An intrinsic mechanism of secreted protein aging and turnover

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The composition and functions of the secreted proteome are controlled by the life spans of different proteins. However, unlike intracellular protein fate, intrinsic factors determining secreted protein aging and turnover have not been identified and characterized. Almost all secreted proteins are posttranslationally modified with the covalent attachment of N-glycans. We have discovered an intrinsic mechanism of secreted protein aging and turnover linked to the stepwise elimination of saccharides attached to the termini of N-glycans. Endogenous glycosidases, including neuraminidase 1 (Neu1), neuraminidase 3 (Neu3), beta-galactosidase 1 (Glb1), and hexosaminidase B (HexB), possess hydrolytic activities that temporally remodel N-glycan structures, progressively exposing different saccharides with increased protein age. Subsequently, endocytic lectins with distinct binding specificities, including the Ashwell–Morell receptor, integrin αM, and macrophage mannose receptor, are engaged in N-glycan ligand recognition and the turnover of secreted proteins. Glycosidase inhibition and lectin deficiencies increased protein life spans and abundance, and the basal rate of N-glycan remodeling varied among distinct proteins, accounting for differences in their life spans. This intrinsic multifactorial mechanism of secreted protein aging and turnover contributes to health and the outcomes of disease.

Significance

In the blood, secreted proteins have different life spans that determine their abundance and function. Measurements of plasma protein composition and biological activities remain important for many clinical diagnoses. However, the molecular mechanisms by which secreted proteins age and turnover have remained unidentified. The findings of this research have established an intrinsic and constitutive mechanism of secreted protein aging and turnover. This mechanism involves multiple factors including circulating glycosidases that progressively remodel the N-glycan links attached to most secreted proteins. N-glycan remodeling with time exposes glycan ligands of various endocytic lectin receptors that then eliminate these aged secreted proteins. This mechanism thereby determines the life spans and abundance of secreted proteins, and modulates the pathogenesis and outcomes of disease.

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The authors declare no conflict of interest.

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We identified Neu isozymes present in the plasma of mice and humans. Both Neu1 and Neu3 were detected in mice of multiple strains, including CD57BL/6J, A/J, and SMJ (Fig. 2A). The SM/J strain is homozygous for a point mutation in the Neu1 allele that reduces Neu activity by 50% (13). In the SM/J strain, an increase in Neu3 abundance was detected with the retention of normal Neu activity (Fig. 2B). Expression of secreted Neu1 and Neu3 was also detected in plasma samples of healthy humans with consistent basal levels of Neu activity (Fig. 2C and D).

Identification of Neuraminidases That Remodel Aging N-Glycans. The contributions of Neu1 and Neu3 were investigated by pharmacological inhibition. The compound 2,3-dehydro-2-deoxy-N-acetylatedaminic acid (DANA) is a broad-spectrum neuraminidase inhibitor of all four Neu isozymes, whereas zanamivir inhibits Neu2, Neu3, and Neu4 but does not significantly inhibit Neu1 (14, 15). The i.v. administration of DANA and zanamivir at multiple dosages and times was studied to achieve and maintain maximal inhibition (Fig. S2). DANA and zanamivir administration (250 mg/kg) every 6 h was required to maintain maximal inhibition. Using DANA, reductions in plasma Neu activity of up to 80% were obtained. Zanamivir administration reduced Neu activity by 50% and 20% of normal in plasma of C57BL/6J and SM/J mice, respectively (Fig. 2F). These results indicated that the majority of plasma Neu activity measured in this assay was due to Neu3.

Inhibition of Neu activity was further investigated in the context of N-glycan remodeling during secreted protein aging. Administration of DANA markedly inhibited the exposure of Gal linkages during secreted protein aging (Fig. 2F and G). Results with zanamivir treatment were similar, consistent with the involvement of Neu3 (Fig. S3). Remarkably, unmasking of GlcNAc and Man linkages was also inhibited by DANA and zanamivir. This finding implied that N-glycan remodeling during secreted protein aging involves the sequential hydrolysis of exposed glycan linkages unmasked by multiple exoglycosidases. The identification and study of two secreted proteins with distinct $t_{1/2}$s were undertaken to compare the effects of N-glycan remodeling on individual proteins. We observed that Neu inhibition by DANA or zanamivir resulted in an increase in circulating alkaline phosphatase activity (Fig. 3A). Measurements of plasma alkaline phosphatase activity indicated that tissue nonspecific alkaline phosphatase (TNAP) and intestinal alkaline phosphatase (IAP) were increased in abundance (Fig. 3B and C). This increase was coincident with elevated $t_{1/2}$s of TNAP and IAP (Fig. 3D and E and Fig. S4). Moreover, the N-glycans of TNAP and IAP were subject to progressive remodeling with increased age, which was significantly reduced by Neu inhibition (Fig. 3F and G and Fig. S4). These findings demonstrated a connection between N-glycan remodeling in secreted protein aging and the rate of protein turnover in circulation, implying the presence of one or more endocytic lectin receptors that participate in determining the $t_{1/2}$ and abundance of secreted proteins.

