J. Biochem. 2012;151(1):57–64 doi:10.1093/jb/mvr114

JB THE JOURNAL OF BIOCHEMISTRY

Comparison of human blood concentrations of collectin kidney 1 and mannan-binding lectin

Received June 30, 2011; accepted August 21, 2011; published online September 4, 2011

Takayuki Yoshizaki[1,*], Katsuki Ohtani[1,*], Wataru Motomura[1], Seong-Jae Jang[1], Ken-ichiro Mori[1], Noritoshi Kitamoto[2], Itsuro Yoshida[1], Yasuhiko Suzuki[3] and Nobutaka Wakamiya[1,†]

[1]Department of Microbiology and Immunochemistry, Asahikawa Medical University, 2-1-1-1 Midorigaoka-Higashi, Asahikawa 078-8510; [2]Department of Microbiology, School of Human Science and Environment, University of Hyogo, Himeji 670-0092; and [3]Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo 060-0818, Japan

*The first two authors contributed equally to this work.

†Nobutaka Wakamiya, Department of Microbiology and Immunochemistry, Asahikawa Medical University, 2-1-1-1 Midorigaoka-Higashi, Asahikawa 078-8510, Japan. Tel: +81 166 68 2393, Fax: +81 166 68 2399, email: wakamiya@asahikawa-med.ac.jp

Mannan-binding lectin (MBL) was first discovered as a collectin in animal blood, and was shown to have such unique characteristics as a collage-like domain and a carbohydrate recognition domain. We recently identified human collectin kidney 1 (CL-K1, *COLEC11*) from a human kidney cDNA library. To quantitate the CL-K1 concentration in blood, we developed several polyclonal and monoclonal antibodies using recombinant human CL-K1 in CHO cells and the CL-K1 fragment in *Escherichia coli*. Using these antibodies, we established a sandwich enzyme-linked immunosorbent assay (ELISA) system. The concentration of CL-K1 in human plasma was 0.34 ± 0.13 μg/ml and that in MBL was 1.72 ± 1.51 μg/ml. Concentrations of MBL are often low due to its single nucleotide polymorphisms (SNPs) which seem to be related to an opsonic defect. However, no low concentrations of CL-K1 were observed on testing over two hundred blood samples. We also found that the blood concentration of CL-K1 was not dependent on gender or age and did not correlate completely with that of MBL. The ELISA system developed in this study will be useful for elucidating the physiological and pathophysiological role of CL-K1 in humans.

Keywords: complement/blood coagulation/lectin/carbohydrate/genetic.

Abbreviations: Ab, antibody; CL-K1, collectin kidney 1; CL-L1, collectin liver 1; CL-P1, collectin placenta 1; CRD, carbohydrate recognition domain; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; MBL, mannan-binding lectin; MoAb, monoclonal Ab; PoAb, polyclonal Ab; SP-A, surfactant protein A; SP-D, surfactant protein D.

Collectins are characterized by a collagen-like sequence and a carbohydrate recognition domain (CRD), and are members of the vertebrate C-type lectin superfamily (*1*). Recently, human collectins have been divided into two groups: three 'classical collectins' consisting of mannan-binding lectin (MBL) (*2*), and surfactant proteins A and D (SP-A and SP-D) (*3*, *4*), and three 'novel collectins' made up of collectin liver 1 (CL-L1) (*5*), collectin placenta 1 (CL-P1) (*6*), and collectin kidney 1 (CL-K1) (*7*).

MBL in serum and liver (*2*, *8*) is associated with the activation of complement (*9*). MBL can also act as an opsonin due to its interaction with a collectin receptor (*10*) or with viral neutralization factors (*11*). It was reported that MBL deficiency is associated with recurrent infections in both infants and adults (*12*, *13*). The concentration of MBL in serum is determined by allelic forms associated with MBL gene mutations in codons of exon 1 encoding the collagen-like domain (*14*) and sequence polymorphisms in the promoter region (*15*). Other 'classical collectins', SP-A and SP-D can also mediate opsonization of bacteria and neutralization of viral growth, and are able to associate directly with macrophages and stimulate phagocytosis or oxidant-dependent microbial clearance (*16*–*18*). Thus, 'classical collectins' are considered to play an important role in innate immunity.

Recently, cDNAs encoding the three 'novel collectins' were isolated and characterized using a reverse genetic approach. CL-L1 is mainly produced in liver as a cytoplasmic protein (*5*). CL-P1 is a scavenger receptor mainly expressed in vascular endothelial cells, which binds to and endocytoses oxidized low density lipoprotein (OxLDL) as well as microbes (*6*, *19*). More recently, we have demonstrated a novel human and murine collectin, CL-K1 (*7*).

CL-K1 was first cloned and deposited as RIKEN cDNA 1010001H16 in 2001 and was later assigned the gene name *COLEC11* (HUGO: collectin sub-family member 11) in 2003. CL-K1 that harbours a 25-amino acid signal sequence was found in human blood as was MBL (*7*). CL-K1 can bind to microbial lipopolysaccharides and lipoteichoic acid and activate the lectin complement pathway as the recognition molecule as well as MBL and ficolin (*7*, *20*), suggesting

that it might play a role in innate immunity. However, the physiological role of CL-K1 in humans has not yet been elucidated. In the current study, we have developed an enzyme-linked immunosorbent assay (ELISA) system for quantifying human plasma CL-K1 and compared the concentration of this collectin with that of MBL.

Experimental procedures

Blood samples

This investigation was approved by the review board of the Asahikawa Medical University Ethics Committee. Blood samples were taken from 220 healthy volunteers after obtaining their informed consent prior to entering this study. Plasma and serum samples were harvested and stored at −80°C.

Expression of recombinant human CL-K1 proteins and preparation of antibodies

To construct two recombinant proteins for the ELISA assay, we produced a CL-K1 fragment protein (CL-K1-neck-CRD) in *Escherichia coli* and the full size CL-K1 protein (CL-K1-full) in Chinese hamster ovary (CHO) cells, as described previously (*7*, *21*). Briefly, CL-K1-neck-CRD, including the neck and CRD domains (amino acids 107–271) together with six histidines, was expressed in *E. coli* GI724 using *pPLH3* expression vector, as described previously (*7*). The CL-K1-neck-CRD protein was extracted and purified with Ni-NTA Agarose (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. CL-K1 stable expression clones were obtained by transfection of *pNOW/CMV-A* containing the CL-K1-full gene into CHO cells and stable clones producing high levels of CL-K1-full protein were established (*7*). CL-K1-full was purified from culture supernatants of stable clone #77 as follows. The culture supernatants were incubated with mannan–agarose (Sigma-Aldrich Co.), then the CL-K1 preparations were eluted with TBS/Ca containing 100 mM D-mannose and stored at 4°C.

