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Charcot-Leyden Crystal Protein/Galectin-10 Interacts with Cationic Ribonucleases and is Required for Eosinophil Granulogenesis

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Abstract:	Background: The human eosinophil Charcot-Leyden Crystal (CLC) protein is a member of the Galectin superfamily and is also known as Galectin-10 (Gal-10). CLC/Gal-10 forms the distinctive hexagonal bipyramidal crystals considered hallmarks of eosinophil participation in allergic responses and related inflammatory reactions; however, the glycan-containing ligands of CLC/Gal-10, its cellular function(s), and its role(s) in allergic diseases are unknown. Objective: We sought to determine the binding partners of CLC/Gal-10 and elucidate its role in eosinophil biology. Methods: Intracellular binding partners were determined by ligand blotting with CLC/Gal-10, followed by co-immunoprecipitation and co-affinity purifications. The role of CLC/Gal-10 in eosinophil function was determined by employing enzyme activity assays, confocal microscopy, and shRNA knock-out of CLC/Gal-10 expression in human CD34 + cord blood hematopoietic progenitors differentiated to eosinophils. Results: CLC/Gal-10 interacts with both human eosinophil granule cationic ribonucleases, eosinophil-derived neurotoxin (EDN, RNS2) and eosinophil cationic protein (ECP, RNS3) , and with murine eosinophil-associated ribonucleases. The interaction is independent of glycosylation and is not inhibitory toward endoribonuclease activity. Activation of eosinophils with INF-γ induces the rapid co-localization of CLC/Gal-10 with EDN/RNS2 and CD63. ShRNA knock-down of CLC/Gal-10 in human cord blood-derived CD34 + progenitor cells impairs eosinophil granulogenesis. Conclusions: CLC/Gal-10 functions as a carrier for the sequestration and vesicular transport of the potent eosinophil granule cationic ribonucleases during both differentiation and degranulation, enabling their intracellular packaging and extracellular functions in allergic inflammation. Clinical Implications: Understanding the crucial role of CLC/Gal-10 in eosinophil differentiation/granulogenesis allows for further insights into potential therapeutic targets to treat allergic diseases.

Charcot-Leyden Crystal Protein/Galectin-10 Interacts with Cationic Ribonucleases and is Required for Eosinophil Granulogenesis

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Capsule summary: Charcot-Leyden protein/Galectin-10, a prominent eosinophil constituent associated with eosinophilic inflammation, functions as a binding partner for cationic ribonucleases and is required for eosinophil differentiation and granulogenesis.

Key Words: eosinophils, galectins, Charcot-Leyden, ribonucleases, EDN, ECP, RNase2, RNase3, granulogenesis

Abbreviations: CLC, Charcot-Leyden crystal; CRD, carbohydrate recognition domain, EDN, eosinophil-derived neurotoxin; ECP, eosinophil cationic protein; EPX, eosinophil peroxidase; EARS, eosinophil-associated ribonucleases; Gal-10, galectin-10; lysophospholipase, LPLase; MBP-1, eosinophil granule major basic protein-1; PMD, piecemeal degranulation; RNase, ribonuclease.

ABSTRACT

Background: The human eosinophil Charcot-Leyden Crystal (CLC) protein is a member of the Galectin superfamily and is also known as Galectin-10 (Gal-10). CLC/Gal-10 forms the distinctive hexagonal bipyramidal crystals considered hallmarks of eosinophil participation in allergic responses and related inflammatory reactions; however, the glycan-containing ligands of CLC/Gal-10, its cellular function(s), and its role(s) in allergic diseases are unknown.

Objective: We sought to determine the binding partners of CLC/Gal-10 and elucidate its role in eosinophil biology.

Methods: Intracellular binding partners were determined by ligand blotting with CLC/Gal-10, followed by co-immunoprecipitation and co-affinity purifications. The role of CLC/Gal-10 in eosinophil function was determined by employing enzyme activity assays, confocal microscopy, and shRNA knock-out of CLC/Gal-10 expression in human $CD34^+$ cord blood hematopoietic progenitors differentiated to eosinophils.

Results: CLC/Gal-10 interacts with both human eosinophil granule cationic ribonucleases, eosinophil-derived neurotoxin (EDN, RNS2) and eosinophil cationic protein (ECP, RNS3) , and with murine eosinophil-associated ribonucleases. The interaction is independent of glycosylation and is not inhibitory toward endoribonuclease activity. Activation of eosinophils with INF-γ induces the rapid co-localization of CLC/Gal-10 with EDN/RNS2 and CD63. ShRNA knock-down of CLC/Gal-10 in human cord blood-derived $CD34^+$ progenitor cells impairs eosinophil granulogenesis.

