In vitro selection of an RNA aptamer yields an interleukin-6/interleukin-6 receptor interaction inhibitor

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Abstract

  Interleukin-6 (IL-6) binds to IL-6 receptor (IL-6R) subunit, related to autoimmune diseases and cytokine storm in COVID-19. In this study we performed Systematic Evolution of Ligands by Exponential enrichment (SELEX) and identified a novel RNA aptamer. This RNA aptamer not only bound to IL-6R with a dissociation constant of 200 nM, but also inhibited the interaction of IL-6R with IL-6.

Word count: 2655

Interleukin-6 (IL-6), a multifunctional cytokine that participates in many inflammatory and immune responses, is a secretory protein composed of 183 amino acid residues (Kang, 2019). IL-6, which shows both anti-inflammatory and pro-inflammatory properties, is expressed and secreted by macrophages, osteoblasts, monocytes, T cells, B cells, fibroblasts etc. IL-6 signaling occurs in IL-6 receptor (IL-6R)-expressing cells, where IL-6 binds to the non-signaling IL-6R subunit and cytokine signal-transducing glycoprotein 130 (gp130) dimer. IL-6R is expressed only in a few types of cells such as macrophages, monocytes, and hepatocytes. Conversely, gp130 is ubiquitously expressed in nearly all types of cells. IL-6R has three extracellular domains, including two fibronectin type III-like domains 2 and 3, which form a cytokine-binding module that interacts with IL-6. Binding of soluble IL-6R (sIL-6R) to IL-6 can also activate cells that do not express membrane-bound IL-6R (mIL-6R). The sIL-6R is secreted by mIL-6R-expressing cells via producing by proteolytic cleavage or alternative splicing of mIL-6R (Baran, 2018).

Interaction of IL-6R and IL-6 is implicated in the progression of several autoimmune diseases including rheumatoid arthritis (RA), Castleman's disease and Crohn's disease (Kim, 2015). RA is a debilitating disease characterized by chronic joint inflammation and progressive bone loss, with a prevalence of approximately 1% in the general population. IL-6R/IL-6 interaction is also reported to be involved in the progression of non-autoimmune diseases including cytokine storm in COVID-19 (Liu, 2020).

Currently two anti-human IL-6R monoclonal antibodies, tocilizumab and salilumab, are used for treatment of autoimmune diseases including RA and Castleman's disease (Huizinga, 2014). Several other inhibitors of IL-6R/IL-6 interaction have also been developed, including an anti-IL-6 monoclonal antibody, siltuximab (Davis, 2015, Gupta, 2014). However, antibodies have disadvantages such as low stability and high production costs (Kim, 2016, Roy, 2015). Therefore alternative IL-6R/IL-6 interaction inhibitors that overcome these shortcomings are needed for treatment of autoimmune diseases (Takamori, 2021).

Small nucleic acid aptamers, introduced in the early 1990s, can be used for discovery of ligands and inhibitors of target biomolecules (Wilson, 2006). Aptamers that interact with target biomolecules can be enriched from a highly diverse library (up to 10^{12}) by an *in vitro* selection method termed Systematic Evolution of Ligands by Exponential enrichment (SELEX) (Ando, 2021a). As with antibodies, nucleic acid aptamers can bind with high specificity to target biomolecules (Ando, 2021b). In addition, nucleic acid aptamers have advantages over antibodies, including their smaller size and lower production costs (Magbanua, 2013). Thus nucleic acid aptamers are promising tools for therapeutic and research applications.

Hahn's group reported IL-6R-binding RNA aptamer, named AIR-3, obtained by *in vitro* selection from an RNA library. AIR-3 had a high affinity for human IL-6R, although AIR-3 did not inhibit the interaction of IL-6R with IL-6 (Meyer, 2012, Hahn, 2017, Szameit,

2016).

In this study, we performed *in vitro* selection of RNA aptamers against an extracellular domain of human IL-6R and discovered a novel IL-6R-binding RNA aptamer that we named IBR. IBR is composed of 80 nucleotides (nt) and bound to IL-6R with a dissociation constant (K_D) of 200 nM. In addition, an IL-6R-pull-down assay demonstrated that IBR inhibits interaction between IL-6R and IL-6. The identified novel IL-6R-binding RNA aptamer, IBR, is potentially a basic tool for studies of IL-6R.

