Article

cKit Ligand (Stem Cell Factor)-Induced Immune Response via an Alternative Receptor in Human Cells

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Abstract: cKit (also known as CD117) is known for stem cell factor (SCF) receptor and it is involved in bone marrow hematopoietic stem cell (HSC) proliferation as well as mast cell growth. We have cloned the cDNA of cKit ligand (cKit-L; also known as SCF) from human keratinocyte HaCaT cells. Recombinant HaCaT cKit-L protein was produced and characterized whether it is active on HMC-1 cell expressing very high level of cKit transcript. Interestingly, HMC-1 cells did not respond to its ligand HaCaT cKit-L, whereas the same recombinant HaCaT cKit-L induced inflammatory chemokine interleukin 8 (IL-8) in human THP-1 monocyte cells that do not express cKit (also known as CD117). We wonder if HMC-1 is defective in the production of inflammatory molecules. Nevertheless, HMC-1 cells produce significant amounts of human IL-8 in response to IL-1α and IL-33. Commercial cKit-L was used to confirm these results, and the activity of commercial cKit-L was very similar to that of HaCaT cKit-L. These data suggest that the known cKit-L receptor cKit is required for cKit-L activity but is not sufficient to complete the immunological activity of cKit-L. It is possible that alternative cKit-L receptors exist that are responsible for inducing IL-8 in human and mouse immune cells.

Keywords: cKit; SCF; cKit ligand; HMC-1; THP-1

1. Introduction

cKit ligand (cKit-L; also known as stem cell factor (SCF)) is a membrane-bound cytokine that interacts with cKit (also known as CD117) to induce various signaling in the early stages of immune cells [1–5]. cKit-L occurs as both a transmembrane protein and a soluble protein [6,7]. Soluble cKit-L is a membrane-bound form that possesses a proteolytic cleavage site in exon 6 and whose cleavage releases the extracellular domain of the protein [8–10]. The transmembrane form of cKit-L is an alternatively spliced transcript without exon 6, while the cleavage site is lost, and it remains in the membrane-bound form. HoweveSSr, both forms of cKit-L interact with cKit and are biologically active. Soluble and transmembrane cKit-L is produced by fibroblasts and endothelial cells. Soluble cKit-L has a molecular mass of 18.5 kDa, forms a dimer, and is detected at 3.3 ng/mL in normal human serum [11–13].

cKit-L has a function in the hematopoietic activity during embryonic advancement. Hematopoietic sites such as fetal liver and bone marrow that express cKit-L serve as direct guides for hematopoietic stem cells (HSCs) and play a critical role in HSC maintenance [14,15]. Point mutations in the cKit-L receptor cause anemia and reduced fertility [16]. cKit-L receptor is expressed in primordial germ cells, spermatogonia, and primordial oocytes s[17]. cKit-L receptor is also expressed in the primordial germ cells of females. cKit-L is expressed along the pathways

that germ cells use to reach their destinations in the body, helping guide cells such as melanoblasts to the appropriate location in the body [18,19].

cKit-L on the stromal cells surrounding HSCs plays a role in regulating HSCs in the bone marrow. cKit-L had been shown to increase HSC survival in vitro and contribute to HSC self-renewal and maintenance in vivo. The cKit-L receptor is expressed at equal levels at all stages of HSC development [19,20]. A small proportion of HSCs regularly leave the bone marrow in the circulation and then return to the intramedullary niche. A concentration gradient of the cKit-L receptor along with the chemokine SDF-1 allows HSCs to return to their niche [21–23].

Mast cells are the only terminally differentiated blood cells expressing the cKit-L receptor. cKit-L or its receptor mutations cause dramatic defects in the production of mast cells. Conversely, injection of cKit-L increases the number of mast cells near the injection site by more than 100-fold. In addition to this cKit-L promotes mast cell attachment, migration, proliferation and survival as well as enhances histamine and tryptase release associated with allergic reactions [24–26].

cKit, a receptor tyrosine kinase, is the receptor for cKit-L. cKit is expressed on HSCs, mast cells, melanocytes, and germ cells, as well as on hematopoietic progenitor cells, including erythrocytes, myeloblasts, and megakaryocytes. On the other hand, mast cell expression decreases as hematopoietic cells mature, and when these cells are fully differentiated, cKit is absent. cKit-L binding to cKit induces cKit to homodimerize and autophosphorylate at tyrosine residues. The activation of cKit triggers multiple signaling cascades, including RAS/ERK, PI3-kinase, Src kinase, and JAK/STAT pathways [1–5,27,28]. HMC-1 cells with high expression of cKit did not respond to newly cloned HaCaT cKit-L and commercial cKit-L. Therefore, our results hypothesize an alternative pathway or receptor for cKit-L activity in THP-1 cells (Graphical Abstract).