Conclusions: CLC/Gal-10 functions as a carrier for the sequestration and vesicular transport of the potent eosinophil granule cationic ribonucleases during both differentiation and degranulation, enabling their intracellular packaging and extracellular functions in allergic inflammation.

Clinical Implications: Understanding the crucial role of CLC/Gal-10 in eosinophil differentiation/granulogenesis allows for further insights into potential therapeutic targets to treat allergic diseases.

INTRODUCTION

Charcot-Leyden crystals (CLC), first identified more than 150 years ago,[1, 2] are found in a variety of tissues, body fluids and secretions as hallmarks of inflammation involving eosinophils and/or basophils in asthma, myeloid leukemias, allergic, parasitic, and other eosinophil-associated diseases and inflammatory reactions.[3, 4] CLC protein autocrystallizes to form distinctive hexagonal bipyramidal crystals[5, 6] and is the sole protein constituent of both native CLC formed *in vivo*[7] and CLC prepared from disrupted eosinophils[5, 6] and basophils[8] *in vitro*. CLC is one of the most abundant eosinophil proteins comprising an estimated 7-10% of total cellular protein[9] and was previously thought to be an eosinophil lysophospholipase,[7, 9-11] but has since been reclassified as a member of the galectin superfamily of animal lectins,[12] and it is hereafter referred to as CLC/Gal-10. However, unlike members of the galectin superfamily, many of which bind lactose and other β-galactoside-containing oligosaccharides, and share 12 highly conserved residues that constitute the carbohydrate recognition domain (CRD),[13-16] CLC/Gal-10 has a putative carbohydrate recognition domain that contains only 7 out of the 12 conserved amino acid residues and does not bind ß-galactosides.[17]

To date, the biologic activities and physiologic function(s) of CLC/Gal-10 in eosinophils (and basophils) with associated inflammatory responses in allergic diseases and host immune responses to parasitic helminths have remained indeterminate. Several studies have shown a direct association of CLC/Gal-10 levels with both airway and gastrointestinal eosinophilic inflammation. A strong correlation was observed between

CLC/Gal-10 levels and the percentage of eosinophils in the sputum of asthmatic patients.[18] Sputum gene expression of CLC/Gal-10 was found to discriminate between inflammatory phenotypes in asthma and to predict response to inhaled corticosteroid.[19] Overexpression of CLC/Gal-10 mRNA was noted in the peripheral blood of patients with aspirin-induced asthma and mRNA levels of CLC/Gal-10 were also shown to be a marker of CRTH2 activation.[20, 21] Genetic variation in CLC/Gal-10 was found to be associated with allergic rhinitis and patients with seasonal allergic rhinitis were found to have elevated levels of CLC/Gal-10 present in nasal lavage fluid during allergy season.[22, 23] Analysis of protein expression patterns in gut biopsies of celiac disease patients found a positive correlation between CLC/Gal-10 levels and tissue damage, and a recent study showed that higher levels of CLC/Gal-10 mRNA distinguished children with eosinophilic esophagitis (EoE) from control children.[24, 25] A recent report by Persson et al., showed that human CLCs administered directly into mouse airways act as a type 2 adjuvant, mimicking many features of human asthma; the effects were readily reversible by CLC-dissolving anti-Gal-10 monoclonal antibodies, suggesting a possible therapeutic approach.[26] Intriguingly, in addition to the considerable amounts present in eosinophils and basophils, CLC/Gal-10 is also expressed in human $CD4^+$ $CD25^+$ regulatory T cells, where it is necessary for the maintenance of immunosuppressive functions.[27] As well, a recent study described a novel subset of $CD16^+$ eosinophils that can suppress T-cells through a mechanism involving CLC/Gal-10.[28]

Eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) are among the major secreted effector proteins of human eosinophils[29] present in the matrix of their large secondary (specific) granules. Both EDN and ECP have structural[30-32] and catalytic[30, 31, 33-36] residues typical of the ribonuclease A superfamily of proteins, and have been designated RNS2 (EDN) and RNS3 (ECP), with EDN having significantly greater ribonuclease activity (~100-fold) than ECP.[37] Both EDN and ECP are highly glycosylated proteins variably decorated with N-linked and O-linked oligosaccharides, and for EDN, C-mannosylation is present as well.[38] EDN and ECP are more than 60% homologous at the amino acid level[35, 36] and their core polypeptides are ~15.5kD with multiple glycosylated isoforms (glycoforms) of molecular masses from 18-21kD and higher.[39] These cationic ribonucleases likely function as anti-parasitic, anti-bacterial and anti-viral agents as part of both innate and adaptive host immune defense mechanisms.[33, 34, 40-44] In addition, EDN and its murine ortholog eosinophil-associated RNase 2 (mEAR2), were reported to function as chemo-attractants for dendritic cells both *in vitro* and *in vivo.*[40] These cationic ribonucleases are secreted by activated eosinophils in part by piecemeal degranulation (PMD),[45] a process that involves vesicular transport from secondary granules to the extracellular space[46] in the absence of classical granule exocytosis, but are also found in extracellular DNA traps.[47, 48]

In the current study, we used ligand (far-Western) blotting to identify possible intracellular glycoprotein ligands for CLC/Gal-10 in the eosinophil, revealing the eosinophil granule cationic ribonucleases (EDN/RNS2 and ECP/RNS3) as major binding partners for this unusual galectin. We demonstrate that CLC/Gal-10 does not

inhibit their ribonuclease activity and show the intracellular movement and co-localization of CLC/Gal-10 with EDN and CD63 during eosinophil activation. Finally, we demonstrate through shRNA knock-down of CLC/Gal-10 in human CD34+ hematopoietic progenitors, a functional role and requirement for this galectin in granulogenesis during IL-5-driven eosinophil differentiation.

MATERIALS AND METHODS

Purification of human blood eosinophils

Blood eosinophils were obtained from normal, non-allergic, non-asthmatic subjects with informed donor consent according to IRB approved protocols at the University of Illinois at Chicago. The purification of eosinophils by magnetic-activated cell sorting (Miltenyi Biotec AutoMACS) was performed as previously described.[15]

Cell lines and culture

The AML14.3D10 eosinophil-differentiated myelocyte cell line[49] was kindly provided by Drs. Cassandra Paul and Michael Baumann (VA Medical Center and Wright State University, Dayton, OH). The AML14.3D10 myelocyte line displays many of the characteristics of mature peripheral blood eosinophils, including constitutive expression of the major protein mediators of the eosinophil such as the granule cationic proteins major basic protein-1 (MBP-1), eosinophil peroxidase (EPX), EDN and ECP, and CLC/Gal-10.[50-52] AML14.3D10 eosinophils express CLC/Gal-10 protein in amounts

comparable to that of peripheral blood eosinophils.[15] Cells were maintained in RPMI 1640 medium supplemented with 2mM L-glutamine, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate and 8% fetal bovine serum. Purified human $CD34^+$ cord blood-derived hematopoeitic progenitor cells were purchased from AllCells (Emeryville, CA) and cultured in IMDM media containing 10% FBS, 2 mM L-glutamine, 50 μM β-mercaptoethanol, and 1% Penicillin-Streptomycin. The media was supplemented with 100 ng/mL of FMS-like tyrosine kinase-3 (FLT-3) ligand, stem cell factor (SCF), and Thrombopoietin (TPO) for the first 48h of culture.

Purification of eosinophil granule cationic proteins

Purified human EDN, ECP, and MBP-1 were generous gifts from Drs. Gerald Gleich and Hirohito Kita (Mayo Clinic, Rochester, MN). The purification procedures for the human eosinophil granule cationic proteins have been described in detail previously.[39, 53] Purified murine eosinophil-associated ribonucleases (EARS)[33, 54-56] were a generous gift from Dr. James J. Lee (Mayo Clinic, Scottsdale, AZ).

Ligand (far-western) blotting with CLC/Gal-10 protein probe

Cell lysates or purified eosinophil granule cationic proteins were resolved by SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% non-fat dry milk, the membranes were incubated for one hour with 10 μg/mL of pure crystal-derived CLC/Gal-10 protein in 100mM Tris-HCL buffer (pH 7.5). The membranes were further probed with affinity-purified rabbit anti-CLC/Gal-10 antibody. The crystal-derived CLC/Gal-10 used for ligand blotting was prepared according to published protocols.[6, 15,

[57] Protein bands identified by binding to CLC/Gal-10 were excised from the membrane and subjected to trypsin digestion, followed by MALDI-TOF/MS analysis on an Applied Biosystems Voyager DE-STR instrument.

