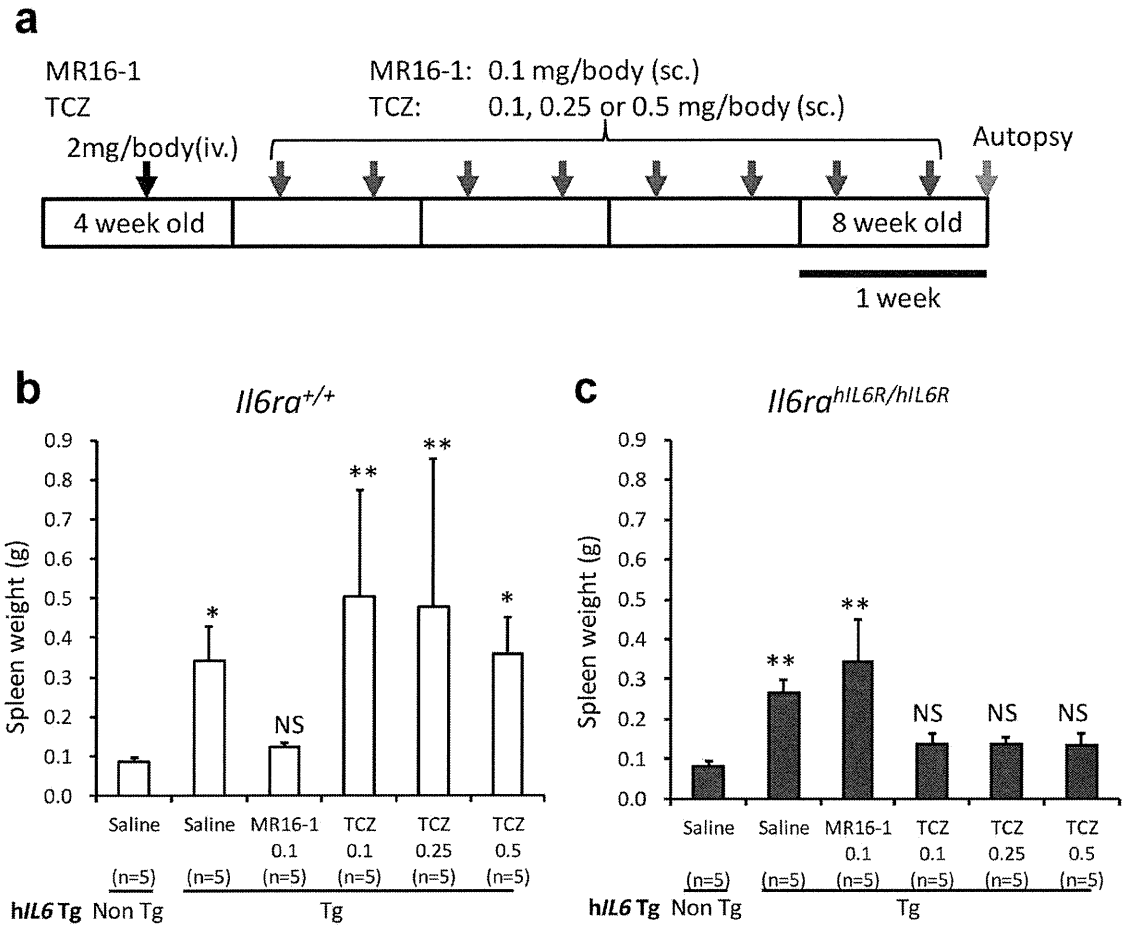

Figure 2 | Treatment with an hIL6R-neutralizing antibody in humanized Castleman's disease model mice. (a) Protocol for 4-week treatment with the anti-mouse Il6ra antibody, MR16-1, or the anti-human IL6R antibody, tocilizumab (TCZ). (iv.), intravenous injection; (sc.) subcutaneous injection. (b) Spleen weights of *Il6ra*$^{+/+}$-h*IL6* transgenic mice and (c) *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice after 4-week treatment (n = 5 per group). Statistical significances were determined by nonparametric comparisons with control using the Dunn method for joint ranking. The data of each treatment group were compared with those of the respective h*IL6* non-transgenic mouse group in each genotype of interleukin-6 receptor, *Il6ra*$^{+/+}$ (b) and *Il6ra*$^{hIL6R/hIL6R}$ (c). *, $p < 0.05$, **, $p < 0.01$ and NS, not significant. Non Tg, h*IL6* non-transgenic mice; Tg, h*IL6* transgenic mice.