Asialoglycoprotein Receptors in the Turnover of Aged Secreted Proteins. The Ashwell–Morell receptor (AMR) of hepatocytes is an endocytic lectin discovered by its rapid ability to eliminate from the circulation i.v.-administered glycoproteins that have been desialylated in vitro by Neu activity (7). The AMR binds exposed Gal linkages with increased avidity in multivalent contexts, as exist among the multiple branches of N-glycans (7, 16, 17). AMR function has been found to eliminate desialylated platelets produced during pneumococcal sepsis and following platelet exposure to reduced temperatures (6, 18, 19).

Among mice lacking either the Asgr1 or Asgr2 protein subunit of the AMR, an accumulation of asialoglycoproteins was detected among secreted proteins by lectin affinity chromatography using nondenaturing conditions (Fig. 4A and B). This accumulation was accompanied by a two- to threefold increase in the amount of plasma protein isolated. In addition, proteins bearing exposed GlcNAc and Man linkages were increased in abundance to a lesser extent. Neu gene and activity levels were unaffected in AMR-deficient mice (Fig. 4C and D). It was evident that the accumulation of asialoglycoproteins in AMR deficiency sought in previous studies had been obscured by the appearance of lectin ligands by lectin blotting following denaturing electrophoresis (20–22). This result was directly demonstrated by lectin blotting of secreted proteins including those that failed to bind by affinity chromatography (Fig. S5).

Proteomic analyses identified 291 secreted proteins elevated in Asgr1 and Asgr2 deficiency, with many increased 10-fold or more (Table S2). A gene ontology analysis revealed that these proteins were linked to proteolysis, coagulation, inflammation, and immunity (Fig. S6). Essentially the same processes were indicated in an identical analysis of asialoglycoproteins accumulating in Asgr2 deficiency. Proteomic analyses confirmed the elevation of TNAP and IAP levels in AMR-deficient mice, consistent with results obtained following Neu inhibition (Fig. 3).

Increased abundance of TNAP and IAP in AMR deficiency occurred with a corresponding elevation of alkaline phosphatase activity (Fig. 4 E and F). No changes were observed in RNA expression encoding TNAP and IAP in multiple tissues, including the liver, bone, kidney, and small intestine (Fig. S7). Circulating TNAP and IAP $t_{1/2}$s were increased in the absence of Asgr1 or Asgr2 with the presence of TNAP and IAP glycoforms bearing increased Gal exposure (Fig. 4 G–J). These findings provided a mechanistic linkage between Neu activity, N-glycan remodeling, and AMR function in the aging and turnover of secreted proteins, including the individual secreted proteins TNAP and IAP.

Sequential Functions of Multiple Glycosidases and Endocytic Lectin Receptors. Unmasking of GlcNAc and Man linkages subsequent to Neu function implied the possibility that additional endocytic lectin receptors with different glycan binding specificities may also control secreted protein $t_{1/2}$s and abundance. Multiple lectins capable of binding multivalent GlcNAc and Man linkages have been identified. For example, integrin αM encoded by the Itgam gene can bind and endocytose proteins bearing exposed GlcNAc linkages such as occurs at the surface of chilled platelets (23). We observed the accumulation of secreted proteins with unmasked GlcNAc by lectin chromatography in plasma of mice lacking integrin αM (Fig. 5A and B). This finding was coincident with an approximate 75% increase in the amount of plasma protein isolated and further implied the presence of β-gal activity.

The β-gal Glb1 was identified in the plasma of healthy mice (Fig. 5C). Additional studies also identified beta-galactosidase 1 (Glb1) in the plasma of healthy humans. The i.v. administration of the galactosidase inhibitor N-nonyl-deoxygalactonojirimycin (NN-DGJ) at optimized doses and times reduced the level of...
plasma β-gal activity to 40% of normal (Fig. 5D and Fig. S8A).
Administration of NN-DGJ further resulted in a reduction in the
unmasking of both GlcNAc and Man linkages (Fig. 5E and
Fig. S8B). These findings were consistent with the stepwise
remodeling of N-glycans during secreted protein aging and
implied that circulating β-galactosidase activity produced li-
gands of integrin αM and possibly other endocytic lectins with
similar binding specificity.

