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The Dual Function Cytokine IL-33 Interacts with the Transcription Factor NF- κ B To Dampen NF- κ B–Stimulated Gene Transcription

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Full-length IL-33 is a member of the IL-1 family of cytokines, which can act in an autocrine or paracrine manner by binding to the IL-33R on several different target cell types. In addition, IL-33 can act in an intracrine fashion by translocating to the nucleus, where it binds to the chromatin and modulates gene expression. In this article, we report that full-length IL-33, but not mature IL-33, interacts with the transcription factor NF- κ B. This interaction occurs between the N-terminal part of IL-33 from aa 66–109 and the N-terminal Rel homology domain of NF- κ B p65. Coimmunoprecipitation experiments in cells overexpressing IL-33 or endogenously expressing IL-33 revealed rhIL-1 β –stimulated association between IL-33 and p65, whereas binding to the p50 subunit was constitutive. The biological consequence of IL-33/NF- κ B complex formation was reduction in NF- κ B p65 binding to its cognate DNA and impairment of p65-triggered transactivation. Overexpression of IL-33 resulted in a reduction and delay in the rhIL-1 β –stimulated expression of endogenous NF- κ B target genes such as *I κ B α* , *TNF- α* , and *C-REL*. We suggest that nuclear IL-33 sequesters nuclear NF- κ B and reduces NF- κ B–triggered gene expression to dampen proinflammatory signaling. *The Journal of Immunology*, 2011, 187: 1609–1616.

Interleukin-33, a member of the IL-1 family of cytokines, was originally described as a nuclear protein of unknown function in canine cerebral arteries (1) and as NF-HEV, an NF expressed in human high endothelial venules in secondary lymphoid organs (2). It was identified as a member of the IL-1 cytokine family when ST2/T1, hitherto an orphan receptor in the IL-1R family, was found to be the receptor for IL-33 (3). IL-33 functions as a classical cytokine by binding to its specific plasma membrane receptor (3), now designated IL-33R α -chain (4), and by recruiting the IL-1R accessory protein into a trimeric complex (5–8), inducing signaling pathways similar to those of IL-1 (5–7). IL-33 acts on many different cell types, including Th2 lymphocytes and mast cells (reviewed in Ref. 9), and is believed to be involved in many diseases (reviewed in Refs. 10, 11), especially those with involvement of Th2 lymphocytes or dominated by a Th2 cytokine profile (3, 12). Although the participation of IL-33 in many acute and chronic inflammatory and autoimmune diseases has become increasingly clear, the source of IL-33 remains elusive. In contrast to other family members, such as IL-1 α , IL-1 β , and IL-18, IL-33 is not mainly produced by immune cells, such as sentinel cells, after stimulation of pattern recognition

receptors in response to microbial challenges but seems to be constitutively produced predominantly by tissue cells such as endothelial cells, keratinocytes, or fibroblasts (reviewed in Ref. 10). IL-33 is produced as a full-length (fl) molecule that is biologically active and does not require processing to a mature form by caspase 1, as originally described (3). However, a mature form (IL-33_{110–266}) is biologically active, and one report describes cleavage to the mature form by calpain in human epithelial cells (13). Proteolytic cleavage of IL-33 by caspase 1 (14) or caspase 3 or 7 (15–17) yields biologically inactive products. Like IL-1 α , IL-1 β , and IL-18, IL-33 does not contain a leader sequence, and how it is released from cells is at present unclear (reviewed in Ref. 18). In fact, only few reports actually show IL-33 release after stimulation (19–21), although increased levels of IL-33 protein in tissues and in circulation have been reported in several diseases (reviewed in Ref. 22).

IL-33 shares several features with IL-1 α : Both are biologically active as fl molecules, and both are found in the nuclei of the producing cells. These characteristics are due to a classical nuclear localization sequence in fl IL-1 α (23) and a bipartite homeo-domain-like helix-loop-helix DNA binding domain residing in N-terminal aa 1–65 of IL-33 (24). High levels of endogenous IL-33 were identified in the nuclei of endothelial cells from different sources (2, 14, 25, 26), in pancreatic stellate cells (27), and in human monocytes (28). Overexpressed fl IL-33 mainly localizes to the nucleus (17, 24).

The biological consequence of the presence of IL-33 in the nucleus is not well understood, and at least two different roles are discussed. First, IL-33 “stored” in the nucleus may function as an alarmin, which is released after cell damage to alert the surrounding tissue and the immune system (14, 16, 29). A recent study proposed a similar function for IL-1 α (30), raising the possibility that IL-33 and IL-1 α are similar to the prototypic alarmin high mobility group box 1 protein (reviewed in Refs. 31, 32). It was suggested that IL-33 is a dual-function cytokine that functions as an alarmin in a paracrine fashion (reviewed in Refs.

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Abbreviations used in this article: CBM, chromatin binding motif; fl, full-length; HA, hemagglutinin; IP, immunoprecipitate; m, murine; MEF, mouse embryonic fibroblast; RHD, Rel homology domain; TAD, transactivation domain; WB, Western blot.

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33, 34). Second, IL-33 was found to be associated with heterochromatin (24, 35), and it was suggested that nuclear IL-33 negatively affects gene transcription in an intracrine, non-IL-33 receptor-mediated manner by a yet to be defined molecular mechanism (24). In summary, IL-33 is a dual-function cytokine exercising its role as an intracellular regulator of gene expression and also as an alarm mediator (reviewed in Ref. 34) that initiates a sterile inflammation in response to cellular destruction.

Inflammatory processes are rapidly amplified by the transcription factor NF- κ B, which triggers expression of proinflammatory cytokines, which are themselves activators of specific receptors in the cell membrane and thus amplify the signaling events and alert neighboring cells. The NF- κ B transcription factor is a key component for the production of many cytokines and also a central mediator of cytokine-triggered effects. All canonical NF- κ B inducers lead to the proteasomal elimination of the inhibitory I κ B proteins and thus release the DNA-binding subunits that immigrate into the nucleus and trigger gene expression (36). The central relevance of NF- κ B for the powerful induction of innate immunity makes this transcription factor a preferred target for manipulation by viral proteins, bacteria, and their products, as well as parasites, to escape from the immune system (37).

In this article, we describe how intracellular fl IL-33 interacts with the NF- κ B p65 subunit in the cytosol and nucleus of the producing cell. This interaction is enhanced after activating the IL-1 signaling pathway. IL-33/NF- κ B p65 protein-protein interaction interfered with NF- κ B-dependent transcription by two mechanisms as IL-33 (1) impaired NF- κ B DNA binding and (2) interfered with p65-mediated transactivation. These findings reveal a novel role for IL-33 as a negative modulator of NF- κ B activity.

Materials and Methods

Mammalian cell culture

Human HEK293RI cells (38) were kindly provided by Z. Cao (Tularik, South San Francisco, CA). Mouse embryonic fibroblasts (MEFs) (39) were kindly provided by W.C. Yeh, Toronto, Canada. NF- κ B p65 (RelA) knockout MEFs (p65^{-/-} MEFs) (40) were kindly provided by E. Burstein (Dallas, TX). Cells were passaged every 2–3 d and maintained in DMEM (PAA Laboratories, Coelbe, Germany) with 10% FCS (PAA Laboratories) at 10% CO₂ in a humidified incubator at 37°C.

