Transcriptional Regulation of Murine IL-33 by TLR and Non-TLR Agonists


*J Immunol* 2012; 189:50-60; Prepublished online 25 May 2012;
doi: 10.4049/jimmunol.1003554
http://www.jimmunol.org/content/189/1/50

**References**

This article cites 68 articles, 36 of which you can access for free at: http://www.jimmunol.org/content/189/1/50.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Transcriptional Regulation of Murine IL-33 by TLR and Non-TLR Agonists

Swamy Kumar Polumuri,* Gift Gunaraj Jayakar,* Kari Ann Shirey,* Zachary J. Roberts,* Darren J. Perkins,* Paula M. Pitha,† and Stefanie N. Vogel*

IL-33, a member of the IL-1 family of cytokines, is produced by many cell types, including macrophages, yet its regulation is largely unknown. Treatment of primary murine macrophages with a panel of TLR (e.g., TLR2, TLR3, TLR4, and TLR9) agonists and non-TLR (e.g., MDA5, RIG-I) agonists revealed a pattern of gene and protein expression consistent with a role for IFN regulatory factor-3 (IRF-3) in the expression of IL-33. Accordingly, induction of IL-33 mRNA was attenuated in IRF-3−/− macrophages and TBK-1−/− mouse embryonic fibroblasts. Despite the fact that all IL-33 agonists were IRF-3 dependent, LPS-induced IL-33 mRNA was fully inducible in IFN-β−/− macrophages, indicating that IL-33 is not dependent on IFN-β as an intermediate. Epinephrine and Bordetella pertussis adenylate cyclase toxin (ACT), cAMP-activating agents, activate CREB and greatly synergize with LPS to induce IL-33 mRNA in macrophages. Both LPS-induced and ACT/LPS-enhanced expression of IL-33 mRNA was partially, but significantly, inhibited by the protein kinase A inhibitor H-89 but not by tyrosine kinase or protein kinase C inhibitors. Two IL-33 mRNA species derived from two alternative promoters encode full-length IL-33; however, the shorter “A” species is preferentially induced by all IL-33–inducing agonists except Newcastle disease virus, a RIG-I agonist that induced expression of both “A” and “B” transcripts. Together, these studies greatly extend what is currently known about the regulation of IL-33 induction in macrophages stimulated by bacterial and viral agonists that engage distinct innate immune signaling pathways. The Journal of Immunology, 2012, 189: 50–60.
umbilical cord blood-derived mast cells (23). In mice, administration of anti-ST2L Ab enhances the Th1 cytokine response and inhibits allergic airway inflammation (24, 25). Thus, although IL-33 can elicit induction of proinflammatory mediators, it favors development of a Th2-biased immune response.

Active secretion of IL-33 from cells has not been reported. Like high mobility group box 1, IL-1α, or IL-1β, the IL-33 gene sequence does not contain a classical secretory leader sequence (26). Also, like IL-1α, IL-33 exhibits dual functions as both a cytokine and nuclear chromatin modulator (2, 27). IL-33 has been found to be chromatin-associated in the nucleus of endothelial cells and has the capacity to regulate transcription (27). IL-33 binds to chromatin in the surface of the nucleosome by docking to the pockets of histone H2A–H2B dimers (28). Despite the fact that IL-33 has been detected in multiple tissues (2), very little is known about how IL-33 production is regulated. Therefore, in the current study, we measured the steady-state levels of IL-33 mRNA or IL-33 protein in macrophages and fibroblasts after exposure to different TLR ligands and to non-TLR ligands including viral pathogens. Our studies revealed that IL-33 is regulated by two transcription factors, IFN regulatory factor-3 (IRF-3) and CREB, but not by protein kinase C (PKC) or tyrosine kinases, as supported by pharmacologic inhibition studies. Importantly, while two mRNA species encoding full-length IL-33 are transcribed, the shorter “A” transcript is preferentially induced by all IL-33 inducers identified except the RIG-I agonist Newcastle disease virus (NDV), which induced both “A” and “B” transcripts. This study provides new insights into the regulation of IL-33 gene expression in macrophages.

Materials and Methods

Reagents

Protein-free Escherichia coli K235 LPS (<0.008% protein) was prepared by modification of the phenol–water extraction method described previously (29). The synthetic lipoprotein S-[2,3-Bis(palmito)xylo-(2-R)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys-4-OH trihydrochloride (PSO) was purchased from EMC Microcollections (Tübingen, Germany). Polyinosinic-polycytidylic acid (poly-IC) was purchased from Amersham Biosciences (Pittsburgh, PA). 5′-Dimethylxanthenone-4-acetic acid (DMXAA) was purchased from Sigma-Aldrich. Anti–p-IRF-3, anti–β-actin, anti–p-STAT1, anti–p-tyrosine mouse mAb (P-tyr-100; no. 9411), and anti–p-CREB (Ser 133), which also detects phosphorylation of the CREB-related protein ATF-2, were purchased from Cell Signaling (Beverly, MA). Anti–total IRF-3 Ab was obtained from Invitrogen (Carlsbad, CA). Adenylate cyclase toxin (ACT), a potent inducer of CAMP, was the kind gift of Dr. Erik Hewlett (University of Virginia, Charlottesville, VA). Tyrosine kinase inhibitors PP2 (Src family of protein tyrosine kinases) and EGF/FGF/IGF-1 receptor tyrosine kinase inhibitor (RTKi), the PKC inhibitor Go 6983, epinephrine (EPI), and the protein kinase A (PKA) inhibitor H-89 were purchased from Calbiochem, EMD Chemicals (Gibbstown, NJ).

