Efficient Secretory Expression of Sequence-Optimized Mouse IL-33 Gene in Mammalian Cells (Postprint)

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Abstract

Objective: Interleukin (IL)-33 plays a crucial role in immune regulation and a significant role in disease pathogenesis. This study aims to achieve high-efficiency expression of IL-33 in mammalian cells through codon optimization, providing a foundation for research on disease mechanisms and vaccine adjuvant applications. Methods: Based on the amino acid sequence of mouse interleukin-33 mature peptide (mIL-33), gene optimization was performed according to codon usage bias in mammalian cells. The optimized mIL-33 gene fragment was chemically synthesized. The nucleotide sequence encoding human CD8 signal peptide was fused to both optimized and unoptimized mIL-33 genes through splice-overlap extension PCR. These constructs were individually cloned with the enhanced green fluorescent protein (EGFP) gene under different promoters into the dual-expression plasmid pBudCE4.1. The recombinant plasmids were transfected into 293FT cells using Lipofectamine 3000 and PEI. Recombinant protein expression was detected by Western blot and ELISA. The expressed mIL-33 was collected to stimulate Raw264.7 macrophages, and TNF levels in the culture supernatant were measured by ELISA to verify the biological activity of IL-33. Results: Restriction enzyme digestion and sequencing analysis confirmed successful construction of the recombinant plasmids. Lipofectamine 3000 exhibited higher transfection efficiency than PEI. Western blot and ELISA results demonstrated that codon-optimized mIL-33 achieved higher expression levels compared to the unoptimized sequence. Expression levels of mIL-33 in 293FT cells were comparable under the EF-1 promoter and CMV promoter. The CD8 signal peptide successfully directed the secretion of mIL-33, and the product exhibited biological activity. Conclusion: Codon optimization significantly improved the expression level of mIL-33 in mammalian cells, establishing a foundation for further research.

Full Text

Preamble

Efficient Secretory Expression of Codon-Optimized Mouse IL-33 Gene in Mammalian Cells

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Abstract

Objective: Interleukin (IL)-33 plays crucial immunoregulatory roles and is implicated in various diseases. This study aimed to achieve high-level expression of IL-33 in mammalian cells through gene optimization to provide a foundation for mechanistic disease research and vaccine adjuvant applications.

Methods: Based on the amino acid sequence of mouse IL-33 mature peptide (mIL-33), we performed codon optimization according to mammalian cell expression preferences. The optimized mIL-33 gene fragments were chemically synthesized and fused with the human CD8 signal peptide coding sequence via bridge PCR. Both optimized and unoptimized versions were constructed into different promoter units of the dual-expression plasmid pBudCE4.1, along with the EGFP gene. Recombinant plasmids were transfected into 293FT cells using either Lipofectamine 3000 or PEI. Recombinant protein expression was detected by Western blot and ELISA. The expressed mIL-33 was collected to stimulate Raw264.7 macrophages, and TNF- levels in the culture supernatant were measured by ELISA to verify biological activity.

Results: Restriction digestion and sequencing confirmed successful construction of recombinant plasmids. Lipofectamine 3000 showed higher transfection efficiency than PEI. Western blot and ELISA results demonstrated that codon-optimized mIL-33 achieved significantly higher expression levels than the unoptimized sequence, with comparable expression under both EF-1 and CMV promoters. The CD8 signal peptide successfully directed mIL-33 secretion, and the expressed product exhibited biological activity.

Conclusion: Codon optimization substantially improved mIL-33 expression levels in mammalian cells, establishing a foundation for further research.

