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Brief Conclusive Report

The mouse *interleukin (Il)33* gene is expressed in a cell type- and stimulus-dependent manner from two alternative promoters

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RECEIVED AUGUST 22, 2011; REVISED SEPTEMBER 14, 2011; ACCEPTED SEPTEMBER 28, 2011. DOI: 10.1189/jlb.0811425

ABSTRACT

GenBank entries for mouse *Il33* reveal the existence of two transcripts, *Il33a* and *Il33b*, with different 5′UTRs but coding for the same protein. We investigated expression of these transcripts in different mouse organs and cell types in basal and inflammatory conditions. *Il33a* and *Il33b* mRNAs start with different noncoding first exons, transcribed from different promoter regions, which both contain a consensus TATA-like sequence. Constitutive *Il33a* mRNA expression was detected in mouse stomach, lung, spleen, and brain, whereas basal *Il33b* mRNA expression was observed only in the stomach. Expression of both transcripts increased after systemic LPS administration. In vitro, we observed high constitutive expression of *Il33* transcripts in MEFs. Constitutive *Il33a* mRNA expression was observed also in BMDCs, where it was preferentially increased in response to poly(I:C), whereas LPS increased levels of *Il33a* and *Il33b* mRNA. In contrast, BMMs and Raw 264.7 cells did not express *Il33* mRNA constitutively, and LPS stimulation selectively induced expression of *Il33b* mRNA in these cells. Our data indicate that the *Il33* gene is expressed from two alternative promoters in the mouse and that the relative expression of *Il33a* and *Il33b* transcripts is cell type- and stimulus-dependent. *J. Leukoc. Biol.* 90: 119–125; 2012.

Introduction

IL-33 is the most recently described cytokine of the IL-1 family (see ref. [1] for review). IL-33, like IL-1α, is a dual-function protein, displaying nuclear and extracellular effects [2, 3]. The latter is mediated by its binding to an IL-1R family member called ST2. Binding of IL-33 to ST2 elicits activation of classical IL-1-like signaling, including activation of NF-κB and MAPK pathways, and induces production of various cytokines and chemokines, cell activation, or differentiation. Consistent with expression of ST2 on different cell types associated with Th2 immunity, IL-33 injection induces or amplifies Th2-type responses in various mouse models [2, 4–7]. In addition, IL-33 displays proinflammatory effects in pathologies, which are independent of Th2 immunity [8–10].

In human tissues, IL-33 is constitutively expressed in endothelial cells and in some epithelial cells [3, 11]. In addition, IL-33 expression is induced in resident and infiltrated inflammatory cells in inflamed tissues [9, 12–16]. Consistently, IL-33 expression is detected in cultured human endothelial end epithelial cells [17, 18], and several studies indicate that IL-33 expression can be increased in vitro in different epithelial and mesenchymal cell types by proinflammatory stimuli [9, 12, 19–24]. Regulated expression of IL-33 has also been reported in cultured mouse and human myeloid cells, such as monocytes/macrophages or DCs [25–30]. In these cells, no or low constitutive levels of IL-33 mRNA or protein are generally reported, and IL-33 expression is enhanced upon stimulation with TLR ligands.

Little information is published to date concerning the regulation of *IL33* gene transcription. GenBank entries for mouse *Il33* reveal the existence of two transcripts with different 5′UTRs but coding for the same protein (GenBank ID: NM_133775.2 and NM_001164724.1). In this study, we investigated expression of these two *Il33* transcripts in different mouse organs and cell types in basal and inflammatory conditions, and we describe expression of mouse *Il33* mRNA from two alternative promoters, which are used in a cell type- and stimulus-dependent manner.

Abbreviations: BMDC=bone marrow-derived DC, BMM=bone marrow-derived macrophage, Ct=comparative threshold, MEF=mouse embryonic fibroblast, MLEC=mouse lung endothelial cell, MyEnd=myocardial endothelial cell, poly(I:C)=polyinosinic:polycytidylic acid, qPCR=quantitative PCR, UTR=untranslated region

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MATERIALS AND METHODS

Material

Media used for cell culture were obtained from Invitrogen Life Technologies (Basel, Switzerland). Purified LPS (*Escherichia coli* 055:B5) was purchased from Fluka (Buchs, Switzerland), poly(I:C) from GE Healthcare Europe GmbH (Glattbrugg, Switzerland), and resiquimod (R848) from InvivoGen (San Diego, CA, USA). Radiolabeled nucleotides were purchased from Hartmann Analytic GmbH (Braunschweig, Germany).