Endogenous lectins that bind Man linkages of N-glycans are also
expressed in mammals. One example is the macrophage mannose
receptor encoded by the *Mmr* gene (24). Previous studies of *Mmr*
deficiency identified elevated serum proteins bearing exposed Man
(25). We also detected their accumulation in *Mmr* deficiency (Fig. 5
F and G). An approximate 50% increase in plasma protein was
isolated by mannose-binding lectin chromatography in the presence of PBS or DANA administered every 6 h. Identical amounts of protein (20 μg) were analyzed by SDS/
PAGE and lectin blotting. (G) Quantification of lectin ligands detected among plasma proteins in F was normalized to time = 0. Data are representative of results
from four to eight separate mouse littermate cohort comparisons and seven human volunteers, and are presented as means ± SEM (***P < 0.001; **P < 0.01; *P < 0.05).

**Fig. 2.** Identification of circulating neuraminidases and linkage of Neu activity to N-glycan remodeling of aged secreted proteins. (A) Antibodies specific to Neu1, Neu2,
Neu3, or Neu4 were used to detect Neu protein abundance in the plasma of mice of indicated strains by ELISA and SDS-PAGE, followed by immunoblotting. M.W.,
molecular weight. (B) Neu activity measured in mouse plasma. U/L, units per liter. (C) Neu protein abundance in the plasma of healthy human adults by ELISA and
SDS-PAGE followed by immunoblotting. (D) Human Neu activity in plasma. (E) Inhibition of Neu activity in plasma following i.v. injection of DANA or zanamivir (250 mg/kg),
compared with saline (PBS), administered at time 0 and every 6 h (arrows) over a 24-h period. (F) Biotinylated plasma proteins from WT C57BL/6J mice were isolated at
indicated times by lectin affinity chromatography in the presence of PBS or DANA administered every 6 h. Identical amounts of protein (20 μg) were analyzed by SDS/
PAGE and lectin blotting. (G) Quantification of lectin ligands detected among plasma proteins in F was normalized to time = 0. Data are representative of results
from four to eight separate mouse littermate cohort comparisons and seven human volunteers, and are presented as means ± SEM (***P < 0.001; **P < 0.01; *P < 0.05).

**Discussion**
An intrinsic multifactorial mechanism controls the composition of the
secreted proteome by integrating the activities of glycosidases and
endocytic lectin receptors with secreted protein aging and turnover.
Over 600 plasma proteins were found with lectin ligands detected
using nondenaturing experimental conditions, whereas different rates
of N-glycan remodeling accounted for different life spans among
individual proteins studied. Inhibition of glycosidase activities im-
paired N-glycan remodeling consistent with the sequential action of
multiple exoglycosidases, whereas lectin receptor deficiencies resul-
ted in the accumulation of aged proteins bearing corresponding
glycan ligands. In both cases, secreted protein life span and abun-
dance were elevated. This accumulation correspondingly aug-
mented the activities of those enzymes examined, indicating that
N-glycan remodeling does not necessarily alter function. This
possibility nevertheless exists among some fraction of secreted
proteins because desialylation of coagulation factor X, for example,
has been demonstrated to diminish its activity (18).

Neu1 and Neu3 operate at the first step in N-glycan remodeling
and accounted for the majority of secreted protein turnover. Most
glycosidases identified in circulation, including Neu1, Glb1,
and HexB, have been localized to lysosomes, where they function
in intracellular glycan catabolism, whereas Neu3, in contrast, is
expressed at the plasma membrane (26, 27). Their presence in the
blood may originate from multiple cell types and likely reflects lys-
osomal secretion, proteolytic processing, or possibly differential

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Alkaline phosphatase was quantified following immunoprecipitation relative to time 0. Biotinylated TNAP and IAP were then analyzed by SDS/PAGE, and lectin affinity chromatography in the presence of PBS or DANA administered every 6 h. Results from six to eight separate littermate cohort comparisons, and are presented as means ± SEM (***P < 0.001; **P < 0.01; *P < 0.05). IP, immunoprecipitation.