An increase in the amount of plasma cells in the marginal zone was observed in all of the saline-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice (Table 1, Fig. 3b) compared to non-transgenic mice (Table 1, Fig. 3a), and aggregates of plasma cells were observed in one of three animals (Table 1, Fig. 3b). Additionally, increased numbers of white pulp was observed in saline-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice (Table 1, Fig. 3e) compared to non-transgenic mice (Table 1, Fig. 3d), which was evidenced by the incidence of white pulp, and this finding was accompanied by enlargement of the total spleen area (Table 1, Fig. 3e). Pathological symptoms of the spleen in tocilizumab-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice were substantially ameliorated upon histological observation at the end of the 4-week treatment (Table 1, Fig. 3, c and f) compared with saline-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice.

Plasma levels of human soluble IL6R and human IL6 were markedly increased at the end of a 4-week tocilizumab treatment in *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice (Fig. 4, a and b). Antibodies to the drug were minimally detected in *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice even after repeated subcutaneous administration of tocilizumab (Fig. 5).

Table 1 | Incidence of histopathological findings of splenic lymphocytes in humanized Castleman's disease model mice with or without tocilizumab treatment

		Il6ra$^{hIL6R/hIL6R}$		
Findings	Severity	Non Tg Saline	h*IL6* Tg Saline	h*IL6* Tg Treated†
*Increased plasma cells	−	3/3	0/3	4/5
	±	0/3	2/3	1/5
	+	0/3	1/3	0/5
**Increased number of white pulp	−	3/3	0/3	1/5
	+	0/3	0/3	4/5
	++	0/3	3/3	0/5

Severity of findings: *±, increased amount of plasma cells in the marginal zone compared to non-transgenic mice; +, aggregates of plasma cells observed in the marginal zone. ** +, increased incidence of white pulp compared to non-transgenic mice; ++, increased incidence of white pulp with enlargement of the total spleen area.
Non Tg, h*IL6* non-transgenic mice; h*IL6* Tg, human *IL6* transgenic mice.
Numerals indicate the number of animals examined.
†, treated with tocilizumab.

Discussion

We have established a line of h*IL6R* knock-in mice, in which endogenous mouse *Il6ra* gene is successfully replaced by h*IL6R* cDNA.

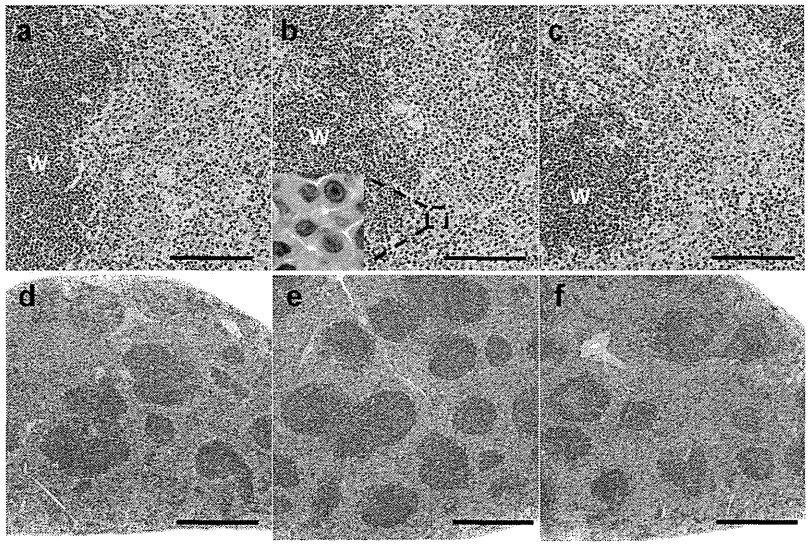

Figure 3 | Spleen tissue of an *Il6ra*$^{hIL6R/hIL6R}$ mouse (a, d), an *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mouse (b, e), and a tocilizumab-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mouse (c, f). Increase of plasma cells and increased numbers of white pulp in the *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mouse (b, e) were ameliorated after 4-week treatment with tocilizumab (c, f). Plasma cells are shown in insert (b). Bars: (a–c), 100 μm; (d–f), 500 μm. W, white pulp.