Expression plasmids

Murine (m) pIL-1 α -Flag was used for the expression of C-terminally Flag-tagged IL-1 α . pMyc-mIL-18-Flag and pMyc-mIL-33₁₋₂₆₆-Flag encode fl mIL-18 and mIL-33, respectively, with a Myc tag at the N terminus and a Flag tag at the C terminus. pMyc-mIL-33_{D175A}-Flag was used for expression of fl IL-33 with point mutation at aa 175 (D175A). pMyc-mIL-33₁₋₁₇₅ encodes N terminally Myc-tagged mIL-33 deletion mutant aa 1–175. pMyc-mIL-33₁₇₆₋₂₆₆-Flag, pMyc-mIL-33₁₁₀₋₂₆₆-Flag, and pMyc-mIL-33₆₆₋₂₆₆-Flag encode N-terminally Myc- and C-terminally Flag-tagged mIL-33 deletion mutants, aa 176–266, aa 110–266, and aa 66–266, respectively. Information on the pGal4-Luc reporter plasmid and the various Gal4-p65 fusion proteins has been published (41), and the EGFP-p65 fusion protein was described in Ref. 42. The p3 \times NF- κ B-Luc reporter plasmid encodes firefly luciferase under the control of three consecutive NF- κ B binding sites and was used for the NF- κ B reporter gene assays, as noted previously (6). p65-hemagglutinin (HA) encodes fl human (h) NF- κ B p65 with an HA tag (43). Primers and cloning details are available on request.

Transient transfection

HEK293RI cells and p65^{-/-} MEFs were transiently transfected by a slightly modified polyethylenimine (Sigma-Aldrich, Munich, Germany) transfection method (44). The total amount of plasmid DNA was always adjusted using the appropriate empty vector.

Coimmunoprecipitation and Western blotting

First, 3.6 \times 10⁶ HEK293RI cells were seeded 24 h before transfection. Cells were transfected with 6 μ g plasmid encoding either mIL-1 α , mIL-33,

or mIL-18 (pmIL-1 α -Flag, pMyc-mIL-18-Flag, pMyc-mIL-33₁₋₂₆₆-Flag, pMyc-mIL-33_{D175A}-Flag, pMyc-mIL-33₁₋₁₇₅, pMyc-mIL-33₁₇₆₋₂₆₆-Flag, or empty vector). For experiments in which more than one protein was coexpressed, 3 μ g plasmid encoding IL-33 (pMyc-mIL-33-Flag, pMyc-mIL-33₁₁₀₋₂₆₆-Flag, or pMyc-mIL-33₆₆₋₂₆₆-Flag) was cotransfected with 3 μ g plasmid encoding the p65 fusion proteins [p65-EGFP, pGal4-p65, pGal4-p65 transactivation domain (TAD), or pGal4-p65 Rel homology domain (RHD)]. The total amount of plasmid DNA was always adjusted to 6 μ g, using empty vector. Transfected cells were kept either unstimulated or stimulated with 1 ng/ml rhIL-1 β (a kind gift from D. Boraschi, Pisa, Italy) for 30 min at 37°C, washed, and lysed with lysis buffer (38) for 30 min at 4°C. Where indicated, nuclei were separated from cytosol, washed, and lysed in lysis buffer by sonication. Cell debris was removed from cytosol and nucleosol before the supernatant was incubated with anti-Flag M2 agarose (Sigma-Aldrich) for 16 h at 4°C with gentle rotation. For anti-Myc immunoprecipitation, 500 μ l conditioned medium from the hybridoma 9E10 was used in combination with 10 μ l Protein G Sepharose slurry (GE Healthcare, Munich, Germany). Washed beads were resuspended in Laemmli sample buffer and heated to 95°C for 10 min. After SDS-PAGE, proteins were detected in a Western blot (WB) with anti-p65 A Ab (Santa Cruz Biotechnology, Heidelberg, Germany), anti-p65 C20 (Santa Cruz Biotechnology), anti-p50 Ab (Santa Cruz Biotechnology), anti-I κ B α Ab (Santa Cruz Biotechnology), anti-c-Myc Ab (Santa Cruz Biotechnology), or anti-Flag bioM2 Ab (Sigma-Aldrich).

Coprecipitation of endogenous IL-33 with p65

MEFs expressing endogenous IL-33 were seeded in 75-cm² flasks. Upon reaching confluency, five 75-cm² flasks were pooled and either left untreated (controls) or stimulated with 1 ng/ml rhIL-1 β for 60 min. Cells were subsequently harvested and lysed. After sonication and removal of cell debris, 2 μ g anti-IL-33 mAb Nesy-1 (Alexis, Lörrach, Germany) was added together with 10 μ l Protein G Sepharose slurry and incubated overnight at 4°C. IL-33 immunoprecipitates (IPs) were washed three times, and then proteins were released from the beads by heating with Laemmli buffer, followed by SDS-PAGE and WB. Precipitated proteins were detected with anti-p65 C20 (Santa Cruz Biotechnology) and anti-IL-33 (rabbit pAb; Alexis), respectively.

EMSA

HEK293RI cells were transfected with 3 μ g plasmid encoding HA-tagged NF- κ B p65 subunit or increasing amounts (0, 2, 4, 6, 9, or 12 μ g) of pMyc-IL-33-Flag, or both (3 μ g p65-HA + 0, 3, 6, or 9 μ g pMyc-IL-33-Flag). The following day, cells were either kept unstimulated or stimulated with 1 ng/ml rhIL-1 β for 30 or 60 min. Cell lysates were prepared by 30 min incubation with lysis buffer at 4°C (38) and subsequent sonication.

Both the sense and antisense DNA oligonucleotides containing NF- κ B binding site (5'-TGACAGAGGGGACTTCCAGAGA-3' and 5'-TCTCTGGAAAGTCCCCTCTGTCA-3') were labeled separately with radioactive [³²P]-phosphate using T4 polynucleotide kinase (Fermentas, St. Leon-Rot, Germany) and [³²P]-ATP (Hartmann, Braunschweig, Germany). The annealed oligonucleotides were incubated with 10 μ g protein from total cell lysates in binding buffer according to the protocol of a nonradioactive labeling kit (Roche Diagnostics, Mannheim, Germany) for 30 min at room temperature. In some of the samples, 2 μ g anti-p65 (Santa Cruz Biotechnology) or anti-p50 (Santa Cruz Biotechnology) was added to achieve a supershift. As specificity control, 125-fold excess concentration of non-labeled oligonucleotides was added. Oligonucleotide-protein complexes were separated on 6% polyacrylamide gels in 0.5 \times Tris-borate-EDTA buffer. Gels were dried and exposed overnight on a high-performance chemiluminescence film (Amersham Biosciences, Freiburg, Germany).