Mice

WT C57BL/6J mice were obtained from Janvier (Le Genest-St-Isle, France) and maintained under conventional conditions. Mice were injected i.p. with NaCl (control) or LPS (2 mg/kg) and killed 4 h after injection. Total RNA was extracted from various organs with Trizol (Invitrogen AG, Basel, Switzerland). Animal studies were approved by the Animal Experimentation Ethics Committee and the Geneva Veterinarian Office and performed according to the appropriate codes of practice.

Cell culture

MEFs were prepared as described previously [31] and cultured in DMEM GlutaMAX medium (4.5 g/L glucose), supplemented with 10% FCS, nonessential amino acids, 5×10^{-5} β-ME, 50 units/ml penicillin, and 50 μg/ml streptomycin. MyEnds and primary MLECs were cultured as described [32, 33]. Primary mouse BMDCs and BMMs were differentiated as described previously [34]. BMDC (>75% $CD11c^+$ $CD11b^+$) were used on Days 8–10. BMMs (>70% $CD11b^+$) were used on Day 6–8. Raw 264.7 cells were cultured in RPMI-1640 GlutaMAX-I medium, supplemented with 10% FCS, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells ($0.5–1\times10^6$ cells/ml) were cultured with or without TLR agonists at indicated concentrations and times. Total RNA was extracted with Trizol or using the RNeasy Mini kit (Qiagen, Valencia, CA, USA).

RT-qPCR

Total RNA (0.5–1 μg), treated with RQ1 DNase (Promega, Madison, WI, USA), in the case of Trizol-extracted samples, was reverse-transcribed using SuperScript II RT (Invitrogen Life Technologies). Total *Il33*, *Il33a*, and *Il33b* mRNA levels were assessed by RT-qPCR using appropriate primers (**Table 1**, and see **Fig. 1A**) and a SYBR Green Master Mix (Eurogentec, Seraing, Belgium) in the StepOne real-time PCR system (Life Technologies, Carlsbad, CA, USA). RNA expression levels were calculated using the Ct method ($2^{-\Delta Ct}$) for relative quantification by normalization to *Gapdh* gene expression.

5′RACE, RPA, and cloning of luciferase reporter plasmids

Extended methods for these experiments are available online.

Cell transfection and luciferase assay

Raw 264.7 cells were transfected with 1 μg firefly luciferase reporter gene plasmid using the Amaxa Cell Line Nucleofector Kit V (Lonza Verviers S.p.r.l., Verviers, Belgium), according to the manufacturer's optimized protocol. Negative controls were performed by transfecting Raw 264.7 cells with 1 μg empty pGL3 enhancer vector. Transfected cells were seeded into duplicate wells and were left unstimulated or stimulated, 24 h after transfection with 1 μg/ml LPS, and harvested 24 h later in passive lysis buffer (Promega). Firefly luciferase activity was assayed on 20 μl cell lysate using luciferase reporter assay reagents from Promega.

Statistical analysis

Significance of differences was evaluated by unpaired Student's *t* test. A difference between experimental groups was considered significant when the *P* value was <0.05.

