Archives of Biochemistry and Biophysics 727 (2022) 109322

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Generation of antagonistic monoclonal antibodies against the neoepitope of active mouse interleukin (IL)-18 cleaved by inflammatory caspases

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ARTICLE INFO

Keywords:
Antagonistic antibody
Epitope mapping
Inflammation
Interleukin-18 (IL-18)
Monoclonal antibody
Mouse IL-18 inhibition assay
Neoepitope
Preclinical studies in mice

ABSTRACT

Interleukin 18 (IL-18) is a member of the IL-1 family and plays an important role in both the innate and acquired immune systems. It is constitutively expressed as an inactive precursor (24 kDa) in various cell types, and the mature IL-18 (18 kDa) cleaved by inflammatory caspase-1/4 binds to the interleukin-18 receptor, thereby activating downstream signaling pathways. We previously generated anti-human IL-18 antibodies that specifically recognize the human IL-18 neoepitope cleaved by inflammatory caspase-1/4. Because the N-terminal amino acid sequences of the neoepitopes are different between human IL-18 and mouse IL-18, the anti-human IL-18 neoepitope antibodies do not recognize mouse mature IL-18. We have now generated novel anti-mouse IL-18 neoepitope antibodies. We also confirmed CXCL2 secretion from P-815 mouse cells by mouse IL-18 stimulation, and established a simple assay to evaluate the activity of mouse IL-18. Using this evaluation system, we confirmed that the anti-mouse IL-18 neoepitope antibodies could inhibit mouse IL-18. By demonstrating the therapeutic efficacy of the anti-mouse IL-18 neoepitope and function-blocking mAbs established in the present study in mouse models, corresponding to human inflammatory diseases in which IL-18 may be involved, such as inflammatory bowel diseases, we can provide the proof-of-concept that the previously established anti-human IL-18 neoepitope and function-blocking mAbs work in human inflammatory disorders corresponding to mouse models.

1. Introduction

Interleukin 18 (IL-18), a member of the IL-1 family, has important functions in both the innate and acquired immune systems [1–3]. IL-18 is constitutively expressed as an inactive precursor (24 kDa) in a wide variety of cell types, including monocytes, macrophages, intestinal epithelial cells, keratinocytes, and synovial fibroblasts [1]. When inflammation is triggered, a large protein complex called the inflammasome is formed and caspase-1/4 is activated. These inflammatory caspases cleave the precursor of IL-18, thus generating mature IL-18 (18 kDa), which is released into the extracellular space via the pore created by gasdermin D [4–6]. Other ways to mature IL-18, in addition to inflammasome-mediated activation by caspase-1 or caspase-4, have also been reported [7–10]. Only mature IL-18 binds specifically to interleukin-18 receptor 1 (IL-18R1) (also known as IL-18 receptor α, IL-18Rα), and this is followed by interleukin-18 receptor accessory protein (IL-18RAcP) (also known as IL-18 receptor β, IL-18Rβ) recruitment and activation of the downstream NF-κB and MAPK pathways [11,

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https://doi.org/10.1016/j.abb.2022.109322
Received 2 January 2022; Received in revised form 23 May 2022; Accepted 9 June 2022
Available online 13 June 2022

12]. Mature IL-18 also binds to IL-18 binding protein (IL-18BP) with higher affinity than IL-18R, and the IL-18/IL-18BP complex is unable to bind to IL-18R [13,14]. Therefore, IL-18 signaling is also regulated by the amount of IL-18BP.

Patients with a variety of diseases have elevated IL-18 levels in their blood, suggesting the involvement of IL-18 in the onset and progression of these diseases, such as COVID-19 [15,16], inflammatory bowel diseases such as Crohn's disease, metabolic syndrome, type II diabetes, atherosclerosis, myocardial infarction, pulmonary diseases such as asthma and chronic obstructive pulmonary disease, and neurological diseases such as multiple sclerosis and amyotrophic lateral sclerosis [2,17,18].

The N-terminus of the active form of IL-18, which is absent in non-inflamed cells, is a novel epitope. Therefore, this neoepitope should provide a unique and sensitive indicator of the inflammatory response, as well as a therapeutic target. In this regard, we previously generated two new anti-human IL-18 neoepitope antibodies that specifically recognize the new N-terminus of human IL-18 after cleavage by inflammatory caspase-1/4 [19]. The N-terminal neoepitope of human IL-18 and mouse IL-18 have different amino acid sequences. To conduct therapeutic experiments using IL-18 neoepitope mAbs in mouse models of inflammatory and other diseases, we have now generated two novel anti-mouse IL-18 neoepitope mAbs for specific detection, as described herein.

