Proteolytic cleavage of antigen extends the durability of an anti-PCSK9 monoclonal antibody

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Abstract  Lilly PCSK9 antibody LY3015014 (LY) is a monoclonal antibody (mAb) that neutralizes proprotein convertase subtilisin-kexin type 9 (PCSK9). LY decreases LDL cholesterol in monkeys and, unlike other PCSK9 mAbs, does not cause an accumulation of intact PCSK9 in serum. Comparing the epitope of LY with other clinically tested PCSK9 mAbs, it was noted that the LY epitope excludes the furin cleavage site in PCSK9, whereas other mAbs span this site. In vitro exposure of PCSK9 to furin resulted in degradation of PCSK9 bound to LY, whereas cleavage was blocked by other mAbs. These other mAbs caused a significant accumulation of serum PCSK9 and displayed a shorter duration of LDL-cholesterol lowering than LY when administered to mice expressing the WT human PCSK9. In mice expressing a noncleavable variant of human PCSK9, LY behaved like a cleavage-blocking mAb, in that it caused significant PCSK9 accumulation, its duration of LDL lowering was reduced, and its clearance (CL) from serum was accelerated. Thus, LY neutralizes PCSK9 and allows its proteolytic degradation to proceed, which limits PCSK9 accumulation, reduces the CL rate of LY, and extends its duration of action. PCSK9 mAbs with this property are likely to achieve longer durability and require lower doses than mAbs that cause antigen to accumulate.—Schroeder, K. M., T. P. Beyer, R. J. Hansen, B. Han, R. T. Pickard, V. J. Wroblewski, M. C. Kowala, and P. I. Eacho. Proteolytic cleavage of antigen extends the durability of an anti-PCSK9 monoclonal antibody. J. Lipid Res. 2015. 56: 2124–2132.

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Proprotein convertase subtilisin-kexin type 9 (PCSK9) is a secreted serine protease that regulates plasma LDL cholesterol (LDL-C) by modulating the levels of LDL receptor (LDLR) and does so independently of its proteolytic activity (1–6). PCSK9 binds to the LDLR and directs it into a lysosomal degradation pathway rather than the recycling pathway. Rare gain-of-function mutations in PCSK9 cause autosomal dominant hypercholesterolemia, a disorder characterized by LDL-C >300 mg/dl and premature atherosclerosis (7, 8). Conversely, loss-of-function mutations are associated with reduced LDL-C and reduced risk of cardiovascular diseases (9–11).

PCSK9 is expressed in multiple tissues, including liver, intestine, kidney, and cerebellum, of which the liver appears to be the major source of the circulating protein (12–14). It is synthesized as a 74 kDa proprotein, which is activated prior to secretion by the autocatalytic cleavage of its N-terminal prodomain (15). PCSK9 that is secreted from cells is comprised of the 14 kDa prodomain associated noncovalently with a 60 kDa mature domain, the latter consisting of an N-terminal catalytic domain and a cysteine- and histidine-rich C-terminal domain. In addition to this 74 kDa form (intact PCSK9), a smaller form (truncated PCSK9) is found in serum in which the N terminus of the catalytic domain is truncated by 7–8 kDa. This truncated PCSK9 represents up to 40% of the total circulating PCSK9 in humans (16–18). The site of cleavage in PCSK9 was identified as Arg218 of the catalytic domain (16, 17). Furin appears to be responsible for most of the cleavage in vivo, based on studies in mice with hepatocyte-specific inactivation of the protease (18). The observation of reduced plasma levels of the truncated PCSK9 in humans heterozygous for the R218S gain-of-function mutation is also consistent with furin-mediated cleavage, as this mutation disrupts the RXXR sequence recognized by furin (18).