RESULTS AND DISCUSSION

Mouse *Il33* gene structure and transcription start sites

GenBank entries for mouse *Il33* reveal the existence of two transcripts, which we call *Il33a* (GenBank ID: NM_133775.2) and *Il33b* (NM_001164724.1). These transcripts contain different 5′UTRs but code for the same protein. Sequence analysis indicates that *Il33a* and *Il33b* mRNAs start with two different noncoding first exons, distant by ~20 kb, which are used alternatively and joined to the first coding exon by alternative splicing (Fig. 1A). We examined the localization of transcription start sites for *Il33a* and *Il33b* by 5′RACE and RPA on lung, spleen, and brain (*Il33a*) or lung, spleen, and stomach (*Il33b*) RNA from control and LPS (50 μg/mouse i.p.; 4 h)-injected mice. Using these two independent approaches, we confirmed the presence of major transcription start sites at bp 29,999,604 of mouse chromosome 19 (NC_000085.5) for *Il33a* and at bp 30,020,280 of mouse chromosome 19 for *Il33b*, indicating that the *Il33a* and *Il33b* mRNA sequences deposited in GenBank

TABLE 1. Primer Information

Experiment	Target	GenBank	Primer	Primer sequence	Anneal	Size
RT-qPCR	*Il-33* cDNA	NM_133775.2	total fwd	5′-ggtgtggatgggaagaagctg-3′	60°C	155 bp
			total rev	5′-gaggacttttgtgaaggacg-3′		
	Il-33a cDNA	NM_133775.2	1a fwd	5′-gctgcagaagggagaaatcacg-3′	60°C	77 bp
			common rev	5′-ggagttggaatacttcattctaggtctcat-3′		
	Il-33b cDNA	NM_001164724.1	1b fwd	5′-ggctcactgcaggaaagtacagca-3′	60°C	97 bp
			common rev	5′-ggagttggaatacttcattctaggtctcat-3′		
	Gapdh cDNA	NM_008084.2	Gapdh fwd	5′-acggccgcatcttcttgtgca-3′	60°C	109 bp
			Gapdh rev	5′-aatggcagccctggtgacca-3′		
5′ RACE	*Il-33* cDNA	NM_133775.2	GSP1	5′-gaggacttttgtgaaggacg-3′		
			GSP2	5′-gaccagggcttcgcctgcggtgctgctgaa-3′		
Il-33b RPA	*Il-33* genomic	NC_000085.5	RPA1	5′-gttaccaaagttgtttaatctgag-3′	55°C	119 bp
			RPA2	5′-gacaggaaatagctggtcttgaatg-3′		
	Il-33b cDNA	NM_001164724.1	RPA3	5′-ggctcactgcaggaaagtacagca-3′	55°C	145 bp
			RPA4	5′-gaccagggcttcgcctgcggtgctgctgaa-3′		

fwd, Forward; rev, reverse; GSP1/2, gene-specific primer 1/2.

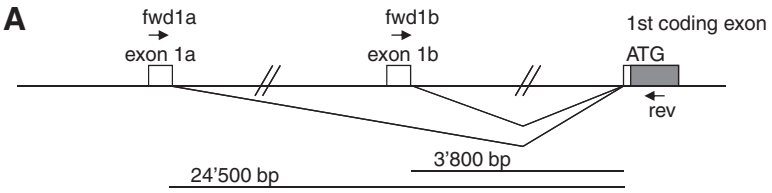

Figure 1. (A) Genomic organization of the mouse *Il33* gene 5′ end. Open boxes indicate 5′UTR sequences; shaded box indicates coding sequence. The noncoding exons 1a and 1b are used alternatively and joined to the first coding exon to generate transcripts *Il33a* or *Il33b*. Location of specific forward (fwd1a and fwd1b) and common reverse (rev) primers used for quantification of *Il33a* and *Il33b* mRNA expression levels by RT-qPCR is indicated. (B) Genomic sequences surrounding the major transcription start sites (bold), as mapped by 5′RACE and RPA, for *Il33a* (upper) and *Il33b* (lower). For each transcript, transcription start sites are located 29 nucleotides downstream of a consensus TATA-like sequence (underlined).

(NM_133775.2; NM_001164724.1) indeed contain the complete 5′ ends of these transcripts. Consistently, a consensus TATAA-like sequence is found 29 bp upstream of each of these transcription start sites (Fig. 1B), suggesting that *Il33a* and *Il33b* are transcribed from classical TATA box-containing promoters.

