were then lysed in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 containing antiproteases). Cell lysates were preclarified with protein G PLUS-agarose beads (Santa Cruz Biotechnology) and equal amounts of lysates were incubated with anti-ST2 mAb (MD Biosciences) or isotype control coupled to protein G PLUS-agarose at 4°C overnight. Beads were then washed three times with lysis buffer and resuspended in reducing sample buffer. Immunoprecipitates were analyzed by SDS-PAGE and transferred to nitrocellulose membranes (BioRad) that were blocked with 5% nonfat milk in Tween/BSA overnight. After four washes in Tween/BSA, they were incubated with HRP-conjugated streptavidin for 1 h. Specific bands were visualized by ECL (Amer sham ECL Plus Western blotting detection reagents).

ELISA

ELISA kits were used to measure the respective concentrations of TNF-α (eBioscience), IL-6 (eBioscience), and IL-1β (R&D Systems) according to the manufacturer’s instructions.

Flow cytometric analyses

To assess the expression of cell surface molecules, we incubated macrophages after different treatments with Fc block for 5 min on ice in PBS supplemented with 1% BSA and containing 0.01% sodium azide (wash buffer). Then, anti-CD14 (eBioscience), anti-MD2 (Abcam), or anti-TLR-4 (Biolegend) was added for 30 min on ice. After washing, the cells were probed with secondary Abs for 30 min. We analyzed the expression of MD2, CD14, or TLR-4 by flow cytometry (Becton Dickinson).
Western blot analyses

Cell membrane or cell lysates were analyzed by SDS-PAGE and transferred to Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad), which were blocked with 5% nonfat milk in Tris buffer saline containing 0.2% Tween 20 at 37°C for 2 h. Blots were immunostained with primary Abs at 4°C overnight and probed with secondary Abs conjugated to HRP. Specific bands were visualized by ECL.

Quantitative real-time PCR

Thioglycolate-elicited peritoneal macrophages were treated in 35-mm plates (5 × 10⁶/plate) (Corning Glass Works) with IL-33 (5 ng/ml) for 2, 4, or 6 h. Two micrograms of total RNA, extracted from cells using TRIzol reagent (Invitrogen) and treated with Turbo DNase I (TURBO DNA kit; Ambion), were used to synthesize cDNA using the SuperScript First-Strand synthesis system for RT-PCR (Invitrogen). Quantitative real-time PCR (qPCR) reactions were performed using LightCycler Fast Start DNA Master Plus SYBR Green I mix (Roche) in a LightCycler 2.0 (Roche) instrument according to the manufacturer’s instructions.

The cycling conditions were 95°C for 10 min followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 20 s. At the end of the last cycle, the temperature was increased from 65°C to 95°C (0.1°C/s) to produce a melting curve. The specificity of amplification was assessed for each sample by melting curve analysis. Each PCR product showed a single peak. The size of the amplicon was checked by electrophoresis. Agarose gel electrophoresis revealed a single product of the expected size. The analysis of the expression of the different genes relative to the β-actin endogenous control was performed with LightCycler software in relative quantification mode following the manufacturer’s instructions and using control cells as calibrators. To demonstrate the specificity of the qPCR results we included a negative control, the ribosomal protein L32 (RPL32), in addition to the β-actin normalization control. This is a commonly used housekeeping gene. Our results show that the Rl32 mRNA is not significantly changed after the treatment of macrophages with IL-33 (α = 3, p > 0.5).

Sequences of all primers used in this study are listed in Table I. The following criteria were applied in the course of designing the primers: product size from 80 to 400 bp, primer size from 17 to 22 bp, and a mean melting temperature of 60°C.