2. Experimental procedures

2.1. Antibodies

The following commercial antibodies were used: rabbit polyclonal anti-mouse IL-18 (B-I-003, mAbProtein, Shimane, Japan), horseradish peroxidase (HRP)-conjugated goat F(ab')$_2$ anti-rabbit (711-1122, Rockland Immunochemicals, Limerick, ME, USA) for Western blot analysis; HRP-conjugated goat anti-mouse IgG (H+L) (115-035-003, Jackson ImmunoResearch Laboratories, West Grove, WV, USA) for ELISA; and mouse monoclonal anti-DLVPR (R-G-001, clone name G196, mAbProtein, Shimane, Japan) as the control mAb for immunoprecipitation.

2.2. Cell culture

P-815 mouse mastocytoma cells (JCRB0078) and Sp2/O-Ag14 myeloma cells (JCRB9084) were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. P-815 cells and Sp2/O-Ag14 cells were grown in RPMI1640 medium (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich). The other cell lines used in this paper are listed in Supplementary Table 1.

2.3. RNA extraction and real-time quantitative PCR

For RNA extraction, 1000 μl of Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) was added to 2×10^6 cells. After 5 min, 100–200 μl of chloroform was added, and the mixture was centrifuged at 20,000×*g* for 5 min at 4 °C. The RNA extracted into the aqueous layer was precipitated by adding an equal volume of isopropanol. After rinsing with 70% ethanol, the RNA was dried and dissolved in DEPC-treated distilled water. Total RNA was reverse-transcribed using an oligo (dT) primer and a ReverTra Ace system (TOYOBO, Osaka, Japan). Quantitative PCR was performed using SYBR premix Ex-Taq (TaKaRa, Shiga, Japan) and the Thermal Cycler Dice Real Time System TP800 (TaKaRa). Primer sequences are shown in Supplementary Table 2. The relative mRNA expression level was defined as the ratio of the target gene expression level to the β-actin expression level, with that of the control sample set as 1.0.

2.4. Protein expression

Synthetic genes encoding mouse IL-18 (UniProt ID P70380-1) and human caspase-4$^{105-377}$ (P49662-1) were optimized for *Escherichia coli* (*E. coli*) codon usage (Fasmac, Kanagawa, Japan). The gene fragments were cloned into pET28-TEV [20], to produce N-terminal His_6-tagged proteins, expressed in *E. coli*, and purified using Ni-NTA agarose (QIAGEN, Valencia, CA, USA) to generate the mature mouse IL-18 protein. A mixture of these proteins (IL-18:caspase-4$^{105-377}$ = 10:1) was incubated at 24 °C overnight and then passed through a Ni-NTA agarose column. The flow-through was collected as the mature mouse IL-18 protein (Fig. 1*B*). The N-terminal Asn of IL-18 was confirmed by Edman degradation sequencing. This study was approved by the recombinant DNA experiment safety committee of Shimane University and performed in accordance with the approved protocol (ID: 539-1).