Differential expression of *Il33a* and *Il33b* mRNA in mouse organs in vivo

To evaluate expression of the two *Il33* transcripts under steady-state and inflammatory conditions in vivo, we assessed the relative abundance of *Il33a* and *Il33b* mRNA in various

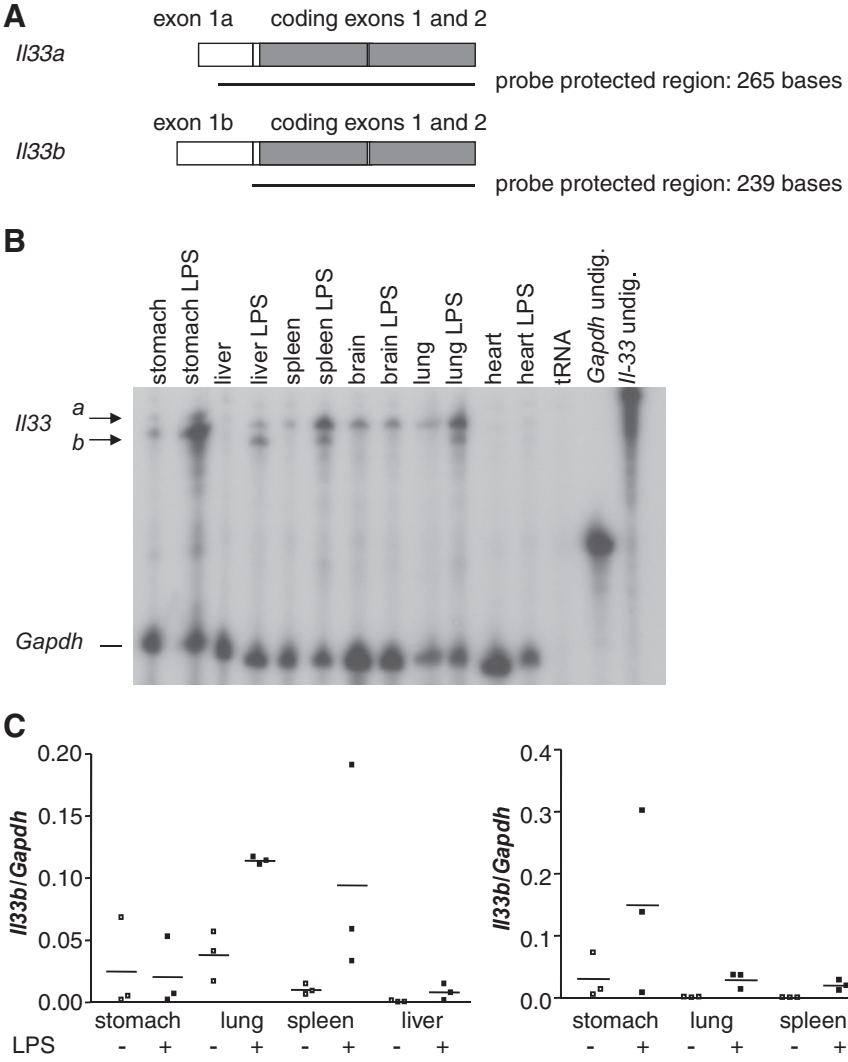

Figure 2. (A) Schematic representation of the three first exons for *Il33a* and *Il33b*. Open boxes indicate 5′UTR sequences; shaded boxes indicate coding sequence. The thick line indicates the location of the probe used for *Il33* mRNA quantification by RPA. (B) Mouse *Il33* and *Gapdh* mRNA expression was quantified by RPA. Mice were injected i.p. with NaCl or with LPS (2 mg/kg), 4 h before various tissues were harvested for RNA extraction. Sample identity is indicated above the corresponding lanes (tRNA, negative control; undig., undigested probe). Location of probe-protected bands for *Il33a*, *Il33b*, and *Gapdh* mRNA is indicated on the left. (C) Mice (n=3/group) were injected with NaCl (–) or LPS (2 mg/kg; +), 4 h before tissues were harvested for RNA extraction. *Il33a* (left panel) and *Il33b* (right panel) mRNA expression was examined by RT-qPCR and quantified relative to *Gapdh* mRNA expression. Results are shown as individual values (■) for each mouse and means (horizontal lines).