Results of RT-PCR analysis indicate that tissue distribution of the knocked-in h*IL6R* expression is well-controlled by endogenous transcription mechanisms (Fig. 1c). Membrane-bound hIL6R expressed on the cell surface in these mice would be normally released to the blood as soluble hIL6R, which lacks the transmembrane and cytoplasmic region[18,19], and the plasma levels of soluble hIL6R are revealed to be similar to those reported in Castleman's disease[13,14], rheumatoid arthritis patients[14] and healthy volunteers[14,15]. According to our survey, two lines of h*IL6R* transgenic mice were previously established[9,10]. Both of them were reported to have higher serum levels of soluble hIL6R than healthy humans. Peters *et al.* established a line of hIL6R transgenic mice, driven by *phosphoenolpyruvate carboxykinase* gene promoter, in which serum concentrations of soluble hIL6R were described to range between 4 and 8 μg/mL[9,20], several hundred times higher than those in human. Moreover, these h*IL6R* transgenic mice express only the soluble type of hIL6R, not the membrane-bound type. Another line of h*IL6R* transgenic mice was established to express the membrane bound type of hIL6R, driven by

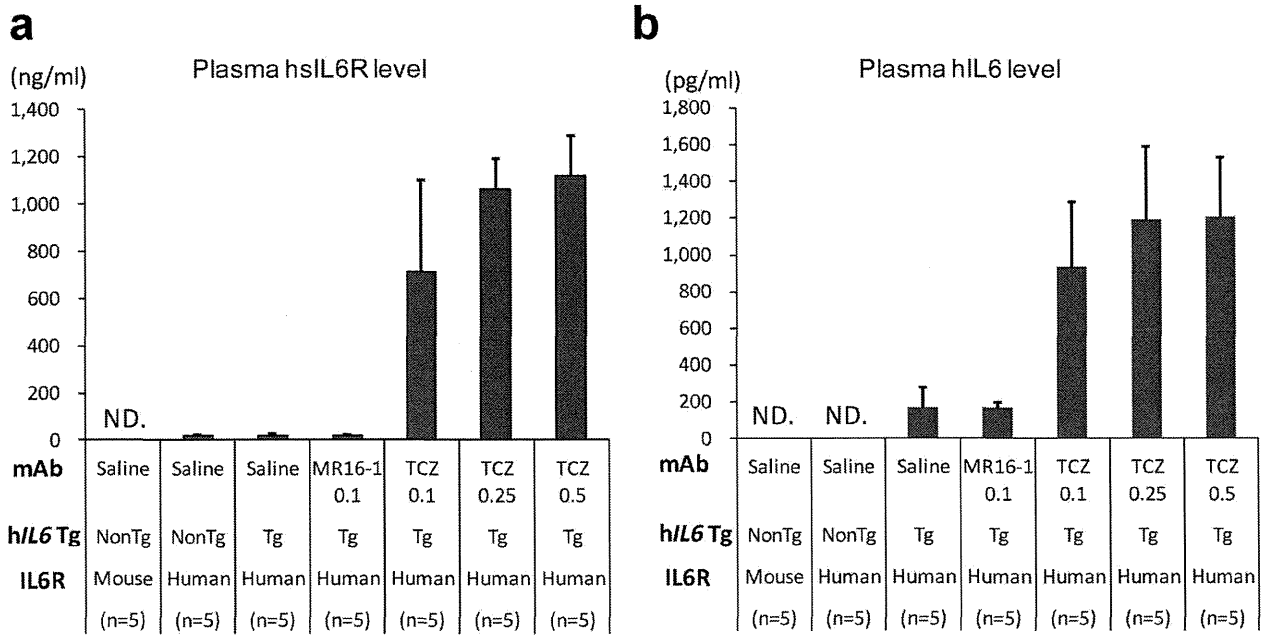