Statistical analyses

Data were analyzed by a paired Student t test to determine the difference between the samples (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Results

ST2 is expressed at the plasma membrane of macrophages

To study the effects of IL-33 on the LPS responses of mouse macrophages, we first tested the expression of ST2 in peritoneal macrophages. As a first approach, we analyzed the cell surface expression of ST2 on macrophages by flow cytometry. Although we found ST2 on P-815 mastocytoma cells, we failed to detect this molecule on the plasma membrane of macrophages. Thus, we used biochemical methods to determine whether macrophages express ST2. We found that ST2 mRNA (Fig. 1A) was present in macrophages. To ensure that ST2 is expressed at the cell surface of macrophages, we biotinylated the surface proteins of macrophages stimulated or not stimulated with LPS. Then, we analyzed the expression of ST2 in cell lysates after immunoprecipitation with a rat anti-mouse ST2 mAb (Fig. 1B). We detected two bands with apparent molecular masses of 60 and 70 kDa, which may correspond to different glycosylated forms of ST2 as described by Tago et al. (26). By quantitative PCR, we found that the level of ST2 was increased by a factor of 2 in LPS-stimulated macrophages as compared with unstimulated ones (Fig. 1D). To determine whether this 2-fold increase in ST2 mRNA has any impact on the amount of cell surface ST2 protein, we quantified and compared the amount of immunoprecipitated ST2 from macrophages stimulated or not stimulated with LPS. As shown in Fig. 1C, the amount of biotin surface-labeled ST2 is 2.7 higher in LPS-stimulated macrophages than in untreated ones. Altogether, our data show that mouse macrophages express ST2 at their cell surface and that the amount of ST2 increases by a factor of 2 to 3 after stimulation with LPS for 24 h.

IL-33 increases the cytokine responses of macrophages to LPS

It was previously shown that ST2 is involved in the induction of LPS desensitization (7). Thus, we determined whether the stimulation by IL-33, a ST2 ligand, induces a decrease in cytokine responses of macrophages triggered by LPS.

To induce LPS desensitization, we used classical protocols in which macrophages were preincubated with 100 ng/ml LPS for 24 h followed by a second stimulation with the same dose of LPS for 24 h. As shown in Fig. 2A, macrophages stimulated with only the optimal dose of LPS for 24 h secreted ~25,000 pg/ml TNF-α, whereas those that were stimulated twice were desensitized and released very low levels of this cytokine after a second treatment with LPS. To test the role of IL-33 on LPS desensitization, we preincubated macrophages with IL-33 for various periods of time and stimulated them with LPS for 24 h. We found that preincubation with IL-33 does not induce LPS desensitization even after 24 h of pretreatment, but does increase the TNF-α responses of macrophages to LPS (Fig. 2B). The maximal increase in the TNF-α response was observed after 6 h of preincubation with IL-33. In most of the following experiments, this preincubation time is used unless stated.
We determined that this increase in TNF-α is not induced by IL-33 stimulation itself. IL-33 alone triggers a weak TNF-α secretion (300–400 pg/ml) (Fig. 2C). The pretreatment of macrophages for 6 h with increasing concentrations of IL-33, followed by LPS stimulation, increases the production of TNF-α compared with LPS stimulation alone. The maximal level of secreted TNF-α is reached at 5 ng/ml of IL-33. The optimal dose of IL-33 varied with the commercial batches, a phenomenon that could be attributed to oxidation as suggested by Palmer et al. (27). It is worth noting that this potentiating effect of IL-33 on LPS responses is found in macrophages obtained from various strains of mice (BALB/C, C57BL/6, and C57BL/10 (ScSn)) and is independent of the age and sex of the animals (data not shown).

We then determined whether other cytokines induced after LPS stimulation are increased by IL-33 pretreatment of macrophages. IL-33 has the same effect on the IL-6 response to LPS stimulation as on that observed for TNF-α (Fig. 3A). We also observed that the production of mature IL-1β is increased by IL-33 pretreatment of macrophages (Fig. 3B). In these experiments, we treated the LPS-primed macrophages with extracellular ATP to promote the processing and release of mature IL-1β. Indeed, it has been established that IL-1β secretion requires several signals (reviewed in Ref. 28); LPS increases pro-IL-1β transcription and biosynthesis, and extracellular ATP induces the processing and release of mature IL-1β. This nucleotide triggers the P2X7 purinergic receptor, inducing a K⁺ efflux leading to the activation of caspase 1, which cleaves pro-IL-1β into the mature IL-1β.