The Fc fusion protein of the extracellular domain (ECD) of human IL-6R was immobilized on Protein G-modified magnetic beads, which was confirmed by SDS-PAGE (Figure S1). We prepared an RNA library by run-off *in vitro* transcription of a synthetic DNA library containing randomized sequences (Figure S2) (Tsukamoto, 2021). The prepared RNA library was then pulled down with IL-6R-non-immobilized beads for removing bead-binding RNAs as negative selection. Next, the RNA library was pulled down with IL-6R-immobilized magnetic beads for the isolation of IL-6R-binding RNAs as positive selection. The recovered RNAs bound to the beads were then reverse-transcribed to form double-stranded complementary DNA (cDNA)/RNA. The reverse-transcribed cDNAs on the beads encoding IL-6R-binding RNAs were amplified by PCR and used for the next round of SELEX. The recovery rate of cDNAs encoding IL-6R-binding RNAs in each round of SELEX was determined by quantitative PCR. Quantitative PCR analysis showed that the recovery rate of cDNA immobilized on human IL-6R-beads increased during SELEX (Figure S3).

After seven rounds of *in vitro* selection, the recovered DNA library was cloned and individual clones were sequenced to identify the recovered RNA sequences. The RNA sequences of the clones and their frequency of appearance are shown in Figure 1. Sequence alignment of 37 clones showed that the RNA library was enriched to a single family containing a similar consensus sequence. Therefore, the most abundant cloned RNA consisting of 80 nt was used in subsequent experiments.

The IL-6R-binding activity of the identified RNA clone was analyzed by pull-down with the human IL-6R-immobilized beads (Figure S4). The most abundant RNA clone was prepared by run-off *in vitro* transcription. Binding efficiency was determined by quantification of the cDNA recovered after incubation with IL-6R-immobilized beads or IL-6R-non-immobilized beads followed by quantitative reverse transcription PCR (qRT-PCR). The significantly higher recovery rate of IL-6R-immobilized beads demonstrated that the cloned RNA binds to IL-6R.

The IL-6R-binding activity of the cloned RNA was also analyzed by chemiluminescence detection using horseradish peroxidase (HRP) as shown in Figure 2A. HRP complexed with the cloned RNA was pulled down with IL-6R-immobilized or non-immobilized beads and assayed by adding HRP's chemiluminescent substrate. Consistent with the result of the qRT-PCR-based pull-down assay, chemiluminescence analysis showed

that the cloned RNA binds to IL-6R. Therefore, the cloned RNA aptamer was named "IBR" (IL-6R-Binding RNA) and further characterized.

Next, the human IL-6R binding constant of IBR was measured using a bio-layer interferometry (BLI) assay (Figure 2B). As a ligand, biotinylated IBR was immobilized on a streptavidin-modified sensor. Various concentrations of free IL-6R were added as an analyte to the IBR-immobilized sensor. The determined dissociation constant (K_D) of IBR calculated from the BLI sensorgrams was 200 ± 200 nM. The k_{on} and k_{off} of IBR were 2×10^4 $M^{-1}s^{-1}$ and 4×10^{-3} s^{-1}, respectively. BLI assay showed that significant binding of random RNA as a control ligand to IL-6R was not observed (Figure S5). By using BLI, we also found that binding of IBR to IL-4R (25% homology), which was used as other interleukin receptor, was not observed (Figure S6). The K_D of secondarily abundant IL-6R-binding RNA clones 2 and 3 (Figure 1) was 50 ± 10 nM and 80 ± 10 nM, respectively, showing that clones 2 and 3 have slightly higher affinity compared to IBR aptamer (clone 1).

To examine the inhibitory effect of IBR on the interaction of IL-6 with IL-6R, we performed an inhibition assay using BLI with an IL-6R-immobilized sensor (Figure 3A). As an analyte, human IL-6 alone or a mixture of IL-6 and IBR was added to IL-6R immobilized on the sensor as a ligand. BLI analysis of IBR alone used as an analyte was also performed as a control. The BLI signal of a mixture of IL-6 and IBR decreased compared to that of IL-6 alone and increased compared to that of IBR alone, indicating that IBR inhibits the interaction of IL-6 with IL-6R (Figure S7).