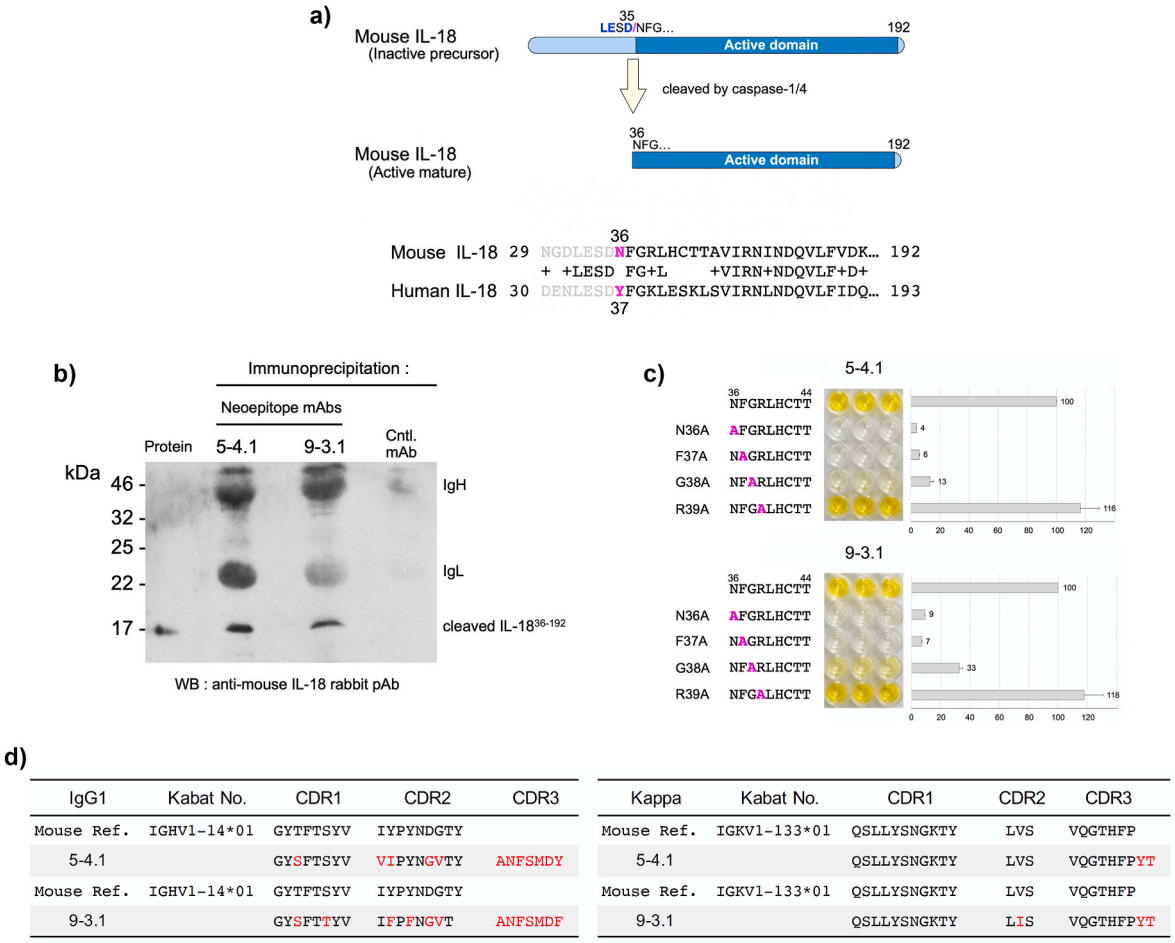
2.5. mAb generation

Mouse mAbs against the neoepitope of mouse IL-18 cleaved by caspase-1/4 were produced by immunizing mice with the neoepitope peptide ^{36}NFGRLHCTT^{44}-C (mAbProtein) conjugated with keyhole-limpet hemocyanin (77666, Thermo Scientific). The sequence of the neoepitope peptide was ^{36}NFGRLHCTT^{44}, corresponding to the N-terminal end of cleaved IL-18, with an additional C-terminal Cys for conjugation. Emulsions were prepared by mixing 150 μL of KLH-conjugated peptide/PBS and 150 μL of Freund's Complete Adjuvant (F5881, Sigma-Aldrich) and inoculated subcutaneously (N = 3). Two weeks later, emulsions of 150 μL of KLH-conjugated peptide/PBS and 150 μL of Freund's Incomplete Adjuvant (F5506, Sigma-Aldrich) were inoculated subcutaneously. Incomplete Adjuvant was used for the third, fourth, and subsequent inoculations, which were given every week. Serum titers were monitored by immunoblotting, using the recombinant mouse IL-18$^{36-192}$ protein. Spleen cells from mice with elevated antibody titers were prepared and fused with Sp2/O-Ag14 cells using polyethylene glycol (3,350, P4338, Sigma-Aldrich), and fused cells were selected with HAT medium (16-808-49, MP Biomedicals, Santa Ana, CA, USA) after two days. Clonal populations of fusion cells were screened by ELISA for antibody production against the neoepitope peptide conjugated with BSA. Productive cells were cloned to monoclonal lines by serial dilution screening. Finally, two types of hybridomas, 5-4.1 and 9-3.1, were selected and established. The antibodies produced by hybridomas 5-4.1 and 9-3.1 are referred to as mAbs 5-4.1 and 9-3.1, respectively. The isotypes of mAbs 5-4.1 and 9-3.1 were confirmed to be IgG1, kappa using an IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche, 11493027001). Highly concentrated mAbs were isolated from murine ascites after intraperitoneal injection of the hybridoma cells. All animal experiments were performed in compliance with the standards established by the International Guiding Principles for Biomedical Research Involving Animals and were approved by the animal care and use committee of Shimane University (ID: IZ29-53).

The antibody gene sequences of the hybridomas were determined according to the manufacturer's protocol (SMARTer Human BCR IgG IgM H/K/L Profiling Kit, Z4466 N, Takara Bio, Shiga, Japan). Amino acid sequences of the variable regions of heavy and light chains of hybridoma clones were aligned, and the complementarity determining regions (CDRs) were analyzed with the antibody gene sequence tool (https://www.imgt.org/IMGT_vquest/vquest). Isotypes of hybridoma clones were analyzed using an IsoStrip Mouse Monoclonal Antibody Isotyping Kit (11493027001, Roche, Mannheim, Germany).