Several reports have addressed the question of whether the truncated PCSK9 is active in the regulation of LDL-C. Studies in furin knockout mice found reduced circulating levels of truncated PCSK9 and less hepatic LDLR than in
WT mice, suggesting that truncated PCSK9 was inactive at LDLR degradation (18). This conclusion was consistent with in vitro studies in which overexpression of a mutant form of PCSK9 with an enhanced furin-cleavage site resulted in complete cleavage of PCSK9, 100% truncated PCSK9, and no reduction of LDLR (16). We recently generated truncated PCSK9, which appeared to be identical in composition to truncated PCSK9 isolated from human serum, and concluded that it was not active (17). In contrast, studies by Lipari et al. (19) indicated that the truncated PCSK9 that they produced was active as a regulator of LDLR degradation. However, truncated PCSK9 in these studies retained the 7.7 kDa N-terminal segment by noncovalent association and had the same 74 kDa mass as secreted PCSK9. Our studies demonstrated that truncated PCSK9, which circulates in humans, does not retain the fragment (17). The nonphysiological retention of the 7.7 kDa fragment, which contains amino acids critical for binding to LDLR (20–22), may explain the reported activity of truncated PCSK9 (19). Based on our results and those cited (16, 17), we have concluded that the circulating, truncated PCSK9 is functionally inactive.

The question of whether truncated PCSK9 is functionally active may be particularly relevant to PCSK9 neutralizing monoclonal antibodies (mAbs), which have shown promise for LDL-C lowering in human clinical trials (23, 24). Considering that up to 40% of human PCSK9 circulates as truncated PCSK9 and is inactive, according to our data (17), an antibody that binds both intact and truncated PCSK9 could be consumed unproductively by truncated PCSK9. Antibodies whose epitopes are in truncated PCSK9, such as those that target the C-terminal domain of PCSK9, would fall into this class (25, 26), as would a recently described catalytic domain antibody (19).

In this report, we describe the therapeutic Lilly PCSK9 antibody LY3015014 (LY). The epitope where LY binds affords unique properties. LY binds to intact but not truncated PCSK9. It inhibits PCSK9 binding to LDLR while permitting the normal proteolytic cleavage of the bound intact PCSK9. As a result, the in vivo administration of LY does not result in the accumulation of PCSK9 that has been observed with other therapeutic anti-PCSK9 mAbs. Additionally, upon cleavage, truncated inactive PCSK9 is released from LY. This property affords LY with prolonged LDL-lowering durability relative to antibodies that block the proteolytic processing of PCSK9 and retain active intact PCSK9. The data provide new information regarding a property of therapeutic mAbs that can influence dose and duration of action.

**MATERIALS AND METHODS**

**Generation of anti-PCSK9 antibodies**

All animal protocols were approved by the Eli Lilly and Co. Animal Care and Use Committee. Anti-PCSK9 mAbs were generated as described in Davies et al. (27). Briefly, male BALB/c mice were immunized with full-length human PCSK9 for C-terminal antibody generation or C-terminal truncated human PCSK9 for catalytic domain antibody generation. The resulting antibodies were recovered and purified, and the amino acid sequences determined. The antibodies were engineered to contain human framework regions surrounding complementarity determining regions (CDRs) derived from the mouse antibody. The epitopes in PCSK9 where the mAbs bound were determined using hydrogen deuterium exchange and peptide binding as in Davies et al. (27). IgG4 mAbs composed of the CDRs of REGN727 (28) and AMG145 (29), which have proven LDL-lowering efficacy in humans (23, 24), were expressed and evaluated. Antibody affinity for human PCSK9 was determined by surface plasmon resonance using the BIAcore Biosensor 2000 and BIAevaluation software with a 1:1 binding with mass transfer model (27).