The inhibition of IL-6/IL-6R interaction by IBR was also analyzed by a pull-down assay (Figure 3B). IL-6R-immoblized beads were incubated with IL-6 or a mixture of IL-6R and IL-6 and then analyzed by SDS-PAGE. Consistent with the result of the BLI assay, CBB staining of SDS-PAGE gels demonstrated that IBR inhibits IL-6/IL-6R interaction.

In vitro selection (SELEX) of RNA aptamers has been done for over 30 years. During this time, a number of RNA aptamers have been identified by *in vitro* selection and used for a variety of purposes not only *in vitro* but also *in vivo* (Meyer, 2012). In this study, we succeeded in identifying a novel IL-6R-binding RNA aptamer (IBR) by using *in vitro* selection and demonstrated that the aptamer inhibits IL-6R/IL-6 interaction. IL-6 is a multifunctional cytokine involved in many immune and inflammatory reactions. A complex of IL-6 and its receptor IL-6R binds to the ubiquitously expressed dimer gp130, causing a hyperimmune reaction leading to autoimmune disease. To the best of our knowledge, this study is the first to report an IL-6R-binding RNA aptamer that inhibits interaction between IL-6 and IL-6R.

The K_D values for human IL-6 binding to human IL-6R have been reported in the range of 0.5–34 nM (Baran, 2018). The IBR we identified had a lower IL-6R-binding affinity (200 nM) than that of IL-6. IBR could inhibit IL-6/IL-6R interaction since a higher amount of IBR than IL-6 was used in the inhibition assay (Figure 3B). Previously reported IL-6R-

binding RNA aptamer, AIR-3 (Figure S8) had a higher affinity for IL-6R with a K_D of 19.7 nM than IBR reported here (Meyer, 2012). Since the IBR binding affinity is insufficient for use as a therapeutic reagent for autoimmune disease, in the future it will be necessary to increase IL-6R-binding affinity through *in vitro* affinity maturation and engineering by mutation.

RNA aptamers generally have the disadvantage of being labile *in vivo* due to their degradation by nucleases. Chemical modification is one effective approach to increasing resistance of RNAs to nuclease degradation (Meyer, 2014, Mittelberger, 2015). Therefore, chemical modification after *in vitro* selection may be also effective in IBR aptamer we discovered to increase its stability.

Our IBR aptamer can be used not only as an IL-6R/IL-6 interaction inhibitor but also as an IL-6R-binding affinity reagent. For example, IBR modified with a fluorescent dye allows for fluorescent imaging of IL-6R. In fact, previous reports demonstrated that receptor-mediated internalization of IL-6R in IL-6R transfected cells could be visualized with Atto645N-fluorescently labeled IL-6R-specific RNA aptamers (Meyer, 2012). In addition, IBR modified with a photosensitizer can be applied to light-dependent inactivation of IL-6R and IL-6R-expressing cells in a precise spatiotemporal manner using so-called chromophore-assisted light inactivation or photodynamic therapy. In fact, a photosensitizer, chlorin e6, conjugated to an IL-6R-aptamer was used to selectively kill IL-6R-presenting cells by optical irradiation (Kruspe, 2014a). IBR modified with a cytotoxic compound can also be used for targeted chemotherapy. It was reported that an IL-6R-binding RNA aptamer comprising cytotoxic 5-Fluoro-2'-deoxyuridine reduced the proliferation rate of IL-6R-presenting cells (Kruspe, 2014b). Therefore, the IBR we discovered can potentially be used in such research applications.

Author's Contribution

T.K. designed the project. T.A., M.Y., Y.T., D.F. and K.T. performed experiments. T.A., M.Y, Y.T., K.T. and T.K analyzed the data. T.A., M.Y, Y.T., K.T. and T.K wrote the manuscript.