Enzymatic Deglycosylation and PAS staining

Blood eosinophil lysates or purified eosinophil granule cationic proteins were subjected to digestion with PNGase F (New England Biolabs, Ipswich, MA), Sialidase A, O-Glycanase, β-(1-4) Galactosidase, and β-N-Acetylglucosaminidase (Prozyme, Hayward, CA) according to the manufacturer's protocols. Staining for glycoproteins was performed using a PAS Glycoprotein Detection Kit™ (Sigma-Aldrich, St. Louis, MO).

Antibodies to native CLC/Gal-10, EDN/ECP, and MBP-1

Affinity purification and preparation of rabbit polyclonal anti-CLC/Gal-10 and rabbit polyclonal anti-EDN/ECP was previously described in detail elsewhere and in the Online Repository Materials.[15, 53] An anti-EDN monoclonal antibody (clone 167-6C5) that does not recognize ECP or any other eosinophil granule cationic protein, and an MBP-1-specific monoclonal antibody were provided by Drs. Gerald Gleich and Hirohito Kita (Mayo Clinic, Rochester, MN).

Co-immunoprecipitation, affinity co-purification and confocal microscopy

Co-immunoprecipitation and affinity co-purification of CLC/Gal-10 and EDN and confocal immunofluorescence microscopy to determine colocalization of CLC/GAL-10 with EDN and CD63 are described in detail in the Online Repository Materials.

Ribonuclease enzyme activity assay

The assay was performed using a cleavable fluoro-labeled substrate (Ambion, Austin, TX) and is further described in the Online Repository Materials.

Knock-down of CLC/Gal-10 in cord blood CD34$^+$ progenitor cells

CD34$^+$ cells were transduced with MISSION lentiviral shRNA transduction particles (Sigma- Aldrich, St. Louis, MO) at an MOI of 5 for 20 hours. Cells were collected, the viruses washed away, and the media supplemented with 100 nM IL-3 and IL-5 (R&D Systems, Minneapolis, MN) for the remainder of the experiment to promote differentiation of the eosinophil lineage. Cells were maintained at a concentration of 0.3-1.0 x 10^6 cell/mL for 14 or 21 days. Six days after the virus particles were removed, 2 µg/mL Puromycin (Sigma- Aldrich, St. Louis, MO) was added to the media in order to select for cells transduced with the shRNA lentiviral particles. Cells were stained using Fast Green/ Neutral Red and May-Grünwald Giemsa (Sigma- Aldrich, St. Louis, MO) according to the manufacturer's protocol. Cell morphology on cytocentrifuge slides (Shandon Cytospin II, Thermo Fisher Scientific, Waltham, MA) was evaluated by differential counts on sequential 40X high power fields, counting at least 200 cells/slide.

Detection of granule proteins in CD34$^+$ progenitor cell-derived eosinophils

CLC/Gal-10, EDN, ECP, EPX and MBP-1 were detected in cell lysates 21 days after transduction of cells with either non-target control or CLC/Gal-10 specific shRNAs. Granule protein levels were detected using double-antibody sandwich ELISAs as

previously described.[58] EPX was detected using a commercially available ELISA kit (Lifespan Bioscience Inc, Seattle, WA).

Degranulation of CD34+ progenitor cell-derived eosinophils

Degranulation of CD34+ progenitor cell-derived in vitro differentiated eosinophils was performed by incubating 0.5 x 10^5 cells/well with 0, 2, 4, and 6 µM PAF (Platelet-activating factor (PAF_{C16}) (Sigma-Aldrich, St. Louis, MO) in cell culture plates previously coated with 3% human serum albumin in PBS. Cells were incubated at 37°C and 5% CO_2 for 4h and protein concentrations of EDN and EPX were measured in cell supernatants as described above.

RESULTS

Identification of a 21kD protein in blood eosinophils that binds CLC/Gal-10

In order to identify a potential intracellular ligand(s) for CLC/Gal-10, we performed CLC/Gal-10 protein ligand blotting (far-western blotting) of blood eosinophil whole cell lysates before or after enzymatic cleavage of N-linked glycans by PNGase F **(Fig. 1A)**. Following incubation of cell lysates with crystal-derived CLC/Gal-10 protein, interacting protein bands were detected by a highly specific affinity-purified anti-CLC/Gal-10 antibody.[59] In addition to detecting the expected endogenous CLC/Gal-10 present in blood eosinophils, this approach also identified a 21kD protein that interacted with CLC/Gal-10 **(Fig. 1A)**. The detected 21kD protein still bound CLC/GAL-10 after being reduced to 18kD following PNGase F digestion**(Fig. 1B)**. The 18kD band became

visible only after carefully removing the membrane section containing monomeric CLC/Gal-10 following membrane transfer during western blotting. Importantly, MALDI-TOF/MS analysis identified the 21kD band as the two eosinophil granule cationic ribonucleases, EDN (RNS2) and ECP (RNS3). The 18kD band that appears after enzymatic cleavage of N-linked glycans was also confirmed by MALDI-TOF/MS to be comprised of EDN and ECP, suggesting them as possible intracellular ligands for this eosinophil galectin.

