A distinctive histidine residue is essential for in vivo glycation-inactivation of human CD59 transgenically expressed in mice erythrocytes: Implications for human diabetes complications

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Abstract Word Count: 208

Text Word Count: 2,493.

No. of Table: None

No. of Figures: 3

Running title: In vivo glycation of the human complement regulator CD59.

Keywords: CD59, Diabetes complication, Protein glycation, Glycation motif.

Abstract:

Clinical and experimental evidences support a link between the complement system and the pathogenesis of diabetes complications. CD59, an extracellular cell membrane-anchored protein, inhibits formation of the membrane attack complex (MAC), the main effector of complement-mediated tissue damage. This complement regulatory activity of human CD59 (hCD59) is inhibited by hyperglycemia-induced ε-amino glycation of Lys^{41}. Biochemical and structural analyses of glycated proteins with known three-dimensional structure revealed that glycation of ε-amino lysyl residues occurs predominantly at "glycation motives" that include lysyl/lysyl pairs or proximity of a histidyl residue, in which the imidazolyl moiety is ≈ 5Å from the ε-amino group. hCD59 contains a distinctive Lys^{41}/His^{44} putative glycation motif within its active site. In a model of transgenic diabetic mice expressing in erythrocytes either the wild type or a H44Q mutant form of hCD59, we demonstrate *in vivo* that the His^{44} is required for Lys^{41} glycation and consequent functional inactivation of hCD59, as evidenced using a mouse erythrocytes hemolytic assay. Since 1) the His^{44} residue is not present in CD59 form other animal species and 2) humans are particularly prone to develop complications of diabetes, our results indicate that the Lys^{41}/His^{44} glycation motif in human CD59 may confer humans a higher risk of developing vascular disease in response to hyperglycemia.

Introduction:

The complement system is an effector of both adaptive and innate immunity. The system can be activated through three different cascades known as the classical, the alternative and the mannose-binding lectin (MBL) pathways[1, 2]. The three pathways share a common late reaction sequence that leads to the generation of the membrane attack complex (MAC), a trans-membrane pore that is the main effector of complement-mediated tissue damage. Insertion of the MAC into red blood cell membranes causes colloidosmotic lysis while insertion into nucleated cells can activate intracellular signaling cascades and trigger the release of cytokines and growth factors that stimulate proliferation, inflammation and thrombosis [3-9] as characteristically seen in the target organs of complement activation.

To protect "self" cells from the devastating consequences of unrestricted complement activation, several soluble and membrane-bound complement regulatory proteins have evolved. CD59, a GPI-linked glycoprotein, is a specific inhibitor of MAC formation that is ubiquitous in mammalian cells. Emerging evidence indicates that the complement system and CD59 are involved in several features of cardiometabolic disease (reviewed in[10]). Three clinical reports describe cases in which absence (mutations) or decreased expression of CD59 increases the risk of cardiovascular or neuropathic disease[11-13]. In animal models, we have reported that ablation of CD59 promoted accelerated atherosclerosis with occlusive coronary disease and premature death, which were attenuated by transgenic overexpression of human CD59 in the endothelium[14].

Regarding diabetes, a body of clinical and experimental evidence supports a pathogenic link between the complement system, CD59, and the pathogenesis and progression of diabetes complications[15-23] and reviewed in[24]. In animal models, we recently reported that diabetic mCD59 deficient mice develop accelerated atherosclerosis as compared with diabetic mCD59 wild type mice[25].

There are different ways in which the "delicate balance" between complement activation and restriction can be potentially broken in diabetes. Autoantibodies to glycated and glycol-oxidized proteins can activate the classical pathway[26, 27], fructosamines and advanced glycation end products (AGEs) reportedly activate the lectin pathway [18, 28]. Of all the putative mechanisms that potentially contribute to complement-mediated tissue damage in diabetes, inactivation by non-enzymatic glycation of hCD59 is the most extensively studied and documented.