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Clone number	Sequence			Frequency
1	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUAGUAU--UCCUACGAAGUAUUAUUCUUCU	UGCGGUGGAGGAGGAGGUAGCUA	8
2	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUAGCAU--UCCUACGAAGUAUUAUUCUUCG	UGCGGUGGAGGAGGAGGUAGCUA	4
3	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-CAU--UCCUAUUUAG-AUGGAGCCUCG	UGCGGUGGAGGAGGAGGUAGCUA	4
4	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-GAU--UCCUAUUUAG-AUGGAGCCUCU	UGCGGUGGAGGAGGAGGUAGCUA	2
5	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-GAU--UCCUAUUUAG-AUGGAGCCUCG	UGCGGUGGAGGAGGAGGUAGCUA	2
6	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-AAU--UCCUAUUUAG-AUGGAGCCUCU	UGCGGUGGAGGAGGAGGUAGCUA	2
7	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-GAU--UCCUAUUUAG-AUGGAGCCCCU	UGCGGUGGAGGAGGAGGUAGCUA	1
8	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-CAU--UCCUAUUUAG-AUGGAGCCUCU	UGCGGUGGAGGAGGAGGUAGCUA	1
9	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-GAU--UCCUAUUUAG-AUGGAGCCUCG	UGCGGUGGAGGAGGAGGUAGCUA	1
10	GGAUUAAGGAGGUGAUAUUUAUG	UAGACGUA-CAU--UCCUAUUUAG-AUGGAGCCUCG	UGCGGUGGAGGAGGAGGUAGCUA	1
11	GGAUUAAGGAGGUGAUAUUUAUG	UAGACGUA-CAU--UCCUAUUUAG-AUGGAGCCUCU	UGCGGUGGAGGAGGAGGUAGCUA	1
12	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-CAU--UCCUAUUUAG-GUGGAGCCUCU	UGCGGUGGAGGAGGAGGUAGCUA	1
13	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-AAU--UCCUAUUUAG-AUGGAGCCUCU	UGCGGUGGAGGAGGAGGUAGCUA	1
14	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-GAU--UCCUAUAUAG-ACGGAGCCUCU	UGCGGUGGAGGAGGAGGUAGCUA	1
15	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-CAU--UCCUAAAAAG-AUGGAGCCUCG	UGCGGUGGAGGAGGAGGUAGCUA	1
16	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUA-GAU--UCCUAUUUAG-AUGGAGCCUCG	UGCGGUGGAGGAGGAGGUAGCUA	1
17	GGAUUAAGGAGGUGAUAUUUAUG	UAGAGGUAGUAU--UCCUAAGAAGUAUUAUUCUUCG	UGCGGUGGAGGAGGAGGUAGCUA	1
18	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUAGCAU--UCCUACGAAGUAUUAUUCUUCU	UGCGGUGGAGGAGGAGGUAGCUA	1
19	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUAGCAU--UCCUAUGAAGUAUCAUUCUUCU	UGCGGUGGAGGAGGAGGUAGCUA	1
20	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUAUUGUAUUCCUACGAAGUAUUAUUCUUCU	UGCGGUGGAGGAGGAGGUAGCUA	1
21	GGAUUAAGGAGGUGAUAUUUAUG	UAGAUGUAUUGUAUUCCUACGAAGUAUUAUUCUUCG	UGCGGUGGAGGAGGAGGUAGCUA	1

FIG. 1.

Alignment analysis of RNA sequences against human IL-6R identified by cloning and sequencing of the corresponding DNA library in the final SELEX round. The selected sequences are boxed with a black frame. Bold indicates the consensus sequence.
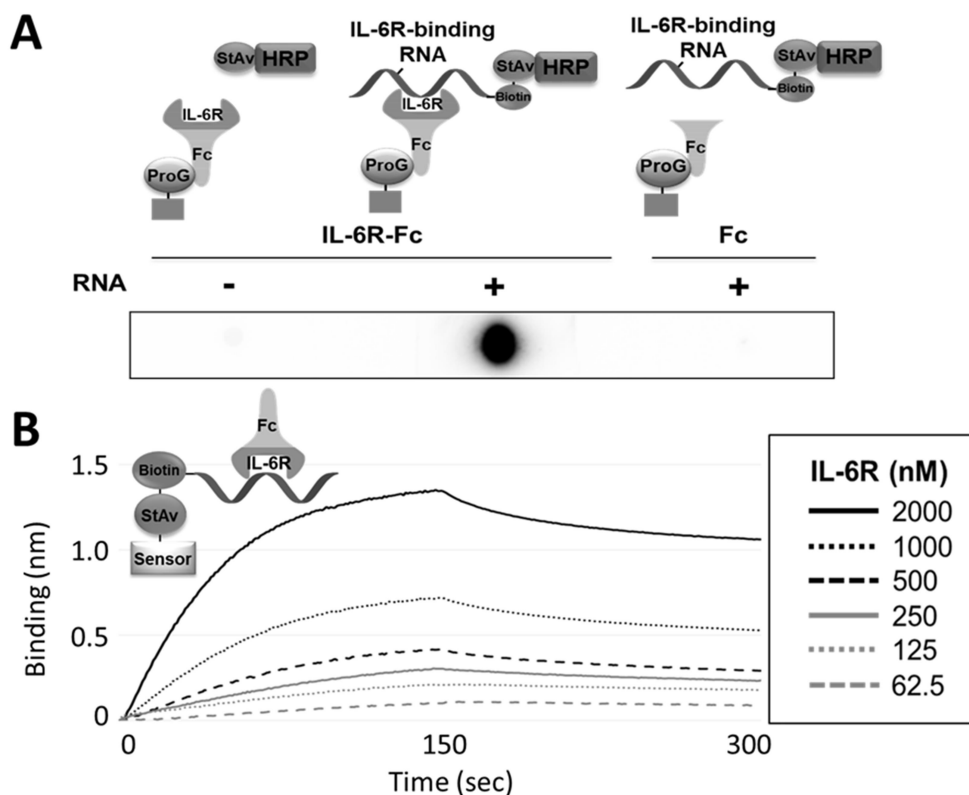
FIG. 2.