Fig. 3. Alkaline phosphatase t1/2 linked rates of N-glycan remodeling. (A-C) Plasma alkaline phosphatase activity, TNAP abundance, and IAP abundance among WT C57BL/6J mice following administration of PBS, DANA, or zanamivir every 6 h. Circulating t1/2 analyses of TNAP (D) and IAP (E) biotinylated in vivo in the presence of either DANA or PBS administered every 6 h and quantified following immunoprecipitation relative to time 0. Biotinylated TNAP (F) and IAP (G) were isolated from WT C57BL/6J mice at indicated times by lectin affinity chromatography in the presence of PBS or DANA administered every 6 h. Identical amounts of TNAP and IAP were then analyzed by SDS/PAGE, and lectin blotting was quantified normalized to time = 0. Data are representative of results from six to eight separate littermate cohort comparisons, and are presented as means ± SEM (***P < 0.001; **P < 0.01; *P < 0.05). IP, immunoprecipitation.

In the regulation of secreted protein homeostasis by biosynthesis and turnover, the most rapid way to achieve changes in protein abundance is by modulating turnover. We have identified an intrinsic multistep mechanism of secreted protein aging and turnover consisting of the sequential glycosidic remodeling of N-glycans in intrinsic multistep mechanism of secreted protein aging and turnover. The unmasking of GlcNAc and Man linkages subsequent to Neu activity requires additional time, suggesting that secreted proteins with extended life spans may be cleared by lectins that bind GlcNAc and Man, such as integrin αM and the Mmr. These endocytic lectins may also be involved in the turnover of secreted proteins that escape clearance by the AMR and other asialoglycoprotein receptors. Mammalian lectins that bind GlcNAc and Man linkages may further represent phylogenetic vestiges of a primordial mechanism of secreted protein aging and turnover because avian species mostly lack Sia linkages. In this regard, it has been demonstrated that the avian AMR homolog specifically binds GlcNAc, which is exposed following hydrolysis of terminal Gal linkages typical of nascent mature avian N-glycans. Glycosidase substrate recognition and binding represent control points determining the life spans of secreted proteins. Multiple mechanisms may regulate this process. N-glycan remodeling may occur by random interactions of glycosidases with secreted proteins determined by their relative concentrations. Nevertheless, the shorter life span of IAP was associated with an increased rate of N-glycan remodeling compared with TNAP, even though IAP is present at a 10-fold lower concentration than TNAP. The number of N-glycan consensus sites also does not correlate with secreted protein life spans. For example, TNAP and IAP have five and three of N-glycan consensus sites conserved in mouse and human homologs, respectively, yet their normal life spans are 12 h and 2 h, respectively. Also relevant are variations in N-glycan site occupancy and the variable presence of bi-, tri-, or tetraantennary N-glycan structures. It is further likely that protein conformation influences lectin binding by the juxtaposition of N-glycans among native and extended life spans may be cleared by lectins that bind GlcNAc and Man in AMR deficiency is consistent with reduced turnover with extended life spans that render them susceptible to further remodeling by galactosidase and glucosaminidase activities.

The AMR is expressed on the mammalian hepatocyte surface and is one of multiple mammalian asialoglycoprotein receptors of the C-type lectin family. Secreted protein turnover controlled by the AMR comprised ~50% of the plasma proteome identified bearing Gal ligands of the related Enyhrina cristalagi lectin. Although it is unlikely that the analytical lectins used are precise indicators of AMR ligands, overlap in proteins modulated in AMR deficiency was noted. The functions of proteins modulated by the AMR span proteolysis, coagulation, inflammation, and immunity. Comparative proteomic studies of Asgr1 and Asgr2 deficiency indicated a similar compilation of secreted proteins accumulated. This finding implied that both Asgr1 and Asgr2 are required in the AMR-dependent turnover of secreted proteins, which may reflect the presence of various heteromeric AMR complexes that operate in determining protein binding and endocytic turnover. The existence of other asialoglycoprotein receptors, including the Kupffer cell receptor, indicates the possibility of additional combinatorial modulation moderating the effects of AMR deficiency. The further analysis of secreted proteins bearing exposed GlcNAc and Man in AMR deficiency is consistent with reduced turnover with extended life spans that render them susceptible to further remodeling by galactosidase and glucosaminidase activities.