Cell culture

Primary murine peritoneal macrophages were obtained by peritoneal lavage from 6- to 8-week-old C57Bl/6d mouse (The Jackson Laboratory, Bar Harbor, ME), IFN-β−/− mice, and IFN-β−/− mice 4 d after i.p. injection with sterile thioglycollate as described previously (30). IFN-β−/− mice (back-crossed ≥ N8 onto a C57Bl/6 background) (31) were bred homozygously at the University of Maryland, Baltimore. IFN-β−/− mice (back-crossed ≥ N8 onto a C57Bl/6 background) were bred homozygously at the University of Massachusetts Medical School, and thioglycollate-elicted macrophages were kindly provided by Dr. Katherine Fitzgerald. Macrophages were cultured in RPMI 1640 supplemented with 2% FCS, 2 mM glutamine, penicillin, and streptomycin as described previously (30). Mouse embryonic fibroblasts (MEFs) from TBK-1−/− and TBK-1−/− mice were a gift of Dr. W.-C. Yeh (University of Toronto, Toronto, ON, Canada). RIG-1 and RIG-1−/− MEFs were the kind gift of Dr. S. Akira (32). Embryonic fibroblasts were cultured in DMEM (BioWhittaker) supplemented with 10% (v/v) FBS (HyClone Laboratories), glutamate (2 mM), penicillin (10,000 U/ml), and streptomycin (10,000 μg/ml) at 37°C in 5% CO2 in air, in 6-well plates. After overnight incubation, culture medium was replaced with fresh medium, and cells were stimulated with medium only, LPS (100 ng/ml), poly-IC (100 μg/ml), PSC (1 μg/ml), or CpG DNA (5 μg/ml) by transfection of poly-IC (10 μg/ml) or CpG DNA (1 μg/ml) poly-IC and transfected into the macrophages (32) or by infection with NDV or vesicular stomatitis virus (VSV) (multiplicity of infection [MOI] = 10) for the times indicated in the figures that accompany this article.

Western analysis

Macrophages were washed with PBS and then lysed in buffer [1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 10 mM Tris with protease inhibitor mixture (Roche) and 1 mM sodium vanadate] and boiled for 5 min with Laemmli lysis buffer for SDS-PAGE and subsequent Western analysis. Twenty micrograms of total protein in Laemmli buffer was boiled for 5 min, resolved by 10% SDS-PAGE in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) from Bio-Rad (Hercules, CA), and then electrotransferred onto Immobilon-P transfer membranes (Millipore, Bedford, MA) at 100 V for 1.5 h (4°C). After blocking for 1 h in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk, membranes were washed three times in TBS-T and probed for 20 h at 4°C with the respective Abs according to the manufacturer’s instructions. After washing in TBS-T, membranes were incubated with secondary HRP-conjugated, anti-rabbit IgG from Cell Signaling (1:2000 dilution) for 1 h at room temperature, washed three times in TBS-T, and bands were detected using ECL plus reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric signals from Western blots were quantified using the ImageJ program from the National Institutes of Health (http://rsbweb.nih.gov/ij/). Measurement of steady-state mRNA by quantitative real-time PCR

Total RNA was isolated by using TRIzol reagent from Invitrogen, as specified by the manufacturer’s instructions, and quantified by spectrophotometric analysis. The cDNA was prepared from 1 μg of total RNA using iScript reverse transcriptase (Bio-Rad) and both oligonucleotide-dT and random primer mix, as recommended by the manufacturer’s instructions. The resulting cDNA was quantified by real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the ABI Prism 7900HT cycler as described previously (33). Primers for detection of IL-33, IFN-β, IL-12 p40, ELAM, ISG-56, IP-10, and GAPDH mRNAs were designed using the Primer Express 2.0 program (Applied Biosystems). The IL-33 sense primer 5′-TGAGACTCCGTTC1GGGCTC-3′ and anti-sense primer 5′-CTTCTTACGTCTGGTGACGCGT-3′ were used for most of the experiments in this study.

Full-length IL-33 is encoded by two mRNA species, “A,” and “B,” which are transcribed from different promoters. To ascertain which mRNA species was induced by IL-33 in macrophages, we designed two specific forward primers that correspond to the two respective 5′ untranslated regions (5′UTRs), 5′-GGGCTC1ACTCGAGGAAAGTTA-3′ (AK075849.1) and 5′-CAGCTGCA-1GAAGGGAAGAAAT-3′ (AK136464.1), and one common reverse primer from the third exon 5′-CATTTAGGTCAGGCACACG-3′. PCR products were cloned into the TA cloning vector (pDrive) and further sequenced by the Biopolymer and Genomics Core Laboratory at the University of Maryland, Baltimore. The real-time PCR primers, “A” mRNA (AK075849.1) forward primer 5′-GGGCTC1ACTCGAGGAAAGTTA-3′, “B” mRNA (AK136464.1) forward primer 5′-CAGCTGCA-1GAAGGGAAGAAAT-3′, and the common reverse primer 5′-GGGACGGGGCTTGGCCT-3′ were used to amplify specific IL-33 transcripts produced in vitro and in vivo.