Reporter gene assay

For NF- κ B-dependent reporter gene assays, 1.25 \times 10⁵ HEK293RI cells were seeded in 1 ml medium per well in 24-well plates. The next day, cells were cotransfected with 55 ng p3 \times NF- κ B-Luc and 0, 69, 139, or 278 ng IL-33 encoding plasmids. At 16 h after transfection, cells were kept either unstimulated or stimulated for 8 h with 50 pg/ml rhIL-1 β or 10 ng/ml rhTNF- α (BASF AG, Ludwigshafen, Germany). For NF- κ B-dependent reporter gene assays in keratinocytes, IL-1RI^{-/-} murine keratinocytes (90 \times 10³) (a kind gift from W. Falk, Regensburg, Germany) were seeded in 1 ml medium per well in 24-well plates. The next day, cells were cotransfected with 110 ng p3 \times NF- κ B-Luc (luciferase reporter plasmid under the control of NF- κ B) and 0, 139, 278, or 556 ng plasmids encoding wild-type fl IL-33 or IL-33 C-terminal fragment (IL-33₁₇₆₋₂₆₆). The total amount of plasmid DNA was always maintained by adding empty vector. Cells were either left unstimulated or stimulated with 500 pg/ml LPS for 8 h.

For Gal4 reporter gene assays, 50×10^4 p65^{-/-} MEFs were seeded in 1 ml medium per well in 24-well plates. The next day, cells were cotransfected with 30 ng pGal4-Luc + 3 ng pGal4-p65 and 0, 158.25, 316.5, or 633 ng of IL-33-encoding plasmids. After transfection, cells were incubated for 16 h at 37°C. Transfected and stimulated cells were washed with PBS and lysed with 100 μ l passive lysis buffer (Promega, Mannheim, Germany), and luciferase activity was measured as described earlier (6).

Analysis of gene expression (real-time PCR)

HEK293RI cells were transfected with either empty vector or fl IL-33-encoding plasmid (pMyc-mIL-33₁₋₂₆₆-Flag). The next day, cells were trypsinized, and all cells transfected with the same plasmid were pooled. Cells were reseeded in 56-cm² Petri dishes. A day after seeding, cells were washed once and allowed to stay for 2 h in serum-free medium. Cells were incubated with rhIL-1 β for 10 min, then washed twice with serum-free medium and incubated for the indicated period. Subsequently, cells were trypsinized, and total RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics) following the manufacturer's recommendations. cDNA was reverse transcribed using M-MuLV H⁻ reverse transcriptase and oligo dT (Fermentas). mRNA transcripts of different genes were quantified by real-time PCR (Mx3005P; Stratagene, Amsterdam, The Netherlands) using a master mixture with SYBR Green (Abgene, Epsom, U.K.). Transcription of each gene was normalized to *GAPDH* (a housekeeping gene) transcription. The following primers were used for real-time PCR analysis. For *I κ B α* : 5'-GTCAAGGAGCTGCAGGAGAT-3' and 5'-CCATGGTCAGTGCCTTTTCT-3'; for *GAPDH*: 5'-ACCACCATGGAG-AAGGC-3' and 5'-GGCATGGACTGTGGTCATGA-3'; for *hTNF- α* : 5'-TGAGCACTGAAAGCATGATCCG-3' and 5'-GAGGGCTGATTAGAGA-GAGGTC-3'; for *hC-REL*: 5'-TACAAGAATAAAGGCAGGAATC-3' and 5'-CAGGAGGAAGAGCAGTCGTC-3'; and for *hPGK1*: 5'-TAAAG-CCGAGCCAGCCAAAATAG-3' and 5'-TCATCAAAAACCCACCAGC-CTTCT-3'.

Results

IL-33 interacts with the p65 and p50 subunits of NF- κ B

The occurrence of IL-33 in the nucleus raises the possibility that this cytokine binds to transcription factors. To test a possible interaction with NF- κ B, human HEK293RI cells were transiently transfected to express fl IL-33 (IL-33₁₋₂₆₆) or an uncleavable mutant of fl IL-33 (IL-33_{D175A}). Cells remained untreated or were stimulated with rhIL-1 β , followed by separation into cytosolic and nucleosolic fractions and coimmunoprecipitation experiments. The fl IL-33 and IL-33_{D175A} occurred in both fractions in untreated and rhIL-1 β -stimulated cells (Fig. 1A, bottom). IL-33 IPs were checked for the presence of the NF- κ B subunits p65 and p50. Both were found to be associated with IL-33 in the cytosol (Fig. 1A, right) and in the nucleosol (Fig. 1A, left), demonstrating an interaction of IL-33 with this transcription factor. rhIL-1 β stimulation resulted in an increase of coprecipitated p65 in cytosolic and nuclear fractions (Fig. 1A, upper panels). Purity of the cytosolic and nuclear fractions was ascertained by demonstrating the exclusive distribution of GAPDH in the cytosol and lamin B in the nucleosol (data not shown). The p50 subunit was consistently found in association with IL-33 in cytosol and nucleosol (Fig. 1A, middle panels); however, in contrast to the p65 signal, the intensity of the p50 signal did not increase after rhIL-1 β stimulation. Usually, NF- κ B p65 and p50 subunits are retained in the cytosol in a complex with the inhibitory subunit I κ B α (45). Thus we tested whether I κ B α could also be detected in IL-33 IPs. However, we never found I κ B α in the complex with IL-33 (data not shown), suggesting that IL-33 interacts only with free and active NF- κ B that was released from I κ B.

Having shown that the interaction of IL-33 and NF- κ B is possible in cells overexpressing IL-33, we wanted to demonstrate the interaction of p65 with endogenous IL-33 under physiological conditions. We tested a series of different cell lines for constitutive IL-33 expression and found one MEF that expressed low but detectable levels of IL-33. These proliferating fibroblasts were either