2. Material and Methods

2.1. RT-PCR of cKit and cKit-L

Briefly, cDNA generation was performed from total RNA from HMC-1, K562, THP-1, U937, HaCaT, PC3, Hela, HEK293T, and A549 cells using the MMLV reverse transcription kit (Millipore Sigma, St. Louis, MO, USA) as described [29] and according to the manufacturer's instructions. Forward and reverse primers were used to amplify cKit (forward: 5′-CGGATCCCATCGCAGCTAC -3′ and reverse: 5′-CTGCTCAGACATCGTCGTGC -3′) and the cKit-L (forward: 5′-ACACCACTGTTTGTGCTGG-3′ and reverse: 5′-CTGTTACCAGCCA ATGTACGA -3′) are shown in Figures 1 and 2A. Normalization was based on GAPDH using the following primers; forward: 5′-ACCACAGTCCATGCCATCAC-3′ and reverse: 5′-TCCACCACCCTGTTGCTGTA-3′.

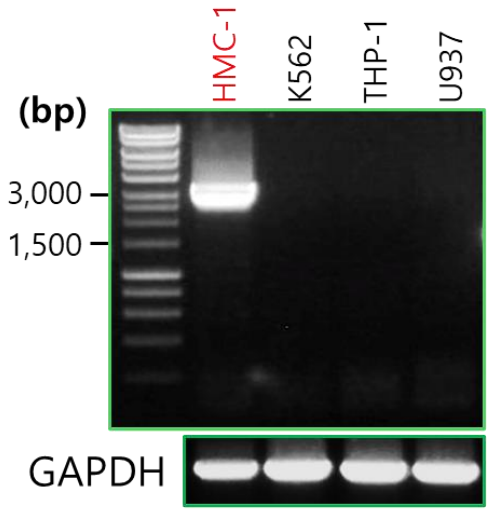

Figure 1. cKit expression in different human cell lines. Expression of cKit was examined by RT-PCR using RNA isolated from four representative human immune cell lines. Each human cell line was indicated at the top of the picture. The expected size of the full-length cKit is 2958 bp.