Flow cytometry analysis

To preclude the need for scraping of cells for flow cytometric studies, thioglycollate-elicted peritoneal macrophages from C57Bl/6d mouse were cultured on 6-well low-cluster and low-adhesion plates (Corning). Washes were carried out in centrifuge tubes, and the cells were replated and treated with medium alone, poly-IC transfection (10 μg/ml), or were infected with NDV (MOI = 10) for 14 h. Cells were harvested for analysis by gentle shaking, washed with PBS, and then fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were blocked and permeabilized for 30 min with PBS (1% BSA, 0.3% Triton X-100) at room temperature. IL-33 was detected using a goat polyclonal Ab (R&D Systems) directed against the protein, followed by secondary and tertiary staining with biotin-conjugated donkey anti-goat IgG and Cy3-conjugated streptavidin, respectively (Jackson ImmunoResearch Laboratories). Cells were washed in PBST and suspended in PBS for immediate analysis using a FACSCalibur.
Induction of IL-33 mRNA in mouse peritoneal macrophages by TLR and non-TLR agonists

Although IL-33 has been shown to be produced by macrophages (34), little is known about its transcriptional regulation by TLR and non-TLR stimuli. Therefore, we initially measured steady-state IL-33 mRNA levels in mouse primary macrophages after treatment with TLR2, TLR3, TLR4, and TLR9 agonists (e.g., P3C, poly-IC, LPS, and CpG DNA, respectively). As shown in Fig. 1A (left panel), stimulation by TLR3 and TLR4 agonists strongly upregulated IL-33 mRNA expression in a time-dependent manner; however, there was no IL-33 mRNA induced when macrophages were stimulated with TLR2 (P3C) or TLR9 (CpG DNA) ligands. The ability of the TLR agonists to induce IL-33 mRNA correlated with their ability to induce IFN-β (Fig. 1B, left panel). Thus, we hypothesized that non-TLR ligands that are strong inducers of IFN-β would also be strong inducers of IL-33 gene expression. Therefore, IL-33 mRNA was measured in macrophages treated with the anti-cancer drug DMXAA, which uses an unknown (UNK), non-TLR, non–RIG-I, and non-MDA5 sensor (35), transfected poly-IC, an MDA5 ligand (32), and NDV infection, a RIG-I ligand (32, 36), all of which are potent inducers of IFN-β (32, 35, 36). Fig. 1A (right panel) shows that IL-33 mRNA was potently induced by transfected poly-IC and NDV and, to a lesser extent, by DMXAA in primary macrophage cultures with kinetics similar to the active TLR agonists and that each of these agents also induced IFN-β mRNA (Fig. 1B, right panel). These findings were confirmed by measuring intracellular IL-33 protein in macrophages by FACS analysis after treatment with transfected poly-IC or NDV infection for 14 h (Fig. 1C). Thus, we found a strong correlation between agents that are known IFN-β inducers and the ability of these agents to induce IL-33.

We also examined the induction of IL-33 in vivo in the livers of mice after lethal challenge with LPS. IL-33 mRNA levels increased over 8 h in liver samples and then diminished, in contrast to TNF-α mRNA levels, which peaked after only 1 h (Fig. 1D). Thus, IL-33 mRNA is induced relatively late in response to LPS in vivo.

Induction of IL-33 mRNA in LPS-stimulated murine peritoneal macrophages is independent of IFN-β production

IFN-β is the primary type I IFN induced by TLR stimulation in macrophages and often functions in an autocrine/paracrine manner through the IFN-α/β receptor to activate transcription factors (e.g., STAT1, STAT2) that induce expression of IFN-β–dependent genes including MCP-5 and inducible NO synthase (37), as well as anti-inflammatory genes such as IL-10, IL-4 (38), and SOCS-1 (35). To determine if LPS-induced IL-33 was dependent on the production and autocrine action of IFN-β, macrophages from wild-type (WT) and IFN-β−/− mice were stimulated by LPS, and induction of IFN-β (control), endothelial leukocyte adhesion molecule [ELAM; IFN-β independent (39)], and IL-33 mRNA were compared (Fig. 2). We confirmed that IFN-β−/− macrophages did not produce IFN-β mRNA in response to LPS, in contrast to WT macrophages (Fig. 2, left panel). Expression of the gene that encodes ELAM is strongly regulated by transcription factor NF-κB (39) and was comparably induced by LPS in WT and IFN-β–deficient macrophages (Fig. 2, middle panel). Similar to ELAM mRNA, IL-33 mRNA was comparably induced by LPS in WT and IFN-β−/− macrophages (Fig. 2, right panel). These data indicate that induction of IL-33 mRNA by LPS is IFN-β independent.