EDN/ECP co-purify and co-immunoprecipitate with CLC/Gal-10

The finding of a CLC/Gal-10-eosinophil ribonuclease interaction by ligand blotting prompted us to determine if EDN and/or ECP co-purify or co-immunoprecipitate with CLC/Gal-10 from eosinophil lysates. EDN co-purified with CLC/gal-10 from both AML14.3D10 eosinophilic myelocytes and blood eosinophils on a column of affinity purified anti-CLC/Gal-10 antibody[15] **(Fig. 1C)**. MBP-1, the most cationic (pI=11.3) granule protein of the eosinophil,[60] was used as a specificity control **(Fig. 1C, bottom panel)**. We next performed cross-immunoprecipitations of AML14.3D10 eosinophil lysates using either anti-CLC/Gal-10 or EDN-specific mouse monoclonal antibody. Both proteins co-immunoprecipitated with one another as detected by Western blotting **(Fig. 1D)**, and the EDN that co-immunoprecipitated was ~15kD, comparable in size to the non-glycosylated core polypeptide.

CLC/Gal-10 binds to purified eosinophil ribonucleases

To confirm the binding and specificity of CLC/Gal-10 for EDN and ECP, we repeated the ligand blotting procedure using purified human eosinophil granule-derived native EDN and ECP proteins, murine eosinophil-associated ribonucleases (EARS), and MBP-1 **(Fig. 2A)**. CLC/Gal-10 bound to EDN, to the mouse EARS (a mixture containing EARS-1, -2, -3, -4, -6, -7 and –8), and to some, but not all glycoforms of ECP. In contrast, CLC/Gal-10 did not bind MBP-1, indicating that the interaction between CLC/Gal-10 and EDN/ECP/EARS was not due to a simple non-specific charge interaction between the slightly acidic CLC/Gal-10 (pI ≈ 5.1-5.7) and the granule cationic proteins.

Oligosaccharides are not required for the CLC/Gal-10 – ribonuclease interactions

To further characterize the mechanism of interaction between CLC/Gal-10 and the eosinophil ribonucleases in terms of galectin-carbohydrate versus protein-protein interactions, we used purified human EDN and ECP, and mouse EARS for CLC/Gal-10 ligand blotting in combination with PNGase F digestion to remove their N-linked sugars. The cleavage of N-linked sugars did not significantly diminish their recognition by CLC/Gal-10 **(Fig. 2A)**. Despite efficient digestion with PNGase F (visible by the size/ mobility shift of the digested bands), both EDN, ECP and the mouse EARS remained PAS positive. This result is consistent with previous findings that the eosinophil ribonucleases also contain O-linked sugars,[39] and that EDN is also C-mannosylated.[38] We extended our studies by performing CLC/Gal-10 ligand blotting on EDN that was digested with PNGase F, followed by sialidase A (to remove sialic acid residues) and finally a series of exoglycosidases (to remove O-linked sugars) **(Fig. 2B)**. Results showed a non-diminished CLC/Gal-10-EDN interaction, again suggesting that the

interaction is independent of glycan binding. However, due to O-glycans being notoriously difficult to remove, EDN was still positive for PAS staining and we did not see the native EDN fully reduced in size to that of the non-glycosylated rEDN. In order to fully clarify whether glycans are necessary for CLC/Gal-10 binding to EDN, we directly compared the ability of eosinophil-derived native glycosylated EDN and recombinant bacterially-expressed non-glycosylated EDN to bind CLC/Gal-10 **(Fig. 2C)**. Increasing amounts of either recombinant or native EDN shows an equal affinity of CLC/Gal-10 for either of these proteins in a dose-response fashion. This relationship further demonstrates that the CLC/Gal-10-EDN interaction is not oligosaccharide-dependent and that oligosaccharides do not change the affinity of CLC/Gal-10 for EDN. In addition, we employed molecular modelling techniques to identify possible amino acids involved in protein-protein interactions between EDN and CLC/Gal-10 (Supplementary Figures S1-S4). A surface patch of ~39 $Å^3$ with hydrophobic residues and electro-positive charge was identified on CLC/Gal-10 and found to be complementary to a similar patch (also ~39 $Å^3$) on EDN. Residues comprising these two hydrophobic surface patches are predicted to be involved in CLC/Gal-10 and EDN protein-protein interactions. Of note, out of the 26 residues identified (see Supplementary materials), only one residue (H53) is located in the putative carbohydrate recognition domain (CRD) of CLC/Gal-10.[17]