Keywords: expression; optimization; mature interleukin-33 (mIL-33); 293FT cells

Introduction

IL-33, first isolated from high endothelial venules by Baekkevold and identified as a novel IL-1 family member by Schmitz in 2005, is constitutively expressed in various tissue cells. As a dual-function factor with both nuclear localization and secretory capacity, IL-33 primarily participates in initiating immune responses and regulating Th1/Th2-type immunity. Under normal conditions, full-length IL-33 (approximately 30 kD) localizes to the nucleus and functions as a transcription factor. However, when tissue cells undergo damage or stress, the biologically active mature fragment of IL-33 is secreted via a non-classical, signal peptide-independent mechanism. Upon release, it binds to its specific receptor ST2 and exerts cytokine functions. As an “alarmin” that triggers downstream immune responses upon sensing external stimuli, IL-33 induces type 2 immune responses in innate lymphoid cells (ILC2), M2 macrophages, and Th2 cells, and acts on neutrophils and eosinophils to initiate and promote inflammation. Additionally, IL-33 can enhance Th1/CTL immune responses, playing important roles in antiviral and antitumor cellular immunity. Recent studies have revealed that IL-33 also promotes differentiation, proliferation, and tumor infiltration of myeloid-derived suppressor cells (MDSCs), thereby contributing to the formation and development of immunosuppressive tumor microenvironments. Given its multifaceted functions and critical regulatory roles in both innate and adaptive immunity, the specific mechanisms through which IL-33 operates in particular diseases such as cancer, inflammatory disorders, autoimmune diseases, and cardiopulmonary conditions remain incompletely understood and require further investigation.

Since IL-33’ s functional outcomes are closely related to its spatiotemporal expression patterns within specific tissues, pathological environments, and disease states, this study aimed to achieve efficient secretory expression of mouse IL-33 mature protein in mammalian cells through gene optimization. This approach provides a foundation for controlled IL-33 expression in specific disease model contexts to investigate its functional mechanisms and immunological influences, while also facilitating its application in nucleic acid and vector-based vaccines to enhance antiviral or antitumor cellular immune responses.

Materials and Methods

1.1 Plasmids, Bacterial Strains, and Cell Lines

The dual-expression plasmid pBudCE4.1 was purchased from Invitrogen. *E. coli* DH5 competent cells were obtained from Beijing TransGen Biotech. The human embryonic kidney cell line 293FT was maintained in our laboratory (Molecular Immunology Laboratory, Institute of Medical Biology, Chinese Academy of Medical Sciences), and the mouse macrophage cell line Raw264.7 was kindly provided by Dr. Chen Bin from the Second Affiliated Hospital of Kunming Medical University.

1.2 Reagents

DNA markers, Taq polymerase, T4 DNA ligase, and restriction enzymes were purchased from TaKaRa (Dalian). Plasmid extraction and DNA gel recovery kits were obtained from Tiangen Biotech (Beijing). Rabbit anti-IL-33 polyclonal antibody and HRP-labeled goat anti-rabbit IgG were from Santa Cruz Biotechnology (USA). Pierce ECL substrate was from Thermo Fisher Scientific (USA). Biotin-labeled goat anti-mouse IL-33 and TNF- antibodies were from eBioscience. HRP-labeled streptavidin was from Beyotime Biotechnology. TMB single-component substrate was from Solarbio. IL-33 standard was from PeproTech. Lipofectamine 3000 transfection reagent was from Invitrogen, and PEI transfection reagent was from Proteintech. DMEM medium and fetal bovine serum were from Biological Industries.

1.3 Design of IL-33 Gene Sequences and Primers

Based on the amino acid sequence from GenBank [AY905582.1], we designed a codon-optimized mouse mature IL-33 gene sequence according to mammalian cell codon preferences. The design avoided rare codons (synonymous codon usage frequency <0.1) and restriction enzyme sites used in cloning, while preferentially selecting optimal codons. Both optimized and unoptimized wild-type gene sequences were synthesized by Sangon Biotech (Shanghai) and cloned into pUC57. Since full-length IL-33 is a nuclear-localized protein released only by stressed cells, we fused the human CD8 signal peptide coding sequence [NM_171827.3] to mIL-33 (S109 to I266) via bridge PCR for cloning into the pBudCE4.1 vector. EGFP primers were designed based on GenBank sequence [LC008492.1] and synthesized by Shanghai Sangon, as listed in .