TLR-induced and non-TLR-induced IL-33 mRNA is TBK-1–dependent in MEFs

Activated TIR domain-containing adaptor inducing IFN-β (TRIF), MDA5, and RIG-I recruit TBK-1, leading ultimately to activation of IRF-3, a key transcription factor that is required for induction of IFN-β and other IRF-3–dependent gene products, for example,
RANTES (32, 36, 40). Because the induction of IL-33 was IFN-β independent, but inducible by stimuli that share the capacity to induce IFN-β through activation of IRF-3, we next sought to determine if IL-33 induction was also dependent on TBK-1, the kinase that leads to IRF-3 activation. Because TBK-1−/− mice do not survive (35), we used TBK-1−/− MEFs for these experiments. Fig. 3A shows that in contrast to effects in WT MEFs, IL-33 mRNA failed to be induced in TBK-1−/− MEFs after stimulation with DMXAA (stimulates IRF-3–induced IFN-β through a UNK sensor), VSV infection (RIG-I), or transfected poly-IC (MDA5), whereas ELAM mRNA expression was induced by these three agonists in both in WT and TBK-1−/− MEFs (data not shown). IL-33 mRNA was also not induced in TBK-1−/− MEFs compared with WT MEFs after stimulation by LPS (data not shown). As an additional control, ISG-56 mRNA, an IFN-β–inducible gene, was also measured in these same samples. Induction of ISG-56 mRNA by these agonists (Fig. 3B) was also abrogated in TBK-1−/− MEFs compared with WT MEFs. Thus, IL-33 mRNA induction by these stimuli in MEFs is TBK-1 dependent.

NDV-induced IL-33 mRNA is RIG-I–dependent in MEFs

Previous studies have shown that NDV infection activates the RIG-I signaling pathway and uses IPS/MAVS, rather than TRIF, to activate TBK-1, IRF-3, and NF-kB (41). Because RIG-I−/− mice also fail to survive (35), we used WT and RIG-I−/− MEFs to determine whether induction of IL-33 mRNA is dependent upon RIG-I. Fig. 3C shows a significant, time-dependent increase in IL-33 mRNA levels after NDV infection of WT MEFs that was completely eliminated in RIG-I−/− MEFs. There was no difference in induction of IL-33 mRNA in RIG-I+/+ and RIG-I−/− MEFs when stimulated with DMXAA, transfected poly-IC, or LPS, ligands that do not activate RIG-I (35) (data not shown).

Induction of IL-33 is IRF-3–dependent in macrophages

Because the induction of IL-33 could not be attributed to the autocrine/paracrine action of IFN-β (Fig. 2) but was dependent on TBK-1 (Fig. 3), we next hypothesized that IL-33 induction was IRF-3 dependent. Therefore, IL-33 mRNA was measured in WT and IRF3−/− peritoneal macrophages that were stimulated with LPS, P3C, poly-IC, or transfected poly-IC, or infected with NDV. Fig. 4A shows that induction of IL-33 mRNA was significantly inhibited in IRF-3−/− macrophages compared with WT macrophages after stimulation with these TLR and non-TLR agonists that activate TBK-1 and induce IFN-β expression (Figs. 1, 3A). IL-33 mRNA failed to be induced in WT or IRF-3−/− macrophages after treatment with P3C (data not shown), consistent with the failure of this TLR2 agonist to activate the “MyD88-independent,” TRIF-dependent pathway (37, 42, 43). However, MyD88–dependent expression of TNF-α mRNA was comparably induced in P3C-treated WT or IRF-3−/− macrophages (data not shown).

Total cell lysates were extracted from WT and IRF-3−/− macrophages treated with LPS, P3C, transfected poly-IC, and NDV infection and were subjected to Western analysis for detection of p–IRF-3, total IRF-3, and β-actin, the latter being used as a protein loading control. These Western blots were subjected to scanning densitometry and quantified after normalization to the β-actin signal, and these data are presented below each Western blot. As seen in Fig. 4B and 4C, relative levels of total IRF-3 protein were comparable in WT macrophages regardless of treatment, and, as expected, IRF-3 protein was not detected in IRF-3−/− macrophages. p–IRF-3 was detected in WT macrophages after stimulation with LPS (Fig. 4B), transfected poly-IC, and NDV infection (Fig. 4C) for 3 h. NDV and transfected poly-IC were more potent activators of p–IRF-3 than LPS at this time point. No phosphorylation of IRF-3 was detected in samples treated with the TLR2 agonist P3C in WT or IRF-3−/− macrophages (Fig. 4B), consistent with the failure of this agonist to induce IL-33 or IFN-β mRNA (Fig. 1). Thus, TBK-1–dependent phosphorylation of IRF-3 is required for induction of IL-33 in macrophages.

We next measured phosphorylation of IRF-3 in macrophages after stimulation with TLR and non-TLR agonists for longer periods of time (i.e., 3 or 6 h). Fig. 4D confirms and extends the data in Fig. 4B that LPS-induced and poly-IC–induced phosphorylation of IRF-3 was transient and relatively weak compared with that induced by transfected poly-IC. LPS-induced phosphorylation of IRF-3 was detected at 3 h, but no longer at 6 h, whereas poly-IC–induced phosphorylation of IRF-3 was detectable at 3 h and continued to increase at 6 h. Robust phosphorylation of IRF-3 by transfected poly-IC was observed at both 3 and 6 h.

Role of cAMP–CREB pathway in the transcriptional regulation of IL-33

The CREBs are transcription factors that bind to DNA sequences called cAMP response elements (CREs) and thereby regulate ex-

**FIGURE 2.** IL-33 mRNA induction in macrophages is independent of IFN-β. IFN-β, ELAM, and IL-33 mRNAs were measured by quantitative real-time PCR at the indicated times after stimulation with LPS (100 ng/ml) in WT and IFN-β−/− macrophages. Data shown are representative of two separate experiments.