Figure 4 | Plasma levels of soluble hIL6R (a) and hIL6 (b) concentration after 4-week treatment with 0.1, 0.25 and 0.5 mg/body of TCZ in each genotype of mouse (n = 5 per group). (a) Plasma soluble hIL6R concentrations were approximately 21 ng/ml in saline-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice, whereas marked elevation of plasma soluble hIL6R levels, approximately 40–50 times higher than those of vehicle control, was observed after 4 weeks in TCZ-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice. (b) Plasma hIL6 was detected at the level of 163 pg/ml in saline-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice, whereas the hIL6 levels were markedly elevated to the levels of 936–1204 pg/ml after 4-week treatment of TCZ. ND, not detected. Non Tg, h*IL6* non-transgenic mice; Tg, h*IL6* transgenic mice.

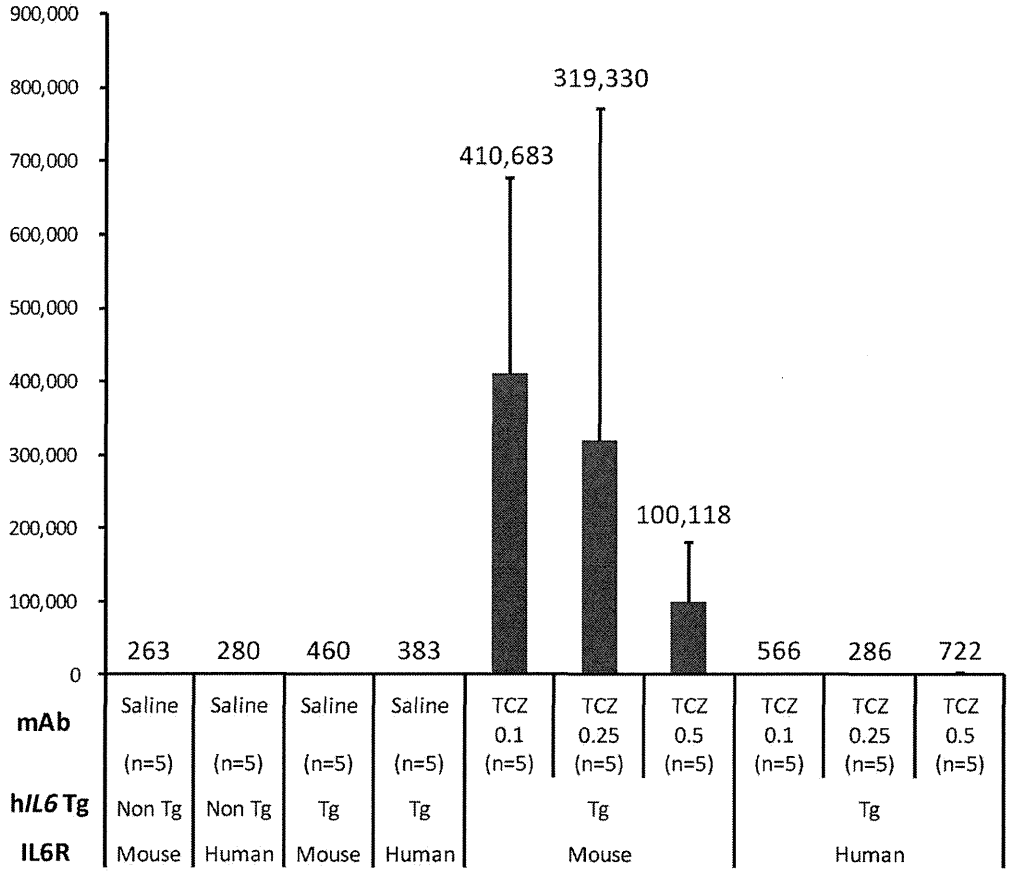

Figure 5 | Titers of plasma anti-drug antibodies after 4-week treatment with 0.1, 0.25 and 0.5 mg/body of TCZ in each genotype of mouse (n = 5 per group). Extremely high levels of plasma anti-TCZ-antibody titers were detected in *Il6ra*$^{+/+}$-h*IL6* transgenic mice, whereas those in TCZ-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice were minimally detected. Non Tg, h*IL6* non-transgenic mice; Tg, h*IL6* transgenic mice.