In conclusion, the pretreatment of macrophages by IL-33 does not induce LPS desensitization but increases the proinflammatory cytokine responses to LPS. IL-33 effect is mediated by ST2

ST2 is the only known receptor for IL-33. It was shown that this receptor is implicated in LPS desensitization of macrophages (7). Because our results show that IL-33 stimulates macrophage responses to LPS and does not lead to hyporesponsiveness to endotoxin, we wondered whether the IL-33 effects were mediated by ST2 or by another receptor. As a first approach, we treated macrophages with two different anti-ST2 Abs and observed that these treatments abolished the potentiating effect of IL-33 on macrophage responses to LPS (Fig. 4A, histograms 2 and 3 vs histogram 1).

We also compared the effect of IL-33 pretreatment upon LPS responses of peritoneal macrophages from five ST2 KO and six wild-type (wt) mice tested individually. This treatment increases LPS responses of macrophages from wt mice but not those of ST2 KO animals (Fig. 4B). We found that identical amounts of TNF-α...
were produced by macrophages of ST2 KO mice stimulated with LPS or IL-33 plus LPS. In contrast, the levels of TNF-α secreted by wt mice macrophages treated with IL-33 and LPS were significantly increased when compared with those of macrophages stimulated with LPS only (p < 0.001).

Overall, these data demonstrate that the increase in LPS responses induced by IL-33 pretreatment of macrophages is mediated by the ST2 receptor.

**IL-33 effect on the LPS receptor complex**

An increase in cell surface expression of the LPS receptor complex may explain the enhancement of the LPS responses of macrophages after IL-33 pretreatment. Thus, we compared the MD2 (myeloid differentiation protein 2), a molecule associated with TLR-4 that is required to generate a functional LPS receptor (29), CD14, and TLR-4 mRNA levels in macrophages treated or not treated with IL-33. In Fig. 5, the expression of CD14 at the mRNA and protein levels is shown. We found a 3-fold increase of CD14 mRNA (Fig. 5A) without any enhancement of the plasma membrane expression of CD14 (Fig. 5B). Because it was previously shown that a soluble form of CD14 (sCD14) allows potentiation of the LPS responses of cells lacking membrane CD14 (30), we determined whether an increase in sCD14 is found in the supernatants of IL-33-stimulated macrophages. As presented in Fig. 5C, there is a 1.7-fold increase in sCD14 after treatment with IL-33. As shown in Fig. 6A, in IL-33-stimulated macrophages the transcripts of MD2 and TLR-4 are increased by a factors of 5.4 and 5.3, respectively. To confirm these data at the protein level, we studied the expression of MD2 and TLR-4 by flow cytometry after 6, 8, or 10 h of IL-33 treatment. As shown in Fig. 6B, the MD2 level is increased after 10 h of treatment, and the TLR-4 level increases from 8 to 10 h. At 6 h, the statistical analyses show that the TLR-4 increase is not significant. These data suggest that the IL-33 signaling increases the level of sCD14 in macrophage supernatants and the level of the MD2/TLR-4 components of the LPS receptor at the plasma membrane of macrophages.

**IL-33 effect on other TLR responses and MyD88**

The members of the TLR-IL1-R superfamily share several molecules in their signaling pathways (31). Interactions between these pathways are well documented (32). Thus, we investigated the effect of IL-33 on the response of macrophages to other TLR ligands. We prestimulated macrophages with IL-33 for 6 h and incubated them with LTA and poly(I:C), specific ligands of TLR-2 and TLR-3, respectively. TLR-2 signaling uses the MyD88 pathway, whereas the TLR-3 pathway is TRIF dependent. IL-33 pre-treatment of macrophages had no effect on the TNF-α production.
induced by poly(I:C). In contrast, we observed an increase of TNF-α secretion (~2-fold) in response to LTA (Fig. 7). Thus, pretreatment of macrophages with IL-33 increases the response to the TLR-2 ligand but not to the TLR-3 ligand. Altogether, our results suggest that the IL-33 enhancing effect on the LPS and LTA responses of mouse macrophages targets the MyD88-dependent pathway of TLR-4 and TLR-2.