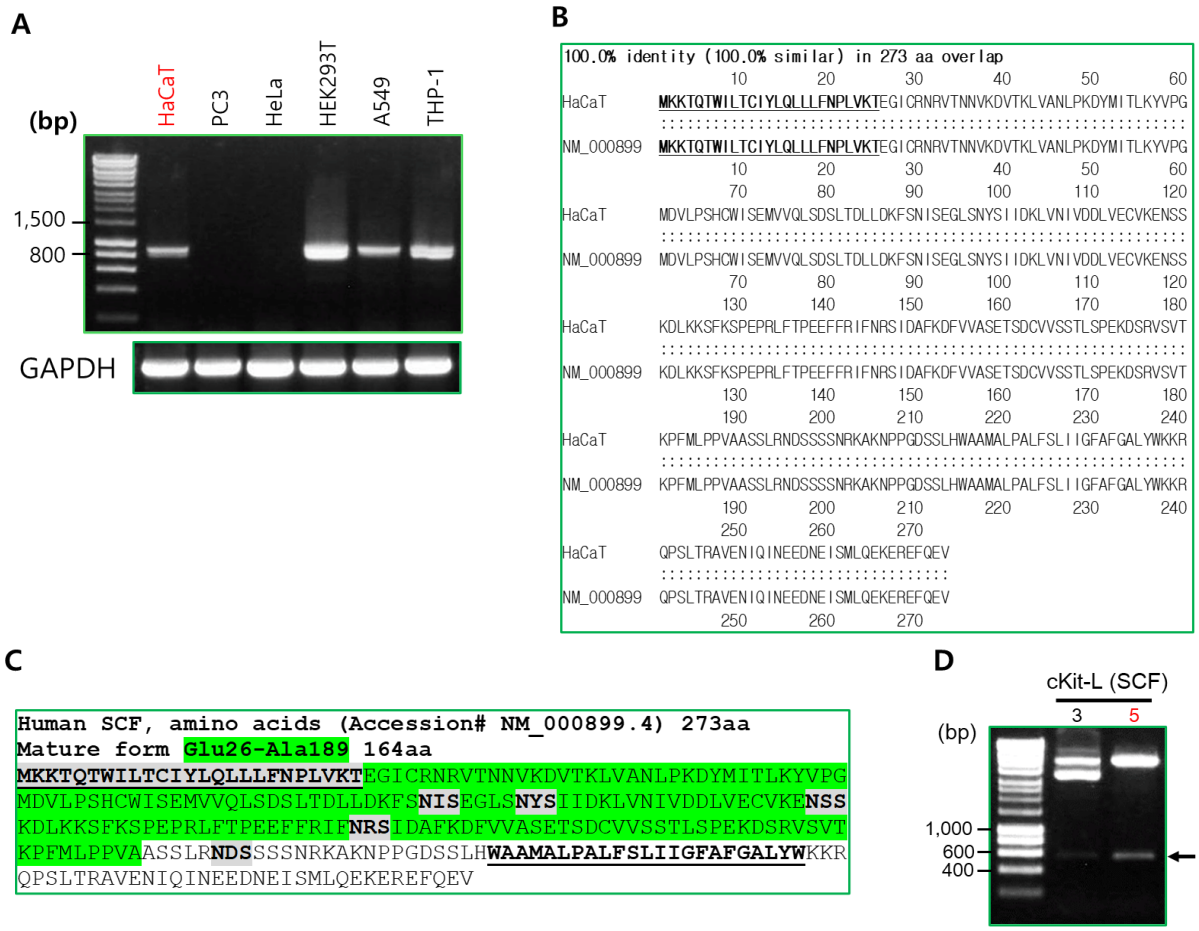

Figure 2. cKit-L expression in different human cell lines. (**A**) Expression of cKit-L was examined by RT-PCR using RNA isolated from six human immune and non-immune cell lines. Each human cell line was indicated at the top of the picture. The expected size of the full-length cKit-L is 910 bp. (**B**) HaCaT cell PCR product was TA cloned to obtain cDNA for recombinant cKit-L. The cloned HaCaT cKit-L was translated into amino acids by DNA sequence analysis and found to be 100% identical to cKit-L in the database (accession no. NM_000899.4). (**C**,**D**) The mature form of cKit-L at Glu26-Ala189 (highlighted in green) was used for recombinant cKit expression in *E. coli*. pET/21a/HaCaT cKit-L plasmid vector digested with restriction enzymes Nde I and Xho I. The potential glycosylation sites are highlighted by bold letters with gray color.

2.2. TA Cloning and Restriction Enzyme Map

The PCR product of the cKit-L was ligated into T&A cloning vector according to the manufacturer's instructions (DonginBiotech, Seoul, Republic of Korea). The positive plasmid vector (2 μg) was digested with Hind III restriction enzyme according to the manufacturer's instructions (Takara, Japan) with provided buffer to release the insert of cKit-L. The insert of cKit-L gene cDNA was detected by 1% agarose (Biobase, Minneapolis, MN, USA) photographed by Vilber lourmat Electronic Ballast transilluminators (Millipore Sigma).

2.3. Mini-Prep and DNA Sequence Analysis

The positive clone from colony PCR screening was cultured in 3 mL volume of LB broth overnight at 37 °C. The bacterial cells were used to isolate plasmid DNA according to manufacturer's instructions (Promega, Madison, WI, USA). The plasmid was digested with Nde I and Xho I restriction enzyme to confirm the insert cKit-L gene. The positive cKit-L plasmid was sent for DNA sequence analysis (Cosmotech, Seoul, Republic of Korea). These expression vectors were transformed into BL21-Codon Plus from Stratagene (San Diego, CA, USA) by heat shock method.