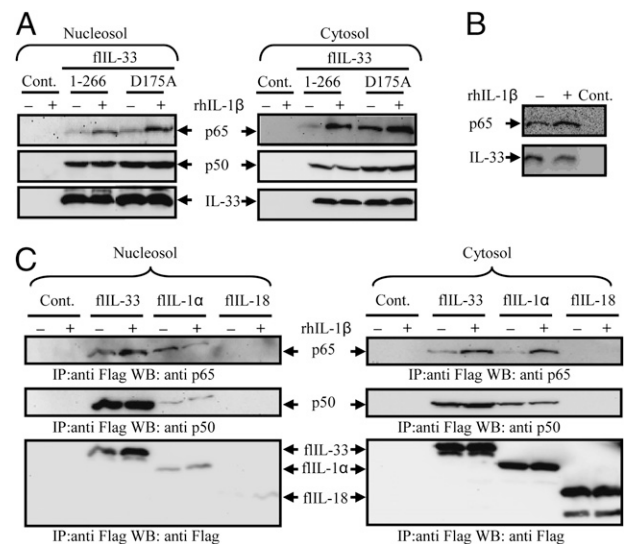


FIGURE 1. The fl IL-33 interacts with NF- κ B p65 and p50 subunits. *A*, Overexpressed fl IL-33 interacts with endogenous NF- κ B p65 and p50 subunits in cytosol, and nucleosol and IL-33/p65 interaction is enhanced by rhIL-1 β stimulation. In HEK293RI cells, epitope-tagged wild-type fl IL-33 (1–266) or a mutated fl IL-33 (1–266_{D175A}) was expressed after transient transfection. Cells were also transfected with empty vector as a control (Cont.). The next day, transfected cells were either left untreated or stimulated with 1 ng/ml rhIL-1 β for 30 min. Cytosol and nucleosol were prepared, and Myc-tagged IL-33 was immunoprecipitated using the anti-Myc mAb 9E10. Precipitated proteins were visualized by WB with anti-p65 (upper), anti-p50 (middle), and anti-Myc (lower) Abs. *B*, Endogenous IL-33 and endogenous NF- κ B p65 interact in a MEF line. A total of 2.5×10^7 cells were left unstimulated or stimulated with 1 ng/ml rhIL-1 β for 60 min. Subsequently, endogenous IL-33 was precipitated from total lysates by overnight incubation with 2 μ g/sample of the anti-IL-33 mAb Nussy-1 and Protein G Sepharose. Proteins were visualized by WB using anti-p65 (upper) and anti-IL-33 (lower) Abs. As control, cell lysates were incubated with Protein G Sepharose and an irrelevant isotype-matched control Ab (Cont.). *C*, The fl IL-1 α and fl IL-18 interact with NF- κ B but not fl IL-18. HEK293RI cells were transiently transfected with empty vector (Cont.) or plasmids encoding Flag-tagged fl versions of IL-33, IL-18, and IL-1 α . Nucleosol and cytosol were prepared from unstimulated and stimulated cells (1 ng/ml rhIL-1 β for 30 min). IL-1 family members were precipitated using anti-Flag M2 agarose. Proteins were detected by WB using anti-p65 (upper), anti-p50 (middle), and anti-Flag (lower) Abs. The results shown in A–C are one representative of five independent experiments with similar results.

left untreated or stimulated with rhIL-1 β and then lysed, and IL-33 was immunoprecipitated from total cell lysates. In unstimulated but proliferating cells, p65 could be faintly detected in IL-33 IPs (Fig. 1B). Upon stimulation with rhIL-1 β , the amount of p65 coprecipitated with endogenous IL-33 increased (Fig. 1B). This result demonstrates that IL-33 interacts with p65 under physiological conditions, that is, without overexpressing either partner molecule. Although the signal was rather weak owing to the fact that these fibroblasts express very little endogenous IL-33, compared with cells transfected with a plasmid encoding fl IL-33 (data not shown), coprecipitation of p65 with IL-33 was always possible.

In addition to coimmunoprecipitation, we also showed colocalization of NF- κ B p65 and fl IL-33–GFP fusion in the nucleus of HeLa cells after stimulation with rhTNF- α by immunostaining followed by microscopy (Supplemental Fig. 1A) and flow cytometry (Supplemental Fig. 1B, 1C). These results support our data obtained by coimmunoprecipitation experiments obtained from cytosol or nuclei of transiently transfected cells, as depicted in Fig. 1.

We then wanted to know whether the interaction of IL-33 with NF- κ B is an exclusive feature of IL-33 or shared by other IL-1 cytokine family members. We transiently transfected HEK293RI cells with vectors encoding fl versions of IL-33, IL-1 α , or IL-18; left the cells unstimulated or stimulated them with rhIL-1 β ; lysed the cells; and separated nucleosol from cytosol. IL-33 was immunoprecipitated, and we tested whether NF- κ B p65 or p50 subunits coprecipitated with the individual cytokine. All three cytokines were expressed in the cytosol (Fig. 1C, *right bottom*), but only fl IL-33 and fl IL-1 α constitutively translocated to the nucleus, whereas fl IL-18 remained in the cytosol. Both NF- κ B subunits could be coprecipitated with fl IL-33 and fl IL-1 α , in both cytosolic and nuclear fractions, respectively, but not with fl IL-18 in either compartment (Fig. 1C). The weak constitutive association of p65 with fl IL-33 or fl IL-1 α increased after rhIL-1 β stimulation of the cells, whereas the p50 interaction showed no rhIL-1 β dependency. In general, the nuclear translocation of fl IL-1 α was less pronounced than that of fl IL-33. These data also suggest that fl IL-1 α is able to interact with NF- κ B, but the physiological consequences of this finding need to be elaborated on in the future.

The N-terminal domain of IL-33 interacts with the N-terminal domain of p65

To map the interaction domains allowing this protein–protein interaction, different truncated versions of IL-33 were transiently overexpressed in HEK293RI cells, immunoprecipitated from lysates, and the IL-33 IPs were tested for the presence of endogenous p65 and p50. Both NF- κ B subunits could be coprecipitated with fl IL-33_{1–266} and the N-terminal caspase 3 cleavage product IL-33_{1–175}, whereas it was not possible to detect NF- κ B subunits in association with the C-terminal caspase 3 cleavage product IL-33_{176–266} (Fig. 2A). The NF- κ B subunits were coprecipitable from the cytosolic and nuclear fractions; however, after rhIL-1 β stimulation of the cells, the signal for p65 associated with fl IL-33_{1–266} or IL-33_{1–175} was much stronger in both compartments, whereas the signal for p50 remained unaffected by prior rhIL-1 β stimulation.

Fine mapping of the domain in IL-33 responsible for the interaction with the p65 NF- κ B subunit was performed by constructing different fragments of IL-33 covering the critical areas in the N-terminal half of the molecule: a Δ N-version of IL-33_{66–266} excluding the N terminus, which contains the homeodomain-like helix-loop-helix DNA binding motif responsible for nuclear translocation of fl IL-33 (24), and the “mature” form of IL-33_{110–266} (3). These forms were transiently expressed in HEK293RI cells alone or in combination with EGFP-p65 (Fig. 2B). NF- κ B p65 could be coprecipitated with fl IL-33_{1–266} and with IL-33_{66–266}, but not with the “mature” form of IL-33_{110–266} (Fig. 2B, *upper panel*). This result maps the site in IL-33 necessary for the interaction with p65 to an N-terminal region between aa 66 and 109, excluding the nuclear localization sequence.

It was then interesting to investigate whether NF- κ B p65 contacts IL-33 via its N-terminal RHD responsible for DNA binding or via the C-terminal TAD. Cells were transiently transfected to express fl IL-33 with two fragments of p65 covering the RHD or the TAD. In these experiments, the Gal4 fusion proteins used were also used in the Gal4 reporter assays discussed below. Coprecipitation studies showed that both fl p65 and a fragment consisting of the N-terminal part of p65 containing the RHD were detected in IL-33 IPs, whereas the transactivating C-terminal part did not significantly bind (Fig. 2C, *lane 6, upper*). From these results we conclude that p65 interacts with its N-terminal region containing the Rel homology domain/DNA binding domain, with a stretch in the N-terminal part of IL-33 defined by aa 66–109. The different

constructs employed and the results of the various coprecipitation studies are summarized schematically in Fig. 2D.