**Pharmacodynamic effects of anti-PCSK9 mAbs in mice expressing human PCSK9**

WT human PCSK9 and the noncleavable (NC) R215A/R218A mutant were expressed in mice under the liver-specific thyroxine binding globulin promoter using a chimeric adeno-associated virus (AAV) vector composed of the inverted terminal repeats of AAV2 packaged with the capsid from AAV8 (ReGenX, Washington, DC). Male C57BL/6JTac mice were injected intravenously with $1 \times 10^{10}$ genomic copies of either WT human PCSK9 AAV or vector containing the NC R215A/R218A variant (18, 19) to achieve stable expression averaging 8–10 μg/ml of PCSK9 4 weeks postinjection. To evaluate the LDL-lowering efficacy of LY, mice expressing WT human PCSK9 were injected subcutaneously with a single 0.3–30 mg/kg dose of LY or 30 mg/kg control human IgG4. Mice expressing LacZ (gene that encodes the bacterial protein beta-galactosidase) from the AAV vector were included as a reference demonstrating normal LDL-C levels in C57Bl6 mice. At 24 h after injection, mice were euthanized using CO$_2$, and serum was collected by retro-orbital puncture for LDL-C analysis. A follow-up experiment was conducted in which IgG4 antibody with CDR sequences of Regeneron PCSK9 antibody REGN727 (RG) was included (10 mg/kg) and LDL-C in serum was measured at 2 and 5 days after treatment (n = 7 per group, same mice in each time point). Mice were anesthetized with isoflurane, and serum was collected by retro-orbital puncture (2 days) or cardiac puncture (5 days). Serum LDL-C was quantified after fractionation on a Superose® 10/300 GL size exclusion column (GE Healthcare Bio-sciences AB), based on the procedure described previously (30). LDLR levels were determined in liver homogenates by sandwich ELISA using two polyclonal LDLR antibodies (R and D Systems, cat# 2255-LD-025).

**Anti-PCSK9 mAb treatment in cynomolgus monkeys**

Normal, chow-fed cynomolgus monkeys were injected intravenously with 5 mg/kg of a nonbinding IgG4 or LY in PBS. At various time points after injection, serum samples were taken for LDL-C and PCSK9 analyses. LDL-C levels were determined with a homogeneous assay (LDL-C plus second generation; Roche Diagnostics) using a Roche P800 Modular Analytics analyzer. Antibody-bound + free PCSK9, including both intact and truncated PCSK9, was determined using a sandwich ELISA method, with an acid-dissociation pretreatment. The curve range was 0.78–75 ng/ml.
with lower and upper limits of quantitation of 1.56 and 50 ng/ml, respectively.

**Anti-PCSK9 mAb effects on furin-mediated cleavage of PCSK9**

Recombinant WT human PCSK9 expressed with a C-terminal His-tag and the serum from mice expressing WT human PCSK9 from an AAV vector were used to study the effects of anti-PCSK9 antibodies on furin-mediated cleavage. C-terminal 6xHis recombinant WT human PCSK9 was generated by expression in HEK293 cells as in Han et al. (17). Recombinant human WT PCSK9 (300 μg/ml) was precomplexed with anti-PCSK9 antibodies at a 1:1 molar ratio in HEPES buffer (50 mM Hepes, 100 mM NaCl, 10 mM CaCl₂, pH 8.0). Complexes were preincubated at 37°C for 30 min followed by addition of a serial dilution of furin. PCSK9, antibody, and furin were incubated at 37°C for 6 h. After the incubation, nonreducing 2x NuPAGE LDS sample buffer (Thermo Fisher Scientific #NP0008) was added to each sample. Samples were mixed and loaded onto NuPage 12% Bis-Tris 12 well gels. After electrophoresis, gels were stained with SimplyBlue SafeStain (Life Technologies) and visualized using the 700 Channel of the Odyssey infrared imaging system (Licor). Furin cleavage of intact PCSK9 was also evaluated ex vivo using serum from mice expressing ~10 μg/ml WT human PCSK9 from an AAV vector. The serum was diluted 2x in HEPES buffer (above) and precomplexed with anti-PCSK9 antibodies at ~1:5 molar ratio. Complexes were preincubated at 37°C for 30 min followed by the addition of a serial dilution of furin. The serum with antibody and furin were incubated at 37°C for 6 h. After the incubation, intact and truncated human PCSK9 were immunoprecipitated using 5 μg of bead-bound C-terminal PCSK9 antibody as in Han et al. (17). After immunoprecipitation, 2x NuPAGE LDS sample buffer was added to each sample. Samples were then heated at 95°C for 5 min followed by PCSK9 Western blot analysis. PCSK9 Western blotting was performed using a PCSK9 polyclonal antibody (R and D Systems, Cat#AF3888) as in Han et al. (17).