1.4 Amplification of CD8 +mIL-33 and EGFP Genes

Using pUC57/IL-33 (Not optimized) as template, PCR amplification was performed with primers IL-33-F1, IL-33-F2, IL-33-F3, and IL-33-R1. Using pUC57/IL-33 (optimized) as template, amplification used primers IL-33-F1, IL-33-F2, IL-33-F4, and IL-33-R2. These reactions yielded optimized and unoptimized IL-33 gene fragments for cloning into the PCMV expression unit of pBudCE4.1. PCR conditions were: 95°C for 3 min; 34 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; final extension at 72°C for 10 min; hold at 12°C. Products were verified by 1% agarose gel electrophoresis.

Additionally, using constructed pBudCE4.1/CD8 +mIL-33 (PCMV) as template, PCR with primers IL33-F5 and IL33-R amplified the optimized IL-33 gene fragment for cloning into the PEF-1 expression unit of pBudCE4.1. Conditions were: 95°C for 3 min; 34 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min; final extension at 72°C for 10 min; hold at 12°C.

Using pLvcT as template, EGFP-F and EGFP-R primers amplified the EGFP gene for insertion into the PEF-1 expression unit of pBudCE4.1. Conditions were: 95°C for 3 min; 34 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; final extension at 72°C for 10 min; hold at 12°C. PCR products were verified by 1% agarose gel electrophoresis.

1.5 Construction and Identification of Recombinant Expression Plasmids

The pBudCE4.1 plasmid was digested with PstI+BamHI and KpnI+BglII. PCR-amplified CD8 +mIL-33 and EGFP fragments were similarly digested. After gel recovery, vector and fragments were ligated with T4 DNA ligase at 16°C overnight, transformed into DH5 competent cells, and plated on Zeocin-containing LB agar. Single colonies were selected, cultured, and plasmids were extracted. Recombinant plasmids were identified by double digestion with PstI+BamHI or KpnI+BglII and 1% agarose electrophoresis, then sequenced by Sangon Biotech (Shanghai).

Specific constructs included: pBudCE4.1/CD8 +mIL-33(PCMV)/EGFP(PEF-1) co-expressing mIL-33 and EGFP ([Figure 1: see original paper]), and pBudCE4.1/CD8 +mIL-33(PEF-1) expressing only mIL-33 ([Figure 2: see original paper]).

[Figure 1: see original paper]
[Figure 2: see original paper]

1.6 Transfection of 293FT Cells

293FT cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. One day before transfection, cells were seeded at 1×10 cells/well in 6-well plates and incubated at 37°C with 5% CO . When cells reached 70-80% confluence, transfection was performed. For Lipofectamine 3000, 7.5 l/well reagent and 2.5 g/well plasmid were diluted in Opti-MEM, mixed 1:1, incubated 15 min, then added to cells. For PEI, 7.5 g/well reagent and 2.5 g/well plasmid were similarly prepared. Plasmids expressing mIL-33 and EGFP, empty vector controls, and blank controls (no plasmid) were transfected. Cells were observed under fluorescence microscopy at 24 h and 48 h, and harvested at 48 h.

1.7 Protein Detection

1.7.1 Western Blot Cell culture supernatants were collected, and cells were lysed with RIPA buffer. Proteins were separated by 12% SDS-PAGE, transferred to PVDF membrane at constant voltage (20 V) for 1 h, blocked with 5% skim milk in TBS (50 mmol/L Tris, pH 7.2) for 2 h at room temperature, then incubated with rabbit anti-mouse IL-33 polyclonal antibody (1:1000) overnight at 4°C. After washing, HRP-labeled goat anti-rabbit secondary antibody (1:8000) was added and incubated for 1 h at room temperature. Bands were visualized by ECL chemiluminescence.

1.7.2 ELISA A sandwich ELISA was performed according to eBioscience protocols. Briefly, 96-well plates were coated with IL-33 capture antibody (1:250) at 50 l/well overnight at 4°C in a humidified chamber. After washing three times with PBST (0.5‰ Tween-20 in PBS, pH 7.2), plates were blocked with 2% BSA in PBST for 2 h at room temperature. Following three washes, 293FT cell lysates and supernatant samples (1:100 dilution) were added with standard curve controls and incubated for 1 h at room temperature. After washing, biotinylated secondary antibody (1:250) was added and incubated at 37°C for 2 h, followed by HRP-labeled streptavidin (1:10,000) for 1 h at 37°C. After final washes, TMB substrate was added and incubated at room temperature for 10-30 min protected from light. Reactions were stopped with 1 M sulfuric acid (50 l/well) and absorbance was measured at 450 nm.