**FIGURE 3.** IL-33 mRNA induction in MEFs is dependent on TBK-1. (A) IL-33 and (B) ISG-56 mRNA were measured by quantitative real-time PCR at the indicated times after stimulation with DMXAA (100 μg/ml), VSV infection (10 MOI), and transfection of poly-IC (10 μg/ml) in WT and TBK1−/− MEFs. (C) IL-33 mRNA was measured by quantitative real-time PCR at the indicated times after NDV infection (MOI = 10) in WT and RIG-I−/− MEFs. Data shown are representative of two separate experiments. Tfp(I:C), Transfected poly-IC.
pression of certain genes. cAMP-activating drugs produce second messengers, such as cAMP or Ca²⁺, which in turn activate PKA, which translocates to the nucleus where it activates CREB (44, 45). In addition, CREB activation can be driven by TLR or non-TLR ligands that activate MAPKs pathways, involving ERK and p38 MAPK signaling (46). Activated CREB binds to a CRE and is then bound by CREB binding protein that coactivates it, leading to increased or decreased transcription (47). Fig. 4D shows that in addition to IRF-3 being phosphorylated, CREB phosphorylation was also upregulated by LPS, poly-IC, and transfected poly-IC; however, the relative degree of CREB activation induced by these agonists differed from their relative abilities to activate IRF-3: LPS-induced and transfected poly-IC–induced phosphorylation of CREB was only slightly greater than that induced by poly-IC. Stimulation of IRF-3⁻/⁻ macrophages with transfected poly-IC, the agonist shown in Fig. 4D to be the most potent of the noninfectious IRF-3–activating agents, resulted in reduced phosphorylation of CREB compared with that in WT macrophages (Fig. 4E), suggesting that IRF-3 may contribute partially to the activation of CREB by transfected poly-IC.

In Th1 cells, cAMP inhibits Th1 cytokine production, whereas cAMP markedly increases Th2 cytokine production in GATA3-expressing cells (48). cAMP also activates p38, which in turn activates GATA3, leading to Th2 cytokine production (49, 50). Because IL-33 has been associated with a Th2 bias, we hypothesized that cAMP might also be involved in regulation of IL-33 production. Therefore, we measured the effects of cAMP agonists on LPS-induced IL-33 mRNA in peritoneal macrophages. Fig. 5A illustrates that LPS-induced IL-33 mRNA was enhanced ~5-fold compared with that in untreated macrophages, but in the presence of...
PKA inhibitor H-89 (10 μM) significantly inhibited LPS-induced IL-33 mRNA expression. Both LPS- and poly-IC-induced IL-33 mRNA was not statistically different from the control (Fig. 5A). We confirmed that PP2 and RTKi were active at concentrations used in Fig. 6A and 6B by showing inhibition of tyrosine phosphorylation (p-Y) induced by LPS or LPS plus ACT by inclusion of either of the two tyrosine kinase inhibitors in the cultures, whereas neither the PKC nor PKA inhibitors had any effect on p-tyrosine induction (Fig. 6C; densitometric analyses of the two major p-tyrosine species, “pY-2” and “pY-1,” are presented to the right of each Western blot). As was observed at the level of IL-33 mRNA, there was no inhibition of p-CREB in the presence of tyrosine kinase inhibitors (PP2 or RTKi), the PKC inhibitor Go 6983, and the PKA inhibitor H-89 (as a control). LPS-induced IL-33 mRNA was not statistically different from the control with any of these inhibitors (Fig. 6D; densitometric analysis is below the blot). Finally, there was no significant inhibition of LPS-induced p–IRF-3 in presence of any of these inhibitors (Fig. 6E; densitometric analysis is below the blot).

To determine if tyrosine kinases or PKC were involved in signaling leading to IL-33 mRNA expression, we next compared induction of IL-33 mRNA by LPS alone (Fig. 6A) or LPS plus ACT (Fig. 6B) in the presence of tyrosine kinase inhibitors (PP2 or RTKi), the PKC inhibitor Go 6983, and the PKA inhibitor H-89 (as a control). LPS-induced IL-33 mRNA was not statistically different from the control (Fig. 6A) or LPS plus ACT by inclusion of either of the two tyrosine kinase inhibitors in the cultures, whereas neither the PKC nor PKA inhibitors had any effect on p-tyrosine induction (Fig. 6C; densitometric analyses of the two major p-tyrosine species, “pY-2” and “pY-1,” are presented to the right of each Western blot). As was observed at the level of IL-33 mRNA, there was no inhibition of p-CREB in the presence of tyrosine kinase inhibitors (PP2 or RTKi), the PKC inhibitor Go 6983, and the PKA inhibitor H-89 (as a control). LPS-induced IL-33 mRNA was not statistically different from the control with any of these inhibitors (Fig. 6D; densitometric analysis is below the blot). Finally, there was no significant inhibition of LPS-induced p–IRF-3 in presence of any of these inhibitors (Fig. 6E; densitometric analysis is below the blot).