tissues of control and LPS-injected mice by RPA (**Fig. 2A** and **B**) and RT-qPCR (Fig. 2C; see Fig. 1A for qPCR primer location). The primers and conditions used for relative quantification of *Il33a* and *Il33b* mRNA expression by RT-qPCR were validated by comparison with the results obtained by the RPA, where quantification of both transcripts is based on hybridization with the same probe (Fig. 2B and C, and data not shown). We observed constitutive expression of *Il33a* mRNA in mouse stomach, lung, spleen, and brain but not in liver or heart (Fig. 2B and C). Expression of *Il33a* mRNA was increased in lung and spleen and to a lesser extent, in liver and stomach, 4 h after systemic administration of LPS (2 mg/kg). Constitutive expression of *Il33b* mRNA was observed in the stomach, where it was enhanced further by LPS. In lung, spleen, and liver, *Il33b* mRNA was only detected after LPS injection.

Il33a and *Il33b* mRNA expression in cultured cells in vitro

We next screened for expression of the two *Il33* variants in primary mesenchymal cells and cell lines. We observed remarkably high constitutive expression of *Il33* transcripts in primary MEFs (total *Il33/Gapdh*: 0.26; *Il33a/Gapdh*: 0.32; *Il33b/Gapdh*: 0.049 arbitrary units). Immortalized MyEnds (total *Il33/Gapdh*: 0.11; *Il33a/Gapdh*: 0.075; *Il33b/Gapdh*: 0.053) and to a lesser extent, MLECs (total *Il33/Gapdh*: 1.2×10^{-3}; *Il33a/Gapdh*: 1.2×10^{-3}; *Il33b/Gapdh*: 2.2×10^{-4}) also expressed *Il33* mRNA constitutively. In addition, as investigated in more detail below, we observed regulated expression of *Il33a* and *Il33b* mRNA in myeloid cells.

Il33a and *Il33b* mRNA expression in DCs

BMDCs constitutively expressed low levels of *Il33a* mRNA, which was selectively, more vigorously increased over *Il33b* mRNA upon stimulation with the TLR3 agonist poly(I:C) (**Fig. 3A**). The increase in *Il33* mRNA expression in response to poly(I:C) was progressive, maximal after 24 h stimulation, and decreased thereafter. Interestingly, induction of *Il33* mRNA expression by the TLR4 agonist LPS followed a different pattern in BMDCs, as LPS increased levels of *Il33a* and *Il33b* transcripts, and the response was more rapid and transient, with maximal induction detectable after 6 h stimulation (Fig. 3B). Finally, the TLR7/8 agonist R848 increased IL-6 production in BMDC cultures when used at 10 or 100 ng/ml but only very marginally enhanced *Il33* mRNA expression (data not shown).

Il33a and *Il33b* mRNA expression in macrophages

In contrast to BMDCs, BMMs did not express *Il33* mRNA constitutively. Interestingly, stimulation with LPS selectively induced a transient expression of transcript *Il33b* in these cells, with a maximal induction after 6 h (**Fig. 4A**). poly(I:C) (50 μg/ml) and R848 (1 μg/ml), used here at concentrations that optimally induced IL-6 production in the same culture conditions, were not efficient in inducing *Il33* expression in BMM (data not shown). Similarly to primary BMM, Raw 264.7 macrophage-like cells did not express *Il33* mRNA constitutively,