**PCSK9 accumulation caused by LY, RG, and AM in mice expressing WT and NC human PCSK9**

Male C57BL/6NTac mice were injected intravenously with 1 × 10⁸ genomic copies of either WT human PCSK9 AAV or NC R215A/R218A AAV to achieve stable expression averaging 400–500 ng/ml of PCSK9 4 weeks postinjection. These mice were given a single subcutaneous 10 μg/kg dose of a control IgG4, LY, RG, or AM (n = 4 per group). Serum was collected 24 h after dosing by retro-orbital puncture under isoflurane anesthesia. Total human intact and truncated PCSK9 (free and antibody bound) in serum was immunoprecipitated from each sample using 5 μg of bead-bound C-terminal domain PCSK9 antibody as in Han et al. (17). Beads were washed with PBS + 0.1% NP-40 and frozen for PCSK9 analysis by MS, as in Han et al. (17).

**Pharmacokinetics of LY and AM in mice expressing WT and NC human PCSK9**

Male C57BL/6NTac mice expressing the WT or NC R215A/R218A mutant human PCSK9 from an AAV vector (1 × 10⁸ genomic copies) were given single intravenous 10 μg/kg dose of a control IgG4, LY, or AM. The serum from 12–16 mice per group was collected for analysis of mAb levels at 1 h and 1, 2, 3, 5, 8, and 14 days after intravenous antibody injection, using the sparse sampling protocol. For the sparse sampling protocol, two blood samples were taken from each mouse, the first retro-orbitally under light anesthesia and the second by cardiac puncture under isoflurane anesthesia, followed by euthanasia. Four mice per subgroup were sampled at 1 h and 2 days, 1, 5, 10, and 30 days after treatment. The final subgroups (IgG4 and LY) in the WT PCSK9 mice were sampled at 14 days posttreatment by cardiac puncture. Serum levels of the mAbs were determined by immunoprecipitation with anti-human IgG antibody (Southern Biotech #2049-08), followed by trypsin digestion and quantification by MS.

**RESULTS**

**Binding and efficacy of LY**

LY is a humanized IgG4 mAb that binds the catalytic domain of human and monkey, but not rodent, PCSK9. The binding kinetics and affinity of LY for recombinant human PCSK9 are shown in supplementary Table 1. mAb LY binds to intact PCSK9 in human serum but not to truncated PCSK9 (supplementary Fig. 1). In contrast, a PCSK9 C-terminal domain mAb binds both the intact PCSK9 and truncated PCSK9 in human serum (supplementary Fig. 1). The selective binding of LY to intact PCSK9 is related to its binding epitope, the linear sequence of amino acids 160–181 of the catalytic domain of human PCSK9 (27), which is absent in truncated PCSK9.

The LDL-lowering efficacy of LY was demonstrated in mice expressing human PCSK9 from an AAV vector. The expression of ~8–10 μg/ml human PCSK9 induces a high LDL-C phenotype in mice, which results from a strong reduction of LDLR in the liver (Fig. 1). LY decreased LDL-C and increased hepatic LDLR in a dose-related manner in this mouse model (Fig. 1).

The efficacy of LY was further demonstrated in normal, chow-fed cynomolgus monkeys. A single intravenous administration of LY at 5 mg/kg decreased LDL-C up to 60% (Fig. 2A). The LDL-C lowering persisted for more than 30 days after a single dose. The serum concentration of PCSK9, which included the antibody-bound and free forms of both intact and truncated PCSK9, was measured in the monkeys as an index of target engagement. Previous studies in anti-PCSK9 mAbs in mice, monkeys and humans have demonstrated as much as 20-fold accumulation of circulating PCSK9 (23, 31, 32), due to slower PCSK9 clearance (CL) when bound to a therapeutic antibody. However, serum PCSK9 concentration did not increase in monkeys treated with LY, similar to monkeys treated with a control IgG4 (Fig. 2B). It was clear that PCSK9 was engaged by LY in the monkeys based on robust LDL-C lowering. Because LY binds to a linear sequence which is 37 amino acids distant (N terminally) from the Arg218 proteolytic cleavage site, we considered the possibility that the different effects of PCSK9 mAbs on antigen accumulation were due to their differential effects on the proteolytic degradation of PCSK9.