Protein glycation is a major mechanism of tissue damage in diabetes [29]. The reaction of glucose with α- or ε-amino groups in proteins starts with the formation of a Schiff base or aldimine, followed by tautomerization to the more stable ketoamine via the Amadori rearrangement [30-32]. It has long been observed that despite the presence of many amino groups on the surface of proteins few ε-amino groups are glycated to a significant extent *in vivo* [33, 34]. Initial attempts to elucidate this intriguing observation suggested that properties of nearby amino acids could play a role in determining whether a given lysine is glycated[33, 34]. Later, identification of ε-amino glycation in some Lys/Lys pairs [31] led to sequence logo statistical analysis of glycated lysine residues as well as proteomic analyses of glycated proteins in plasma and erythrocytes. These efforts generated sequence-based glycation predictors derived from the primary structure of

glycated proteins [35, 36]. However, sequence-based predictors of ε-amino lysine glycation fail to predict the three-dimensional structure of proteins and the potential involvement of a catalytic amino acid residue that is proximal in the three-dimensional protein structure (3D) but distant in the primary sequence[35, 36]. The identification of glycated amino groups in proteins with known 3D revealed that ε-amino glycation preferably occurs at "glycation motives" such as Lys/His in which an imidazolyl moiety located at ≈ 5Å from the ε-amino group of a Lys provides acid–base catalysis for the aldimine/ketoamine tautomerization [31, 32, 37, 38]. For example, the β chain of hemoglobin displays Lys^{61}, Lys^{65}, and Lys^{66} residues but only the ε- amino group of Lys^{66}, which is in proximity to His^{63}, is preferentially glycated *in vivo* [33]. Similarly, in horse liver alcohol dehydrogenase, *in vivo* glycation preferentially occurs at Lys^{231} that is close to the imidazole group of His^{348}[39].

Analyzing the NMR structure of human CD59 [40], we recognized the presence of a putative glycation-motif formed by amino acid residues Lys^{41} and His^{44}. To test experimentally the presence of a glycation motif in human CD59, we expressed recombinant human CD59 (hCD59WT) and a mutant lacking the H44 residue (hCD59H44Q), and showed that hCD59WT but not the hCD59H44Q mutant was inhibited by glycation. It is known, however, that *in vitro* protein glycation studies require concentrations of glucose that are extremely high (0.5- 1M) [41] as compared with either the physiological glucose concentration (0.005M) or the pathological glucose levels observed in individuals with diabetes. For this reason, *in vitro* glycation studies provide potentially useful information but their actual pathophysiological and potentially therapeutic implications need confirmation *in vivo*. Here we report experiments in transgenic mice showing that *in vivo*

inactivation of hCD59 by preferential glycation of the Lys^{41} residue does require the proximity of the His^{44} residue. This His^{44} residue that conforms the glycation motif in hCD59 is not present in CD59 from any other species sequenced to date, a remarkable observation because humans are particularly prone to develop complications of diabetes that are not seen in the same intensity and distribution in animal models of the diabetes.

The results reported here provide the first *in vivo* demonstration of the glycation-motif concept; they suggest that the glycation motif in hCD59 may confer humans a higher risk of developing vascular disease in response to hyperglycemia.

Methods:

Animals.

Animal studies were approved by the Harvard Medical School's Institutional Animal Care and Use Committee (IACUC). Mice were housed in micro-isolator cages in a pathogen-free animal facility, fed a regular chow diet and kept on 12 hours light/12 hours dark cycle.

Generation of transgenic mice expressing human CD59.

Transgenic mice expressing either wild type (Tg-hCD59WT) or H44Q mutant (Tg-hCD59H44Q) human CD59 in erythrocytes were generated using the construct described previously[42]. The transgenic vector consisted of the human locus control region (LCR), alpha globin gene promoter, and hCD59 cDNA (either WT or H44Q mutant, from 5' to 3'). These mice were originally generated in a mixed genetic background and were backcrossed for more than 10 generations to C57BL/6J background. Age-matched non-transgenic C57BL/6J mice (Non-Tg) were used as controls. The successful generation of

these mice was determined by both Northern blot and FACS analysis of erythrocytes, as we previously described in details[43].

Induction of diabetes.