2.4. Cloning and Recombinant Protein Expression

cKit-L from the TA cloned HaCat cDNA was cloned into pET/21a from Takara (Shiga, Japan) as previously described [30]. Briefly, BL21-Codon Plus cells from Stratagene (San Diego, CA, USA) were transformed via heat

shock method with the expression vectors. pET/21a/cKit-L plasmid into was used to express in *E. coli* with 4 h IPTG induction at 37 °C. pET/21a/cKit-L were purified with a TALON® Magnetic Bead (Takara) using his^6-tag at the C-terminus of cKit-L proteins. The affinity-purified recombinant cKit-L protein was subjected to high-performance liquid chromatography (HPLC-RP) column from Grace (Stockbridge, GA, USA). After that, we checked its concentration by silver staining and Bradford assay. The bands of purified recombinant cKit-L proteins were examined. The recombinant cKit-L protein was tested with LAL chromogenic endotoxin quantitation kit from Thermo fisher (Waltham, MA, USA). The endotoxin level was below 0.5 EU per 1 μg of recombinant cKit-L proteins, that is approximately 0.02 ng in 1 μg of cKit-L. Westen blots of recombinant HaCat cKit-L were compared to commercial cKit-L using an anti-cKit-L antibody from MyBioSource (San Diego, CA, USA).

2.5. Cell Culture and Cytokine Assays

Human HMC-1, K562, U937, THP-1, and mouse Raw 264.7 cell lines are obtained from ATCC (Manassas, VA). HMC-1 cells were cultured in Iscove's Modified Dulbecco medium, and other cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 μg/mL streptomycin. The culture condition was the same as follows: under humidified 5% CO_2 at 37 °C. Human cells (2.5×10^4/well) whereas mouse Raw 264.7 cells (5.0×10^4/well) were seeded in 96-well plate in 100 μL volume. The cells were treated with increasing concentrations of purified HaCaT and commercial cKit-L from BioLegend San Diego, CA (100 ng/mL, 200 ng/mL, and 500 ng/mL) in 100 μL volume media and control is media alone. The 200 ng/mL of other stimuli LPS (LPS O127:B8, Millipore Sigma), human IL-1α (YbdYbiotech, Seoul, Republic of Korea), and human IL-33 (YbdYbiotech) were added to 100 μL volume medium, and the control was medium alone. After 18 h of stimulation, supernatants from different cell lines were assessed for human IL-8 and mouse TNFα measurements. The cytokines were determined by ELISA kits from R&D system, Minneapolis, MN.

2.6. Statistical Analysis

All data were analyzed by Graph Pad Prism v.9 to perform one-way or two-way ANOVA followed by Tukey's post-hoc analysis, p values < 0.05 were considered statistically significant and were indicated in the figure legends.

3. Result

3.1. cKit and cKit-L Expression in Different Human Cells

As a result of examining the expression of cKit in four human immune cell lines by RT-PCR, a large amount of human cKit transcript was confirmed in HMC-1 cells as expected, but cKit was not detected in the other three immune cells, K562, THP-1, and U937. (Figure 1). cKit high-expressing human HMC-1 mast cell line is distinct from the other three immune cell lines. The K562 cell line is of the erythroleukemic type, while the THP-1 and U937 cell lines are of the monocytic type. Normalization was based on the *GAPDH* housekeeping gene, resulting in each sample containing equal amounts of total transcripts.

Six different human cell lines were used for examining cKit-L expressions. As shown in Figure 2A, significant amounts of cKit-L transcript were detected by RT-PCR in HaCaT, HEK293T, A549, and THP-1 cell lines, whereas PC3 and Hela cell lines did not express it. The PCR product of HaCaT, indicated in red letters, was used for TA cloning to obtain cKit-L cDNA for expressing recombinant cKit-L. The cloned HaCaT cKit-L cDNA was DNA sequencing, and the translated amino acid sequence was aligned with the know cKit-L (accession No NM_000899.4). The results show that HaCaT cKit-L shares 100% homology with the cKit-L sequence in the database. The 25 amino acids of the N-terminal signal peptide are indicated in bold (Figure 2B). Eventually, the mature form of human cKit-L, marked with green highlighting Glu26-Ala189, lacking the N-terminal signal peptide and also excluding the C-terminal membrane-associated region, was cloned into an *E. coli* expression vector (Figure 2C). Mini-preps of the pET21a/HaCaT cKit-L clones 3 and 5 were digested with restriction enzymes Nde I and Xho I. Clone 3 (red letters) released an insert of the correct size (577 bp) and this clone was sent for DNA sequence analysis (Figure 2D).