a strong promoter cassette pCAGGS[21], in which serum concentrations of soluble hIL6R were described to range between 80 and 100 ng/mL[22], about 4–5 times higher than those in human. Taken together, for the first time we have succeeded in genetically humanizing *IL6R* in mice to produce blood levels of soluble hIL6R similar to those in human, with plasma concentrations of soluble hIL6R ranging between 15 and 30 ng/mL (Fig. 1d).

No apparent abnormalities were observed in human *IL6R* knock-in mice, a fact which shows that human *IL6R* expression does not affect animal health under normal breeding conditions. Histological observation revealed that the spleens of *Il6ra*$^{hIL6R/hIL6R}$ mice did not show any abnormalities (Fig. 3a). Homozygous *Il6ra*$^{hIL6R/hIL6R}$ mice deplete the downstream signal from IL6R because human IL6R cannot bind to mouse endogenous Il6[19,23], but this lack of IL6 signaling would not have apparent effects on animal health in normal conditions, as described in several reports that either *Il6* or *Il6ra* gene-disrupted mice are viable and have normal appearance[24–27]. We have detected elevated plasma SAA levels that confirm the species-specific ligand responses after intraperitoneal injection of mouse Il6 or human IL6 to the h*IL6R* knock-in mice. Homozygous *Il6ra*$^{hIL6R/hIL6R}$ mice exclusively respond to human IL6 only, and not to mouse Il6 (Fig. 1e). These responses are compatible with the fact that mouse Il6ra can respond to both mouse Il6 and human IL6, whereas hIL6R can respond to human IL6 only[19,23]. These results strongly suggest that our h*IL6R* knock-in mouse expresses not mouse endogenous Il6ra, but a functional hIL6R molecule that can transduce the downstream signals normally.

Recently, either albumin-expressing hepatocyte-specific or lysozyme M-expressing macrophage/granulocyte-specific *Il6ra* gene knockout mice have been established by crossing mice having floxed alleles of *Il6ra* (*Il6ra*$^{fl/fl}$) with mice expressing Cre recombinase under the control of albumin (*AlbCre*) or lysozyme M promoter (*LysCre*)[27]. McFarland-Mancini *et al.* demonstrated that soluble Il6ra level in plasma was more dependent on immune cell secretion than hepatic production by showing that *AlbCre*$^{+/+}$/*Il6ra*$^{fl/fl}$ mice had higher levels of soluble Il6ra (67.95% of *Cre*$^{-/-}$/*Il6ra*$^{fl/fl}$) than *LysCre*$^{+/-}$/*Il6ra*$^{fl/fl}$ mice (39.95%)[27]. However, SAA production after challenge with turpentine in *AlbCre*$^{+/+}$/*Il6ra*$^{fl/fl}$ mice was severely inhibited, whereas plasma SAA level in *LysCre*$^{+/-}$/*Il6ra*$^{fl/fl}$ mice was similar to that of wild-type mice. Consequently, membrane-bound Il6ra on the hepatocytes makes a critical contribution to hepatic SAA production, meaning that trans-signaling by soluble Il6ra may not have a significant role in SAA production. We have demonstrated that *Il6ra*$^{hIL6R/hIL6R}$ mice can respond to exogenous hIL6 to produce SAA, which strongly suggests that *Il6ra*$^{hIL6R/hIL6R}$ mice express intact membrane-bound hIL6R, at least on the hepatocytes.