New Zealand White rabbits were injected three times at 2-week intervals with 200 or 100 μg of the above two CL-K1 proteins in complete or incomplete Freund's adjuvant. After immunization, whole sera from rabbits were applied to HiTrap Protein G HP (GE Healthcare) and anti-CL-K1 rabbit IgG fractions were eluted with 0.1 M glycine–HCL buffer at pH 2.5. In addition, the anti-CL-K1 IgG was affinity-purified using a CL-K1-neck-CRD or CL-K1-full conjugated antigen column, HiTrap NHS-activated HP (GE Healthcare). Two types of polyclonal antibodies were prepared, polyclonal anti-CL-K1-neck-CRD antibody (PoAb-CRD) and polyclonal anti-CL-K1-full (PoAb-full). The IgG fraction which was passed through the CL-K1 antigen column was used as the control IgG. Monoclonal antibodies (MoAb) were established by immunizing with CL-K1-neck-CRD. Twenty monoclonal antibody-producing cell clones (clones 47, 118, 129, 145, 211, 325, 412, 611, 710, 718, 821, 1011, 1311, 1412, 1516, 1520, 2711, 2814 and 5112) were cultured with IS GROTM (Irvine Scientific) in serum-free medium for 7 days and their culture supernatants were purified on a Protein G HP column (GE Healthcare). These CL-K1 monoclonal antibodies were prepared as MoAbs-47, 118, 129, 145, 211, 325, 412, 611, 710, 718, 821, 1011, 1311, 1412, 1516, 1520, 2711, 2814 and 5112. All experiments were carried out in accordance with the rules and guidelines of the Animal Experiment Committee of Asahikawa Medical University.

SDS–PAGE and western blotting analyses

Western blotting was performed to identify which of several antibodies were able to react with CL-K1 proteins. Various amounts of recombinant CL-K1 proteins or 1 μl of plasma and serum solution treated with serial dilutions were dissolved in SDS–PAGE sample buffer and subjected to 4–20% SDS–PAGE. The gel was then stained with Coomassie blue, or proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Co., Bedford, MA) using a semi-dry transfer apparatus (*7*). After blocking with blockace (Dainippon Pharmaceutical, Osaka, Japan) at 4°C overnight, the membrane was incubated with 5 μg/ml CL-K1 rabbit polyclonal antibodies or 1 μg/ml monoclonal antibodies for 1 h. After washing with TBS containing 0.05% Tween 20 (TTBS), the membrane was incubated with horseradish–peroxidase (HRP)-conjugated anti-rabbit-IgG or anti-mouse-IgG (Chemicon International, Inc., Temecula, CA, USA). An ECL chemiluminescence system (Amersham Life Sciences, Buckinghamshire, UK) was used to visualize bands on the blots which were analysed using an LAS-3000 (FUJIFILM, Tokyo, Japan).

CL-K1 and MBL in plasma were extracted by microtitre-well-based affinity chromatography (*22*). Wells were coated with anti-CL-K1 monoclonal antibody (MoAb-2814) or anti-MBL monoclonal antibody (MBLMoAb-224) and then incubated with diluted normal human plasma and washed, then bound material was eluted with SDS–PAGE sample buffer. The plasma samples and diluted recombinant CL-K1 and MBL were analysed by western blotting using anti-CL-K1 polyclonal antibody (PoAb-full) or anti-MBL polyclonal antibody (MBLPoAb), as previous described (*23*).

Enzyme-linked immunosorbent assay

The ELISA procedure employed here was developed to determine the expression levels of the above two proteins in human blood. Our sandwich ELISA procedure employed rabbit polyclonal antibodies and murine monoclonal antibodies. All the above antibodies were tested as to which pair made the best match for the capture antibody and the biotinylated detection antibody. Wells of 96-well microtitre plates were coated with the capture antibody at a concentration of 1 μg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05% NaN_3, pH 9.6) and incubated overnight at 4°C. TTBS/Ca (TTBS containing 5 mM $CaCl_2$, pH 7.4) was used in all of the washing steps. After washing, the plates were blocked with Block Ace and washed again. The plates were then incubated at 37°C for 1 h with culture fluid diluted with TTBS with or without $CaCl_2$ or EDTA at concentrations of 5 mM. The plates were washed and incubated with the biotinylated detection antibody at a concentration of 1 μg/ml in TTBS/Ca. After washing, the plates were incubated at 37°C for 1 h using a VECTASTAIN *Elite* ABC kit (Vector Lab. Inc., CA, USA). Finally, 100 μl of SureBlue were applied to each well (TMB Microwell Peroxidase Substrate, Kirkegaard & Perry Lab. Inc. (KPL), MD, USA). After the addition of 100 μl of 1 M phosphoric acid, the absorbance at 450 nm was read with a Model 450 Microplate Reader (Bio-Rad Lab.). We next established a standard curve using the best pair of PoAb-full-1 and biotinylated MoAb-1011. The CL-K1-full or pooled plasma as the standard was set at a concentration of 30, 15, 7.5, 3.75, 1.875, 0.9375 and 0.46875 ng/ml, PoAb-full-1 as the capture antibody was used at 1 or 10 μg/ml, and biotinylated MoAb-1011 as the detection antibody was used at 1 μg/ml. Human blood samples from the healthy volunteers were collected as plasma and sera for the ELISA assay. The concentrations of CL-K1 and MBL were measured using the CL-K1 and MBL ELISA systems (*23*). The MBL genotype had already been determined using blood sera, as previously described (*24*).

Results

Volunteer characteristics

We recruited 220 healthy volunteers, aged 61.9 ± 5.6, with both sexes equally represented. The ages of males ranged from 18 to 87 years (median age, 69 years) and those of the females ranged from 18 to 89 years (median age, 64 years) (Table I).

Characterization of recombinant CL-K1 proteins and antibodies

To generate antibodies, we produced recombinant CL-K1 proteins, untagged CL-K1-full in CHO cells and CL-K1-neck-CRD containing histidine tag in *E. coli*. These CL-K1 proteins were applied to SDS–PAGE and gels were stained with Coomassie blue (Fig. 1A). The PAGE of CL-K1-full under reducing condition revealed a 32 kDa band of the complete monomer, which was identified as human CL-K1 with the anti-human CL-K1-neck-CRD antibody (PoAb-CRD), with the anti-human CL-K1-full

Table I. Characteristic of healthy volunteer.