3.2. Expression of Recombinant cKit-L Protein

N/C-terminally trimmed mature HaCaT cKit-L Glu26-Ala189 was cloned at the C-terminus 6 × his-tag pET21a *E. coli* expression vector and transformed into BL21-$codon^+$ competent cells for recombinant cKit protein expression. Recombinant mature HaCaT cKit-L Glu26-Ala189 was purified by Talon affinity column and

visualized by Coomassie blue staining (Figure 3A). In performing the experiment, the elution fraction from the mini-Talon column was first measured using Bradford analysis at bottom, and then the molecular weight and purity of the protein expressed under non-reducing (left) and reducing (right) conditions were confirmed using Coomassie blue staining. The majority of expressed recombinant mature HaCaT cKit-L Glu26-Ala189 eluted at approximately 1 mg in a 1 mL volume in elution 2, which is indicated at the bottom. The predicted molecular mass of mature HaCaT cKit-L Glu26-Ala189 is 19.7 kDa (indicated by arrow) was observed under both non-reducing and reducing conditions, whereas before and after Talon column total lysate containing all sizes of *E. coli* proteins (Figure 3A).

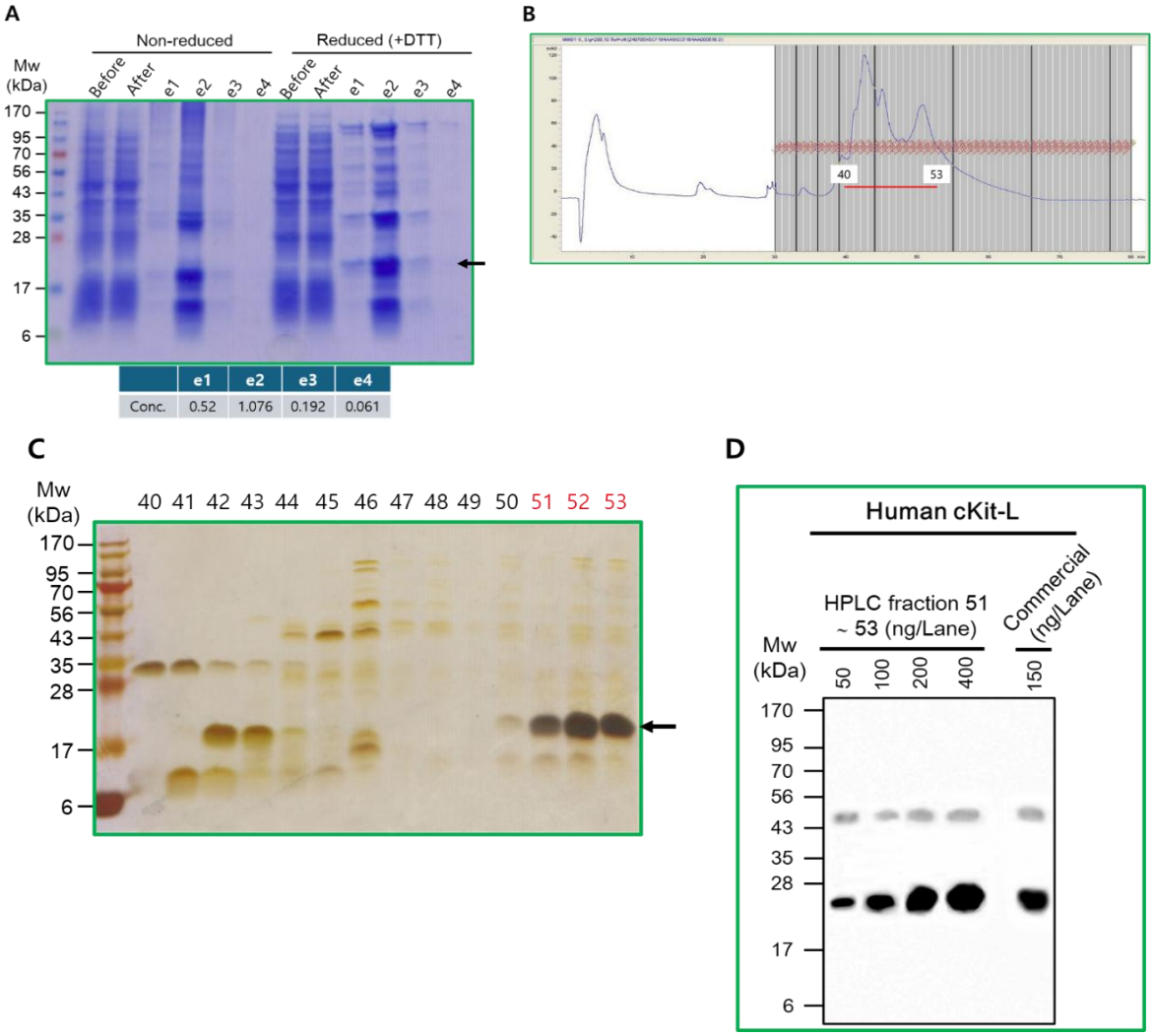

Figure 3. Recombinant cKit-L protein expression. (**A**) Coomassie blue staining of HaCaT cKit-L after Talon affinity purification. The predicted molecular mass is 19.7 kDa (indicated by arrow) and the theoretical isoelectric point (pI) is 5.78. Bradford analysis of recombinant cKit-L from each elution is shown at the bottom. (**B**) The three fractions e1, e2, and e3 were collected and subjected to secondary purification using high-performance liquid chromatography (HPLC). Ultraviolet (UV) absorption at 280 nm is the method used to label fractions 40–53 containing recombinant cKit-L, indicated by red bars. (**C**) HPLC 40–53 fractions were lyophilized and visualized by silver staining. Fractions 51–53 represented the mature cKit-L recombinant protein indicated by arrow. (**D**) Pooled HaCaT cKit-L HPLC fractions 51, 52, and 53 were loaded into each lane at the indicated concentrations to compare commercial cKit-L. Western blot showed similar protein concentrations and molecular weights.