Mice were made diabetic by multiple low dose injections of Streptozotocin (STZ; Sigma-Aldrich, MO, USA), following the protocol recommended by NIH's Animal Models of Diabetic Complications Consortium (https://www.diacomp.org/shared/showFile.aspx?doctypeid=3&docid=19). Briefly, 8 weeks old mice, randomly assigned into two groups, received either STZ (50 mg/kg body weight) or vehicle injected once daily for 5 consecutive days. Blood glucose was measured once a week from a blood drop transferred directly from the tail tip into the glucometer. Mice were considered diabetic if blood glucose reached > 200 mg/dl 2 weeks after the first STZ administration.

FACS analysis.

Blood was collected from tail tip in heparinized capillary and mouse red blood cells (mRBCs) were isolated. Cells were washed twice with phosphate- buffered saline (PBS) and resuspended in PBS containing 2% bovine serum albumin (BSA). Cells were incubated with mouse anti-human CD59 antibody (BRIC 229, IBGRL, Bristol, UK), followed by a FITC-conjugated anti-mouse IgG (Abcam, Cambridge, MA). The cells were washed in PBS three times before analyzing the fluorescence intensity using a FACS Canto II analyzer (Becton Dickinson, Franklin Lakes, NJ).

Hemolytic Assay.

The protective activity of hCD59 against human MAC mediated lysis of mRBCs was determined by a hemolysis protection assay using mRBCs exposed to the human

membrane attack complex (MAC), as described in[16]. Briefly, a suspension of mRBCs in gelatin veronal buffer with calcium and magnesium (GVB^{++}) was challenged with purified human C5b-6, C7, C8, and C9 (Complement Technologies, Texas, USA) that were added sequentially to form human MAC. MAC-mediated lysis of mRBCs was quantitated by measuring the absorbance of released hemoglobin at 405 nm. Results were expressed as percent of mRBC lysis.

Western blot analysis.

Mouse ghost RBC lysate was prepared as described[44] with little modifications. Briefly, RBCs from diabetic or non-diabetic mice were washed twice with PBS and lysed in 5 mM sodium phosphate buffer, pH 8.0 containing protease inhibitor cocktail (Calbiochem, La Jolla, CA) at 4°C overnight. Ghost membranes were pelleted by centrifugation at 15,000 x g, for 30 min at 4°C and solubilized in 0.1% SDS for 30 min at 37°C. Proteins were separated on SDS-PAGE and transferred to PVDF membrane. Membrane was probed with mouse anti-Lys41 glycated hCD59 monoclonal antibody (kindly provided by Mellitus LLC, Cambridge, MA) followed by IRDye 800 conjugated secondary antibody (Rockland Immunochemicals, PA, USA). The same membrane was reprobed with a goat anti-hCD59 antibody (R&D Systems, Minneapolis, USA), followed by IRDye 700 conjugated secondary antibody and scanned on an Odyssey scanner (LI-COR, Lincoln, NE). The specificity of the mouse anti-Lys41 glycated hCD59 monoclonal antibody was assessed by a) Western blot analysis using non-glycated and glycated hCD59 purified from human urine, as reported in[23], b) direct ELISA titers against the glycated antigenic peptide used to raise the antibody as compared with titers against a non-glycated peptide of identical sequence, and c) by real-time analysis of affinity for non-

glycated recombinant human CD59 and for glycated CD59 affinity purified from diabetic urine using a OCTET system (Pall ForteBio, Menlo Park, CA) (data not shown).

Statistical analysis.

Experimental results are shown as the Mean ± S.D. The difference between two groups was evaluated with 2-tailed Student's t-test for continuous variables and with nonparametric Mann-Whitney test for categorical variables. Difference between groups were considered significant when $p< 0.05$ (two tailed).

Results:

STZ-induced hyperglycemia in mice.

To assess *in vivo* the preferential glycation of a Lys^{41} residue in proximity to His^{44} within a glycation motif in hCD59, we generated transgenic mice expressing in red blood cells (mRBC) human CD59, either the glycation-sensitive wild type (Tg-hCD59WT) or the glycation resistant H44Q mutant (Tg-hCD59H44Q) designed to break the putative glycation motif while preserving the Lys^{41} (Figure S1). The transgenic mice were made diabetic by multiple low-dose injection of streptozotocin. Expression of either wild type or H44Q mutant hCD59 did not affect the rate or extent of hyperglycemia in transgenic mice when compare to the non-transgenic animals (Figure 1A). Eight weeks after STZ injection (blood glucose ≈ 400 mg/dL), the mRBC were tested to assess both 1) *in vivo* glycation of hCD59 by Western blot analysis with a monoclonal antibody highly specific for Lys^{41} glycated hCD59 (Figure S2 and Materials and Methods), and 2) functional activity of transgenic hCD59 protecting mRBC from human MAC-mediated lysis.