	Male ($n = 110$)	Female ($n = 110$)	Total ($n = 220$)
Age, median (range)	69 (18–87)	64 (18–89)	65.5 (18–89)
CL-K1, mean, μg/ml (range)	0.32 ± 0.10 (0.13–0.64)	0.35 ± 0.15 (0.15–1.39)	0.34 ± 0.13 (0.13–1.39)
MBL, mean, μg/ml (range)	1.66 ± 1.40 (0.07–5.25)	1.79 ± 1.61 (0.04–9.05)	1.72 ± 1.51 (0.04–9.05)

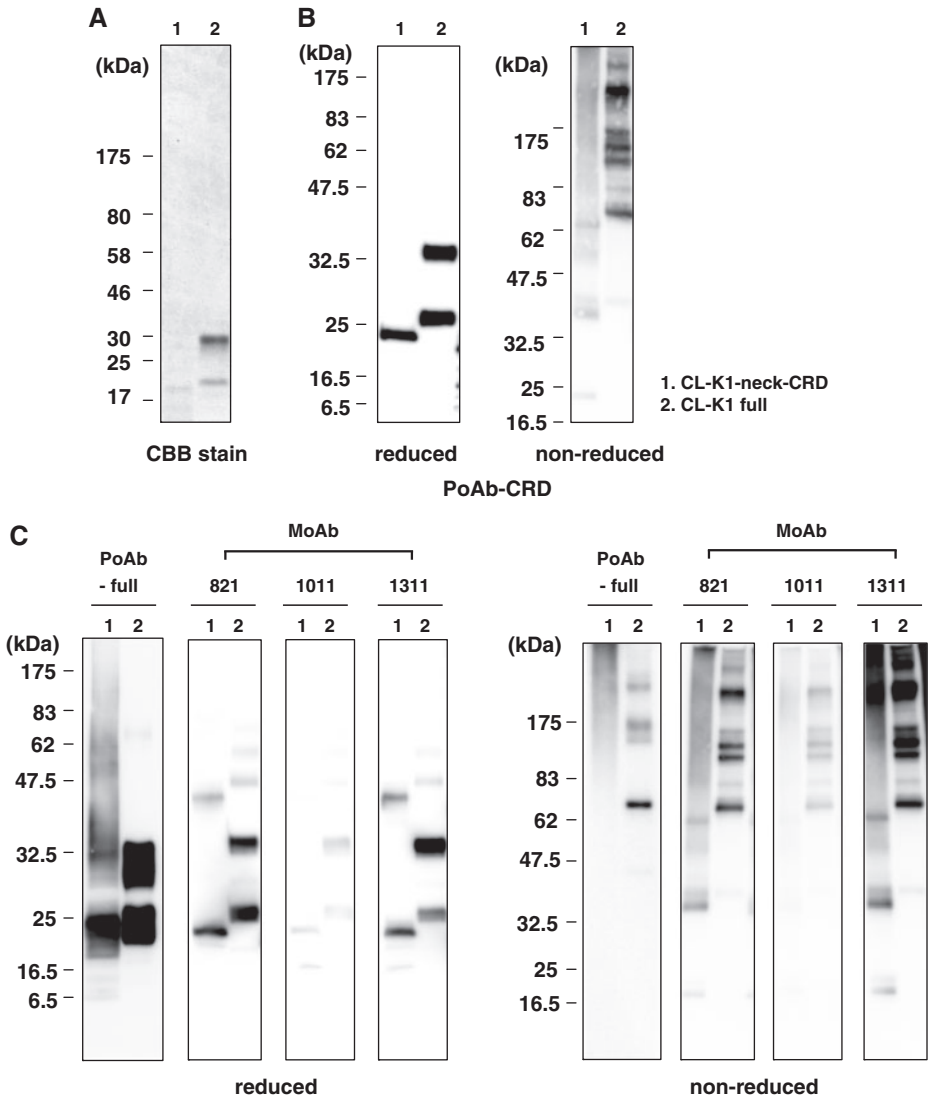

Fig. 1 The antibodies used here were confirmed by immunoblotting. Polyacrylamide gel electrophoresis analyses of two recombinant CL-K1 proteins (CL-K1-neck-CRD, CL-K1-full) were performed with/without western blotting with three antibodies (PoAb-CRD, PoAb-full and MoAb). (**A**) The recombinant CL-P1 protein samples were treated with SDS with β-mercaptoethanol and loaded onto a 4–20% polyacrylamide gel, the gel was stained with Coomassie blue. (**B** and **C**) The same samples were subjected to immunoblotting with the above three antibodies.

antibody (PoAb-full), and with the anti-human CL-K1 monoclonal antibody (MonoAb-821, 1011, 1311) by immunoblotting (Fig. 1B and C). The PAGE of CL-K1-full under non-reducing condition by same immunoblotting demonstrated several higher-molecular weight bands as most likely representing CL-K1 oligomers. The CL-K1-neck-CRD was also observed as a 24-KDa band of the truncated form in the immunoblotting.

Detection of CL-K1 in human plasma

To search for the presence of CL-K1 or MBL in blood, we used Ab-coated microwells to affinity-purify the two collectins from plasma, and these were then analysed by western blotting. Bands at the positions expected for CL-K1 and MBL were seen in lanes containing the eluate from wells coated with the anti-CL-K1 or anti-MBL antibody, as well as in lanes containing the directly loaded recombinant CL-K1 or MBL