**mAb effects on cleavage of PCSK9 by furin**

To determine the impact of the mAbs on PCSK9 cleavage, recombinant human PCSK9 was exposed to furin after preincubation with one of several PCSK9 mAbs or a nonbinding control IgG4. In the presence of the nonbinding IgG4 or the C-terminal domain mAb 595, as observed
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of these mice contained both intact and truncated PCSK9, as we demonstrated previously (17). Coincubation of the serum with LY and increasing concentrations of furin resulted in cleavage of PCSK9, observed as a concentration-dependent decrease of the 60 kDa mature PCSK9 band on the Western blot, which was similar to the effect of the control IgG4 (Fig. 4). In contrast, mAb RG blocked the cleavage of the PCSK9 (Fig. 4).

Proteolytic cleavage of PCSK9 limits its accumulation in mAb-treated mice

The accumulation of PCSK9 in vivo was explored by administering 1 mg/kg of mAb LY, RG, or AM to mice expressing 0.4–0.5 g/l H9262 WT human PCSK9 from an AAV vector (WT mice). Human PCSK9 (free and antibody bound, including both intact and truncated PCSK9) on a gel, furin caused a concentration-dependent decrease in the 60 kDa mature PCSK9 band and an increase of the 52 kDa truncated PCSK9 band, demonstrating intact PCSK9 cleavage to truncated PCSK9 by furin (Fig. 3). In contrast, a catalytic domain mAb, H2a3, markedly inhibited the cleavage of PCSK9. IgG4 mAbs composed of the CDRs of REGN727 (RG) and AMG145 (AM), which have proven LDL-lowering efficacy in humans (23, 24), also inhibited the furin-cleavage of PCSK9. We originally tested mAb A2 (Fig. 3), a variant of LY that binds the same epitope, and subsequently LY (supplementary Fig. 2), and found they did not block the cleavage of PCSK9 by furin. The effect of LY on the cleavage process was further evaluated ex vivo by adding furin to serum taken from mice expressing human PCSK9 from an AAV vector. The serum of these mice contained both intact and truncated PCSK9, as we demonstrated previously (17). Coincubation of the serum with LY and increasing concentrations of furin resulted in cleavage of PCSK9, observed as a concentration-dependent decrease of the 60 kDa mature PCSK9 band on the Western blot, which was similar to the effect of the control IgG4 (Fig. 4). In contrast, mAb RG blocked the cleavage of the PCSK9 (Fig. 4).

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The accumulation of PCSK9 in vivo was explored by administering 1 mg/kg of mAb LY, RG, or AM to mice expressing 0.4–0.5 μg/ml WT human PCSK9 from an AAV vector (WT mice). Human PCSK9 (free and antibody bound, including both intact and truncated PCSK9) in

Fig. 1. LDL-C lowering efficacy of LY in mice expressing human PCSK9. Mice expressing WT human PCSK9 from AAV vector were given single intravenous administration of a nonbinding IgG4 or LY at the doses indicated. Mice expressing LacZ from the AAV vector were included as a reference demonstrating normal LDL-C levels in C57Bl6 mice. Twenty-four hours after treatment, serum was taken for analysis of LDL-C (A) and liver for the analysis of LDLR (B). The values represent the mean of seven to eight mice per treatment group and four in the LacZ group. Error bars represent the SEM, and asterisks denote a significant difference from the IgG4 control group, P < 0.05.