In the regulation of secreted protein homeostasis by biosynthesis and turnover, the most rapid way to achieve changes in protein abundance is by modulating turnover. We have identified an intrinsic multistep mechanism of secreted protein aging and turnover consisting of the sequential glycosidic remodeling of N-glycans thereby unmasking ligands of endogenous endocytic lectin receptors. The synthesis and phylogenic conservation of multiantennary N-glycan structures lends itself to the progressive formation of multivalent ligands with increased binding affinities for lectins. This mechanism may further extend to cell surface proteins as N-glycan number and branching can influence cell surface protein residency.
Moreover, the aging and turnover of blood cells and platelets may also be similarly controlled. The reported presence of asialo-platelets in AMR deficiency (34) may represent platelets of increased age. Because altered levels of secreted glycosidases have been associated with various diseases, including diabetes, pancreatitis, cancer, myocardial infarction, renal deficiency, cirrhosis, and viral hepatitis (9), it is reasonable to anticipate that the targeted modulation of secreted protein aging and turnover is involved in these diseases. In this regard, microbial targeting of neuraminidase activity to host platelets and specific coagulation factors, which determines the outcome of infection during pneumococcal sepsis (6, 18), can be reinterpreted as a focused modulation and acceleration of secreted protein aging and turnover.

### Materials and Methods

Detailed methods, including descriptions of reagents, are included in SI Materials and Methods. All methods are routine and have been previously validated.
published multiple times in the referenced literature, including affinity chromatography, lectin blotting, Western blotting, and enzyme activity assays, with further details provided in the figure legends. Data were analyzed and presented as means ± SEM unless otherwise stated. ANOVA on ranks or the Student’s t test was used to determine statistical significance. p values of less than 0.05 were considered significant. The Institutional Animal Care and Use Committees of the University of California, Santa Barbara and the Sanford–Burnham Medical Research Institute approved studies undertaken herein. Informed consent was obtained for blood collected from human subjects.

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Supporting Information

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SI Materials and Methods

Laboratory Animals and Human Blood Acquisition. Mice bearing null alleles of Asgr1, Asgr2, and Mmpr (21, 25, 35) were backcrossed six or more generations into the C57BL/6J background before study. Mice of the SMJ and A/J inbred strains and mice bearing null alleles of Igam (36) were acquired from The Jackson Laboratory. Littermates bearing normal allele loci were used as controls. All mice analyzed were 8–12 wk of age and were provided sterile pellet food and water ad libitum.

Isolation of Aged Blood Proteins. N-hydroxysuccinimide-biotin (10 mg/kg of mouse body weight; Pierce) was injected i.v. into adult mice (4). Subsequently, whole blood was collected at indicated times into lithium-heparin Microtainer Plasma Separator Tubes (BD Biosciences) by tail bleeds of anesthetized mice. Platelet-poor plasma was collected after centrifugation at 2,000 × g for 10 min. Biotinylated plasma glycoproteins were isolated by affinity chromatography using immobilized monomeric avidin gel and eluted in the presence of d-biotin (Pierce). Time 0 was defined as 1 h after reactive biotin injection, when biotinylation stopped and proteins were maximally biotinylated (100% relative biotinylation).

Isolation of Lectin Ligands. Plasma protein samples were diluted 1:10 in Tris-buffered saline (TBS) and incubated with lectin-agarose affinity matrices of Erythrina cristagalli (ECA), Ricinus communis agglutinin-I (RCA), peanut agglutinin (PNA), Maackia amurensis-II (MAL-II), Sambucus nigra (SNA), wheat germ agglutinin (WGA), Griffonia simplicifolia-II (GSL-II), Con A, or Lens culinaris (LCA) (Vector Laboratories). After incubation overnight at 4 °C on a rotating wheel, the mixture was applied to Poly-Prep gravity-flow columns (Bio-Rad) and washed five times with 10 mL of 0.05% Tween 20 in TBS to remove unbound proteins. Proteins bound were eluted with TBS in the presence of either of 0.2 M Gal and lactose (ECA), 0.2 M Gal (RCA and PNA), 0.2 M Sia (MAL-II and SNA), 0.2 M GlcNac (WGA and GSL-II), or 0.2 M Man (Con A and LCA). Protein concentrations were determined by Bradford assay (Bio-Rad).