IL-33 interferes with the binding of p65 to κ B consensus binding sites and reduces its activity as a transcription factor

We next addressed the question of whether IL-33 affects the transcription factor activity of NF- κ B. EMSAs were performed to ascertain whether the binding of IL-33 to p65 affects the DNA binding of the transcription factor. Lysates from HEK293RI cells overexpressing p65 in the absence or presence of increasing concentrations of coexpressed IL-33 were incubated with radioactive oligonucleotides containing the classical κ B binding site of the I κ B α -promoter sequence (46, 47). Under these experimental conditions, a reproducible inhibition of p65 binding to the oligonucleotide was observed at high concentrations of IL-33 (Fig. 3A, *left*). If only IL-33 was overexpressed in HEK293RI cells and the DNA binding of endogenous p65 was measured, a reduction of the rhIL-1 β -stimulated binding of p65 was also observed with increasing concentration of IL-33 expression (Fig. 3A, *right*). The strongest effect of IL-33 on protein binding to the radioactive probe was seen in a weakly detectable complex migrating much faster than homo- or heterodimers of the NF- κ B family (Fig. 3A, \dagger). At present, the nature of this protein is not known; however, it shows a clear rhIL-1 β dependency in binding, quite similar to that of NF- κ B. The two bands migrating most slowly in the gel were identified as complexes containing p65 by supershift using anti p65 Ab (Fig. 3A, *left, lane 3*) and by performing WB analysis after nonradioactive EMSA (data not shown). The constitutive NF- κ B-binding activity occurring under these conditions, possibly owing to recombination signal sequence binding protein κ (RBP- κ) (47), was not supershifted with NF- κ B-specific Abs and not affected by rhIL-1 β stimulation (Fig. 3A, $*$). Supershifts due to IL-33 binding to p65 bound to the oligonucleotide were not observed, and IL-33 could not be detected in the immunoblots of the p65/oligonucleotide complexes (data not shown). These results demonstrate that the binding of IL-33 to NF- κ B impaired the interaction of p65 with its consensus binding sequence on the DNA probe. As IL-33 could not be detected together with p65 bound to the DNA, it must be assumed that IL-33 masks a critical area in p65 required for DNA binding. This observation was corroborated by a transcription factor ELISA, which measures NF- κ B binding to an oligonucleotide in which fl IL-33, but not IL-33_{176–266}, reduced NF- κ B binding (data not shown).

We then ascertained whether the inhibitory effect of IL-33 on DNA binding of p65 has consequences for its function as transactivator by performing different types of reporter gene assays. We cotransfected a reporter plasmid that encoded luciferase under the control of three NF- κ B consensus binding sequences with increasing amounts of a plasmid encoding either fl IL-33, the N-terminal part of IL-33_{1–175}, or the uncleavable mutant IL-33_{D175A}. We activated the NF- κ B pathway by stimulation with rhIL-1 β and measured luciferase activity in lysates from these cells. We observed a concentration-dependent reduction of NF- κ B-driven reporter gene production by fl IL-33, by IL-33_{D175A}, and by the N-terminal IL-33_{1–175}, but not by the C-terminal IL-33_{176–266}, which had no effect in this reporter gene assay (Fig. 3B). In addition to rhIL-1 β , we also activated the NF- κ B pathway by rhTNF- α in HEK293RI cells. We show that rhTNF- α -stimulated NF- κ B activity (Fig. 3C) is reduced in a specific and concentration-dependent manner, comparable to the effect achieved with rhIL-1 β (Fig. 3B). In addition, we stimulated the NF- κ B pathway with LPS. However, these experiments were performed in a different cell type, as HEK293RI cells do not express TLR4. These results were achieved in a murine keratinocyte cell line. The fl IL-33,