2.6. Immunoprecipitation and Western blot analysis

Immunoprecipitation was conducted by pre-conjugating the antibodies with anti-mouse IgG agarose beads (A6531, Sigma-Aldrich). The samples were mixed with SDS buffer (125 mM Tris-HCl, pH 6.8, 5% glycerol, 4% SDS, 0.005% bromophenol blue, and 10% 2-



d)

IgG1	Kabat No.	CDR1	CDR2	CDR3
Mouse Ref.	IGHV1-14*01	GYTFTSYV	IYPYNDGTY	
5-4.1		GYSFTSYV	VIPYNGVTY	ANFSMDY
Mouse Ref.	IGHV1-14*01	GYTFTSYV	IYPYNDGTY	
9-3.1		GYSFTTYV	IFPFNGVT	ANFSMDF

Kappa	Kabat No.	CDR1	CDR2	CDR3
Mouse Ref.	IGKV1-133*01	QSLLYSNGKTY	LVS	VQGTHFP
5-4.1		QSLLYSNGKTY	LVS	VQGTHFPYT
Mouse Ref.	IGKV1-133*01	QSLLYSNGKTY	LVS	VQGTHFP
9-3.1		QSLLYSNGKTY	LIS	VQGTHFPYT

Fig. 1. Generation of anti-mouse IL-18 neoepitope mAbs
(A) Comparison of neoepitopes between human and mouse IL-18. (B) Immunoprecipitation of the bacterially expressed mouse IL-18 cleaved by caspase-4 with mAbs 5-4.1 and 9-3.1. Negative control (cntl.) mAb, G196. A representative result of two independent experiments is shown. (C) Epitope mapping of mAbs 5-4.1 and 9–3.1 by ELISA. Each position of the neoepitope peptide ^{36}NFGRLHCTT^{44} was substituted individually with Ala. The mean and standard deviation of three independent ELISA experiments is shown as 100% of the mean value of wild-type peptide (right panel). (D) Immunogenetic analysis of the heavy- and light-chain variable regions of anti-mouse IL-18 neoepitope mAbs. Amino acid sequences of mAbs 5-4.1 and 9-3.1 are shown. The CDR sequences of individual mAb clones were analyzed using the IMGT/V-QUEST tool. The unique amino acid sequences for each clone are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mercaptoethanol), subjected to 12% SDS-polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene difluoride membrane (Immobilon-P, IPVH00010, Merck Millipore, Darmstadt, Germany). After blocking with 5% nonfat dry milk for 30 min, the membranes were incubated with the indicated antibodies for 1 h at room temperature, and then with secondary antibodies (HRP-conjugated anti-mouse or anti-rabbit IgG at the appropriate dilutions) for 45 min at room temperature. Antibody binding was detected with an enhanced chemiluminescence detection system (PerkinElmer, Waltham, MA, USA).

2.7. Epitope mapping

The neoepitope peptide ^{36}NFGRLHCTT^{44} was synthesized based on C-terminal biocytin (a conjugate of D-biotin and L-lysine) (Mimotopes Pty Ltd, Victoria, Australia). Epitope mapping of the neoepitope mAbs 5-4.1 and 9-3.1 was achieved by individually substituting each position of the neoepitope peptide with Ala (Mimotopes). These neoepitope peptides with C-terminal biocytin were easily bound to NeutrAvidin Coated Plates (15123, ThermoFisher Scientific), and subjected to an enzyme-linked immunosorbent assay (ELISA) for epitope mapping.

2.8. CXCL2 induction assay and mouse IL-18 inhibition assay

Mouse IL-18 proteins were examined for the ability to induce CXCL2 production from P-815 mouse mastocytoma cells at 37 °C (Fig. 2C). The concentrations of mouse CXCL2 in P-815 cells supernatants were measured by ELISA, using a mouse CXCL2/MIP-2 Quantikine ELISA kit (MM200, R&D Systems, Minneapolis, USA). A FilterMax F5 multi-mode microplate reader (Molecular Devices, San Jose, USA) was used to determine the absorbance (OD_{450nm} -OD_{570nm}). The 50% inhibitory concentration (IC_{50}) values were determined using the nonlinear regression curve fit analysis of Prism 9.3.1 (GraphPad Software Inc., San Diego, CA, USA).

2.9. Preclinical studies in mice

Preclinical studies in mice and data analysis were performed by contract research organizations (DIMS Institute of Medical Science, Inc., Aichi, Japan for the single-dose toxicity test (Test No. 21546), and the Safety Research Institute for Chemical Compounds Co., Ltd., Hokkaido, Japan for the half-life of mAb 5-4.1 in a single intravenous infusion study (Test No. SR21138)) based on the study protocols, on behalf of our research group supported by AMED under Grant Number