A similar trend was observed when macrophages were treated with LPS and poly-IC, but not P3C, in the absence or presence of EPI (Fig. 5B). These data support the possible involvement of the PKA–CREB pathway in IL-33 mRNA induced by LPS and other IL-33–inducing agonists in macrophages.

In contrast to the synergy observed between LPS and either cAMP-activating agent at the level of IL-33 mRNA expression, LPS-induced CREB phosphorylation was only slightly enhanced by the presence of ACT. Nonetheless, H-89 also inhibited phosphorylation of CREB induced by LPS alone, ACT alone, or both (Fig. 5C), as indicated by decreased signal in the blots and in the corresponding normalized densitometric scan. Consistent with a role for PKA in the induction of IL-33 by LPS, H-8, a PKA inhibitor that is ∼10-fold less potent than H-89 (51-53), inhibited LPS-, ACT-, and LPS/ACT-induced p-CREB much less than H-89 at the same concentration (data not shown).

To determine if tyrosine kinases or PKC were involved in signaling leading to IL-33 mRNA expression, we next compared induction of IL-33 mRNA by LPS alone (Fig. 6A) or LPS plus ACT (Fig. 6B) in the presence of tyrosine kinase inhibitors (PP2 or RTKi), the PKC inhibitor Go 6983, and the PKA inhibitor H-89 (as a control). LPS-induced IL-33 mRNA was not statistically different from the control (Fig. 6A) or LPS plus ACT by inclusion of either of the two tyrosine kinase inhibitors in the cultures, whereas neither the PKC nor PKA inhibitors had any effect on p-tyrosine induction (Fig. 6C; densitometric analyses of the two major p-tyrosine species, “pY-2” and “pY-1,” are presented to the right of each Western blot). As was observed at the level of IL-33 mRNA, there was no inhibition of p-CREB in the presence of tyrosine kinase inhibitors (PP2 or RTKi), the PKC inhibitor Go 6983, and the PKA inhibitor H-89 (as a control). LPS-induced IL-33 mRNA was not statistically different from the control with any of these inhibitors (Fig. 6D; densitometric analysis is below the blot). Finally, there was no significant inhibition of LPS-induced p–IRF-3 in presence of any of these inhibitors (Fig. 6E; densitometric analysis is below the blot).

**Mouse gene IL-33 organization and induction of specific IL-33 transcripts**

Originally, Baekkevold et al. (1) published that the mouse IL-33 genomic structure was composed of seven exons. The major difference between human and mouse IL-33 genes was the size of the first intron, which was estimated to be 9 kb in the human IL-33 gene but only 2 kb in mouse ortholog. However, recent advances in sequencing analysis and aceView (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=mouse&term=IL-33&submit=Go) showed that the first exon identified in the study by Baekkevold et al. (1) is actually exon 2. The IL-33 gene is localized on mouse chromosome 19, and the sequence of the mouse IL-33 gene has been defined by 188 gene bank accessions from 185 cDNA clones from different mouse tissues. The IL-33 gene contains 11 different GT AG introns and is transcribed into seven different mRNA species, with five alternatively spliced variants and two unspliced forms. Thus, there are three possible alternative promoters and four validated altered polyadenylation sites. Two of these mRNAs, transcripts “A” and “B” (Fig. 7A), are derived from two alternative promoters and are both predicted to encode a full-length IL-33 protein encoded by eight exons. The other splice variants are not predicted to result in a full-length protein and were therefore not studied further.

**FIGURE 5.** Evidence for the cAMP–CREB pathway in regulation of IL-33 mRNA in peritoneal macrophages. (A) IL-33 mRNA was measured by real-time PCR at the indicated times after stimulation with LPS (100 ng/ml), without or with cAMP-activating drugs EPI (10 μM) or ACT (20 nM), in the absence or presence of the PKA inhibitor H-89 (10 μM). Data shown are the average ± SEM of three separate experiments. *p ≤ 0.05, ***p ≤ 0.001. (B) IL-33 mRNA was measured as described in (A) after stimulation of macrophages with LPS (100 ng/ml), P3C (1 μg/ml), or poly-IC (100 μg/ml), without or with cAMP-activating drug EPI (10 μM), in the absence or presence of the PKA inhibitor H-89 (10 μM). Data shown are the average ± SEM of three separate experiments. *p ≤ 0.05. (C) Whole-cell lysates were prepared after stimulation of macrophages for 2 h with LPS (100 ng/ml), without or with ACT (20 nM), in the absence or presence of the PKA inhibitor H-89 (10 μM). Whole-cell lysates were analyzed by Western blot using anti-p-CREB and anti–β-actin Abs. β-Actin was used as loading control. The panel below the Western blot represents the densitometry data quantified after normalization to β-actin. Data shown are representative of two separate experiments. M, Medium (unstimulated control); p(I:C), poly-IC.
The shorter transcript, “A,” is 14,915 bp (AK075849.1), and the longer transcript, “B,” is 35,609 bp (AK163464.1). Transcripts “A” and “B” are identical with the exception of the 5’UTR. We designed two specific forward primers that correspond to the two respective 5’UTRs, 5’-GGGGCTCACTGCAGGAAAGTA-3’ (AK075849.1) and 5’-CAGCTGCAGAAGGGAGAAAT-3’ (AK163464.1), and