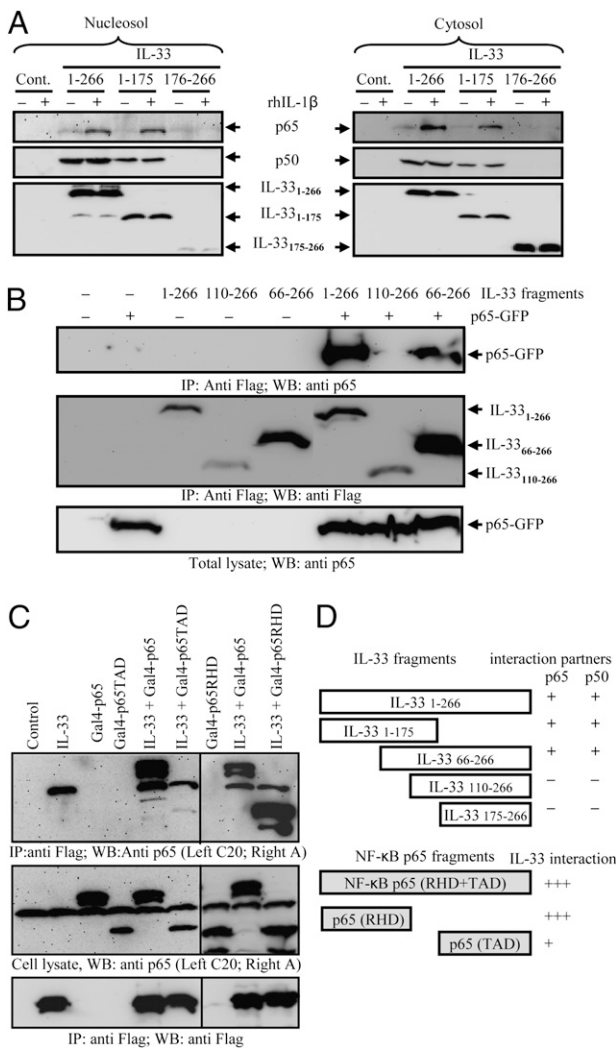


FIGURE 2. Mapping of specific domains in IL-33 and NF-κB p65 responsible for the IL-33/NF-κB interaction. *A*, IL-33/NF-κB interaction is mediated by the N-terminal part of IL-33. Myc-tagged versions of fl IL-33₁₋₂₆₆, the N-terminal fragment IL-33₁₋₁₇₅, or the C-terminal fragment IL-33₁₇₅₋₂₆₆ were expressed in HEK293RI cells. Control cells were transfected with empty vector (Cont.). The next day, transfected cells were left unstimulated or stimulated with 1 ng/ml rhIL-1β for 30 min. Cytosol and nucleosol were prepared, and Myc-tagged IL-33 proteins were immunoprecipitated using the anti-Myc mAb 9E10. Proteins were visualized by WB with anti-p65 (*upper*), anti-p50 (*middle*), and anti-Myc (*lower*) Abs. *B*, A stretch in the N-terminal part of IL-33 defined by aa 66–109 is sufficient for the interaction with NF-κB p65. HEK293RI cells were transiently transfected with plasmids encoding Flag-tagged versions of IL-33 (fl IL-33: 1–266; mature IL-33: 110–266; and N-terminally truncated IL-33: 66–266) alone or in combination with a plasmid encoding a NF-κB p65-GFP fusion protein. The following day, IL-33 proteins were immunoprecipitated with anti-Flag M2 agarose from total cell lysates. Proteins were detected by WB using anti-p65 (*upper*) and anti-Flag (*middle*) Abs. The expression of the NF-κB p65-GFP fusion proteins was controlled using the anti-p65 Ab (*lower*). *C*, NF-κB p65 interacts with IL-33 with its RHD. HEK293RI cells were transiently transfected with plasmids encoding Gal4 fusion proteins, including fl p65 (Gal4-p65), the C-terminal p65 TAD (Gal4-p65 TAD), or the N-terminal p65 RHD (Gal4-p65 RHD), alone or in combination with a plasmid encoding Flag-tagged fl IL-33. On the next day, cells were stimulated with 1 ng/ml rhIL-1β for 30 min. IL-33 was precipitated from lysates using anti-Flag M2 agarose. Proteins were detected by WB with anti-p65 Ab (*upper*) and subsequently with anti-Flag mAb (*lower*). The expression of p65 fragments was analyzed from the total cell lysate by WB (*middle*) using an anti-p65 Ab recognizing the C terminus (C20, *left*) or an anti-p65 Ab specific for the N terminus (*A*, *right*).

but not the C-terminal fragment, reduced, in a concentration-dependent manner, LPS-stimulated NF-κB activity in a reporter gene assay (Fig. 3D). In summary, we show that in two different cellular systems, after stimulating the NF-κB pathway with the three stimuli rhIL-1β, TNF-α, and LPS, which are known activators of the NF-κB pathway, fl IL-33 specifically and in a concentration-dependent way reduced NF-κB transactivation activity.

To test the impact of IL-33 on p65-dependent transactivation, one-hybrid experiments using Gal4-p65 fusion proteins were performed. This assay is independent of NF-κB DNA binding, as DNA contact is mediated by Gal4. Increasing concentrations of fl IL-33 and of the N-terminal IL-33₁₋₁₇₅, but not of the C-terminal IL-33₁₇₆₋₂₆₆ (Fig. 3E), negatively affected transactivation from the p65 TAD domain.

IL-33 reduces and delays NF-κB-mediated gene expression

To measure the effect of IL-33 on expression of endogenous NF-κB target genes, HEK293RI cells were transiently transfected with fl IL-33 and then stimulated with rhIL-1β for different periods to activate the NF-κB pathway. For a prototypic gene product directly regulated by NF-κB, we measured the induction of IκBα mRNA over time. In cells overexpressing IL-33, the rhIL-1β-stimulated increase of IκBα mRNA expression (Fig. 4A, *left*) was delayed and reduced in extent, whereas housekeeping genes like *hPGK1* (Fig. 4A, *right*) or *hGUSB* (data not shown) were not affected. This delay in rhIL-1β-stimulated mRNA expression in the presence of IL-33 was also observed for *hTNF-α* (Fig. 4A, *middle left*) and *hC-REL* (Fig. 4A, *middle right*), both genes known to be regulated by NF-κB.

Downregulation of IκBα-mRNA upon rhIL-1β stimulation was followed on the protein level (Fig. 4B, *lower* [original data] and *upper* [normalized values]). In most experiments, the IL-33 effect on mRNA expression resembled a delay in time rather than an absolute reduction of the total gene induction. The effect of IL-33 on NF-κB activity seemed most prominent in situations when p65 NF-κB was not in excess, such as after overexpression or massive stimulation with high concentrations of rhIL-1β, suggesting that the dampening effect of IL-33 is most effective when few p65 molecules are liberated from the quiescent complex in the cytosol. To address this aspect in more detail, HEK293RI cells were transiently transfected with either empty vector or with a vector encoding fl IL-33. Then rhIL-1β was added at increasing concentrations for 60 min. Subsequently, mRNA was prepared and analyzed by quantitative PCR. In this type of experiment the rhIL-1β-dependent IκBα expression was shifted toward higher concentrations of rhIL-1β in the cells overexpressing IL-33 compared with the control cells (Fig. 4C). However, at maximum concentrations of the rhIL-1β stimulus, a comparable level of mRNA expression was reached in cells overexpressing IL-33 and control cells. These data suggest that IL-33 negatively affects NF-κB activity to the greatest extent when few p65 molecules enter the nucleus.

Discussion

IL-33 came into the spotlight of cytokine research by the end of 2005, when it was identified as a member of the IL-1 family of cytokines binding to an orphan receptor of the IL-1R family (3). Further research mainly concentrated on revealing the molecules

The results shown in A–C are representative of at least three independent experiments with similar results. *D*, Summary of regions in IL-33 and NF-κB p65 responsible for interaction.