CLC/Gal-10 does not bind to any known mammalian glycans

In order to identify specific glycan ligands for CLC/Gal-10, we performed glycan microarray screening in collaboration with the Consortium for Functional Glycomics

(CFG). The CFG's Protein-Glycan Interaction Core (formerly Core H) probed the CFG mammalian glycan array with crystal-derived CLC/Gal-10, bacterially expressed recombinant CLC/Gal-10, and a recombinant 5-amino acid mutant of CLC/Gal-10 (Q55N, C57R, R61T, Q75E, E77R) engineered to have a consensus galectin CRD. Several detection strategies were employed; however, neither native, recombinant wild type, nor the mutant rCLC/Gal-10 showed any significant binding to the glycan ligands displayed on the microarrays **(Table S1)**. The mammalian glycan array data is discussed in detail in the Online Repository materials.

CLC/Gal-10 does not inhibit EDN ribonuclease activity

To determine whether CLC/Gal-10 might function as an EDN/ECP RNase activity inhibitor during their mobilization and secretion during PMD by the activated eosinophil, we performed RNase activity assays **(Fig. 3)**. Crystal-derived CLC/Gal-10 protein neither inhibited nor enhanced the ribonuclease activity of a constant amount of EDN (80 pg) regardless of the amount of CLC/Gal-10 protein added, whereas placental ribonuclease inhibitor blocked the RNase activity of EDN in a dose-dependent fashion **(Fig. 3A)**. Likewise, there was no difference in the RNase activity of increasing amounts of EDN assayed in the presence of a fixed amount of CLC/Gal-10 (400 pg) **(Fig. 3B)**, whereas a constant amount of RNase inhibitor blocked the RNase activity of all concentrations of EDN.

CLC/Gal-10 co-localizes with EDN and CD63 in IFN-γ activated eosinophils

In freshly purified unstimulated blood eosinophils, CLC/Gal-10 was localized principally in the cytosolic compartment, mainly in the agranular regions immediately beneath the plasma membrane, while EDN was present within the eosinophil secondary granule compartment throughout the cell and was not co-localized with CLC/Gal-10 (**Fig. 4A**, 10 min and 30 min controls). In contrast, when eosinophils were activated with IFN-γ, a potent eosinophil secretagogue that induces the vesicular transport and secretion of EDN and ECP by the process of PMD,[46, 61] much of the CLC/Gal-10 became co-localized with EDN within the cytosolic compartment **(Fig. 4A and Supplementary Figure S7A)**. The CLC/Gal-10-EDN co-localization reached a peak ~30 minutes after stimulation with IFN-γ, with bright cytosolic "pockets" of the co-localized proteins apparent in agranular regions of the cell distinct from the secondary granules. Approximately 60 min after eosinophil activation with IFN-γ, the staining pattern for CLC/Gal-10 began to approximate its original cytosolic localization (not shown). To determine whether eosinophil activation induces the association of CLC/Gal-10 with the tetraspanin CD63, a transmembrane protein of eosinophil secondary granules[62] associated with vesicular transport and mediator release during eosinophil PMD,[63] normal blood eosinophils were activated with IFN-γ as performed above **(Fig. 4B)**. Within 10 minutes of activation, CLC/Gal-10 and CD63 showed a pattern of co-localization within a discrete subset of secondary granules within the cytosol (**Fig. 4B**, 10 and 30 min.), with continued co-localization evident after 60 min (**Supplementary Figure S7B**).

shRNA knock-down of CLC/Gal-10 in eosinophil progenitors impairs eosinophil differentiation/granulogenesis