Transgenic expression of hCD59 on mouse RBC membrane.

RBC membranes from either Tg-hCD59WT or Tg-hCD59H44Q mice expressed comparable amounts of hCD59, as documented by FACS and Western blot analysis of mRBC membranes (Figure 1B, and 1C, bottom blot). Further, hyperglycemia did not affect the membrane density of the transgenic proteins (Figure 1B). Remarkably, glycation of hCD59 occurred only in mRBC membranes expressing Tg-hCD59WT (Figure 1C, top blot). In contrast, glycated hCD59 was not detected in diabetic mRBC transgenically expressing the hCD59H44Q mutant. These results provide the first *in vivo* experimental demonstration that a histidine residue fosters ε-amino glycation of a nearby lysine conforming a glycation motif.

Hemolytic assay with human MAC.

The Lys^{41}/His^{44} glycation motif in hCD59 is located within the active site of the protein that centers around Trp^{40}[45, 46]. To assess the functional consequence of hCD59 glycation *in vivo*, we established a hemolytic assay to measure hCD59 activity with a slight modification of the one described in reference [16]. Briefly, the assay measures the activity of transgenically expressed hCD59 through the hemolysis of isolated mRBC following the sequential addition of purified human terminal complement components (C5b-6, C7, C8, and C9). Added in this sequence, the terminal complement components lyse mRBC by formation of the human MAC (Figure 2A). Due to the high species specificity of CD59 (mouse CD59 is active against mouse complement but does not protect mouse cells against human complement), mRBC are highly sensitive to lysis by human MAC (Figure 2B, black open box). Addition of purified recombinant hCD59 (Figure 2B, black filled box) or the transgenic expression of either WT or H44Q mutant human CD59 rescues mRBC from human MAC-mediated lysis (Figure 2B, red open box

and blue open box, respectively). Activity of hCD59 in the transgenic mRBC, as measured by protection of mRBC against human MAC-mediated lysis was similar when the mRBC expressed either the Tg-hCD59WT or the mutant Tg-hCD59H44Q (Figure 2, red open box and blue open box, respectively). The observed protection of transgenic mRBC from human MAC-mediated lysis was specifically due to the transgenic expression of hCD59 because it was abrogated by incubation with BRIC 229 Ab, a mouse anti-human CD59 monoclonal antibody known to neutralize hCD59 inhibitory activity against human MAC (Figure 2, red filled box and blue filled box, respectively).

Glycation and inactivation of wild type hCD59 in vivo.

Figure 3 shows that the protection conferred by Tg-hCD59 to mRBC exposed to human MAC was lost in diabetic mice expressing Tg-hCD59WT (red filled box) but preserved in mRBCs expressing the Tg-hCD59H44Q mutant (blue filled box). The increased lysis of mRBC observed in diabetic transgenic mice expressing Tg-hCD59WT was due to loss of hCD59 protecting activity as 1) it was rescued by *in vitro* addition of purified hCD59 (Figure 3, red hatched box), 2) hyperglycemia did not affect the membrane density of transgenic hCD59, as shown by FACS analysis in Figure 1B, and 3) neither the expression of the transgenic proteins nor hyperglycemia affected the osmotic fragility of the mRBC (Figure S3).