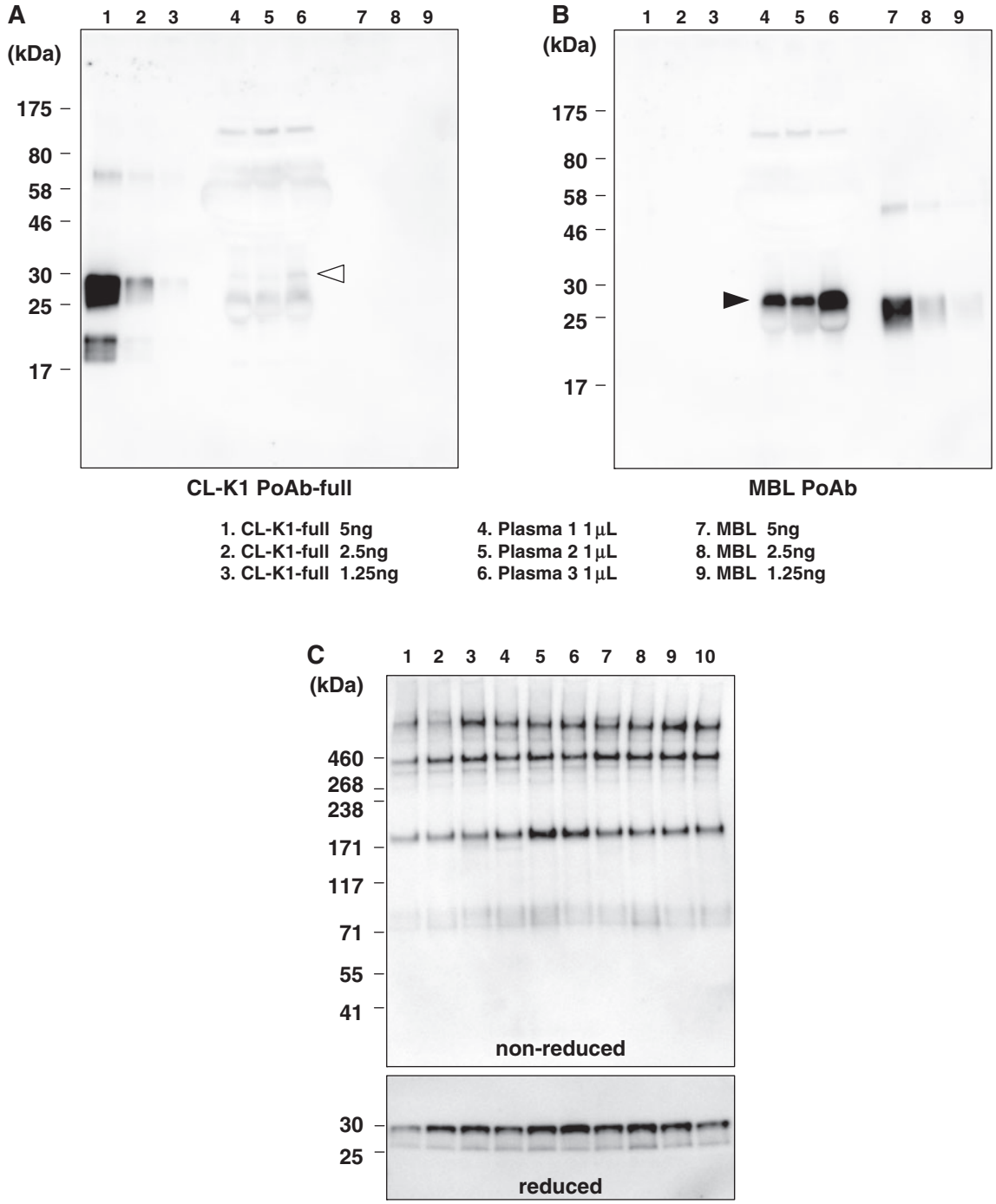

Fig. 2 Immunoblotting of CL-K1 and MBL extracted by microtitre-well-based affinity chromatography. (A and B) The extracted collectins and the recombinant CL-K1-full and MBL as the standard collectin were serially diluted (1.25, 2.5, 5 ng/lane) and levels were measured by immunoblotting with PoAb-full or MBLPoAb. (C) Ten of the extracted samples were subjected to immunoblotting for CL-K1.

proteins (Fig. 2A–C). We observed dose-dependent signals in lanes containing serially diluted CL-K1 or MBL, but no signal in lanes using the reverse combination assay systems with collectin and antibody. Figure 2A and B shows that the plasma concentration of CL-K1 was lower than that of MBL. These results demonstrated that there was no CL-K1 deficiency in these ten volunteers as was also the case on MBL analysis (Fig. 2C).

Development of an ELISA system for determination of plasma CL-K1

We tried to establish a sandwich ELISA system that provided the best match of two specific antibodies. For this purpose, we performed crisscross analysis using 3 polyclonal rabbit antibodies and 20 mouse monoclonal antibodies (Fig. 3A). The best match proved to be PoAb-full-1 as the capture antibody and MoAb-1011 as the detection antibody. Using