The *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mouse established in this study showed basically typical Castleman's disease symptoms (enlargement of systemic lymph nodes and splenomegaly) similar to those previously reported in *Il6ra*$^{+/+}$-h*IL6* transgenic mice[7,8]. Histological observation revealed that the number of white pulps is also increased in *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice. White pulp consists of an accumulation of lymphocytes, mostly B-cells; therefore, these results indicate that the knocked-in hIL6R can respond normally to human

IL6 to cause B-cell differentiation and proliferation in white pulp in vivo in the same way as endogenous mouse Il6ra in wild-type mice. Extramedullary hematopoiesis was also observed in the spleen of *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice (data not shown) as previously reported in *Il6ra*$^{+/+}$-h*IL6* transgenic mice[7,8].

We have also examined the therapeutic efficacy of an hIL6R-specific neutralizing antibody on the Castleman's disease-like symptoms in this mouse model. As far as we know, this humanized Castleman's disease mouse model is the first small rodent that can be used to evaluate in vivo efficacy of a therapeutic antibody specific to human IL6R. Our results suggest that sufficient efficacy was observed at a low dose, 0.1 mg/body of tocilizumab, when administered to this mouse model in our dosing scheme. Even when we increased the dose of tocilizumab to 0.25 or 0.5 mg/body, further reduction of spleen weights was not observed; therefore, it may be possible to decrease the dose of tocilizumab further to find the minimal dose level. We would like to define the therapeutic window of tocilizumab, as well as the improved antibodies[28] described below, in a future study. Marked elevation of plasma soluble hIL6R and human IL6 levels was also observed in our mouse model similar to that reported by Nishimoto *et al.* in the patients with Castleman's disease or rheumatoid arthritis that had been treated with tocilizumab[14]. Nishimoto *et al.* concluded that it was likely that soluble hIL6R increased because the formation of a tocilizumab/soluble hIL6R immune complex prolonged its elimination half-life, and that free serum IL6 increased because IL6R-mediated consumption of IL6 was inhibited by the lack of tocilizumab-free IL6R[14]. We consider that increased plasma levels of soluble hIL6R and human IL6 in tocilizumab-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice could be caused by a mechanism similar to that in humans, and that our humanized Castleman's disease model would substantially reflect the clinical outcomes seen in the tocilizumab-treated patients.

Nowadays various technologies for optimizing therapeutic antibodies (in other words, antibody-engineering technologies) have been intensively developed by leading researchers, and improving the pharmacokinetics of these expensive therapeutic antibodies to reduce the dose or dosing frequency will be an increasingly important issue[28]. It is necessary to determine the therapeutic window, dosing frequency and route of administration while fully understanding the binding affinity to antigen, the pharmacokinetics and the biodistribution of each antibody modified with various sorts of functions[28]. We propose that our mouse model, expressing a physiological level of hIL6R, will be well-suited for preclinical studies assessing a modified function added to the backbone of new therapeutic antibodies. Our mouse model also has the merit of being smaller in body size than other animal species, such as primates, so smaller amounts of candidate agents would be sufficient for evaluation.

Antibody titers to the drug tocilizumab were only minimally detected, despite the repeated and frequent subcutaneous administration (Fig. 5), so that evaluation of in vivo efficacy of humanized hIL6R-neutralizing antibody was possible after 4-week treatment in this novel Castleman's disease mouse model. Although the cause of these low titer levels remains to be investigated, we are currently considering two possibilities. The first is that tolerance might be successfully induced by relatively higher first dosing (2 mg/body) of humanized antibody intravenously. This possibility is suggested by two recent reports using MR16-1, a rat antibody to mouse Il6ra. Yoshida *et al.* reported that first intravenous dosing (2 mg/body) inhibited the production of antibodies to MR16-1 after repetitive intraperitoneal or subcutaneous injections of MR16-1 in NZB/NZW^{F} F1 mice[29]. Sakurai *et al.* also suggested the possibility that tolerance induction would inhibit the production of antibody to drug after finding that antibodies to MR16-1 were detected in some mice treated with 15 mg/kg of MR16-1 intravenously every 3 days but not detected in 50 mg/kg groups[30]. In our study, however, in *Il6ra*$^{+/+}$-h*IL6* transgenic mice expressing only mouse Il6ra, the same doses of tocilizumab produced extremely high titers of anti-tocilizumab antibodies, suggesting that there might be some other mechanism than tolerance induction from a first higher dosing. Therefore we would like to propose a second possibility: that IL6 signal blockade by tocilizumab itself might also suppress the production of antibodies to tocilizumab. In *Il6ra*$^{+/+}$-hIL6 transgenic mice, which express mouse Il6ra but not human IL6R, tocilizumab cannot inhibit IL6 signaling. Therefore, tocilizumab would be treated as nothing more than a foreign substance, not as a therapeutic agent, and might stimulate systemic inflammation induced by hIL6 as well as a strong immune response. As a result, extremely high titers of antibodies to tocilizumab were detected in tocilizumab-treated *Il6ra*$^{+/+}$-hIL6 transgenic mice (Fig. 5). We also speculate that there would be considerable interindividual variability in the exacerbation of systemic inflammatory response, which could cause the large interindividual variation of spleen weights seen in *Il6ra*$^{+/+}$-hIL6 transgenic mice (Fig. 2). In summary, at least two mechanisms, namely tolerance induction (with relatively higher first dosing) and IL6 signal blockade, might be necessary to inhibit the production of antibodies to tocilizumab.