Binding analysis of the RNA aptamer clone discovered by *in vitro* selection against IL-6R. (A) Target specificity of the cloned RNA aptamer was assayed by IL-6R pull-down and chemiluminescence detection using HRP labeling. (B) Binding kinetics of IL-6R-binding RNA aptamer was analyzed using the BLI assay. Representative sensorgram obtained using BLI is shown. The dissociation constant (K_D) of the IL-6R-binding RNA aptamer was determined from the resulting BLI sensorgrams. StAv denotes streptavidin. HRP, horseradish peroxidase; BLI, Bio-layer interferometry.
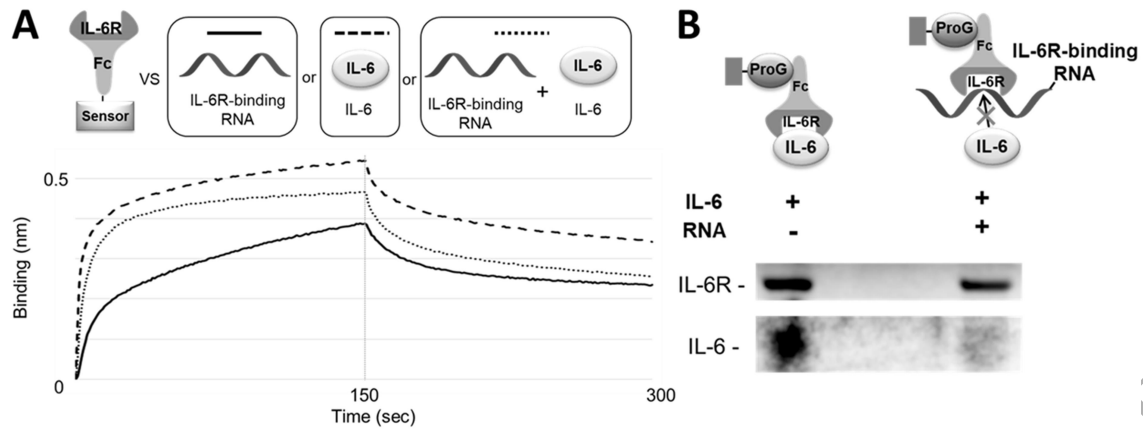
FIG. 3.

IL-6R/IL-6 interaction inhibition analysis of the IL-6R-binding RNA (IBR). (A) IL-6R/IL-6 interaction inhibition by IBR was analyzed using the BLI assay. IBR alone, IL-6 alone, or a mixture of IL-6 and IBR was added to IL-6R immobilized on the BLI sensor. (B) IL-6R/IL-6 interaction inhibition by IBR was analyzed by IL-6R-pull-down of IL-6 in the presence or absence of IBR followed by SDS-PAGE. Proteins were visualized by CBB staining.

Data Availability Statement

The data underlying this article are available in the article and in its online supplementary material.