Discussion:

The results presented here provide the first *in vivo* experimental demonstration of the operation of a glycation motif in a human protein, a concept initially derived from biochemical and structural analysis of glycated proteins. From a human diabetes and

complications perspective, the significance of the glycation motif in hCD59 is highlighted by 1) the emerging clinical and experimental evidence revealing that the complement system and glycation of CD59 play a role in the pathogenesis of the multiple complications of human diabetes, reviewed in[24]), and 2) the fact that the His^{44} residue of hCD59 is absent in CD59 from any other animal species sequenced to date[45] (Table S1). This confluence of structure, function, and pathology is remarkable because humans are particularly prone to develop complications of diabetes, and no single animal model of the disease recapitulates the extension and intensity of the vascular disease that commonly complicates human diabetes[47]. From the *in vivo* evidence that the H44 residue in human CD59 is required for its glycation and abrogation of its activity as a complement inhibitor combined with the emerging evidence for a role of complement in the pathogenesis of diabetes complications, it is tempting to speculate that the Lys^{41}/His^{44} glycation motif in hCD59 may confer humans a higher risk of developing extensive vascular disease in response to hyperglycemia.

Source of Funding:

This work was supported by the National Heart, Lung, and Blood Institute of the National Institute of Heath grants DK60979, HL107762 to JAH.

Figure legends:

Figure 1. (A) Blood glucose measurement of non-transgenic and hCD59 transgenic mice after STZ treatment. Average blood glucose level of diabetic non-transgenic (Non-Tg) or transgenic mice expressing either wild type (Tg-hCD59WT), or H44Q

mutant (Tg-hCD59H44Q) protein after STZ administration. Error bars represent means ± SD, n=10 each group. **(B) Effect of hyperglycemia on expression of hCD59 in transgenic mRBCs.** Representative histograms of hCD59 expression measured by flow cytometry in mRBCs from non-transgenic (Non-Tg) or transgenic mice expressing either wild type (Tg-hCD59WT) or H44Q mutant (Tg-hCD59H44Q) protein. ND- non-diabetic; D- diabetic. **(C) Effect of hyperglycemia on glycation of hCD59 in transgenic mRBCs.** Lysates of mRBC membrane from non-transgenic (Non-Tg) or transgenic mice expressing either wild type (Tg-hCD59WT) or H44Q mutant (Tg-hCD59H44Q) protein was analyzed by Western blot using anti-glycated Lys41 hCD59 antibody (upper blot). The same blot was re-probed with anti- hCD59 antibody (lower blot). Lysates were prepared from pooled mBRCs from 6 mice for each group. Reported blot is representative of three independent experiments. ND: non-diabetic, D: diabetic.

Figure 2. (A) Schematic representation of the hemolytic assay used to test activity of hCD59. Human terminal complement components (C5b-6, C7, C8, and C9) added sequentially lyse mRBC by formation of human MAC pores. Human CD59 transgenically expressed on mRBC protects against human MAC mediated lysis. Lysis of mRBC by human MAC in the absence or presence of transgenically expressed human CD59 represents the activity of the transgenic protein. **(B) Functional human CD59 protects mouse RBC against human MAC mediated lysis.** Box/whisker style plots representing lysis of mRBCs by human MAC formed by sequential addition of purified human terminal complement components (C5b-6, C7, C8, and C9). Black boxes: Lysis of non-transgenic mRBC in the absence (open box) or presence (filled box) of purified

human CD59. Red Boxes: Lysis of mRBC transgenically expressing hCD59WT (wild type) in the absence (open box) or presence (filled box) of the neutralizing anti-hCD59 monoclonal antibody BRIC 229. Blue Boxes: Lysis of mRBC transgenically expressing hCD59H44Q mutant in the absence (open box) or presence (filled box) of the neutralizing anti-hCD59 monoclonal antibody BRIC 229. Boxes are representative of three experiments (n= 10 mice per group). **p< 0.005.

Figure 3. The activity of transgenic hCD59WT but not of the transgenic hCD59H44Q44 mutant is inhibited in diabetic mice. Mice were made diabetic by injection of STZ. After two weeks, the activity of transgenic hCD59 was assessed *ex vivo* using the hemolytic assay shown in Figure 2A. The figure is a box/whisker style plots representing lysis of mRBC by human MAC form non-diabetic (ND) and diabetic mice (D). Red Boxes: Lysis of mRBC from non-diabetic (open box) or diabetic (filled boxes) mice transgenically expressing wild type hCD59. The red hatched box represents lysis of mRBC from diabetic hCD59WT transgenic mice exposed *in vitro* to purified hCD59. Blue Boxes: Lysis of mRBC from non-diabetic (open box) or diabetic (filled boxes) mice transgenically expressing the hCD59H44Q non-glycatable mutant. Boxes are representative of three experiments (n= 10 mice per group). **p< 0.005, ns- non-significant.