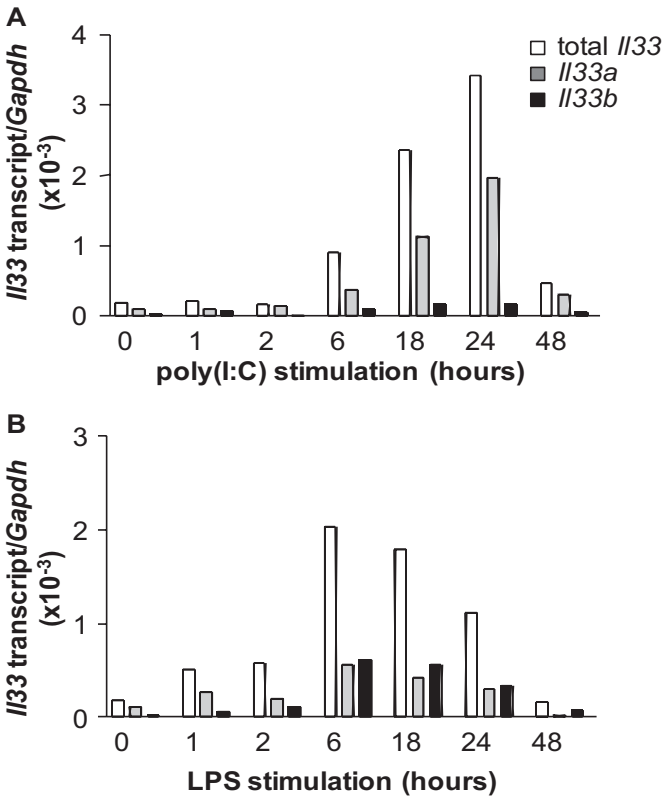

Figure 3. Expression levels of total *Il33* (open columns), *Il33a* (shaded columns), and *Il33b* (solid columns) mRNA, normalized to *Gapdh* mRNA levels are shown for mouse primary BMDCs, stimulated or not for indicated times with poly(I:C) (50 μg/ml) (A) or LPS (100 ng/ml) (B). Data shown are representative of three independent experiments.

and LPS stimulation selectively induced expression of transcript *Il33b* (Fig. 4B), with maximal expression at 6 h. R848 (100 ng/ml) also induced selective induction of *Il33b* in Raw 267.4 cells but at low expression levels and with slower kinetics as compared with LPS. Maximal induction was observed after 24–48 h [R848 (100 ng/ml), 24 h: total *Il33/Gapdh*, 7.6×10^{-5}; *Il33a/Gapdh*, 2.3×10^{-7}; *Il33b/Gapdh*, 8.2×10^{-5}]. poly(I:C) (50 μg/ml) was not efficient in inducing *Il33* expression in Raw 264.7 cells (data not shown).

Finally, consistently with selective induction of *Il33b* mRNA expression by LPS in Raw 264.7 cells, luciferase reporter gene assays revealed LPS-inducible promoter activity of the proximal 733-bp promoter region located 5′ upstream of *Il33* exon 1b but not of the proximal 959 bp located 5′ upstream of exon 1a in Raw 264.7 cells (Fig. 4C).

In this study, we investigated expression of *Il33a* and *Il33b* mRNA in different mouse organs and cell types in basal and inflammatory conditions. We describe transcription of mouse *Il33* from two alternative promoters, which are used in a cell type- and stimulus-specific manner. In the particular case of myeloid cells, *Il33a* mRNA was constitutively expressed at low levels in BMDC but was enhanced by the TLR3 agonist poly(I:C). In contrast, LPS enhanced expression of *Il33a* and *Il33b* transcripts. In BMM and Raw 264.7 cells, no basal expression