**FIGURE 6.** Role of receptor tyrosine kinase, PKC, and PKA in the induction of IL-33 mRNA in macrophages. (A) IL-33 mRNA was measured by quantitative real-time PCR after stimulation with LPS (100 ng/ml), in the absence or presence of receptor tyrosine kinase inhibitors PP2 (2.5 μM) or RTKi (1 μM), the PKC inhibitor Go 6983 (5 μM), or the PKA inhibitor H-89 (10 μM). Data shown are the average ± SEM of three separate experiments (*p ≤ 0.05). (B) IL-33 mRNA was measured by quantitative real-time PCR after stimulation with LPS (100 ng/ml) plus ACT (20 nM), in the absence or presence of receptor tyrosine kinase inhibitors PP2 (2.5 μM) or RTKi (1 μM), the PKC inhibitor Go 6983 (5 μM), or the PKA inhibitor H-89 (10 μM). Data shown are the average ± SEM of three separate experiments (**p ≤ 0.01). (C) Whole-cell lysates were prepared after stimulation of macrophages after stimulation with LPS (100 ng/ml), without or with ACT (20 nM), in the absence or presence of receptor tyrosine kinase inhibitors PP2 (2.5 μM) or RTKi (1 μM), the PKC inhibitor Go 6983 (5 μM), or the PKA inhibitor H-89 (10 μM) for 1 h. The whole-cell lysates were analyzed by Western blot using anti-p-tyrosine (pY) Ab. The panels to the right represent the densitometry data quantified after normalization of the two major bands, pY-2 and pY-1, to β-actin. Data shown are the average ± SEM of three separate experiments. *p ≤ 0.05, **p ≤ 0.01. (D) The same gel shown in (C) was reprobed with anti-p-CREB Ab and (E) anti-p–IRF-3 Ab. The panels below (D) and (E) represent the densitometry data quantified after normalization to β-actin. Data shown are the average ± SEM of three separate experiments (*p ≤ 0.05). M, Medium (unstimulated control).
one common reverse primer from the third exon (5'-CTTATGGTGAGGCCAGAACG-3') to identify these two mRNA species in murine macrophages. These two sets of primers amplified two predominant (i.e., each primer set amplified a single PCR product) PCR products from LPS-stimulated macrophage cDNA. These two PCR products were gel purified and cloned into the TA cloning vector (pDrive). Sequence analyses confirmed that both “A” and “B” mRNA was measured by using the primers derived from sequences in exon 1 and exon 2. (B) IL-33 mRNA (total IL-33, “A” mRNA and “B” mRNA) was measured by real-time PCR at the indicated times after stimulation with LPS (100 ng/ml), P3C (1 µg/ml), poly-IC (100 µg/ml), transfection of poly-IC (10 µg/ml), or NDV infection (MOI = 10) of peritoneal macrophages. (C) IL-33 mRNA (total IL-33, “A” mRNA, and “B” mRNA) was measured by real-time PCR in liver samples, at the indicated times, after lethal injection of LPS (35 mg/kg body weight). (D) Total IL-33 and “A” mRNA was measured by quantitative real-time PCR at the indicated times after stimulation with LPS (100 ng/ml), without or with ACT (20 nM), in absence or presence of the PKA inhibitor H-89 (20 µM). Data shown are the average ± SEM of three separate experiments. *p ≤ 0.05. M, Medium (unstimulated control); p(I:C), poly-IC; Tfp(I:C), transfected poly-IC.
Discussion

Relatively little is known about the molecular regulation of IL-33 gene expression. To approach this problem, we studied the expression of IL-33 mRNA in murine macrophages and fibroblasts after stimulation with a diverse panel of TLR and non-TLR ligands. Although these various stimuli elicit signaling through distinct membrane-associated, endosome-associated, or cytosolic receptors, our data suggest that a common pathway for IL-33 gene expression is the activation of TBK-1, which leads to activation of the transcription factor IRF-3 (Figs. 3A, 4A). IRF-3 is a key transcriptional activator and is central to the transcriptional up-regulation of IFN-β and other genes (54). Once produced, IFN-β acts in an autocrine/paracrine fashion to activate the IFN-α/β receptor, leading to transcriptional induction of >300 IFN-stimulated genes (55). Although induction of IL-33 in macrophages was entirely IRF-3 dependent (Fig. 4A), the failure to observe any difference in LPS-induced IL-33 mRNA in LPS-stimulated WT and IFN-β−/− macrophages indicates that induction of IL-33 is not IFN-β dependent. This suggests the possibility that activated IRF-3 might bind directly to regulatory regions within the IL-33 gene to drive IL-33 transcription.