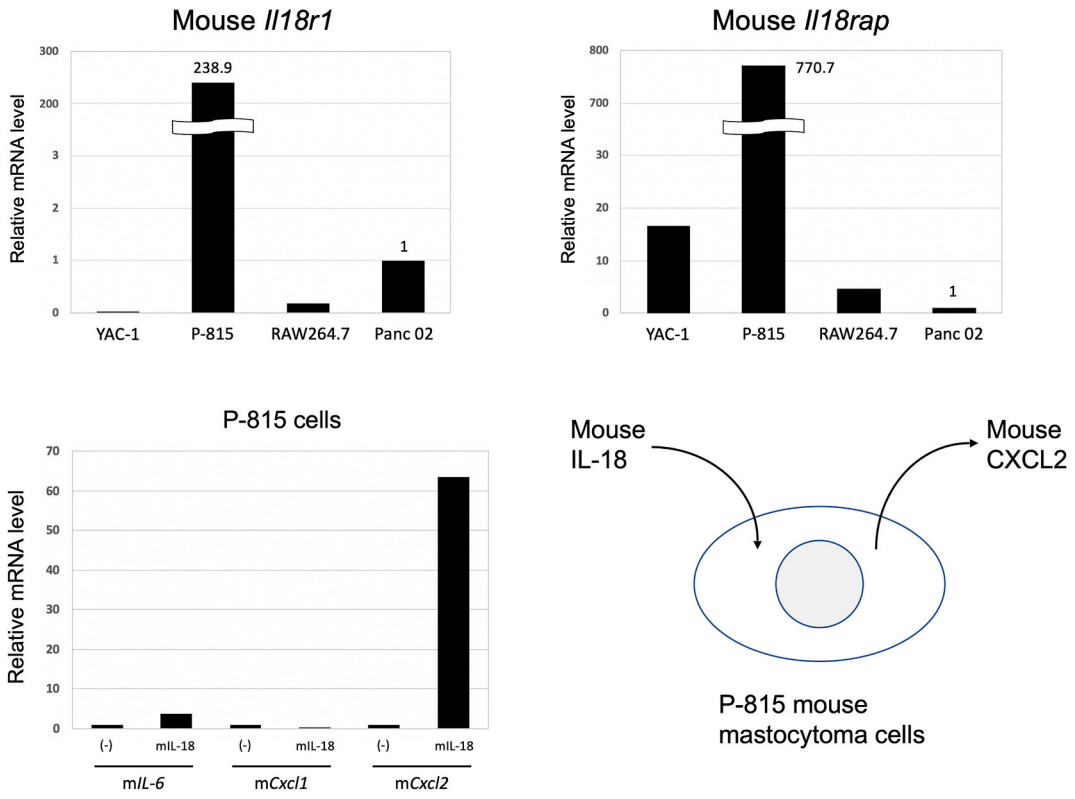


Fig. 2. Development of a functional evaluation system for mouse IL-18
(A) mRNA expression levels of mouse *Il18r1* and *Il18rap* genes in representative mouse cell lines, determined by RT-qPCR. Left panel, *Il18r1*; right panel, *Il18rap*. (B) mRNA expression levels of mouse *Cxcl1*, *Cxcl2*, and *IL-6* in P-815 mouse mastocytoma cells, quantified by RT-qPCR in the presence or absence of mature mouse IL-18. The mean and standard deviation of two independent RT-qPCR experiments are shown as the ratio of the target gene expression level to the β-actin expression level, with that of the control sample set as 1.0. (C) CXCL2 secretion induced by mouse IL-18 from mouse P-815 cells. The ELISA experiments were performed in triplicate. The mean and standard errors of two independent experiments are shown. (D) Outline of a functional evaluation system for mouse IL-18.

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A Bartlett's equal-variance test was performed at the 5% significance level for the difference between the means of body weight in the control IgG group and the 25, 75, and 200 μg/Body mAb 5-4.1 treatment groups (each group, N = 5). Since they were equally distributed, a parametric Dunnett's two-tailed test was performed.

The half-life of the anti-mouse IL-18 neoepitope mAb 5-4.1 was determined by ELISA, using the mouse IL-18 neoepitope peptide and the nonlinear regression curve fit analysis (one phase decay) of the GraphPad Prism software v. 9.3.1. The data and study reports were reviewed and commented upon by the authors of this paper, who also participated in writing the manuscript. The decision to publish was taken jointly by the authors. This does not alter our adherence to all of the Archives of Biochemistry and Biophysics policies on sharing data and materials.

3. Results

3.1. Novel anti-mouse IL-18 neoepitope mAbs

We have recently reported several anti-human IL-18 neoepitope and function-blocking monoclonal antibodies (mAbs) that specifically recognize the new N-terminal ^{37}YFGKLESK^{44} residues of human IL-18 cleaved by inflammatory caspase-1/4 [19]. The neoepitopes between human and mouse IL-18 are different (Fig. 1*A*). To provide the proof-of-concept that anti-human IL-18 neoepitope and function-blocking mAbs will be very useful for therapeutic strategies for inflammatory diseases, we needed to generate anti-mouse IL-18 neoepitope and function-blocking mAbs for therapeutic experiments in inflammatory model mice. We therefore immunized mice with the neoepitope peptide ^{36}NFGRLHCTT^{44}, corresponding to the N-terminal end of mouse IL-18 cleaved by inflammatory caspase-1/4.