Fig. 2. LDL-C and PCSK9 in the serum of monkeys given LY. Normal, chow-fed cynomolgus monkeys were given a single 5 mg/kg intravenous dose of the control IgG4 (gray circles) or LY (open symbols), and serum samples were taken at the times indicated for the analysis of LDL-C (A), presented as the % of baseline levels, and PCSK9 (B). Baseline LDL-C, determined prior to treatment, was 68 ± 7 mg/dl in the IgG4 group (n = 3) and 68 ± 18 mg/dl in the LY group (n = 4). The points on the graph represent the mean and errors bars represent the SD.
In contrast to the WT mice, treatment of NC mice with LY resulted in a significant 4-fold accumulation of total PCSK9 relative to NC mice administered the control IgG4, which was comparable to the increases in PCSK9 induced by RG and AM (Fig. 5, right panel). The data indicate that the unique property of LY, permitting the cleavage of bound PCSK9, accounts for its resistance to PCSK9 accumulation.

Proteolytic cleavage of PCSK9 impacts mAb duration of action

To determine whether the proteolytic cleavage of PCSK9 impacts the duration of action of the mAbs, WT mice and NC mice were administered a single 10 mg/kg dose of LY or AM, and LDL-C was assessed after 2, 5, and 8 days (Fig. 6). In order to achieve a high LDL-C phenotype, the AAV vector was dosed to provide baseline human PCSK9 levels of 10–260 g/ml, compared with the more human-like level of 0.4–0.5 g/ml in the mice represented in Fig. 5. In WT mice, LY decreased LDL-C significantly at all three time points, with a maximum reduction of 63% (Fig. 6). The durability of AM was shorter than LY in WT mice. AM decreased LDL-C by 62% on day 2 but did not decrease LDL-C on days 5 and 8. In the NC mice, both mAbs lowered LDL-C at day 2, but neither lowered LDL-C on days 5 and 8. Thus, in the NC mice the duration of action of LY was similar to that of AM. The reproducibility of the LDL-lowering data was confirmed in a second study that included RG (Fig. 7). LY showed a durability advantage over AM and RG in WT mice, which was lost in the NC mice. The data support the hypothesis that the longer duration of LDL-lowering by LY was attributable to the cleavage of the bound PCSK9.
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DISCUSSION

In this report, we have described a novel mechanism that provides extended therapeutic durability to an anti-PCSK9 mAb. LY binds to a narrow epitope toward the N terminus of the PCSK9 catalytic domain that does not include the site of furin cleavage. LY binds to intact PCSK9 and blocks its interaction with the LDLR while allowing the normal proteolytic cleavage of the PCSK9. Thus, LY neutralizes PCSK9 and permits its proteolytic CL to proceed.

Proteolytic cleavage of PCSK9 slows the CL of LY from serum

To determine whether the differential cleavage of circulating PCSK9 impacted the pharmacokinetics (PK) of the therapeutic mAbs, the serum CL of LY and AM was assessed in WT and NC mice administered a 10 mg/kg dose, as in Fig. 6. The control IgG4 was cleared slowly, 0.16–0.19 ml/h/kg, in WT and NC mice (Fig. 8 and supplementary Table 2). The CL of LY in WT mice was more rapid than the control IgG4, 0.41 ml/h/kg, likely reflecting some degree of antigen-mediated drug disposition of LY. However, AM was cleared even more rapidly than LY in the WT mice, 1.04 ml/h/kg, consistent with its shorter duration of LDL lowering. The PK curves of AM were essentially identical in WT and NC mice, whereas the CL of LY was considerably greater in the NC mice than in WT mice (Fig. 8). The data from the PK study indicate that each of the mAbs is subject to accelerated CL when bound to PCSK9, but this effect is mitigated by the proteolytic cleavage of PCSK9 when bound to LY.

Fig. 6. LDL-C lowering efficacy of LY and AM in mice expressing WT and NC human PCSK9. Mice expressing the WT (A) or NC R213A/R218A mutant human PCSK9 (B) from an AAV vector were given single intravenous 10 mg/kg dose of a control IgG4, LY, or AM. LDL-C in serum was measured at 2, 5, and 8 days after treatment. The bars represent the mean of four mice, and the error bars represent the SEM. The day posttreatment is indicated in italics at the bottom of each bar. Asterisks denote a significant difference from the IgG4 control group, P < 0.05.