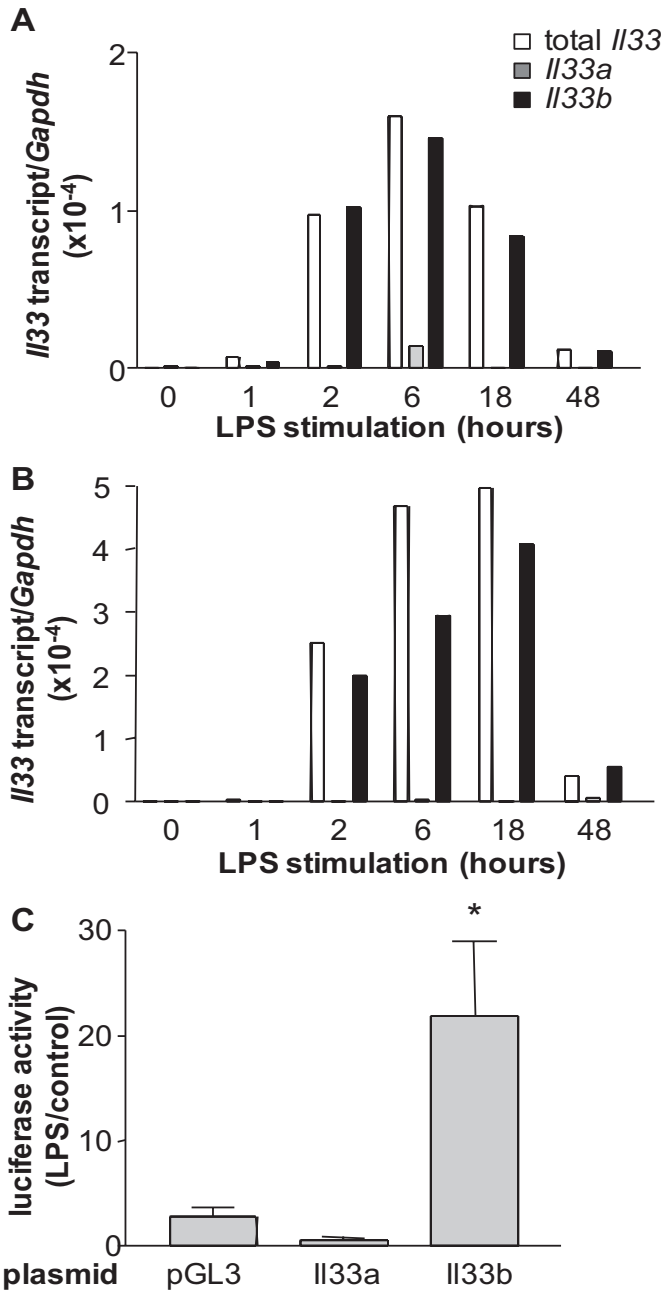

Figure 4. Expression levels of total *Il33* (open columns), *Il33a* (shaded columns), and *Il33b* (solid columns) mRNA, normalized to *Gapdh* mRNA levels are shown for mouse primary BMMs, stimulated or not for indicated times with LPS (100 ng/ml) (A), or for Raw 264.7 cells, stimulated or not for indicated times with LPS (1 μg/ml) (B). Data shown are representative of three to five independent experiments. (C) Luciferase activity of *Il33a* and *Il33b* promoter reporter gene constructs was assessed in Raw 264.7 cells, stimulated or not for 24 h with LPS (1 μg/ml). Promoter activity is expressed as fold increase of luciferase activity in LPS stimulated over unstimulated cells. Results are shown as mean + SEM of eight independent experiments for the *Il33b* promoter construct and as the mean of two independent experiments for the *Il33a* promoter construct. $*P < 0.05$, as compared with empty pGL3 vector control.

of *Il33* was observed, and LPS induced expression selectively and vigorously only from the *Il33b* promoter.

Similarly to the situation described herein for the mouse *Il33* gene, GenBank entries also point to alternative exon use in the 5′UTR of the *IL33* transcript in humans. However, in addition to exons 1a and 1b, the human *IL33* gene contains a third 5′-untranslated exon, which we call exon 1a′ and which can be used alternatively to exon 1a or exon 1b or spliced to a splice acceptor site upstream of exon 1a to create a transcript containing exons 1a′ and 1a (GenBank ID: AK295908.1, DA439244, and DA713547, and our unpublished observations). This additional 5′ exon is also found in the chimpanzee (GenBank ID: XM_001142058), suggesting conservation of this feature in primates. Finally, in a number of other species (rat, dog, cow), EST entries reflect the existence of several *IL33* transcript variants, with further variability in location and use of 5′-noncoding exons.