We established two hybridoma clones, 5-4.1 and 9-3.1, which immunoprecipitated the bacterially expressed mouse IL-18 cleaved by caspase-4 (Fig. 1*B*). In contrast, the IgG1 isotype control mAb did not immunoprecipitate the protein.

Next, we performed alanine substitution scanning on the neoepitope peptide to determine which N-terminal amino acid residues of the neoepitope are important for mAb recognition (Fig. 1*C*). The single substitutions N36A and F37A resulted in the most profound effects, reducing the detection levels by around 95%. The G38A substitution had less pronounced effects (85% and 65% reductions, respectively), and the R39A substitution had none. These results indicated that the nine amino acid residues ^{36}NFGRLHCTT^{44} are the epitope of mAbs 5-4.1 and 9-3.2, and that the N-terminal N36, F37, and G38 residues of the neoepitope are required for recognition by these mAbs.

Next, the amino acid sequences of the individual mAb clones were analyzed for further characterization. The sequences of each CDR1, CDR2, and CDR3 of the heavy and light chains were aligned and compared using the IMGT/V-QUEST tool, which revealed that mAbs 5-4.1 and 9–3.2 have unique amino acid sequences (Fig. 1D). The isotypes were further determined using a commercially available isotyping kit. The results showed that the isotype of the two mAb clones was IgG1, kappa.

3.2. Il18r1 and Il18rap gene expression levels in mouse cell lines

For human IL-18, there is an excellent bioassay using the IFN-γ induction system with KG-1 human acute myeloblastic leukemia cells [19, 21]. In this system, the IFN-γ production induced by mouse IL-18 is at

least 100-fold lower than that elicited by human IL-18 [21]. Therefore, to prove the function-blocking activity of the established mAbs, we should develop a simple and sensitive bioassay for mouse IL-18.

The mouse IL18 receptor consists of two components: IL-18 receptor 1 (IL-18R1) and IL-18 receptor accessory protein (IL-18RAcP). IL-18R1 is responsible for binding IL18, and IL-18RAcP is required for IL-18-mediated signaling [22–24]. First, we analyzed the expression of the mouse *Il18r1* and *IL18rap* genes in our mouse cell lines by real-time quantitative reverse transcription-PCR (RT-qPCR). Among these cell lines, the P-815 mouse mastocytoma cell line expressed the highest levels of both the *Il18r1* and *IL18rap* genes (Fig. 2*A*).

3.3. Mouse IL-18 induces CXCL2 expression and release in P-815 mouse mastocytoma cells

P-815 mouse mastocytoma cells were used for further analyses. We performed a real-time RT-qPCR analysis of several genes with high potential for up-regulation by IL-18, covering a range of different levels of regulation [25]. Representative mouse genes included in the analysis were *Cxcl1*, *Cxcl2*, and, as a positive control gene, *IL-6*. The levels of up-regulation varied, but *Cxcl2* was the highest (more than 60-fold) up-regulated gene in the presence of mouse mature IL-18 in P-815 cells (Fig. 2*B*).

An ELISA analysis confirmed that mouse IL-18 induces C-X-C motif chemokine 2 (CXCL2; also known as macrophage inflammatory protein-2, MIP-2) release from the mouse P-815 cells in a dose-dependent manner (Fig. 2*C*). In this system, mouse IL-18 induced at least 100-fold higher CXCL2 production than that elicited by human IL-18 (Fig. 2*C*).

3.4. mAbs 5-4.1 and 9-3.1 inhibit IL-18-induced CXCL2 release from P-815 cells

As a functional evaluation system for mouse IL-18, we developed a CXCL2 induction assay using P-815 mouse mastocytoma cells (Fig. 2*D*). We utilized this assay to assess the function-blocking activities of the established anti-mouse IL-18 neoepitope mAbs. The mouse IL-18-elicited CXCL2 release was dose-dependently inhibited by mAbs 5-4.1 and 9-3.1, with IC_{50} values of 255 nM and 199 nM, respectively (Fig. 3).

3.5. Preclinical studies in mice: single-dose toxicity test and half-life of the antibody 5-4.1 in a single intravenous infusion

To investigate the systemic toxicity of the anti-mouse IL-18 neoepitope mAb 5-4.1, it was administered intravenously to C57BL/6NJcl mice at doses of 25, 75, and 200 μg/Body (each group, N = 5). On the day of the test substances administration and throughout the subsequent rearing period, there were no deaths in either the control IgG antibody group or the anti-mouse IL-18 neoepitope mAb groups, and no changes in the general health conditions of any of the animals. There was no statistical difference in body weights between the IgG and anti-mouse IL-18 neoepitope mAb groups at either 3 or 14 days post-dose. At the time of necropsy 2 weeks after dosing, no abnormalities were found in the grossly observed organs and tissues of the bodies. These results indicate that a single intravenous administration of 200 μg/Body of the anti-mouse IL-18 neoepitope mAb 5–4.1 did not cause any toxic effects in mice.