Because IL-33 potentiates TLR-2 responses, we determined whether TLR-2 expression is increased by IL-33 stimulation of macrophages as observed for TLR-4. We found by qPCR (Fig. 8A) and Western blotting (Fig. 8, B and C) that IL-33 treatment increased TLR-2 levels by factors of 4.1 and 7, respectively.

As it is well established that MyD88 plays a major role in TLR signaling (33) and that IL-33 seems to target this molecule, we determined whether the increase in the LPS receptor complex after IL-33 pretreatment is coupled with an increase of MyD88. As shown in Fig. 8, MyD88 mRNA is increased by a factor of 5.2 after 6 h of treatment with IL-33 (Fig. 8A), whereas the level of MyD88 protein is enhanced 3-fold (Fig. 8, B and C). These results suggest a potential role for MyD88 in the increased response of macrophages to LPS after pretreatment with IL-33.

Discussion

In the present study, we show that IL-33 enhances the LPS responses of mouse peritoneal macrophages. We demonstrate that the biological effects of IL-33 are mediated by the ST2 receptor, which triggers an increase in the expression of the LPS receptor components (MD2/CD14 and TLR-4) and the MyD88 adaptor molecule.

By flow cytometry, we were unable to detect ST2 on the surface of macrophages. Thus, we surface labeled peritoneal macrophages and immunoprecipitated ST2 from cell lysates with rat anti-mouse ST2 mAb. Our results clearly show that ST2 is expressed on the cell surface of macrophages not stimulated with LPS (Fig. 1). These findings are at variance with those of Brint et al., who detected ST2 on LPS stimulated macrophages but not on naive ones (7). This discrepancy may be due to the method of detection used, flow cytometry vs surface biotinylation, the latter being more sensitive.

The IL-33 receptor ST2 has been shown to be involved in the phenomenon of endotoxin desensitization (7), so we hypothesized that ST2 stimulation by its natural ligand IL-33 could induce macrophage desensitization to LPS. Thus, we studied the role of IL-33 in the desensitization to LPS, i.e., we measured the levels of TNF-α, IL-6, and IL-1β secreted by macrophages in response to endotoxin. We found that IL-33 treatment does not inhibit the TNF-α response to LPS but increases its secretion by a factor of 3 to 4. This augmentation in TNF-α is also found for IL-6 and IL-1β (Fig. 2). One possible explanation for these observations could be that the IL-33 commercial preparations were contaminated by suboptimal doses of LPS, which could prime the LPS response of macrophages (34). However, ST2 KO macrophages pretreated or not pretreated with IL-33 secrete identical amounts of cytokines in response to LPS. These results rule out that the IL-33 effect is due to a contamination by LPS or other molecules.

We found that the enhancing effect of IL-33 is time dependent. A preincubation of macrophages with IL-33 for 6–8 h is the optimal length of time for obtaining the highest secretion of LPS-induced cytokines. Endotoxin responses returned to normal levels when macrophages were pretreated with IL-33 for longer periods of time. This was not due to IL-33 degradation or depletion, because the addition of fresh IL-33 after 6 h did not restore the enhancing effect of IL-33 on the LPS response (data not shown). To explain the decrease of LPS responses at 24 h following IL-33 treatment, we hypothesized that macrophages released anti-inflammatory cytokines such as IL-10. We did not detect IL-10 in culture supernatants of macrophages stimulated with IL-33 for 24 or 48 h (data not shown). However, we cannot rule out the implication of other mediators such as TGF-β (2).