3.3. Purification and Qualification of Recombinant cKit-L Protein

The elution 1–3 was subjected to a second purification due to multiple bands in the first Talon chromatography. HPLC with ultraviolet (UV) absorption at 280 nm detects mature HaCaT cKit-L Glu26-Ala189 in fractions 40–53, which contain recombinant HaCaT cKit-L, indicated by red bars (Figure 3B). HPLC fractions 40–53 was lyophilized and loaded on 10%-SDS-PAGE under reducing conditions. Two-step purification mature recombinant HaCaT cKit-L Glu26-Ala189 (fractions 51–53) is visualized by silver staining. Mature HaCaT cKit-

L Glu26-Ala189 recombinant protein indicated by arrow (Figure 3C). HPLC fractions 51, 52, and 53 were individually determined for protein concentration by Bradford assay prior to use in cell stimulation assays. Estimated protein concentrations of pooled HPLC fractions 51, 52, and 53 were loaded for Western blot analysis and detected with anti-cKit-L antibody (Figure 3D). Western blot showed that the HaCaT cKit-L Glu26-Ala189 recombinant protein was very similar to the commercial recombinant cKit-L protein in molecular weight as well as concentration.

3.4. The Biological Activity of Commercial and Mature HaCaT cKit-L Glu26-Ala189

The mature HaCaT cKit-L Glu26-Ala189 and commercial cKit-L were used to stimulate three different human cell lines: HMC-1, THP-1, and U937. First, the commercial cKit-L was used to stimulate cKit high expression cell, human mast HMC-1 cells, as well as THP-1 and U937 cells that do not express cKit, known as cKit-L receptor (Figure 1). Interestingly, cKit high expression cell, human mast HMC-1 cells do not respond to HaCaT cKit-L Glu26-Ala189 whereas human monocytic THP-1 cells do respond to HaCaT cKit-L Glu26-Ala189 at a high concentration of 500 ng/mL (Figure 4A). To confirm this result, we repeated the experiment using the commercial cKit-L. As shown in Figure 4B, IL-8 induction in THP-1 cells was reproducible.