A

Coating antibodies on wells	Detection antibodies labeled with biotin: MoAb 47	118	129	145	211	325	412	611	620	710	718	821	1011	1311	1412	1516	1520	2711	2814	5112	PoAb CRD	1	2
MoAb 47	0.361	0.254	0.218	0.287	0.265	0.204	0.279	0.204	0.257	0.217	0.247	0.309	0.354	0.324	0.343	0.223	0.235	0.250	0.228	0.252	0.231	0.300	0.296
MoAb 118	0.349	0.209	0.197	0.197	0.198	0.199	0.194	0.187	0.193	0.182	0.182	0.229	0.239	0.228	0.218	0.214	0.208	0.207	0.213	0.229	0.212	0.238	0.227
MoAb 129	0.238	0.195	0.189	0.202	0.194	0.175	0.194	0.179	0.192	0.179	0.189	0.199	0.267	0.233	0.237	0.207	0.208	0.203	0.200	0.213	0.218	0.253	0.225
MoAb 145	0.299	0.204	0.200	0.216	0.234	0.180	0.215	0.191	0.223	0.178	0.198	0.203	0.328	0.298	0.257	0.201	0.207	0.221	0.212	0.237	0.217	0.810	0.702
MoAb 211	0.333	0.216	0.207	0.405	0.204	0.175	0.390	0.187	0.384	0.205	0.281	0.226	0.507	0.484	0.388	0.211	0.278	0.211	0.222	0.339	0.243	0.646	0.579
MoAb 325	0.250	0.208	0.200	0.188	0.179	0.185	0.190	0.176	0.190	0.186	0.191	0.453	0.240	0.235	0.214	0.222	0.208	0.203	0.206	0.211	0.206	0.478	0.459
MoAb 412	0.282	0.213	0.216	0.243	0.257	0.196	0.227	0.201	0.238	0.200	0.213	0.210	0.338	0.318	0.264	0.231	0.247	0.221	0.230	0.240	0.242	0.851	0.848
MoAb 611	0.248	0.253	0.243	0.226	0.226	0.218	0.234	0.193	0.215	0.191	0.191	0.196	0.280	0.258	0.227	0.228	0.255	0.247	0.246	0.248	0.241	0.283	0.303
MoAb 620	0.266	0.252	0.237	0.260	0.248	0.219	0.242	0.203	0.262	0.212	0.220	0.250	0.291	0.266	0.261	0.201	0.220	0.221	0.215	0.223	0.206	0.232	0.269
MoAb 710	0.260	0.207	0.199	0.208	0.223	0.189	0.221	0.191	0.216	0.186	0.198	0.246	0.262	0.226	0.213	0.222	0.197	0.191	0.194	0.208	0.186	0.217	0.224
MoAb 718	0.257	0.190	0.193	0.201	0.207	0.179	0.198	0.180	0.212	0.190	0.197	0.214	0.236	0.235	0.213	0.181	0.192	0.193	0.185	0.207	0.192	0.224	0.220
MoAb 821	0.266	0.190	0.191	0.209	0.198	0.172	0.204	0.187	0.205	0.182	0.165	0.259	0.243	0.219	0.210	0.177	0.193	0.185	0.183	0.199	0.192	0.467	0.217
MoAb 1011	0.252	0.191	0.194	0.210	0.202	0.185	0.223	0.182	0.231	0.176	0.193	0.229	0.246	0.222	0.222	0.185	0.191	0.180	0.183	0.216	0.243	0.479	0.405
MoAb 1311	0.240	0.192	0.194	0.191	0.198	0.177	0.197	0.178	0.194	0.190	0.201	0.266	0.253	0.221	0.234	0.196	0.196	0.189	0.191	0.199	0.192	0.282	0.297
MoAb 1412	0.276	0.203	0.200	0.224	0.261	0.194	0.237	0.223	0.264	0.184	0.280	0.258	0.296	0.283	0.245	0.220	0.205	0.193	0.185	0.217	0.203	0.670	0.652
MoAb 1516	0.239	0.214	0.190	0.218	0.233	0.219	0.238	0.207	0.243	0.194	0.202	0.238	0.200	0.196	0.196	0.191	0.192	0.191	0.179	0.181	0.180	0.963	0.985
MoAb 1520	0.251	0.223	0.231	0.220	0.191	0.214	0.212	0.188	0.204	0.226	0.195	0.318	0.278	0.285	0.292	0.215	0.231	0.250	0.204	0.227	0.246	0.890	0.800
MoAb 2711	0.229	0.206	0.197	0.210	0.203	0.199	0.204	0.190	0.209	0.201	0.201	0.261	0.268	0.250	0.239	0.211	0.233	0.233	0.217	0.237	0.237	0.232	0.252
MoAb 2814	0.207	0.196	0.192	0.194	0.192	0.181	0.197	0.173	0.208	0.186	0.189	0.190	0.294	0.247	0.230	0.197	0.196	0.199	0.183	0.209	0.199	0.220	0.244
MoAb 5112	0.227	0.210	0.199	0.189	0.214	0.185	0.194	0.180	0.181	0.185	0.194	0.188	0.324	0.218	0.207	0.200	0.200	0.195	0.189	0.194	0.188	0.942	0.964
PoAb CRD	0.488	0.220	0.230	0.688	0.668	0.221	0.732	0.314	0.682	0.302	0.387	0.267	0.965	0.863	0.708	0.250	0.380	0.223	0.244	0.488	0.280	0.520	0.617
PoAb 1	2.067	0.474	0.269	2.581	2.491	0.271	2.847	0.460	2.563	0.972	1.291	0.814	2.951	2.253	2.723	0.344	0.697	0.262	0.635	0.762	0.484	1.315	1.302
PoAb 2	0.964	0.300	0.275	1.409	1.333	0.265	1.460	0.411	1.360	0.365	0.650	0.362	2.099	1.659	1.373	0.325	0.499	0.255	0.334	0.707	0.351	1.012	1.197

Red: >2.0, Blue: >1.0, Green: >0.5

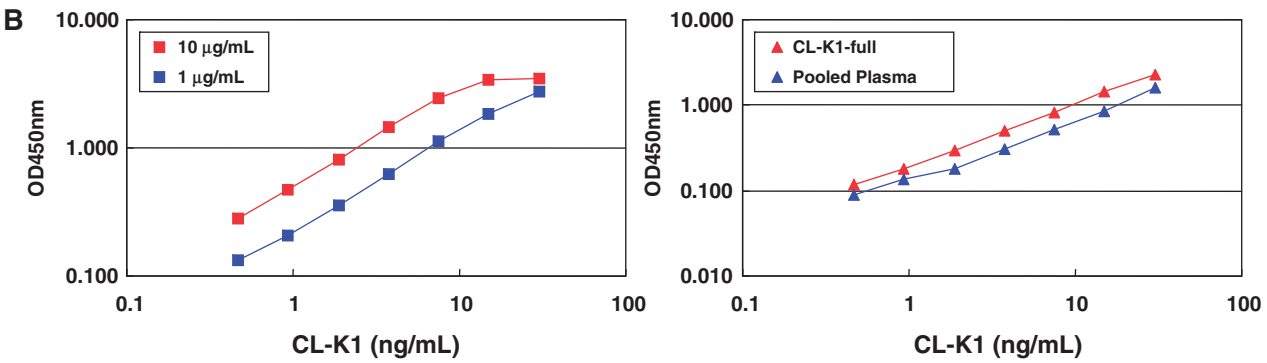

Fig. 3 Establishment of a novel CL-K1 sandwich ELISA system. (A) The best matching set (PoAb-full-1/coated antibody and MoAb-1011/detection antibody) was determined by crisscross analysis using twenty MoAb, two PoAb-full and PoAb-CRD. Absorbance was measured at 450 nm and scored as <0.5 (with green), 1–2 (with blue), or >2 (with red), and the best matching set is indicated in yellow. (B) The two standard curves in the ELISA were obtained using CL-K1-full as CL-K1 standard and the capture antibody of PoAb-full-1 at concentrations of 1 and 10 μg/ml in the left panel and using CL-K1-full or the pooled plasma (its CL-K1 amount measured previously) as CL-K1 standard and the capture antibody of PoAb-full-1 at concentrations of 1 μg/ml in the right panel.

this set, we established a CL-K1 sandwich ELISA system and obtained a good standard curve with a serial dilution of recombinant CL-K1 from CHO cells or pooled plasma which concentration was previously measured by SDS–PAGE and Coomassie blue staining or the ELISA (Fig. 3B). The CL-K1 concentration ranged from 0.47 to 30.00 ng/ml. We determined levels of CL-K1 and MBL in over two hundred plasma samples using the above ELISA system (Fig. 4A–C). The concentration of CL-K1 was 0.34 ± 0.13 μg/ml (ranging from 0.13 to 1.39 μg/ml) and that of MBL was 1.72 ± 1.51 μg/ml (ranging from 0.04 to 9.05 μg/ml). These results were consistent with the results of CL-K1 amount lower than that of MBL shown in SDS–PAGE and western blot analysis (Fig. 2B). The standard deviations of CL-K1 and MBL were 0.13 and 1.51 μg/ml respectively. These results indicate that the concentration of CL-K1 is less variable among individuals compared with that of MBL. Figure 5A and Table I demonstrate that there was no correlation between the amounts of CL-K1 and MBL in plasma. Figure 5B shows that plasma CL-K1 levels in males were similar to those in females as was the case with MBL (0.32 ± 0.10 versus 0.35 ± 0.15 μg/ml). In addition, concentrations of both CL-K1 and MBL were not dependent on age (Fig. 5B).