Glycoprotein Immunoprecipitation, Western Blotting, and Lectin Blotting. Protein samples were diluted in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS] supplemented with complete protease inhibitor mixture (Roche) and incubated overnight at 4 °C on a rotating wheel with either 2 μg/mL TNP antibody (N-18; Santa Cruz Biotechnology) or a 1:100 dilution of IAP-specific antiserum (37), followed by 2 h of incubation in the presence of protein A/G PLUS-agarose (Santa Cruz Biotechnology). Immunoprecipitates were washed five times with RIPA buffer and eluted with SDS sample buffer for SDS/PAGE. Protein samples eluted from lectin-agarose affinity chromatography or immunoprecipitation were subjected to SDS/PAGE, transferred to nitrocellulose membranes, and incubated with 2% (vol/vol) BSA in TBS. They were then analyzed by Western blotting using either 1 μg/mL anti-TNP antibody (ab65834; Abcam), anti-Neu1 (H-300; Santa Cruz Biotechnology), anti-Neu2 (M-13; Santa Cruz Biotechnology), anti-Neu3 (M-50; Santa Cruz Biotechnology), anti-Neu4 (N-14; Santa Cruz Biotechnology), anti-Gib1 (C-20; Santa Cruz Biotechnology), anti-HexB (M-40; Santa Cruz Biotechnology), or a 1:1,000 dilution of anti-IAP antiserum, or by lectin blotting with HRP-conjugated ECA (0.5 μg/mL), RCA (0.1 μg/mL), PNA (1 μg/mL), MAL-II (0.2 μg/mL), SNA (0.1 μg/mL), WGA (0.5 μg/mL), GSL-II (0.5 μg/mL), Con A (0.2 μg/mL), or LCA (0.2 μg/mL) (EY Laboratories). Signals detected by chemiluminescence (GE Healthcare) were analyzed by integrated optical density using Labworks software (UVP Bioimaging Systems). Parallel protein samples were visualized with silver staining (Bio-Rad).

ELISA. ELISA plates (Nunc) were coated with antibodies to either TNP (N-18), Neu1 (H-300), Neu2 (M-13), Neu3 (M-50), Neu4 antibody (N-14), or IAP antiserum, and blocked with BSA before incubation with serial dilutions of mouse plasma samples that were biotinylated using 1 mg/mL N-hydroxysuccinimide-biotin (Pierce). Antigens were detected following the addition of HRP-streptavidin (BD Biosciences) and 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma–Aldrich). Lectin binding was determined in parallel by the addition of HRP-conjugated ECA, RCA, PNA, MAL-II, or SNA, followed by TMB, and changes in glycan linkages were detected by comparing lectin binding among identical amounts of biotinylated TNP and IAP antigen calculated as described (8). Alkaline phosphatase activity was measured using the p-nitrophenyl phosphate substrate (Sigma–Aldrich) as previously described (38).

Proteomic Analyses. Proteins were digested using the filter-aided sample preparation method (39). The 2D LC-tandem MS (MS/MS) analyses were carried in triplicate using an EASY-nLC 1000 HPLC instrument coupled to a Q Exactive Plus mass spectrometer (all from Thermo Fisher Scientific). A 180-min gradient consisting of 5–16% (vol/vol) B (100% acetonitrile) in 140 min, 16–28% (vol/vol) B in 70 min, 28–38% (vol/vol) B in 10 min, and 38–85% (vol/vol) B in 5 min is used to separate the peptides. The Q Exactive Plus is set to scan precursors at a resolution of 70,000, followed by data-dependent MS/MS at a resolution of 17,500 of the top 12 precursors. The 2D LC-MS/MS analysis was carried out by high-pH reversed-phase peptide fractionation (12 fractions) in the first dimension, which was connected online with the second dimension low-pH reversed-phase chromatography using a 2D nanoACQUITY Ultra Performance Liquid Chromatography system (Waters Corp.) coupled to an Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific). Peptides were eluted from the second dimension using a 65-min gradient from 3–28% (vol/vol) B (100% acetonitrile) at 400 nL min−1. The mass spectrometer was operated in positive data-dependent acquisition mode on the top 10 most abundant precursors.

All raw files were analyzed with MaxQuant software version 1.5.0.25. MS/MS spectra were searched against the human UniProt protein sequence database (version downloaded in October 2014). The target decay-based false discovery rate (FDR) filter for spectrum and protein identification was set to 1%. MaxQuant-calculated peptide intensities were log2-transformed and normalized (locally weighted scatterplot smoothing normalization) across samples to account for systematic errors. Protein-level quantification and testing for differential abundance were performed using the MStats bioconductor package (40) based on a linear mixed-effects model.

Blood Chemistry. Blood plasma analyses, including measurements of alkaline phosphatase activity, were acquired with a VetScan Comprehensive Diagnostic Profile reagent rotor (Abaxis) as previously reported (18).