The half-life ($t_{½}$) of the anti-mouse IL-18 neoepitope mAb 5–4.1 during a single intravenous infusion was examined, to provide a reference for the administration schedule of antibody treatment experiments in mouse models. The neoepitope mAb or control IgG antibody was administered intravenously to C57BL/6NJcl mice, at a dose of 200 μg/Body. Blood samples were collected on days 1, 3, 7, and 14 after the administration of the test substance, and the half-life was determined by ELISA, using the mouse IL-18 neoepitope peptide to identify the anti-mouse IL-18 neoepitope mAb. The half-live of the mAb was 1.29 days

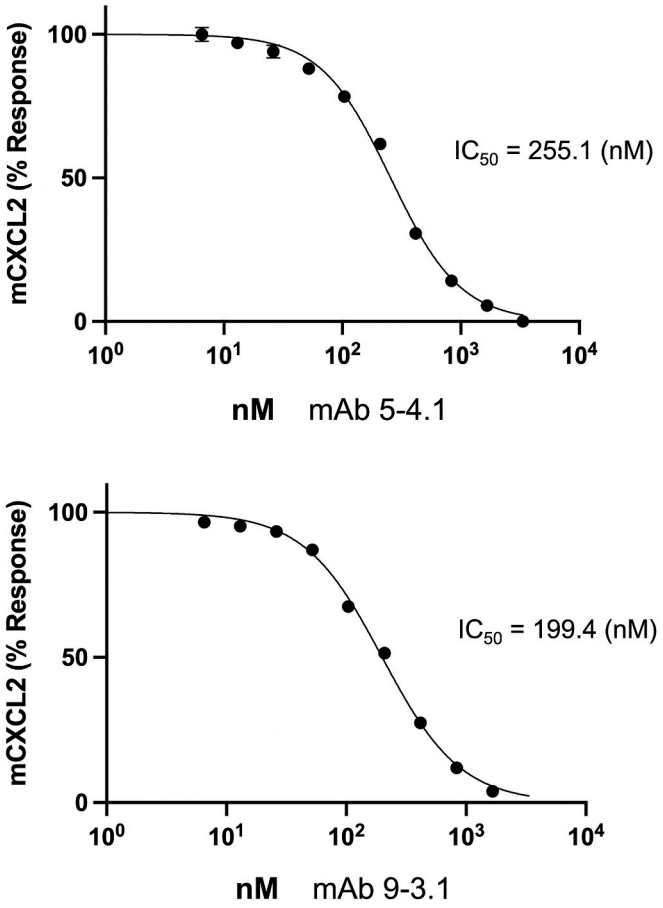

Fig. 3. Function inhibition assay of mouse IL-18 using anti-mouse IL-18 neoepitope mAbs
The concentrations of mouse CXCL2 in P-815 cell supernatants were measured by ELISA. The ELISA experiments were performed in triplicate. Data points are the mean ± SEM of two technical replicates. The 50% inhibitory concentration (IC_{50}) values were determined using the nonlinear sigmoid regression curve fit analysis by the GraphPad Prism software v. 9.3.1. (A) mAb 5-4.1. (B) mAb 9-3.1.

(Fig. 4). Control IgG antibodies could not be detected at any time, indicating that the ELISA using the mouse the IL-18 neoepitope peptide is a highly specific detection system.

4. Discussion

The risks of unexpected safety issues with mAbs in human clinical trials are lower than those for many other types of therapeutics, because mAbs are generally more stable and specific. For these reasons, mAbs are nowadays often the first product candidates to advance to clinical trials. Successful initial proof-of-concept studies of mAb-based therapies in animal models can lead to rapid progress toward clinical implementation [26]. Human IL-18 is known to be associated with many diseases, as described in the introduction section, and thus is an appealing candidate for their treatment. The precursor of IL-18 and the mature IL-18/IL-18BP complex have no activity because they cannot bind to the receptor. Information obtained from the molecular structures of IL-18, IL-18BP, and the epitope of the neoepitope mAb revealed that the neoepitope mAb is an effective therapeutic strategy because it should not bind to the IL-18/IL-18BP complex [19]. Unfortunately, the anti-human IL-18 neoepitope mAbs could not recognize the mouse IL-18 neoepitope because the sequences of the human and mouse neoepitopes, especially in the N-terminal regions, are different. Therefore, mouse IL-18 neoepitope mAbs were required for the initial proof-of-concept study of