Discussion

We previously reported that in human and mice, CL-K1 protein is produced in various tissues and that found in blood is mainly produced by the liver

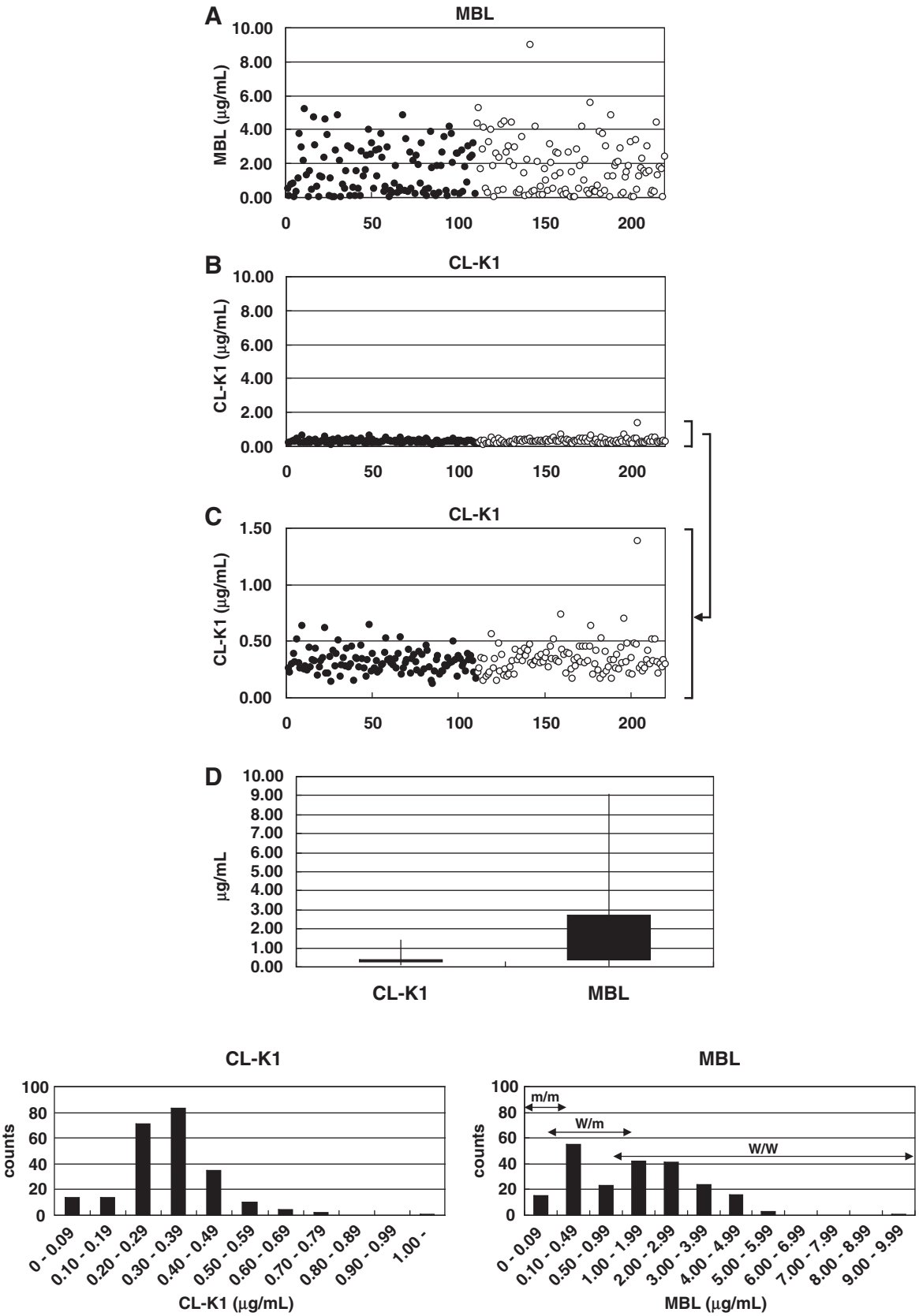

Fig. 4 Comparison of plasma concentration between the CL-K1 and MBL using the new ELISA system. The CL-K1 ELISA and MBL ELISA were performed with over two hundred human plasma samples (closed circle indicates male and open circle indicates female). (A–C) The concentration of CL-K1 was 0.34 ± 0.13 μg/ml (ranging from 0.13 to 1.39 μg/ml) and that of MBL was 1.72 ± 1.51 μg/ml (ranging from 0.04 to 9.05 μg/ml). (D) The histogram of the concentrations of CL-K1 and MBL was obtained using the above ELISA results. Three genotypes indicate that W/W has both wild alleles, W/m has wild and mutant alleles, and m/m has both mutant alleles.