Given the multiple reports of the association of IL-33 with asthma, we explored the possibility that other transcription factors were associated with IL-33 gene expression. NF-κB, AP-1, NF-AT, CREB, and STATs have been strongly implicated in genes expressed in allergic asthma (56). Phosphorylated CREB was significantly increased in patients with bronchial asthma, and its induction correlated with the inflammatory status (57). Augmentation of p-CREB was suppressed upon inhaled glucocorticoid treatment in an experimental model of asthma (57). Both TLR and non-TLR ligands have been reported to activate second signals that ultimately activate PKA and its subsequent translocation to the nucleus where it activates CREB (44, 45). Therefore, the effects of cAMP-activating agents and the PKA inhibitor H-89 were tested for their effects on inducible IL-33 mRNA expression. Both EPI and ACT, known cAMP agonists (58, 59), had little effect on IL-33 mRNA levels alone, but synergistically increased levels of LPS-induced IL-33 mRNA. Both LPS-induced and LPS plus EPI/ACT-induced IL-33 mRNA were sensitive to pharmacologic inhibition of PKA, as was poly-IC and poly-IC plus EPI-induced IL-33 mRNA (Fig. 5A, 5B). These data support the role of the transcription factor CREB in the induction of IL-33 mRNA. It is interesting to note that in IRF-3−/− macrophages, CREB phosphorylation induced by transfected poly-IC was diminished (Fig. 7C), suggesting yet another mechanism by which IRF-3 may regulate IL-33 gene expression.

Transcription of IL-33 is complex, with seven different mRNA species, five alternatively spliced variants, and two unspliced forms (http://www.ncbi.nlm.nih.gov/IEB/Research/Assembly/av.cgi?db=mouse&term=IL-33&submit=Go). Only two transcripts, “A” and “B” (Fig. 7A), encode the full-length IL-33 protein. Although our initial cloning experiments revealed that both “A” and “B” mRNA variants are produced in stimulated primary murine macrophages, all of the stimuli tested, except for NDV, resulted in preferential induction of the shorter IL-33 “A” mRNA variant (Fig. 7B). In contrast, NDV infection induced a mixture of both “A” and “B” transcripts. These data confirm and significantly extend a very recent report by Talabot-Ayer et al. (60), who also found expression of IL-33 gene from two alternative promoters (i.e., “IL33a” and “IL33b”) mRNA, designated in our study as “B” and “A,” respectively, consistent with the nomenclature used by the database http://www.ncbi.nlm.nih.gov/IEB/Research/Assembly/av.cgi?db=mouse&q=IL33), whose expression in the mouse were organ-specific and inducible by LPS and poly-IC. In this report, we have significantly extended their findings by showing that additional TLR and non-TLR agonists that share the ability to induce TBK-1 and IRF-3 by distinct pathways elicit the shorter “A” mRNA species preferentially in primary murine macrophages, with the exception of NDV, which induces both “A” and “B” transcripts. In addition, we provide evidence that this preferential induction of the “A” mRNA species is reflected in vivo as well as in liver samples after administration of a lethal dose of LPS to mice. Finally, our data indicate that the cAMP-activating drug ACT enhanced LPS-induced “A” mRNA (Fig. 7D), but not the “B” mRNA (data not shown), and that this induction was PKA dependent. The significance of these two mRNA species and of preferential induction of the “A” species by all agonists but NDV (RIG-I) will await a complete elucidation of regulatory elements in the promoter and possible intrinsic regulatory sequences in this gene.

Thymic stromal lymphopoietin (TSLP) is produced by epithelial cells in response to certain microbial products (e.g., peptidoglycan, lipoteichoic acid, and dsRNA), injury, or inflammatory cytokines (e.g., IL-1β and TNF-α) (61). TSLP is detected in the airways of asthmatic patients, and the level of TSLP mRNA was shown to be proportional to the severity of the disease (62). Both IL-33 and TSLP activate Th2 cells to produce Th2 cytokines (63, 64). These findings suggest the possibility that viral infection and recruitment of Th2 cytokine-producing cells may amplify Th2 inflammation through the production of IL-33 and TSLP (65) in asthmatic airways. Viral infection of epithelial cells ultimately results in tissue damage and, eventually, release of full-length IL-33 from necrotic cells (66). In chronic asthma, continual inflammation also results in tissue disruption and release of IL-33 (64). This released IL-33 acts on different cells to produce Th2 cytokines as well as proinflammatory cytokines to increase severity of asthma (64). In Th1 cells, cAMP inhibits Th1 cytokine production, whereas cAMP markedly increases Th2 cytokine production in GATA3-expressing cells (48). cAMP also activates p38, which in turn activates GATA3, leading to Th2 cytokine production (49, 50). Because IL-33 has been associated with a Th2 bias, increased cAMP may be involved in regulation of IL-33 production. These observations are strengthened by our findings showing that cAMP agonists significantly enhanced LPS-induced IL-33 mRNA in macrophages through a PKA-dependent process.

Lastly, IL-33 has also been shown to be chromatin-associated in the nuclei of endothelial cells and has the capacity to regulate transcription (27). IL-33 binds to chromatin in the surface of the nucleosome by docking to the pockets of the histone H2A–H2B dimer (28). Therefore, IL-33 is a dual-function protein, in that it acts as both a cytokine and an intracellular nuclear protein. IL-33 may be an important cytokine involved in initiation and perpetuation of inflammation in the case of asthma (64). Depending on the tissue type, IL-33 may contribute to the resolution of inflammation associated with artherosclerosis and cardiac function (21, 67), expulsion of the intestinal-dwelling nematode, or infection-induced tissue damage (66, 68). Thus, depending on the immune mechanism underlying the pathogenesis of each disease condition, IL-33 has the potential as a therapeutic target in various diseases.

Acknowledgments

We thank Drs. Leah Cole and Vladimir Toshchakov for help and advice during these studies.

Disclosures

The authors have no financial conflicts of interest.
References