References:

1. Morgan BP, Harris CL. Complement Regulatory Proteins. London: Academic Press; 1999.

2. Walport MJ. Complement. First of two parts. N Engl J Med 2001;344:1058-1066.

3. Halperin JA, Taratuska A, Nicholson-Weller A. Terminal complement complex C5b-9 stimulates mitogenesis in 3T3 cells. J Clin Invest 1993;91:1974-1978.

4. Halperin JA, Taratuska A, Rynkiewicz M, et al. Transient changes in erythrocyte membrane permeability are induced by sublytic amounts of the complement membrane attack complex (C5b-9). Blood 1993;81:200-205.

5. Acosta JA, Benzaquen LR, Goldstein DJ, et al. The transient pore formed by homologous terminal complement complexes functions as a bidirectional route for the transport of autocrine and paracrine signals across human cell membranes. Mol Med 1996;2:755-765.

6. Viedt C, Hansch GM, Brandes RP, et al. The terminal complement complex C5b-9 stimulates interleukin-6 production in human smooth muscle cells through activation of transcription factors NF-kappaB and AP-1 [In Process Citation]. Faseb J 2000;14:2370-2372.

7. Hattori R, Hamilton KK, McEver RP, et al. Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. J Biol Chem 1989;264:9053-9060.

8. Kilgore KS, Schmid E, Shanley TP, et al. Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte

chemoattractant protein-1 through nuclear factor-kB activation. Am J Pathol 1997;150:2019-2031.

9. Benzaquen LR, Nicholson-Weller A, Halperin JA. Terminal complement proteins C5b-9 release basic fibroblast growth factor and platelet-derived growth factor from endothelial cells. J Exp Med 1994;179:985-992.

10. Hertle E, Stehouwer CD, van Greevenbroek MM. The complement system in human cardiometabolic disease. Mol Immunol 2014;61:135-148.

11. Yamashina M, Ueda E, Kinoshita T, et al. Inherited complete deficiency of 20-kilodalton homologous restriction factor (CD59) as a cause of paroxysmal nocturnal hemoglobinuria. N Engl J Med 1990;323:1184-1189.

12. Nevo Y, Ben-Zeev B, Tabib A, et al. CD59 deficiency is associated with chronic hemolysis and childhood relapsing immune-mediated polyneuropathy. Blood 2013;121:129-135.

13. Emin M, Wang G, Castagna F, et al. Increased internalization of complement inhibitor CD59 may contribute to endothelial inflammation in obstructive sleep apnea. Science translational medicine 2016;8:320ra321.

14. Wu G, Hu W, Shahsafaei A, et al. Complement regulator CD59 protects against atherosclerosis by restricting the formation of complement membrane attack complex. Circ Res 2009;104:550-558.

15. Falk RJ, Sisson SP, Dalmasso AP, et al. Ultrastructural localization of the membrane attack complex of complement in human renal tissues. Am J Kidney Dis 1987;9:121-128.

16. Acosta J, Hettinga J, Fluckiger R, et al. Molecular basis for a link between complement and the vascular complications of diabetes. Proc Natl Acad Sci U S A 2000;97:5450-5455.

17. Rosoklija GB, Dwork AJ, Younger DS, et al. Local activation of the complement system in endoneurial microvessels of diabetic neuropathy. Acta Neuropathol (Berl) 2000;99:55-62.

18. Flyvbjerg A. Diabetic angiopathy, the complement system and the tumor necrosis factor superfamily. Nat Rev Endocrinol 2010;6:94-101.

19. Mellbin LG, Bjerre M, Thiel S, et al. Complement activation and prognosis in patients with type 2 diabetes and myocardial infarction: a report from the DIGAMI 2 trial. Diabetes Care 2012;35:911-917.

20. Hansen TK, Tarnow L, Thiel S, et al. Association between mannose-binding lectin and vascular complications in type 1 diabetes. Diabetes 2004;53:1570-1576.

21. Woroniecka KI, Park AS, Mohtat D, et al. Transcriptome analysis of human diabetic kidney disease. Diabetes 2011;60:2354-2369.