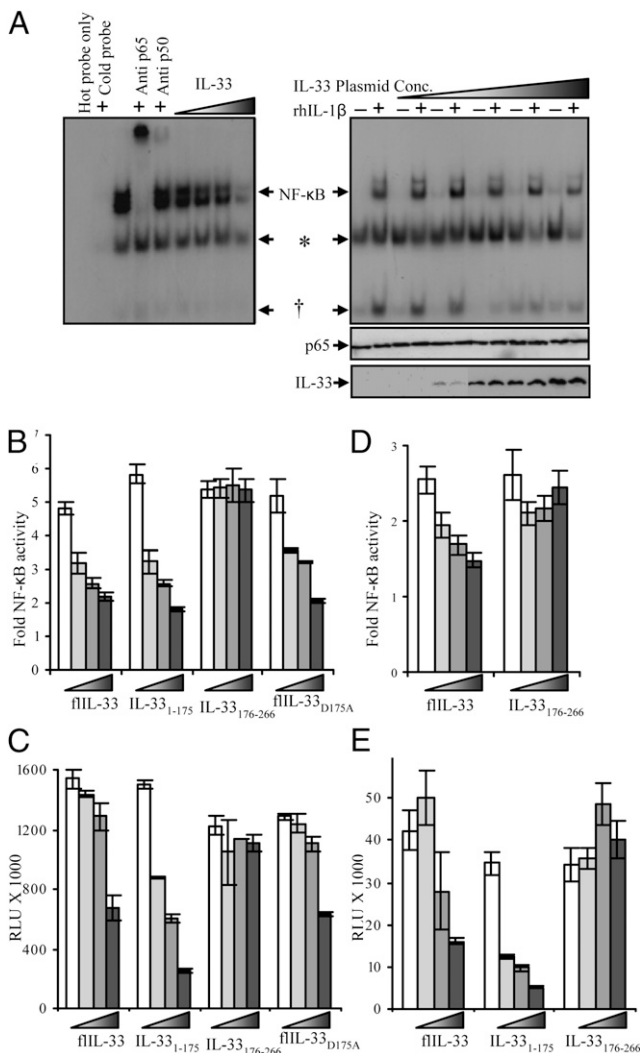


FIGURE 3. IL-33 affects DNA binding and transactivation activities of NF- κ B. **A**, IL-33 reduces binding of NF- κ B to an oligonucleotide containing a κ B binding site. Increasing amounts of a plasmid encoding IL-33 were transiently transfected alone (*right*) or with constant amounts of a plasmid encoding NF- κ B p65 (*left*) in HEK293RI cells. The next day, cells were either left untreated (-) or stimulated with 1 ng/ml rhIL-1 β (+ and all left) for 30 min. Cells were lysed, and EMSAs were performed using a [³²P]-labeled probe (*upper panels*). Expression of NF- κ B p65 and IL-33 was analyzed in parallel in total cell lysates by WB using anti-p65 (*middle right*) and anti-Flag (*lower right*) Abs. Data shown are from one representative experiment from a series of four with comparable results. **B**, IL-33 inhibits transactivation activity of NF- κ B in reporter gene assays. HEK293RI cells were transiently transfected with increasing amounts of plasmids encoding wild-type fl IL-33, the fl IL-33_{D175A} mutant, and IL-33 fragments (N-terminal IL-33₁₋₁₇₅, C-terminal IL-33₁₇₆₋₂₆₆) in combination with a luciferase reporter plasmid under the control of NF- κ B. Cells were either left unstimulated or stimulated with 50 pg/ml rhIL-1 β for 8 h. Subsequently, cells were lysed, and luciferase activity was determined. Depicted is fold induction calculated by dividing relative light unit (RLU) values of stimulated samples by the RLU values of controls. **C**, IL-33 expression reduces rhTNF- α -induced NF- κ B-dependent reporter gene activity. HEK293RI cells were transiently transfected with increasing amounts of plasmids encoding fl IL-33, the fl IL-33_{D175A} mutant, and IL-33 fragments (N-terminal IL-33₁₋₁₇₅, C-terminal IL-33₁₇₆₋₂₆₆) in combination with a luciferase reporter plasmid under the control of NF- κ B. Cells were stimulated with 10 ng/ml rhTNF- α for 8 h. Subsequently, cells were lysed, and luciferase activity was determined. Depicted are RLU values of stimulated samples. **D**, IL-33 expression reduces LPS-induced NF- κ B-dependent reporter gene activity. IL-1RI^{-/-} murine keratinocytes were cotransfected with p3 \times NF- κ B-Luc and increasing amounts of plasmids

and mechanisms mediating the function of IL-33 as a classical cytokine that functions as a mediator between cells (3, 5–7; reviewed in Refs. 9–11). IL-33 was first identified in 1999 as an NF induced in canine brain arteries (1) and in 2003 in high venule endothelial cells (2). Several recent reports confirmed nuclear localization of IL-33 in different cell types (25–28, 48). Recent results provide evidence for the importance of an intracellular function of IL-33 (2, 24, 35). Endogenous IL-33 was shown to interact with heterochromatin and mitotic chromosomes in endothelial cells (24), but also in the nuclei of nonproliferating endothelial cells (25). Nuclear localization of ectopically expressed fl IL-33 critically depends on a homeodomain-like helix-turn-helix motif in its N terminus (24), a region that also functions as a chromatin binding motif (CBM) (aa 40–58) (35). The CBM mediates binding to histone H2A-H2B dimers on the surface of nucleosomes and alters the chromatin architecture (35), thus explaining the ability of IL-33 to repress gene transcription (24).

In this article, we show that IL-33 interacts with the transcription factor NF- κ B and dampens its activity. This interaction depends on aa 66–109 in the N-terminal part of the mIL-33 molecule, an area that is distinct from the CBM (aa 40–58) in hIL-33 and mIL-33 (35). This study shows binding of IL-33 to the prototypical NF- κ B p50 and p65 subunits. Binding between IL-33 and p65 occurred in the cytosol and the nucleus. The weak constitutive p65 binding in unstimulated cells was strongly enhanced upon stimulation with rhIL-1 β , which is a classical activator of the NF- κ B pathway. This observation was supported by two different technical approaches in a different cell type after stimulation with rhTNF- α . After stimulation, colocalization of fl IL-33 and p65 was demonstrated by immunostaining and flow cytometry.

These data are compatible with a model indicating that IL-33 interacts with the p65 region that is required for the interaction with I κ B α . This model suggests that rhIL-1 β -triggered I κ B α degradation results in increased p65/IL-33 interactions. Along this line, we were unable to coprecipitate I κ B α with IL-33 from quiescent or stimulated cells. The constitutive binding of IL-33 to p50 might be due to the relatively high abundance of p50. The rhIL-1 β dependence of p65/IL-33 interaction is supported by structural data and NMR spectroscopy, as both p50 and p65 can contact I κ B α , but the most prominent interaction occurs between the p65 DNA binding domain and the I κ B α PEST sequence (49, 50).

The interaction domains in both molecules were mapped to their respective N-terminal regions. Association of p65 was found with an N-terminal fragment comprising aa 1–175, aa 175 being in the center of the caspase 3 cleavage site in mIL-33 (14, 16, 17). The N-terminal region in fl IL-33 (aa 1–65 in hIL-33) is responsible for nuclear translocation and interaction with the heterochromatin (24, 35). An IL-33 mutant consisting of aa 66–266 lacks the CBM (35) but still binds p65, whereas the published mature form of IL-

encoding wild-type fl IL-33 and IL-33 C-terminal fragment (IL-33₁₇₆₋₂₆₆). Cells were either left unstimulated or stimulated with 500 pg/ml LPS for 8 h. Subsequently, cells were lysed, and luciferase activity was determined. Depicted is fold induction calculated by dividing RLU values of stimulated samples by the RLU values of controls. **E**, The fl IL-33 and N-terminal IL-33₁₋₁₇₅ interfere with the transactivation of NF- κ B. Embryonal fibroblasts from p65 knockout mice (p65^{-/-} MEFs) were transiently transfected with increasing amounts of plasmids encoding the forms of IL-33 explained in **B**, in combination with a luciferase reporter plasmid with a Gal4 DNA binding site. In addition, a Gal4-p65 fusion protein was overexpressed. The influence of the different IL-33 forms on the NF- κ B-mediated constitutive transactivation of the luciferase reporter gene was measured in lysates the next day. Data shown in **B** and **C** are triplicates of one representative experiment from a series of three with comparable results.