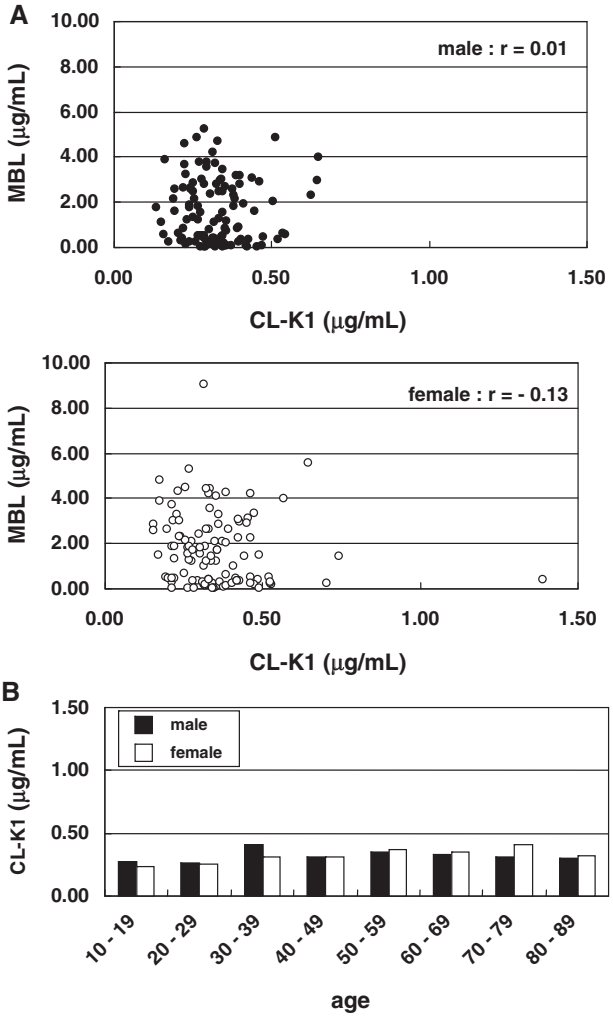

Fig. 5 Characteristics of the plasma CL-K1 compared with plasma MBL. (A) An analysis of the correlation between CL-K1 and MBL concentrations in human plasma was performed, and that of plasma CL-K1 did not correlate with that of MBL (male: $r = 0.01$, female: $r = -0.13$). (B) The histogram of the concentrations of CL-K1 and MBL in males and females was plotted relative to age.

as is MBL (*7*, *20*). In addition, we isolated a CL-K1 cDNA fragment from a cDNA library of human kidney tissue (*7*). Keshi first observed CL-K1 in blood by western blotting and Holmskov confirmed the purified protein by mass spectrometry and western blotting (*20*). He also demonstrated that CL-K1 was the third molecule to be able to activate the complement pathway as well as MBL and ficolin (*20*). In the present study, we established a novel ELISA system using a best-match antibody set which was selected after evaluating a large number of antibodies. This ELISA exhibited good correlations using recombinant CK-K1-full and pooled control plasma. The amount of CL-K1 in plasma was lower than that of MBL, as seen with a semi-quantitative analysis by microtitre-well-based affinity chromatography. These results were consistent in that the concentration of CL-K1 was 0.34 ± 0.13 μg/ml, lower than that of MBL which was 1.72 ± 1.51 μg/ml. Furthermore, the concentration of CL-K1 was less variable among individuals compared with MBL concentration, which is predominantly determined by single nucleotide polymorphisms (SNPs) in the exon coding for collagen-like domain and in the promoter region (*15*).

The functions of CL-K1 have not been completely elucidated although very recently human cases of a CL-K1 deficiency due to mutations in *COLEC11* and *MASP1* genes have been reported as the 3MC syndrome (*25*, *26*). These authors described the human cases of the deficiency due to mutations in Collec11 and MASP1, but it was seemed to be caused by actual deficiency of MASP3 although they described MASP1 deficiency in these papers.

The features of this syndrome result from rare autosomal recessive disorders and include a characteristic facial dysmorphism, a cleft lip and/or a cleft palate, craniosynostosis, learning disabilities and hearing loss. The physiological investigation could not explain how these various features arose. However, this report as well as that of Thiel suggests that CL-K1 together with MASP-1 acts not only as an innate immune factor but also influences fundamental development processes (*27*). Further analysis using animal model such as gene knockout mice should elucidate the true functions of this collectin. Furthermore, by applying this ELISA system in future studies, we can assess CL-K1 levels in both healthy and unhealthy peoples in order to demonstrate the role of CL-K1 in other disorders.

In summary, we established a CL-K1 ELISA system and demonstrated that the concentration of CL-K1 in blood was not only lower than that of MBL but also that it had no correlation with that of MBL. Furthermore, the protein level of CL-K1 was strictly regulated regardless of age or sex. The ELISA system developed in this study will be useful for elucidating the physiological and pathophysiological role of CL-K1 in humans.

Acknowledgements

The authors thank Professors T. Azuma and Y. Ito for useful discussions.

Funding

This work was supported by grants from Grants-in-Aid for Scientific Research (19390227, 22390113, 22590259) and from the 'Knowledge Cluster Initiative' (2nd Stage, 'Sapporo Biocluster Bio-S'), a project of the Japan Ministry of Education, Culture, Sports, Science, and Technology, by a grant from Core Research for Evolutional Science and Technology allocated by the Japan Society for the Promotion of Science, and by a grant from the Japan Health Sciences Foundation KH21011. This work was also supported by grants from Fuso Pharmaceutical Industry, Co., the Fugaku Trust for Medical Research, the Smoking Research Foundation, the Akiyama Foundation, Hokkaido Heart Association Grants for Research, and the Takeda Science Foundation.

Conflict of interest

None declared.