To further determine the role of CLC/Gal-10 in eosinophil biology, we employed targeted shRNA knock-down of CLC/Gal-10 in purified human CD34+ cord blood-derived progenitor cells. These hematopoietic progenitors were then driven to differentiate to the eosinophil lineage with IL-5, resulting in mature (or nearly mature) eosinophils that do not express CLC/Gal-10. Immunofluorescence staining of cells at day 14 showed an almost complete loss of CLC/Gal-10 expression in cells treated with CLC/Gal-10 specific shRNA lentiviral particles **(Fig. 5A, top panel)**. Fast Green/Neutral red staining of cells showed characteristic features of eosinophils, including red nuclei, pink cytoplasm and green granules; confirming that the cells were successfully differentiated toward the eosinophil lineage (**Fig. 5A**). After 14 days, the CLC/Gal-10 knock-down cells showed a significant reduction in the number of Fast Green stained secondary granules, and cells stained on day 21 showed increasing differences in cellular morphology as compared to control cells **(Fig. 5A, bottom panel)**. Specifically, the size of the granules in the CLC/Gal-10 knock-down cells was substantially larger than those of either the non-transduced or non-target control shRNA cells. In addition, many of these granules did not stain with Fast Green at all, suggesting that they do not contain any secondary granule proteins and represent only large empty containers formed by fusion of empty granules **(Fig. 5B)**.

The CLC/Gal-10 knock-down cells displayed an ~42% decrease in the average number of secondary granules as compared to the non-target shRNA transduced cells **(Fig.**

391 **6A)**, mostly manifested by a dramatic increase of cells with no detectable granules at
392 all, and a strikingly smaller number of cells with more than 15 granules **(Fig. 6B).** Cells
393 transfected with CLC/Gal-10 shRNA also presented as a non-proliferating phenotype. In
394 these cells, no cell proliferation was observed for the duration of the experiment, in
395 contrast to non-transfected (untreated) cells and non-target shRNA transfected control
396 cells that both increased ~10-fold in numbers during the same time span (Fig. 6C). Cell
397 viability remained high (≥ 90%) in all cell groups for the entire duration of the
398 experiment. Analysis of cell lysates 14 days after CLC/Gal-10 knock-down
399 demonstrated the expected lack of CLC/Gal-10 expression both by western blotting
400 **(Online Repository Figure S6)** and ELISA **(Fig. 6D)**. Interestingly, CLC/Gal-10 knock-
401 down also caused a significant decrease in detected MBP-1 levels, but no change in
402 EDN, ECP or EPX expression **(Fig. 6D)**.
403 To determine the effect of CLC/Gal-10 knock-down on degranulation of in vitro-
404 differentiated eosinophils, we incubated the cells with 2, 4, and 6μM Platelet Activating
405 Factor (PAF) (a known eosinophil secretagogue) and measured levels of EPX and
406 EDN in cell supernatants following 4h of PAF stimulation (**Fig. 6E and 6F**). The PAF-
407 activated CLC-deficient eosinophils exhibited dose-dependent degranulation with
408 secretion of both EPX and EDN. The secretion of EPX by CLC-deficient eosinophils
409 was not significantly different from that observed for untreated cells or non-target
410 shRNA controls (**Fig. 6F**); however, the amount of secreted EDN was significantly
411 higher in CLC-deficient eosinophils (**Fig 6E**). Of interest, the CLC-deficient eosinophils
412 “leaked” EDN into the culture media even in the absence of the PAF secretagogue (Fig.
413 6E at 0μM PAF).

DISCUSSION

Although clearly a member of the galectin superfamily, CLC/Gal-10 possesses a modified CRD that does not specifically bind lactose or other galactosamine-containing glycans. The oligosaccharides, if any, that bind to the CLC/Gal-10 CRD remain unknown, and in our current study we present data from glycan microarray probes demonstrating that CLC/Gal-10 does not bind any known mammalian glycan structures, thus leaving the function(s) of CLC protein as a galectin in eosinophil (and basophil) biology unresolved. One of our earlier structural studies showed binding of mannose to the CLC/Gal-10 carbohydrate recognition domain in the crystal, but in an unusual, non-standard β-half chair conformation.[64] Subsequent studies demonstrated that CLC/Gal-10 can weakly (non-specifically) bind to carbohydrate under several conditions, like in its monomeric state.[65, 66] In addition, recent findings indicate that Gal-10 dimerizes with a novel global shape that is different from that of other prototype galectins (e.g., Gal-1, -2 and -7) and may essentially inhibit disaccharide binding.[65, 67] The lack of binding to ß-galactosides was also recently demonstrated for one other galectin family member, Galectin-13, which exhibits approximately 54% amino acid identity with CLC/Gal-10.[68]

Our results further show that CLC/Gal-10 interacts with the glycosylated human eosinophil granule cationic ribonucleases, EDN (RNS2) and ECP (RNS3), but that this interaction is not dependent on binding via N- or O-linked sugars, and occurs even with bacterially expressed recombinant EDN devoid of any glycosylation. Of interest, CLC/Gal-10 also binds avidly to the large family of murine eosinophil-associated-

ribonucleases (EARS), despite the lack of a CLC/Gal-10 ortholog in the mouse genome. This might simply reflect similarities of sequence and/or structure between EDN/ECP and the murine EARS or suggests the existence of an as yet unidentified CLC/Gal-10 paralog in the mouse.