Glycosidase Activity and Inhibition. Neuraminidase activity was measured in total plasma using the Amplex Red Neuraminidase Assay Kit according to the manufacturer’s instructions (Molecular
For inhibition of plasma neuraminidase activity, a dose of 250 mg/kg of either DANA (Calbiochem) (41) or zanamivir (Sigma–Aldrich) (14) was prepared in 200 μL of PBS and injected i.v. at multiple times. β-Gal activity was measured as described (42) with minor modifications. Plasma (10 μL) was mixed with 40 μL of 0.1 M Tris buffer at pH 7 and 30 μL of 20 mM p-nitrophenyl-β-d-galactopyranoside (Sigma–Aldrich) in 0.1 M Tris buffer at pH 7. The mixtures were incubated for 60 min at 37 °C, and the reactions were stopped by adding 200 μL of 0.2 M borate buffer at pH 9.8. Product measurements of activity were analyzed by amounts of released p-nitrophenol detected at 405 nm (43). For inhibition of plasma β-gal activity, a dose of 50 mg/kg NN-DGJ (Santa Cruz Biotechnology) (44) was prepared in 200 μL of the dosing vehicle consisting of 20% (vol/vol) DMSO and 80% (vol/vol) water and injected i.v. at multiple times. β-N-acetyl-glucosaminidase (β-GlcNAcase) activity was measured in total plasma using a β-GlcNAcase assay according to the manufacturer’s instructions (Sigma–Aldrich) at pH 7. For inhibition of plasma β-GlcNAcase activity, a dose of 25 mg/kg 2-acetoamido-1,2-dideoxyxojirimycin (Abcam) (45) was prepared in 200 μL of PBS and injected i.v. at multiple times. Plasma samples were taken by tail bleeds at multiple times after administration of the inhibitors for activity measurements.

mRNA Analyses. Total RNA was isolated from cells and tissues using TRIzol (Invitrogen) and subjected to reverse transcription (RT) using SuperScript III (Invitrogen). Quantitative real-time PCR was performed using Brilliant SYBR Green Reagents with the Mx3000P QPCR System (Stratagene). Primers used to detect mouse sequences were TNAP-RT-forward (F) (5'-CCC-AGACACAAGCATTTCCACTAT-3'), TNAP-RT-reverse (R) (5'-CACCATCTCGAGAGCG-3'), IAP-RT-F (5'-CTCATCTC-CAACATGGAC-3'), IAP-RT-R (5'-TGCTTAGACCTTTCACGG-3'), GAPDH-RT-F (5'-TGGTGAAAGTGGTGTTGAAC-3'), and GAPDH-RT-R (5'-AGTGATGGCATGTGACTGTGG-3'). Relative mRNA levels were normalized to littermate WT control expression of GAPDH RNA.