References

1. Drickamer, K. (1988) Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* **263**, 9557–9560

2. Kawasaki, N., Kawasaki, T., and Yamashina, I. (1983) Isolation and characterization of a mannan-binding protein from human serum. *J. Biochem.* **94**, 937–847
3. White, R.T., Damm, D., Miller, J, Spratt, K., Schilling, J., Hawgood, S., Benson, B., and Cordell, B. (1985) Isolation and characterization of the human pulmonary surfactant apoprotein gene. *Nature* **317**, 361–363
4. Persson, A., Chang, D., Rust, K., Moxley, M., Longmore, W., and Crouch, E. (1989) Purification and biochemical characterization of CP4 (SP-D), a collagenous surfactant-associated protein. *Biochemistry* **28**, 6361–6367
5. Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Yamazaki, H., Shimada, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T., and Wakamiya, N. (1999) Molecular cloning of a novel human collectin from liver (CL-L1). *J. Biol. Chem.* **274**, 13681–13689
6. Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T., Itabe, H., Suzutani, T., Ogasawara, M., Yoshida, I., and Wakamiya, N. (2001) The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. *J. Biol. Chem.* **276**, 44222–44228
7. Keshi, H., Sakamoto, T., Kawai, T., Ohtani, K., Katoh, T., Seong-Jae, J., Motomura, W., Yoshizaki, T., Fukuda, M., Koyama, S., Fukuzawa, J., Fukuoh, A., Yoshida, I., Suzuki, Y., and Wakamiya, N. (2006) Identification and characterization of a novel human collectin CL-K1. *Microbiol. Immunol.* **50**, 1001–1013
8. Kawasaki, T., Etoh, R., and Yamashina, I. (1978) Isolation and characterization of a mannan-binding protein from rabbit liver. *Biochem. Biophys. Res. Commun.* **81**, 1018–1024
9. Ikeda, K., Sannoh, T., Kawasaki, N., Kawasaki, T., and Yamashina, I. (1987) Serum lectin with known structure activates complement through the classical pathway. *J. Biol. Chem.* **262**, 7451–7452
10. Malhotra, R., Thiel, S., Reid, K.B., and Sim, R.B. (1990) Human leukocyte C1q receptor binds other soluble proteins with collagen domains. *J. Exp. Med.* **172**, 955–959
11. Kase, T., Suzuki, Y., Kawai, T., Sakamoto, T., Ohtani, K., Eda, S., Maeda, A., Okuno, Y., Kurimura, T., and Wakamiya, N. (1999) Human mannan-binding lectin inhibits the infection of influenza A virus without complement. *Immunology* **97**, 385–392
12. Super, M., Thiel, S., Lu, J., Levinsky, R.J., and Turner, M.W. (1989) Association of low levels of mannan-binding protein with a common defect of opsonisation. *Lancet* **2**, 1236–1239
13. Summerfield, J.A., Ryder, S., Sumiya, M., Thursz, M., Gorchein, A., Monteil, M.A., and Turner, M.W. (1995) Mannose binding protein gene mutations associated with unusual and severe infections in adults. *Lancet* **345**, 886–889
14. Sumiya, M., Super, M., Tabona, P., Levinsky, R.J., Arai, T., Turner, M.W., and Summerfield, J.A. (1991) Molecular basis of opsonic defect in immunedeficient children. *Lancet* **337**, 1569–1570
15. Madsen, H.O., Garred, P., Thiel, S., Kurtzhals, J.A., Lamm, L.U., Ryder, L.P., and Svejgaard, A. (1995) Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *J. Immunol.* **155**, 3013–3020
16. Eda, S., Suzuki, Y., Kase, T., Kawai, T., Ohtani, K., Sakamoto, T., Kurimura, T., and Wakamiya, N. (1996) Recombinant bovine conglutinin, lacking the N-terminal and collagenous domains, has less conglutination activity but is able to inhibit haemagglutination by influenza A virus. *Biochem. J.* **316**, 43–48
17. Eda, S., Suzuki, Y., Kawai, T., Ohtani, K., Kase, T., Fujinaga, Y., Sakamoto, T., Kurimura, T., and Wakamiya, N. (1997) Structure of a truncated human surfactant protein D is less effective in agglutinating bacteria than the native structure and fails to inhibit haemagglutination by influenza A virus. *Biochem. J.* **323**, 393–399
18. Sano, H. and Kuroki, Y. (2005) The lung collectins, SP-A and SP-D, modulate pulmonary innate immunity. *Mol. Immunol.* **42**, 279–287
19. Jang, SJ., Ohtani, K., Fukuoh, A., Yoshizaki, T., Fukuda, M., Motomura, W., Mori, K., Fukuzawa, J., Kitamaoto, N., Yoshida, I., Suzuki, Y., and Wakamiya, N. (2009) Scavenger receptor collectin placenta 1 (CL-P1) predominantly mediates zymosan phagocytosis by human vascular endothelial cells. *J. Biol. Chem.* **284**, 3956–3965
20. Hansen, S., Selman, L, Palaniyar, N., Ziegler, K., Brandt, J., Kliem, A., Jonasson, M., Skjoedt, M.O., Nielsen, O., Hartshorn, K., Jørgensen, T.J., Skjødt, K., and Holmskov, U. (2010) Collectin 11 (CL-11, CL-K1) is a MASP-1/3-associated plasma collectin with microbial-binding activity. *J Immunol.* **185**, 6096–6104
21. Motomura, W., Yoshizaki, T., Ohtani, K., Okumura, T., Fukuda, M., Fukuzawa, J., Mori, K., Jang, SJ., Nomura, N., Yoshida, I., Suzuki, Y., Kohgo, Y., and Wakamiya, N. (2008) Immunolocalization of a novel collectin CL-K1 in murine tissues. *J. Histochem. Cytochem.* **56**, 243–252
22. Degn, S.E., Hansen, A.G., Steffensen, R., Jacobsen, C., Jensenius, J.C., and Thiel, S. (2009) MAp44, a human protein associated with pattern recognition molecules of the complement system and regulating the lectin pathway of complement activation. *J Immunol.* **183**, 7371–7378
23. Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Keshi, H., Sakai, Y., Yamamoto, S., Sakamoto, T., and Wakamiya, N. (1999) High-level and effective production of human mannan-binding lectin (MBL) in Chinese hamster ovary (CHO) cells. *J. Immunol. Methods* **222**, 135–144
24. Hokozaki, Y., Yoshiba, M., Sekiyama, K., Seike, E., Iwamoto, J., Mitani, K., Masafumi, M., Morizone, T., Ohtani, K., Suzuki, Y., and Wakamiya, N. (2002) Mannose-binding lectin and the prognosis of fulminant hepatic failure caused by HBV infection. *Liver* **22**, 29–34
25. Rooryck, C., Diaz-Font, A., Osborn, D.P., Chabchoub, E., Hernandez-Hernandez, V., Shamseldin, H., Kenny, J., Waters, A., Jenkins, D., Kaissi, A.A., Leal, G.F., Dallapiccola, B., Carnevale, F., Bitner-Glindzicz, M., Lees, M., Hennekam, R., Stanier, P., Burns, A.J., Peeters, H., Alkuraya, F.S., and Beales, P.L. (2011) Mutations in lectin complement pathway genes COLEC11 and MASP1 cause 3MC syndrome. *Nat. Genet.* **43**, 197–203
26. Sirmaci, A., Walsh, T., Akay, H., Spiliopoulos, M., Sakalar, Y.B., Hasanefendioğlu-Bayrak, A., Duman, D., Farooq, A., King, M.C., and Tekin, M. (2010) MASP1 mutations in patients with facial, umbilical, coccygeal, and auditory findings of Carnevale, Malpuech, OSA, and Michels syndromes. *Am. J. Hum Genet.* **87**, 679–686
27. Degn, S.E., Jensenius, J.C., and Thiel, S. (2011) Disease-causing mutations in genes of the complement system. *Am. J. Hum Genet.* **88**, 689–705