1.8 Biological Activity Assay of mIL-33

Commercial recombinant mouse IL-33 and eukaryotically expressed mIL-33 (quantified by ELISA) were serially diluted from 50 ng/ml to stimulate Raw264.7 macrophages (1×10 cells/ml) for 12 h. Supernatants were collected and TNF- levels were measured by sandwich ELISA (eBioscience) as described in 1.7.2 to calculate the half-maximal effective concentration (EC).

Results

2.1 Codon Optimization of mIL-33 Coding Sequence

Based on the IL-33 amino acid sequence, we generated a codon-optimized gene sequence for mammalian cell expression. A comparison of unoptimized and optimized sequences is shown in . To analyze the relationship between codon distribution patterns and protein expression levels, we first referenced codon usage frequency tables for IL-33 from an online database (http://www.enscript.com/cgi-bin/tools/codon_req_table), then analyzed codon distribution using another web tool (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis). The codon quality distribution for mIL-33 sequences in mammalian cells is displayed in [Figure 3: see original paper], where the x-axis indicates codon preference quality. Additionally, mRNA folding free energy and GC content were predicted using http://rna.tbi.univie.ac.at/ (). The optimized gene contained over 80% optimal codons and >95% codons with quality scores above 70, compared to ~45% and ~65% respectively for the unoptimized sequence, which also contained several rare codons. The optimized sequence exhibited higher, more uniform GC content and lower free energy, indicating greater mRNA stability.

[Figure 3: see original paper]

2.2 Identification of Recombinant Expression Plasmids

Restriction digestion analysis showed that pBudCE4.1/CD8 +mIL-33/EGFP and pBudCE4.1/CD8 +mIL-33 digested with PstI+BamHI or KpnI+BglII yielded bands of approximately 550 bp and 750 bp, matching the expected sizes of 552 bp for CD8 +mIL-33 and 738 bp for EGFP ([Figure 4: see original paper]). Sequencing confirmed correct insertion and sequence fidelity of the CD8 +mIL-33 and EGFP fragments.

[Figure 4: see original paper]

2.3 Transfection of Recombinant Plasmids into 293FT Cells

Empty pBudCE4.1 vector and recombinant plasmids pBudCE4.1/CD8 +mIL-33 and pBudCE4.1/CD8 +mIL-33/EGFP were transfected into 293FT cells. Green fluorescence was observed at 24 h and 48 h post-transfection, with increasing fluorescent cell proportions over time. Lipofectamine 3000 demonstrated higher transfection efficiency than PEI ([Figure 5: see original paper]).

[Figure 5: see original paper]

2.4 Expression Product Identification

Western blot analysis of transfected 293FT cell lysates showed specific mIL-33 bands at the expected molecular weight in cells transfected with pBudCE4.1/CD8 +mIL-33 (Not optimized/optimized)(PCMV)/EGFP and pBudCE4.1/CD8 +mIL-33 (optimized)(PEF-1), but not in empty vector controls ([Figure 6: see original paper]). Expression from optimized plasmids was markedly enhanced compared to unoptimized versions, with comparable expression levels between EF-1 and CMV promoter units.

[Figure 6: see original paper]

ELISA quantification of transfected cell lysates and culture supernatants revealed mIL-33 concentrations of 561.15 ng/ml vs. 1553.5 ng/ml in lysates, and 21.97 ng/ml vs. 78.63 ng/ml in supernatants for unoptimized versus optimized sequences, respectively. Codon-optimized mIL-33 expression in 293FT cells was approximately 3-fold higher than unoptimized sequences (statistically significant by one-way ANOVA). Detection of IL-33 in culture supernatants confirmed successful secretion mediated by the CD8 signal peptide ([Figure 7: see original paper]).