Statistical Analysis. Data were analyzed as mean ± SEM unless otherwise stated. ANOVA on ranks or the Student t test was used to determine statistical significance in some experiments. P values of less than 0.05 were considered significant. For proteomic studies, peptide identification output from MaxQuant was used for label-free protein quantitative analysis. First, calculated peptide intensities were log2-transformed and normalized across samples to account for systematic errors. A total of eight normalization approaches were deployed [locally weighted scatterplot smoothing (LOWESS), robust linear regression, variance stabilization and normalization, total intensity, median intensity, average intensity, NormFinder, and quantile], and their performance was assessed to determine the optimal normalization method (herein, LOESS normalization). Following normalization, all peptide sequences nonunique to a single protein were removed from the list. Protein-level quantification and testing for differential abundance were performed using the MSstats bioconductor package based on a linear mixed-effects model. The model decomposes log intensities into the effects of technical and biological replicates, peptides, and statistical interactions. For each genotype comparison made, MSstats provided model-based estimates of fold changes, as well as P values that were adjusted to control the FDR at the cutoff of 0.01.
Accumulation of asialoglycoproteins, agalactoglycoproteins, and Man-terminated N-glycans during blood protein aging. (A) Protein biotinylation was performed first in vivo among WT C57BL/6J mice using i.v. injection of biotin, followed by biotinylated protein isolation at multiple times using avidin chromatography. Time 0 is defined as 60 min postbiotin injection, when the in vivo biotinylation of proteins reached maximum and no further biotinylation was detected. Identical amounts (20 μg) of plasma proteins isolated at indicated times postbiotin injection were analyzed by SDS/PAGE and visualized by total protein staining with silver (Left) or by streptavidin binding (Middle). (Right) Total and biotinylated protein concentrations were plotted at the indicated times after in vivo biotinylation. (B) Biotinylated blood plasma proteins from A isolated at the indicated times following i.v. in vivo biotinylation and lectin affinity chromatography were analyzed by lectin blotting (Upper); silver staining (Middle); and by the addition during blotting of 0.2 M Sia, Gal, GlcNAc, or Man (i.e., the relevant glycan competitor) for confirming lectin specificity (Bottom). M.W., molecular weight.
Fig. S2. Pharmacological inhibition of plasma neuraminidase (Neu) activity. (Left) Neuraminidase inhibitor DANA (A) or zanamivir (B) was i.v. injected into WT C57BL/6J mice as the indicated doses and plasma neuraminidase activity were measured after 6 h of DANA or zanamivir injection. (Right) DANA (250 mg/kg) or zanamivir (250 mg/kg) was injected, and plasma neuraminidase activity was measured at the indicated time points. Data are representative of results from four to six littermate cohort comparisons, and are presented as means ± SEM. U/L, units per liter.
Fig. S3. Zanamivir inhibition of N-glycan remodeling during plasma protein aging. (A) Biotinylated blood plasma proteins from WT C57BL/6J mice were isolated at the indicated times by lectin affinity chromatography in the presence of PBS or zanamivir (250 mg/kg) administered every 6 h. Identical amounts of proteins were analyzed by SDS/PAGE and lectin blotting (Top) or total protein staining with silver (Bottom). (B) Measurements of multivalent glycan linkages by lectin binding following administration of PBS or zanamivir calculated relative to time 0. Data are representative of results from four to six littermate cohort comparisons, and are presented as means ± SEM (***P < 0.01; *P < 0.05).
Fig. S4. TNAP and IAP $t_{1/2}$s and N-glycan remodeling following zanamivir treatment. (A and B) Expression and $t_{1/2}$s in blood circulation of biotinylated TNAP and IAP proteins at indicated times in WT mice administered either PBS or zanamivir. (C and D) Lectin blotting analyses from identical amounts of biotinylated, biotin affinity-isolated, and immunoprecipitated TNAP and IAP normalized to time 0 and at indicated times following administration of PBS or zanamivir every 6 h. Data are representative of results from six to eight littermate cohort comparisons, and are presented as means ± SEM (***P < 0.001; **P < 0.01; *P < 0.05). IP, immunoprecipitation.
Fig. S5. Lectin blotting of total plasma proteins in AMR deficiency. (A) Equivalent amounts of total plasma proteins were analyzed by SDS/PAGE and lectin blotting. (B) Results of lectin blotting in A were quantified relative to results obtained from WT littermates. In separate studies, total blood plasma proteins were subjected to lectin affinity chromatography. (C) Those proteins that did not bind during lectin chromatography (flow-through fraction) were quantified, and equal amounts were analyzed by SDS/PAGE followed by lectin blotting. (D) Results of lectin blotting in C were quantified relative to results obtained from WT littermates. Data are representative of results from four to six separate littermate cohort comparisons, and are presented as means ± SEM.
Biological processes associated with proteins elevated in AMR deficiency. Proteins elevated in mice lacking either Asgr1 or Asgr2 were subjected to a functional enrichment analysis by gene ontology (GO) terms using the FGnet package in Bioconductor. The 25 top-scoring biological processes are shown.

TNAP and IAP mRNA abundance in AMR deficiency. (A) Relative TNAP mRNA expression in liver, bone, and kidney tissues from mice of indicated genotypes was measured by quantitative RT-PCR (qRT-PCR). (B) Relative IAP mRNA level from duodenum tissue from mice of indicated genotypes as measured by qRT-PCR. Data are representative of results from six to eight separate littermate cohort comparisons, and are presented as means ± SEM.
Pharmacological inhibition of plasma β-gal activity and β-GlcNAcase activity in secreted protein abundance and turnover. (A) Inhibition of β-gal activity following i.v. administration of NN-DGJ with varying dosage and measurements of activity at multiple times after a single administration. (B) β-Gal inhibitor NN-DGJ was i.v. injected into WT C57BL/6J mice (50 mg/kg) every 12 h. Identical amounts of biotinylated glycoproteins isolated from plasma using lectin affinity chromatography were separated by SDS/PAGE and analyzed by lectin binding (Left) or by protein staining with silver (Right). (C) Inhibition of β-GlcNAcase activity following i.v. administration of ADN with varying dosage and measurements of activity at multiple times after single administration. (D) β-GlcNAcase inhibitor ADN was i.v. injected into WT C57BL/6J mice (25 mg/kg) every 12 h. Identical amounts of biotinylated glycoproteins isolated from plasma using lectin affinity chromatography were separated by SDS/PAGE and analyzed by lectin binding (Left) or by protein staining with silver (Right). Data are representative of results from four to six littermate cohort comparisons, and are presented as means ± SEM.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)