IL6 is a multifunctional cytokine that has a wide range of biological activities in various target cells. Therefore not only Castleman's disease and rheumatoid arthritis but many other diseases and disorders, such as multiple myeloma, sepsis, mesangial proliferative glomerulonephritis, and cancer cachexia, may also be associated with IL6 over-production and subsequent uncontrolled IL6 signaling[18,19,31]. It is predicted that an increasing number of researchers in the future will continue to develop many therapeutic agents molecularly-designed to target hIL6R. Finally, we expect that our mouse model provides a novel system for evaluating in vivo efficacy of the next generation of hIL6R-specific therapeutic agents and also for assessing antibody-engineering technologies to treat patients with Castleman's disease, rheumatoid arthritis, and other diseases caused by abnormalities in IL6 signaling.

Methods

Generation of human IL6R gene knock-in mice. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical Co. Ltd.

A line of human *IL6R* gene knock-in mice was established basically by the protocol we reported previously[12,32–34]. The methods are briefly described as follows. The targeting vector, constructed by the seamless insertion of human IL6R gene cDNA (GenBank # NM_000565) into the mouse Il6ra genomic locus on the BAC clone with pRed/ET system (Quick and Easy BAC Modification kit, GeneBridges GmbH, Heidelberg) as shown in Fig. 1a, was introduced by electroporation to the 129/SvEv mouse ES cells. The ES cells were selected in a culture medium containing G418. Homologous recombinant ES cell clones were injected into C57BL/6J (B6) mouse (CLEA Japan, Inc., Tokyo) blastocysts to produce chimera mice. Chimera mice were bred with B6 females to generate offspring. After confirmation of germline transmission, *neo* gene cassette was removed from the knock-in allele by pronuclear microinjection of the Cre recombinase expression vector[12]. Removal of the *neo* gene cassette was confirmed by PCR using the primers m10355 (5′-TCTGCAGTAGCCTTCAAAGAGC-3′) and m11166R (5′-AACCAGACAGTGTCACATTCC-3′). *Neo*-deleted allele was determined at 2.7 kb, whereas *neo*-intact allele and wild-type allele were detected at 4.2 kb and 0.8 kb, respectively (Fig. 1b). Heterozygous mice were intercrossed to produce homozygous mice.

RT-PCR analysis was performed to determine tissue distribution of human and mouse IL-6R expression. Total RNA samples extracted from tissue samples with Isogen reagents (Nippon Gene Co. Ltd., Tokyo, Japan) were reverse-transcribed with SuperScript III reverse-transcriptase (Invitrogen) to synthesize cDNA. PCRs were performed with a common forward primer 6RIK-s1 (5′-CCCGGCTGCGGAGCCGCTCTGC -3′) set in 5′ untranslated region and species-specific reverse primers in the coding sequences: 6RLIcA2 (5′-AGCAACACCGTGAACTCCTTTG-3′) for mouse Il6ra and RLI6-a1 (5′-ACAGTGATGCTGGAGGTCCTT-3′) for human IL6R, respectively. Serum concentrations of soluble-type receptors were determined as described below.