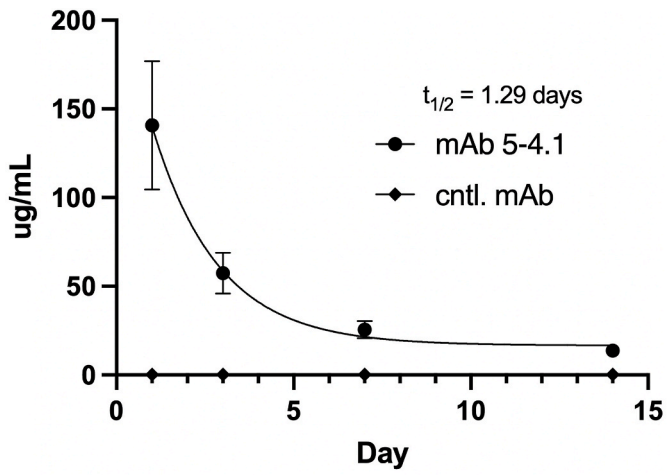

Fig. 4. Half-life of the anti-mouse IL-18 neoepitope mAb 5-4.1
The neoepitope mAb5-4.1 or isotype control IgG antibody (cntl. mAb) was administered intravenously to C57BL/6NJcl mice at a dose of 200 μg/Body (each group, N = 6). The half-life of the anti-mouse IL-18 neoepitope mAb 5-4.1 or control IgG antibody was determined by ELISA, using the mouse IL-18 neoepitope peptide and the nonlinear regression curve fit analysis (one phase decay) by the GraphPad Prism software v. 9.3.1.

mAb-based therapy for animal models, and these antibodies were established in this study. The generated mAbs against the mouse IL-18 neoepitope also specifically recognize the N-terminus of the neoepitope (Fig. 1*C*).

For human IL-18, there is a very simple functional ELISA system that detects INF-γ induction, using KG-1 human acute myeloblastic leukemia cells [19,21]. However, in this system, even the same amount of mouse IL-18 is detected as 100-fold less active, and thus the activity could not be evaluated accurately. Therefore, we investigated the IL-18 receptor expression in mouse cell lines at the mRNA level, confirmed the induction of downstream molecules at the mRNA and protein levels, and established a new functional evaluation ELISA system for mouse IL-18 that detects CXCL2 induction in P-815 cells. This newly developed functional evaluation system for mouse IL-18 allowed us to accurately evaluate the function-blocking activity of the newly developed mAbs.

The half-life of human IgG ranges from 7 to 21 days, depending on the IgG subclasses [27]. The half-life of mouse IgG is about 6–8 days [28], depending on the mouse strains, and experiments with FcRn-deficient mice demonstrated that the half-life of an IgG that cannot bind FcRn is reduced to about 1.7 days. Since the half-life of mAb 5-4.1 is about 1.3 days, it is highly likely that it cannot bind to FcRn. In this study, we measured the half-life in blood by intravenous administration, which is a possible route in clinical applications. We plan to choose intraperitoneal administration as the route of administration for therapeutic experiments of the antagonistic anti-mouse IL-18 neoepitope antibody in model mice. In the case of intraperitoneal administration, a longer time is required for the antibody to enter the bloodstream as compared to intravenous administration, and considering the half-life of this antibody, an intraperitoneal administration schedule of two to three times a week is considered appropriate for therapeutic experiments in mouse models.

In this study, we utilized an ELISA with the neoepitope peptide to measure the amount of antibody in the blood. This is a very simple method with a low background. In many cases, anti-idiotype antibodies are used to study the pharmacokinetics of antibodies [29]. In the case of highly specific antibodies that recognize neoepitope peptides, peptide-based evaluation systems are also sufficient. By changing to peptides for human use, the pharmacokinetics of humanized antibodies can be obtained in the same way.

Author contributions

Conceived and designed the experiments: TU. Performed the experiments: YU, YN, EO, TK, TU, and HK. Analyzed the data: YU, YN, EO, YT, TK, AK, TU, and HK. Wrote the paper: YU, TU, and HK. All authors contributed to the preparation and writing of the manuscript.

Declaration of competing interest

TU is employed by Shimane University and is currently a co-founder and Chief Medical & Scientific Officer of mAbProtein, a biotech company focusing on the development and commercial utilization of mAbs for inflammation research, diagnosis, and treatment. EO is employed by Shimane University and specially appointed researcher of mAbProtein. All other authors have declared no conflicts of interest. The potential conflict of interest by EO and TU does not alter the authors' adherence to all of the Archives of Biochemistry and Biophysics policies on sharing data and materials, as detailed online in the guide for authors.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank SKYBAY Scientific Editing for editing this paper. We would like to acknowledge the technical expertise of the Center for Integrated Research in Science, Shimane University. This study was supported by Grants-in-Aid for Scientific Research (20K16443 to YU, 20H010079A to YN, 21K08753 to YT, 21K08438 to TU, and 20K07635 to HK), the SUIGAN project (to TU), Shimane University, Japan; and AMED, Japan under Grant Number 21ek0109501h0001 (to AK, TK, TU).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2022.109322.

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