Fig. 7. LDL-C lowering efficacy of LY, RG, and AM in mice expressing WT and NC human PCSK9. Mice expressing the WT (A) or the NC human PCSK9 (B) from an AAV vector were given single intravenous 10 mg/kg dose of a control IgG4, LY, RG, or AM. LDL-C in serum was measured at 2 and 5 days after treatment. The bars represent the mean of four mice, and the error bars represent the SEM. The day posttreatment is indicated in italics at the bottom of each bar. Asterisks denote a significant difference from the IgG4 control group, P < 0.05.
supplementary Table 2. Error bars represent the SEM. The PK parameters are displayed in the sparse sampling protocol described in Materials and Methods. Five, eight, and 14 days after intravenous antibody injection, using the per group was collected for analysis of mAb levels at 1 h and 1, 2, 3, (blue curves), or AM (red curves). The serum from 12 to 16 mice

Fig. 8. PK of IgG4, LY, and AM in mice expressing WT and NC human PCSK9. Mice expressing WT (open symbols) or NC human PCSK9 (closed symbols) from an AAV vector were given single intravenous 10 mg/kg dose of a control IgG4 (black curves), LY (blue curves), or AM (red curves). The serum from 12 to 16 mice per group was collected for analysis of mAb levels at 1 h and 1, 2, 3, 5, 8, and 14 days after intravenous antibody injection, using the sparse sampling protocol described in Materials and Methods. Each data point represents the mean of three to four mice, and error bars represent the SEM. The PK parameters are displayed in supplementary Table 2.

PCSK9 remains intact and accumulates in the circulation, which reduces the duration of action of anti-PCSK9 mAbs (discussed below). Accumulation of circulating PCSK9 was observed in animal studies in multiple anti-PCSK9 mAbs (31, 32), including an increase of >20-fold in monkeys. A significant accumulation of total PCSK9 was also observed in humans treated with an anti-PCSK9 mAb (23). LY treatment in monkeys at a dose that reduced LDL-C >50% caused no accumulation of PCSK9.

We used an animal model in which the WT human PCSK9 or an NC variant was expressed in mice to study the impact of PCSK9 cleavage on the durability of LDL lowering, mAb CL, and PCSK9 accumulation. mAbs RG and AM, both of which blocked furin cleavage of PCSK9, caused accumulation of intact PCSK9 in the serum of the mice expressing either WT or NC human PCSK9. LY, in contrast, did not cause significant accumulation of PCSK9 in WT mice. The finding that LY did accumulate PCSK9 in mice expressing the NC variant indicates that the proteolytic cleavage limits PCSK9 accumulation.

In addition to limiting PCSK9 accumulation, we found that the cleavage of LY-bound PCSK9 also prolonged the duration of LDL-lowering by LY. Whereas, the duration of action of mAbs RG and AM was similar in mice expressing the WT and NC forms of human PCSK9, the LDL-lowering durability of LY was reduced in mice expressing the NC variant. Thus, when the possibility of PCSK9 cleavage was eliminated, the duration of action of LY was similar to that of RG and AM.