Based on EST sequences deposited in GenBank, there is evidence for the existence of mouse and human *IL33* splice variants lacking selected coding exons (mouse: BI739671.1; human: AK303943, AK295908). Such selective exon skipping within the coding region would lead to the generation of shorter IL-33 protein isoforms, which might be expected to exhibit altered function. In agreement with this hypothesis, a recent study described expression of a shorter splice variant of IL-33 in a human hepatocarcinoma cell line, which displayed distinct functional properties as compared with full-length IL-33 [35]. We thus wondered whether the use of alternative promoters and first exons in a given cell type or in response to a particular stimulus would be associated with exon skipping in the downstream coding sequence. However, by RT-PCR analysis and sequencing of *Il33a* and *Il33b* cDNAs generated from stomach, lung, liver, spleen, MEF, primary MLECs, BMDCs, and Raw 264.7 cells in basal and in inflammatory conditions, we exclusively detected transcripts comprising the complete coding region for both *Il33* mRNA variants. Furthermore, by immunocytochemistry, we exclusively detected nuclear IL-33 protein expression in different cultured cell types examined, including TLR agonist-stimulated BMDCs, BMMs, and Raw 264.7 cells, primary fibroblasts, and endothelial cell lines, suggesting that the intracellular localization of the IL-33 protein was not affected by differential promoter use. We thus found no evidence for the association of transcription from either promoter with the production of protein variants, suggesting that the existence of two differentially regulated promoter regions for IL-33 expression mainly contributes to fine-tune the regulation of IL-33 production, rather than to generate functionally different proteins. In fact, in the samples tested in this study, constitutive *Il33* mRNA expression was generally associated with transcription from promoter *Il33a*, whereas transcript *Il33b* was detected preferentially in activated cells.

In mouse tissues in vivo, basal *Il33* mRNA expression was observed in the stomach and lung, reflecting constitutive IL-33 protein expression observed in epithelial cells, as well as in spleen, where IL-33 protein is expressed in stromal

cells (DTA, CG, and GP; unpublished observations). In vitro, primary fibroblasts and immortalized endothelial cells displayed high constitutive *Il33* mRNA expression, although IL-33 production is generally weak or absent in fibroblasts and endothelial cells in normal mouse tissues (DTA, CG, and GP; unpublished observations), suggesting, respectively, that passaging, immortalization, and/or culture conditions induce *Il33* gene expression in these cells. Finally and consistently with previous reports, our data confirm induction of *Il33* mRNA expression in macrophages and DCs by various TLR agonists [25–30, 36]. The biological function of IL-33 produced by myeloid cells upon exposure to pathogen-associated molecules remains to be fully elucidated. However, recent in vivo data have linked the induction of IL-33 production in alveolar macrophages in response to influenza virus with the activation of natural helper cells and the development of airway hyper-reactivity in mice, suggesting that induction of IL-33 expression in myeloid cells may be relevant to disease pathogenesis or in other instances, to host defense [30].

In this study, we tested the effects of different TLR agonists, which activate cell surface (LPS/TLR4) or vesicular [poly(I:C)/TLR3, R848/TLR7/8] TLRs and use different adaptor molecules and signaling pathways. Interestingly, in BMDCs, we observed differential effects of TLR3 and TLR4 activation on *Il33a* and *Il33b* mRNA expression. It remains to be investigated how these effects relate to differences in signaling induced by the different TLR agonists. We also observed differential effects of a given agonist according to the cell type examined, with LPS, for instance, increasing *Il33a* mRNA expression in BMDCs but exerting no effect on this transcript in macrophages. Further studies are required to examine whether this relates to cell-specific signaling or whether *Il33a* transcription is globally repressed in macrophages, as a result, for instance, of a closed chromatin context in these cells.

In conclusion, we describe expression of the *Il33* gene from two alternative promoters in the mouse. Our data further indicate that the expression of transcripts *Il33a* and *Il33b* is cell type- and stimulus-dependent.

AUTHORSHIP

D.T-A., N.C., S.V., and C.L. did experiments and analyzed data; C.G. planned studies, analyzed data, and wrote the manuscript; and G.P. supervised the project, planned studies, analyzed data, and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from the Swiss National Science Foundation (310030_135195 CG and 310030_134691 to G.P.), the Rheumasearch Foundation, the Institute of Arthritis Research, and the de Reuter Foundation. We thank Praxedis Martin and Sarah Garrido-Urbani for providing cells and cDNAs and Walter Reith for helpful discussions.

DISCLOSURES

The authors declare no competing financial interests.

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KEY WORDS:
dendritic cells · inflammation · macrophages · myeloid cells · TLR ligands