Species-specific ligand-receptor reaction was examined by plasma levels of SAA after intraperitoneal injection of human and mouse IL-6. In this experiment *Il6ra*$^{hIL6R/hIL6R}$ mice and *Il6ra*$^{+/+}$ mice were used. Plasma SAA levels were determined by the commercially available ELISA kit (Invitrogen), according to the manufacturer's protocol.

Establishment of humanized Castleman's disease model mice. The h*IL6R* knock-in mice were crossed with the *H-2L*^{d}-h*IL6* transgenic mice to establish double transgenic mice, that is, *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice, which have homozygous alleles for

the knocked-in hIL6R and the *H-2L^d*-h*IL6* transgene. These mice were maintained under specific pathogen-free conditions and fed standard laboratory chow (CE-2, CLEA Japan, Inc.) *ad libitum*. Genotypes for these mice were determined by PCRs for knocked-in h*IL6R* allele and *H-2L^d*-h*IL6* transgene. Genotyping for knocked-in h*IL6R* allele was performed by the PCR analysis mentioned above. *H-2L^d*-h*IL6* transgene was detected by PCR with the forward primer (5′- ACCTCTTCAGA-ACGAATTGACAAA -3′) and the reverse primer (5′- AGCTGCGCAGAATGAG-ATGAGTTGT -3′). After an initial denaturation at 94 degrees C for 4 min, 35 cycles of 94 degrees C for 30 sec, 65 degrees C for 30 sec and 72 degrees C for 30 sec were run with TaKaRa Ex Taq (TaKaRa). *H-2L^d*-h*IL6* transgene is detected as a signal at approximately 450 bp.

Treatment with an hIL6R-neutralizing antibody in humanized Castleman's disease model mice. These humanized Castleman's disease model mice were injected with 2 mg/body of humanized mAb to human IL6R (tocilizumab), rat mAb to mouse Il6ra (MR16-1) or physiological saline used for vehicle, intravenously, once at 4 weeks of age. Then from the week after the first injection, mice were given 0.1, 0.25 or 0.5 mg/body of tocilizumab or 0.1 mg/body of MR16-1 subcutaneously twice weekly. In the treatment regimen, first dosing was set at a relatively high amount to attempt to induce tolerance in mice to mAbs originating from other species[29]. Under isoflurane anesthesia, whole blood samples were collected from the inferior vena cava. Spleens were removed, weighed and fixed with 10% neutral buffered formalin for histological examination. *Il6ra$^{+/+}$*-h*IL6* transgenic mice, previously reported by Katsume *et al.* as a Castleman's disease model[8], were used as a disease control. Additionally *Il6ra$^{+/+}$* and *Il6ra$^{hIL6R/hIL6R}$* mice were used for healthy control.

Soluble human and mouse IL-6R-specific ELISA. The plasma levels of soluble human and mouse IL-6R were determined by using a commercially available kit (R&D Systems) according to the manufacturer's protocols.

Human and mouse IL-6-specific ELISA. The blood levels of human and mouse IL-6 were determined using a commercially available IL-6-specific ELISA kit (Invitrogen) according to the manufacturer's instruction.

Measurement of antibody titer to drug. Plasma samples were incubated with biotin-labeled tocilizumab and SULFO-TAG-labeled tocilizumab overnight. These mixtures were placed in the wells of MSD SA plates and incubated for 2 hours. After washing and addition of the read buffer, chemiluminescence was determined immediately by SECTOR PR 400 (Meso Scale Discovery, Maryland).

Statistical analysis. Statistical analysis was performed using JMP 9.02 (SAS Institute Japan, Tokyo, Japan). Statistical significance in spleen weights was determined by nonparametric comparisons with control using Dunn method for joint ranking. $P < 0.05$ was regarded as statistically significant.

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Author contributions

K.J. and O.U. conceived and designed the experiments and coordinated the work presented. O.U., H.T., Y.H., E.F., A.K., Y.K., N.A.W., T.T., M.Kakefuda, C.G. and M.Kawaharada performed the experiments. S.S. and K.H. commented on the manuscript. O.U. and K.J. wrote the manuscript.

Additional information

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