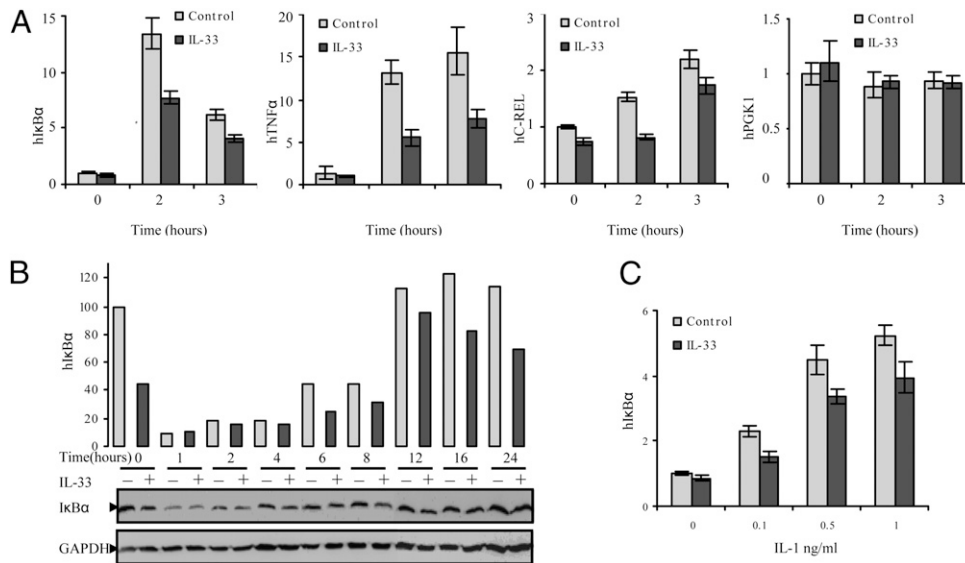


FIGURE 4. The fl intracellular IL-33 delays expression of NF- κ B-regulated genes. *A*, IL-33 delays expression of the NF- κ B-dependent genes *I κ B α* , *hTNF- α* , and *hC-REL*, but not of *hPGK1* after rhIL-1 β stimulation. HEK293RI cells were transiently transfected either with empty vector (Control; gray bars) or with a plasmid encoding fl IL-33 (IL-33; black bars). Corresponding cells were pooled the next day and reseeded overnight. Then cells were stimulated with 1 ng/ml rhIL-1 β for the times indicated, and RNA was isolated. Gene transcripts (*hIkB α* [left], *hTNF- α* [middle left], *hC-REL* [middle right], and *hPGK1* [right]) were analyzed by quantitative real-time PCR. *B*, IL-33 reduces rhIL-1 β -stimulated expression of *I κ B α* . HEK293RI cells were transiently transfected with a plasmid encoding fl IL-33 (+; black bars) or an empty vector as control (-; gray bars). Corresponding cells were pooled the next day and reseeded overnight. The cells were stimulated for the indicated time with 1 ng/ml rhIL-1 β and lysed. Total *I κ B α* and GAPDH protein were analyzed by WB (lower). Relative *I κ B α* and GAPDH protein in each band was quantified, and *I κ B α* protein concentration was normalized with respective GAPDH protein concentrations, using GeneTools software from SynGene (upper). *C*, IL-33 expression inhibits the induction of *I κ B α* gene transcription in a concentration-dependent manner. HEK293RI cells were transfected, pooled, and reseeded as described above. The cells were stimulated with the indicated concentrations of rhIL-1 β for 60 min, and RNA was isolated. *I κ B α* mRNA expression was analyzed by quantitative PCR, and results were normalized with GAPDH mRNA expression. Data in *A* and *C* show relative gene expression on mRNA level normalized to the transcripts of GAPDH.

33 (aa 110–266) did not. These results map the area in IL-33 required for the interaction with p65 to the stretch between aa 66 and 109. This truncated protein lacks the homeodomain-like HTH motif, which was reported to be necessary for nuclear accumulation and transcriptional repression (24, 35). In summary, these results suggest that the N terminus of fl IL-33 contains two different functional domains: the CBM (aa 40–58) required for interaction with the histones and the domain interacting with p65 (aa 66–109). The interaction of IL-33 with the RHD of p65 impairs NF- κ B activity in at least two different ways: IL-33 interferes with the DNA-binding activity of NF- κ B by a molecular mechanism that needs to be investigated in the future. In addition, one-hybrid experiments showed that IL-33 impairs p65-driven transactivation. This effect may be due to the ability of IL-33 to deny recruitment of further molecules required for transactivation. In p65 the domain that interacts with IL-33 resides in the N-terminal RHD, as this area coprecipitated with IL-33. However, we also observed a very weak interaction with the TAD domain of p65, which at present we cannot explain.

The interaction of p65 with IL-33 also had biological consequences in cellular responses to rhIL-1 β stimulation. We showed that prototypic NF- κ B response genes like *I κ B α* , *TNF- α* , and *C-REL* were negatively affected in their expression by the presence of IL-33, whereas the expression of housekeeping genes remained unaffected. Our results suggest not only that the reported repressive activity of fl IL-33 on gene expression is a general phenomenon, as suggested previously (24), but that, in addition, IL-33 interferes with the regulation of NF- κ B target genes.

Of interest, the modulatory effect of IL-33 on NF- κ B activity in cells was never so pronounced as in the reporter gene assays or DNA binding assays. We always observed that the NF- κ B-trig-

gered target gene expression was primarily affected at later time points after stimulation. Stimulation with saturating concentrations of rhIL-1 β was able to override the effects of IL-33, suggesting that IL-33 dampens NF- κ B activity induced by suboptimal stimuli. IL-33 shows the best NF- κ B-repressing activity in situations where relatively few p65 molecules are liberated from the complex with *I κ B α* . Accordingly, overexpression of p65 in amounts that exceed the inhibitory capacity of endogenous *I κ B α* also escaped from IL-33 inhibition. This suggests that a given concentration of IL-33 has only a limited capacity for binding active NF- κ B and retaining it from binding to κ B binding sites. Strong signals resulting in the activation of many p65 subunits can therefore override the dampening IL-33 effect and allow the induction of full NF- κ B activity.

The critical importance of NF- κ B signaling for many different processes reinforces the need to control and restrict its function in many ways. Multiple mechanisms ensure the organized termination of NF- κ B signaling by autoregulatory feedback loops and the control of NF- κ B thresholds (51).

This study adds IL-33 to the list of NF- κ B regulators, thus bringing a new facet to the complex role of this multifunctional cytokine. This aspect of IL-33 may explain why it is found in the nucleus of such cells as endothelial cells. These cells are exposed to many signals originating from the bloodstream that may elicit short pulses of NF- κ B stimulation, which are undesirable, as they could result in unwanted activation of inflammatory processes. Under physiological conditions, nuclear IL-33 can dampen such incoming tonic NF- κ B signals, a mechanism that is reminiscent of the hyporesponsiveness of epithelial mucosal cells in the gut (52). Strong activation of NF- κ B signaling in endothelial cells will override the inhibitory activity of IL-33 and allow full

upregulation of adhesion molecules, cytokines, and inflammatory mediators. In this respect, it is interesting that proinflammatory cytokines cause downregulation of nuclear IL-33 in resting endothelial cells (25). Alternatively, severe cell damage can lead to the release of IL-33, which can then act as an alarmin, showing that one given cytokine can function in various intracellular or extracellular ways to control, induce, and terminate the inflammatory reaction.

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Disclosures

The authors have no financial conflicts of interest.

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