[Figure 7: see original paper]

2.5 Biological Activity Detection of IL-33

Commercial recombinant IL-33 and eukaryotically expressed mIL-33 (quantified by ELISA) were serially diluted from 50 ng/ml to stimulate Raw264.7 macrophages. TNF- levels in culture supernatants were measured to generate dose-response curves. The EC values were approximately 3.037 ng/ml for

IL-33 standard and 6.152 ng/ml for recombinant expressed IL-33, demonstrating that 293FT cell-expressed IL-33 mature protein possesses biological activity ([Figure 8: see original paper]).

[Figure 8: see original paper]

Discussion

IL-33, an IL-1 family cytokine constitutively expressed in the nuclei of epithelial and endothelial cells, functions as an “alarmin” that is highly expressed and released extracellularly under cellular damage, stress, or necrotic pressure. It plays crucial regulatory roles in both innate and adaptive immunity. Early IL-33 research focused on allergic inflammation and asthma, identifying it as a key driver of Th2 responses. Recent studies demonstrate that IL-33 significantly enhances Th1 and CD8 T cell immune responses in tumor vaccine research, inducing antitumor immunity, and IL-33 signaling is essential for CD8 T cell-mediated viral clearance. However, IL-33 also promotes MDSC differentiation, proliferation, and tumor infiltration, contributing to immunosuppressive tumor microenvironments. Thus, IL-33 is a multifunctional, disease-critical regulatory factor with potential as a clinical intervention target or therapeutic agent.

This study established efficient IL-33 expression in mammalian systems through codon optimization to facilitate mechanistic studies and vaccine adjuvant applications in mouse models. Since codon usage frequency is non-uniform across species, with most organisms preferentially utilizing a subset of codons, codon optimization exploits this bias to enhance expression. Host cell tRNA abundance correlates with codon usage frequency, favoring high-level protein synthesis. Codon optimization also alters mRNA composition and secondary structure, affecting folding and stability. While mRNA secondary structure prediction for expression optimization lacks reliable theoretical guidance, some reports suggest free energy prediction can estimate protein expression levels. We analyzed GC content, distribution, and free energy of unoptimized and optimized sequences, finding that reduced free energy (enhancing mRNA stability) and uniform GC distribution within an optimal range likely benefit high-level expression.

Promoter selection is critical for eukaryotic gene expression, determining transcription levels. However, different promoters exhibit varying transcriptional activity across cell types due to methylation and other regulatory factors. We therefore compared IL-33 expression under CMV and EF-1 promoters in 293FT cells, finding similar high-level expression from both. We also compared Lipofectamine 3000 and PEI transfection efficiency, using co-expressed EGFP as a transfection marker. While Lipofectamine 3000 showed higher efficiency ([Figure 5: see original paper]), PEI’ s lower cost provides a scalable alternative for large-scale transfections.

Western blot confirmed correct IL-33 molecular weight, and ELISA demonstrated successful secretion of mature IL-33 under CD8 signal peptide guidance. Previous studies reported recombinant IL-33 secretion levels of ~20,000 pg/ml using IgE signal peptide and ~600 pg/ml using full-length IL-33 in human rhabdomyosarcoma (RD) cells. Our unoptimized sequence achieved 21.97 ng/ml secretion, similar to these reports, suggesting signal peptide type does not dramatically affect expression levels. However, our optimized sequence reached 78.63 ng/ml, significantly higher than both our unoptimized version and literature values, confirming codon optimization as an effective strategy for enhancing heterologous gene expression. Furthermore, stimulation of RAW264.7 macrophages with expressed IL-33 induced dose-dependent TNF- production, confirming biological activity.

In summary, we successfully constructed a pBudCE4.1 eukaryotic expression plasmid for high-level secretory expression of biologically active IL-33 mature protein through codon optimization. This work establishes a foundation for functional studies and immunoadjuvant evaluation of IL-33, while providing a reference for efficient expression of foreign genes in mammalian cells.

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Note: Figure translations are in progress. See original paper for figures.