The cleavage of PCSK9 while bound to LY will likely prolong the therapeutic durability of LY by two mechanisms. The first relates to the lack of significant accumulation of intact PCSK9. Assuming similar serum free antibody concentrations and similar binding affinity, an antibody that accumulates intact, antibody-bound PCSK9 will give rise to higher levels of “free” intact PCSK9, with which it is in equilibrium, than an antibody that does not accumulate intact PCSK9. Thus, an antibody that permits cleavage and reduces intact PCSK9 accumulation will require a lower dose to achieve maximum lowering of “free” intact PCSK9. After the cleavage of PCSK9, the 7.7 kDa N-terminal fragment is likely competent to bind to LY. However, it appears to have little impact on the LDL-lowering durability of LY because of this lack of accumulation of intact PCSK9. A second possible mechanism by which the cleavage of LY-bound PCSK9 prolongs therapeutic durability is through reducing the antigen-mediated drug disposition of the mAb from the serum. Our study in WT mice demonstrated that LY was cleared more rapidly than control IgG4, suggesting some degree of PCSK9-mediated CL. However, the CL of LY was greatly accelerated in mice expressing NC PCSK9, demonstrating that the intact PCSK9 facilitates mAb disposition. This conclusion was supported by the observation that the CL of AM, which blocked PCSK9 cleavage, was equally rapid in the WT and NC mice. Thus, the mAb disposition mediated by PCSK9 cleavage, was equal rapid in the WT and NC mice. It appears that the cleavage of LY-bound PCSK9 prolongs therapeutic durability by two mechanisms.
was challenged in a recent report, which found APLP2 was not involved in PCSK9-mediated degradation of LDLR (37). The finding of Devay and coworkers (34, 35) of most relevance to our work was that an anti-PCSK9 antibody was transported with PCSK9 to lysosomes by the APLP2-mediated pathway. Our preliminary studies confirmed that PCSK9 binds via its C-terminal domain to APLP2 at acidic pH (K. M. Schroeder, unpublished observations). Importantly, mAbs LY and AM in complex with PCSK9 bound to APLP2 in an ELISA-based assay and the addition of furin reduced the binding of LY but not AM (K. M. Schroeder, unpublished observations). Our preliminary findings support the hypothesis proposed by Devay and coworkers (34, 35) that APLP2 mediates the CL of anti-PCSK9 antibodies that are bound to PCSK9. We are pursuing the hypothesis that LY avoids the APLP2-dependent, antigen-mediated CL pathway by permitting the proteolytic cleavage of PCSK9. The cleavage releases the 52 kDa form of PCSK9 containing the C-terminal domain from LY, eliminating the substrate for transport by APLP2 (Fig. 9). It remains possible that other mediators of PCSK9 disposition exist, and these may contribute to the PCSK9-dependent CL of the therapeutic antibodies.

In summary, the novelty of LY is that in addition to binding and blocking the activity of its target antigen, it also permits the normal proteolytic degradation and inactivation of the antigen. This second property endows LY with more efficient PCSK9-neutralizing activity than mAbs that block the cleavage and allow intact, active PCSK9 to accumulate. In addition, upon cleavage of PCSK9, LY avoids antigen-mediated CL from the serum. The impact on therapeutic durability is similar, although by a distinct mechanism, to antibodies that bind antigen in a pH-dependent manner (33, 38). Such antibodies bind antigen poorly at the acidic pH of the endosome, which results in the intracellular degradation of antigen and recycling of antibody. The biology associated with LY neutralization of PCSK9 points out the need to consider antigen processing in the evaluation and selection of therapeutic mAbs. It seems possible that the proteolytic degradation of bound antigen may also impact the durability of therapeutic mAbs in other classes, especially if they target high-concentration or rapid-turnover antigens. A therapeutic mAb that permits antigen degradation is likely to require lower doses, lower dosing volumes, and achieve longer durability than mAbs that allow intact antigen to accumulate.

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**Fig. 9.** Schematic representation of the proposed mechanism by which the LDL-lowering durability of the anti-PCSK9 mAb LY is extended by the proteolytic cleavage PCSK9. A: PCSK9 is depicted in blue (prodomain not shown) with its N-terminal catalytic domain (N) and C-terminal domain (C). It is bound at its catalytic domain to mAb LY. The bound LY prevents PCSK9 binding to LDLR but permits the proteolytic cleavage of PCSK9 at arginine 218 (R218). The cleaved 7.7 kDa N-terminal fragment of PCSK9, which includes the LY binding epitope, is competent to bind LY, but has minimal impact on the LDL-lowering durability of LY, because of the lack of accumulation of intact PCSK9. B: mAbs such as H2a3, RG, and AM block furin cleavage of PCSK9, which results in the retention and accumulation of intact PCSK9 in the circulation. C: mAbs that block the furin cleavage of PCSK9 are especially susceptible to PCSK9-mediated CL. The PCSK9 CL pathway could be mediated by APLP2, which binds to the C-terminal domain of PCSK9 even in the presence of the catalytic domain mAbs (34, 35). D: Following the proteolytic cleavage of PCSK9, LY avoids antigen-mediated CL because the domains required for this CL pathway are found in the 52 kDa truncated form of PCSK9.

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