We wonder whether cell lines that did not respond to cKit-L are defective in IL-8 induction. Therefore, HMC-1, THP-1 and U937 cells were treated with different stimuli: IL-1α, LPS, and IL-33. IL-1α induced IL-8 in all three cell lines, and LPS induced IL-8 in both THP-1 and U937 but failed to induce IL-8 in HMC-1 cells. In contrast to IL-1α and LPS, the Th2 cytokine-related mast cell activation known as IL-33 induced significant amounts of IL-8 in human mast HMC-1 cells. (Figure 4C). Additionally, both HaCaT cKit-L Glu26-Ala189 and commercial cKit-L were tested with mouse macrophage Raw 264.7 cells. Both HaCaT cKit-L Glu26-Ala189 and commercial cKit-L induced mouse TNFα in a dose-dependent manner (Figure 4D). The other three stimuli were also active in inducing mouse TNFα in mouse macrophage Raw 264.7 cells (Figure 4E).

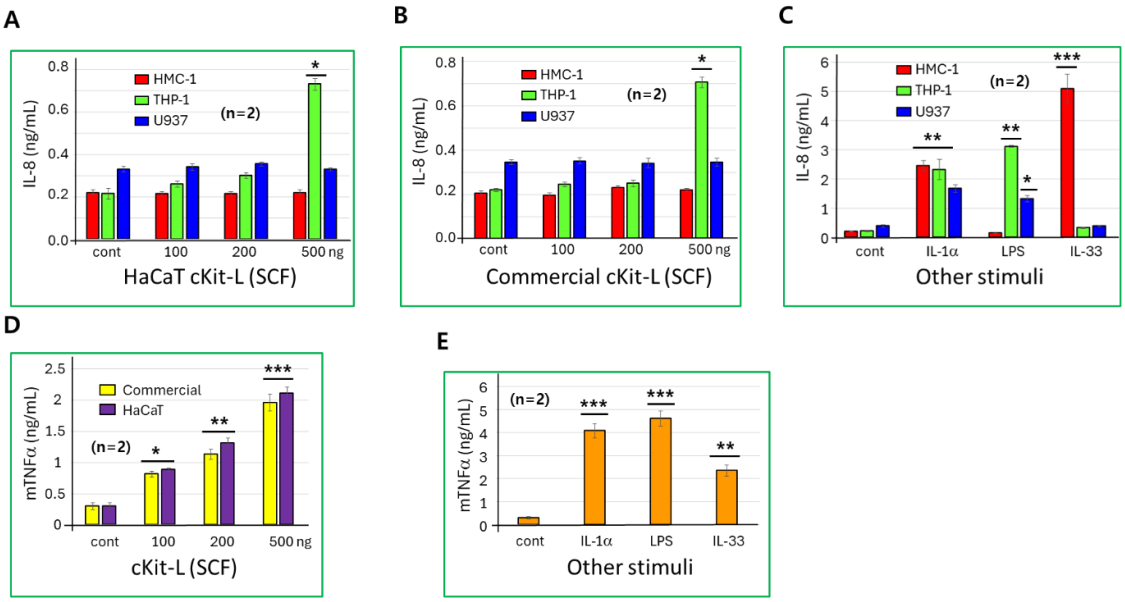

Figure 4. Biological activity of recombinant cKit-L protein. (**A**) HaCaT cKit-L and (**B**) Commercial cKit-L were used to stimulate three human immune cell lines: HMC-1, THP-1, and U937. (**C**) Other stimuli (LPS, IL-1α, and IL-33 200 ng/mL) were used to treat the same human immune cell lines. (**D**) HaCaT/Commercial cKit-L and (**E**) other stimuli (LPS, IL-1α, and IL-33 200 ng/mL) were used to treat mouse macrophage Raw 264.7 cell. The concentration. The graph shows cytokine levels compared to untreated controls, mean ± SEM (n = 2) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

cKit was discovered as an orphan receptor belonging to the tyrosine kinase receptor type III and, like other cell growth factor receptors, contains a long cytoplasmic kinase domain [31]. cKit, like other members of the receptor tyrosine kinase III family, consists of an extracellular domain (ECD), a transmembrane (TM) domain, and an intracellular tyrosine kinase (TK) domain. The ECD is composed of five immunoglobulin (Ig)-like domains,

and the protein kinase (TK) domain is interrupted by a hydrophilic insert sequence of about 80 amino acids. The ligand stem cell factor binds via the second and third immunoglobulin domains [32–34].