The LPS responses of macrophages pretreated with IL-33 are superior or equal to those observed with LPS stimulations only. These results show that IL-33 cannot induce LPS desensitization of mouse macrophages. Because ST2 was shown to be involved in endotoxin desensitization while IL-33 treatment of macrophages enhances LPS responses, we tested whether the stimulatory properties of IL-33 could be due to another unidentified IL-33 receptor present on macrophages. To test this hypothesis, we used two strategies. We treated macrophages with anti-ST2 blocking Abs and stimulated macrophages from wt and ST2 KO mice with IL-33 and LPS. Our results clearly demonstrate that IL-33 enhancing effects are mediated by ST2, because they were blocked by anti-ST2 Abs and not found in ST2 KO macrophages. To identify the mechanisms by which IL-33 potentiates the secretion of cytokines by LPS-stimulated macrophages, we quantified the expression of several molecules involved in the TLR-4 signaling pathway. We found an increase of the mRNAs encoding the components of the LPS receptor (CD14/MD2/TLR-4) and the principal adaptor molecule MyD88. Furthermore, we observed that the amounts of MD2 and TLR-4 present at the cell surface of macrophages increased after pretreatment with IL-33. In contrast, although CD14 mRNA increased after IL-33 treatment, the CD14 level at the macrophage surface was not affected by this treatment. One potential explanation for this discrepancy is that CD14 is produced in a soluble form. Indeed, we found that the amount of sCD14 is increased in the supernatants of IL-33-stimulated macrophages. Altogether, the increase in expression of the components of the LPS receptor complex and MyD88 strongly suggests that they contribute to the enhanced LPS response of macrophages.

Because the receptors of the Toll-like family share numerous signaling molecules, we tested the effect of IL-33 on the response of other TLRs. We observed an increase of the cytokine response to LPS (MyD88 dependent and independent) and LTA (TLR-2, MyD88 dependent), but not to poly(I:C) (TLR-3, MyD88 independent). Thus, our experiments suggest that IL-33 affects the MyD88-dependent pathway of the TLRs. The lack of influence on the TLR-3 response could be explained by two hypotheses. The first possibility is that IL-33 affects the MyD88-dependent pathway of the TLRs. The lack of influence on the TLR-3 response could be explained by two hypotheses. The first possibility is that IL-33 affects the MyD88-dependent pathway of the TLRs. The lack of influence on the TLR-3 response could be explained by two hypotheses. The first possibility is that IL-33 affects the MyD88-dependent pathway of the TLRs. The lack of influence on the TLR-3 response could be explained by two hypotheses. The first possibility is that IL-33 affects the MyD88-dependent pathway of the TLRs. The lack of influence on the TLR-3 response could be explained by two hypotheses.
Several studies on soluble ST2 have shown that it inhibits inflammatory responses. Indeed, soluble ST2 is implicated in the modulation of numerous inflammatory reactions such as acute lung inflammation (37), arthritis (38), asthma (39), and other diseases (40). Recently, soluble ST2 was shown to block IL-33 signaling (41). The inhibitory effect of soluble ST2 may be due to its ability to trap IL-33 and prevent it from binding to ST2 on the surface of various cells. Another possibility is based on the work of Sweet et al. who showed that soluble ST2 binds to macrophages and induces NF-κB activation associated with a down-modulation of TLR-4 and TLR-1 (42). These results suggest that soluble ST2 interacts with an undefined membrane receptor involved in macrophage desensitization. Thus, the role of ST2 in LPS desensitization observed by Brint et al. (7) may be due to the absence of soluble ST2 and not to the deletion of its membrane form.

Numerous studies have shown that IL-33 leads to the amplification of Th2 responses. Indeed, upon IL-33 binding, Th2 lymphocytes and mast cells secrete Th2 cytokines such as IL-4, IL-5, and IL-13. However, recently Smithgall et al. have shown that IL-33 can activate human NK and invariant NKT (iNKT) cells to produce Th1 cytokines (43). Stimulation of iNKT cells by the IL-33 receptor complex. J. Immunol. 179: 2551–2555.


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