22. Qin X, Goldfine A, Krumrei N, et al. Glycation inactivation of the complement regulatory protein CD59: a possible role in the pathogenesis of the vascular complications of human diabetes. Diabetes 2004;53:2653-2661.

23. Ghosh P, Sahoo R, Vaidya A, et al. A specific and sensitive assay for blood levels of glycated CD59: a novel biomarker for diabetes. Am J Hematol 2013;88:670-676.

24. Ghosh P, Sahoo R, Vaidya A, et al. Role of complement and complement regulatory proteins in the complications of diabetes. Endocr Rev 2015;36:272-288.

25. Liu F, Sahoo R, Ge X, et al. Deficiency of the complement regulatory protein CD59 accelerates the development of diabetes-induced atherosclerosis in mice. J Diabetes Complications 2017;31:311-317.

26. Orchard TJ, Virella G, Forrest KY, et al. Antibodies to oxidized LDL predict coronary artery disease in type 1 diabetes: a nested case-control study from the Pittsburgh Epidemiology of Diabetes Complications Study. Diabetes 1999;48:1454-1458.

27. Witztum JL, Steinbrecher UP, Fisher M, et al. Nonenzymatic glucosylation of homologous low density lipoprotein and albumin renders them immunogenic in the guinea pig. Proc Natl Acad Sci U S A 1983;80:2757-2761.

28. Fortpied J, Vertommen D, Van Schaftingen E. Binding of mannose-binding lectin to fructosamines: a potential link between hyperglycaemia and complement activation in diabetes. Diabetes Metab Res Rev 2010;26:254-260.

29. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 1988;318:1315-1321.

30. Bunn HF, Higgins PJ. Reaction of monosaccharides with proteins: possible evolutionary significance. Science 1981;213:222-224.

31. Baynes JW, Watkins NG, Fisher CI, et al. The Amadori product on protein: structure and reactions. Prog Clin Biol Res 1989;304:43-67.

32. Shilton BH, Campbell RL, Walton DJ. Site specificity of glycation of horse liver alcohol dehydrogenase in vitro. Eur J Biochem 1993;215:567-572.

33. Shapiro R, McManus MJ, Zalut C, et al. Sites of nonenzymatic glycosylation of human hemoglobin A. J Biol Chem 1980;255:3120-3127.

34. Watkins NG, Thorpe SR, Baynes JW. Glycation of amino groups in protein. Studies on the specificity of modification of RNase by glucose. J Biol Chem 1985;260:10629-10636.

35. Johansen MB, Kiemer L, Brunak S. Analysis and prediction of mammalian protein glycation. Glycobiology 2006;16:844-853.

36. Zhang Q, Monroe ME, Schepmoes AA, et al. Comprehensive identification of glycated peptides and their glycation motifs in plasma and erythrocytes of control and diabetic subjects. Journal of proteome research 2011;10:3076-3088.

37. Iberg N, Fluckiger R. Nonenzymatic glycosylation of albumin in vivo. Identification of multiple glycosylated sites. J Biol Chem 1986;261:13542-13545.

38. Calvo C, Ulloa N, Campos M, et al. The preferential site of non-enzymatic glycation of human apolipoprotein A-I in vivo. Clin Chim Acta 1993;217:193-198.

39. Shilton BH, Walton DJ. Sites of glycation of human and horse liver alcohol dehydrogenase in vivo. J Biol Chem 1991;266:5587-5592.

40. Fletcher CM, Harrison RA, Lachmann PJ, et al. Structure of a soluble, glycosylated form of the human complement regulatory protein CD59. Structure 1994;2:185-199.

41. Lapolla A, Fedele D, Traldi P. The role of mass spectrometry in the study of non-enzymatic protein glycation in diabetes. Mass Spectrom Rev 2000;19:279-304.

42. Kooyman DL, Byrne GW, McClellan S, et al. In vivo transfer of GPI-linked complement restriction factors from erythrocytes to the endothelium. Science 1995;269:89-92.