Shortly after the cloning of cKit, its ligand was discovered using knowledge of the genetic mutation of steel locus (*Sl*) affect the same cellular targets as mutations at the white spotting locus (*W*) that is allelic with the c-kit proto-oncogene [5,31]. Mast cell growth factor (MGF also known as cKit-L) was shown to be a ligand for cKit by cross-linking ^{125}I-labeled MGF to cKit expressing cells with subsequent immunoprecipitation of the complex with antiserum specific for the C-terminus of cKit [30]. These results were focused on cell proliferation and oncogenic activity of both cKit-L and cKit rather than derived from immunological activity of cKit-L.

Before the discovery of cKit cDNA at the protein level of cKit was first identified as a cell surface marker on human acute myeloid leukemia (AML) cells using monoclonal antibodies (mAbs) YB5.B8 [35]. It was subsequently shown to be identified as a 145 ± 150 kDa molecule in cell membranes [36] expressed by normal haemopoietic progenitor cells (colony-forming units-culture, CFU-c) [37] and prominently, by mast cells in human tissue [38]. The mAb YB5.B8 monoclonal antibody has inhibitory activity on haemopoietic colony assays [37,39]. These data suggested that the identified molecules are growth factor receptors. Mapping of cKit to the white spot locus (W) locus [40,41] suggested that mAb YB5.B8 recognizes human cKit protein levels, which was later demonstrated [42].

So far, research on cKit-L and cKit has focused on cell proliferation and mast cell differentiation. However, the results of this study show that human mast HMC-1 cells, which express very high levels of cKit, did not react immunologically to cKit-L at all (Figures 1 and 4). In this study, cKit-L expression was confirmed by RT-PCR. As a result, HaCaT, HEK293T, A549, and THP-1 cells expressed cKit-L sufficiently, although there were differences in degree, but PC3 and Hela cells did not express cKit-L (Figure 2A). To investigate the immunological activity of cKit-L, HaCaT cell cKit-L was cloned, and its sequence was confirmed with 100% homology to a known cKit-L in the database (accession number NM_000899.4) (Figure 2B). HaCaT cKit-L was cloned in *E. coli* expression vector to produce recombinant HaCaT cKit-L protein. Lipopolysaccharide-free, two-step purified recombinant HaCaT cKit-L protein was used to treat a variety of human and mouse immune cells. Interestingly, THP-1 cells responded to both HaCaT and commercial cKit-L, whereas cKit high-expressing human mast HMC-1 cells did not respond to cKit-L (Figure 4A,B).

IL-33 is the well-known Th2 cytokine stimulating HMC-1 cell [43]. As shown Figure 4C, IL-33 induced significant amounts of IL-8 in HMC-1 cells, suggesting that the immunological cellular components of HMC-1 cells are functioning properly. Eventually, fully differentiated mouse macrophage Raw 264.7 cells were stimulated with both HaCaT and commercial cKit-L including other stimuli. Compared to THP-1 monocyte macrophages, Raw 264.7 cells were highly responsive to both HaCaT and commercial cKit-L in a dose-dependent manner (Figure 4D). Raw 264.7 cells were also highly sensitive to other stimuli (Figure 4E). Further studies investigating the immunological activity of cKit-L and finding unknown cKit-L receptors in the graphic abstract may help to understand cKit-L function in the immune system.

Abbreviations

bp, Base pair; CD, Cluster of differentiation; HPLC, High-performance liquid chromatography; HSC, Hematopoietic stem cell; L, Ligand; Ig, Immunoglobulin; IL, Interleukin; JAK/STAT, Janus kinase/Signal transducer and activator of transcription; kDa, kilodalton; mAb, Monoclonal antibody; Mw, Molecular weight; PI3-kinase, Phosphatidylinositol 3-kinase; RAS/ERK Redundant acronym syndrome/Extracellular signal-regulated kinase; SCF, Stem cell factor; SDF-1, Stromal cell-derived factor 1; TM, Transmembrane; UV, Ultraviolet.

Author Contributions: J.H., A.R., A.S., S.S., S.K., H.K., M.Y., S.L., D.S., H.Y., S.H. and M.C. designed and analyzed the data. J.H., A.R., A.S., S.S., S.K. and H.K. performed the experiments. Funding acquisition was carried out by S.K., S.H., and J.M.B. J.H., A.R., A.S., S.S., S.K., H.K. and S.K. examined the data. S.K. and J.M.B. edited the manuscript. S.K. and J.M.B. designed the study, supervised the project, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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