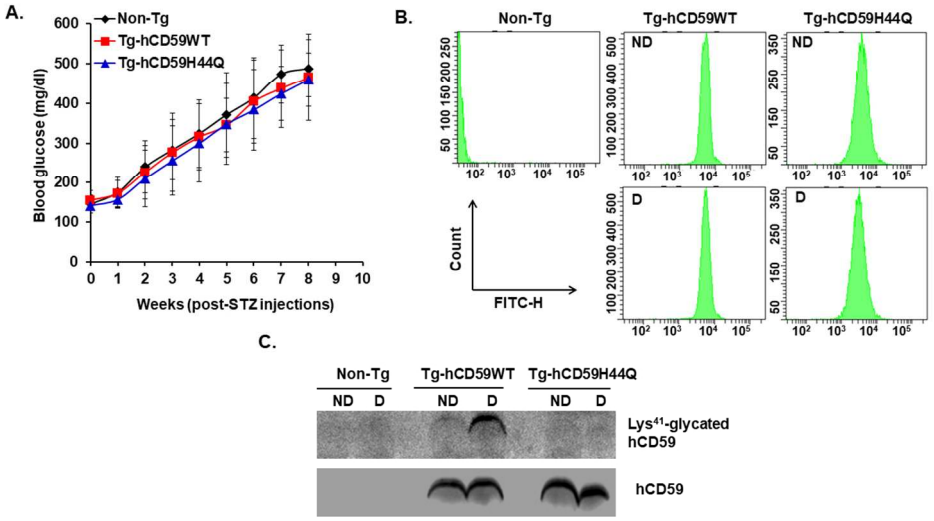
43. Hu W, Ferris SP, Tweten RK, et al. Rapid conditional targeted ablation of cells expressing human CD59 in transgenic mice by intermedilysin. Nat Med 2008;14:98-103.

44. Miller JA, Gravallese E, Bunn F. Nonenzymatic glycosylation of erythrocyte membrane proteins: relevance to diabetes. J Clin Invest 1980;65:896-901.

45. Bodian DL, Davis SJ, Morgan BP, et al. Mutational analysis of the active site and antibody epitopes of the complement-inhibitory glycoprotein, CD59. J Exp Med 1997;185:507-516.

46. Yu J, Abagyan R, Dong S, et al. Mapping the active site of CD59. J Exp Med 1997;185:745-753.

47. Vischer UM. Hyperglycemia and the pathogenesis of atherosclerosis: lessons from murine models. Eur J Endocrinol 1999;140:1-3.



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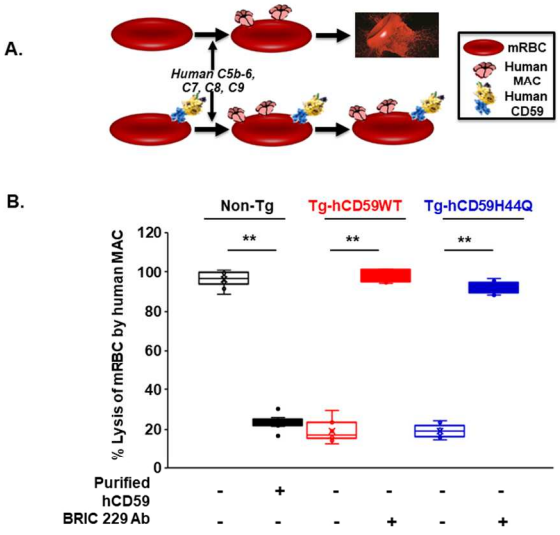

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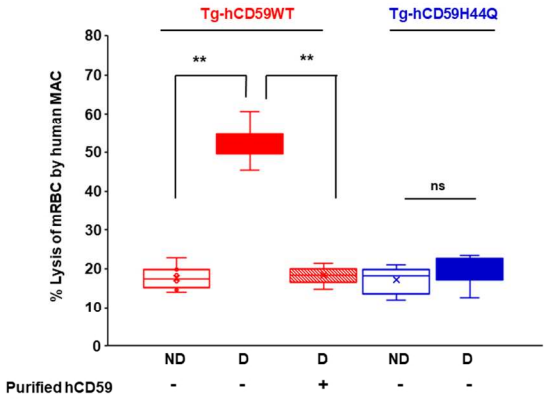

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