We demonstrate that CLC/Gal-10 does not function as an inhibitor of the granule cationic endoribonucleases, and instead find that IFN-γ activation of blood eosinophils induces rapid intracellular movement and co-localization of CLC/Gal-10 with EDN and CD63. A number of studies have implicated the tetraspanin CD63 in the process of agonist-induced eosinophil secretion as part of the selective mobilization of eosinophil-expressed cytokines and granule cationic proteins via vesicular transport from eosinophil secondary granules.[61, 63, 69, 70] As a result, CD63 has been proposed as a marker of PMD in eosinophils.[45] Our finding that CLC/Gal-10 rapidly becomes associated with both CD63-positive secondary granules and EDN during the process of IFN-γ -induced eosinophil activation is indicative of a possible role for CLC/Gal-10 in the vesicular transport of the cationic ribonucleases. Extended studies in actively secreting eosinophils on the co-localization of CLC/Gal-10 with components of the vesicular transport pathway expressed by eosinophils (including VAMP-2, VAMP-7, VAMP-8, syntaxin-4, and SNAP-23)[71-73] will further elucidate the role of CLC/Gal-10 in the process of PMD.

The absence of a murine ortholog or paralog of CLC/Gal-10 knockout studies in the mouse to address its functions in eosinophil biology and/or roles in eosinophil-

460 associated inflammatory responses. However, we extended the current studies by
461 utilizing lentiviral shRNA to knock-down CLC/Gal-10 in cord blood CD34-positive
462 hematopoietic progenitors driven to differentiate into eosinophils. While knockdown of
463 CLC/Gal-10 did not inhibit eosinophil progenitor proliferation, granulogenesis was
464 significantly impaired, with decreased formation of Fast Green positive secondary
465 granules. Knockdown cells also contained increased numbers of large empty granule
466 containers and very large empty granules, suggesting fusion of empty granule
467 containers in CLC/Gal-10 deficient eosinophils. These findings indicate a role for
468 CLC/Gal-10 in granulogenesis during eosinophil differentiation, and are consistent with
469 our hypothesis that CLC/Gal-10 is involved in vesicular transport of the ribonucleases
470 during both PMD and eosinophilopoiesis. We would expect CLC/Gal-10 to function in
471 this role throughout differentiation, from granulogenesis until mediator secretion from
472 the mature cell. Unlike our findings for CLC/Gal-10, previous reports on the
473 consequences of eosinophil granule protein gene knockout demonstrated that the only
474 baseline consequence of losing either MBP-1 or EPX in single gene knockout mice is
475 the generation of peripheral blood eosinophils devoid of the respective secondary
476 granule protein[74, 75] or with a relative reduction in granule outer matrix volume.[76] In
477 contrast, MBP-1$^{-/-}$/EPX$^{-/-}$ double knockout mice were viable but had significantly fewer
478 circulating peripheral blood eosinophils, a consequence of concomitant loss of
479 eosinophil lineage-committed progenitors in the marrow due to targeted disruption of
480 eosinophilopoiesis. The authors hypothesized that granule protein gene expression
481 and/or granule formation is a checkpoint for survival of developing EoPs (eosinophil
482 progenitors).[77] However, an alternative explanation for the dysfunctional granulogenesis

in MBP-1$^{-/-}$/ EPX$^{-/-}$ double knockout mice could also involve the aberrant intracellular release of a toxicant, the mouse eosinophil-associated ribonucleases (EARs), which are capable of rapidly degrading intracellular RNA, thus leading to the observed cell-autonomous defect.[78] Therefore, we hypothesize that CLC/Gal-10 may function as a carrier (chaperone) for the sequestration and vesicular transport of these potent ribonucleases and cationic toxins during eosinophil activation and secretion by PMD. In support of this hypothesis, beside data presented in this study, are a number of other considerations: the abundance of CLC/Gal-10 in the eosinophil (~7-10% of total cellular protein), the very early expression of CLC/Gal-10 mRNA during eosinophil differentiation, and the status of CLC/Gal-10 as the second most abundant mRNA next to MBP-1 in the developing eosinophil progenitor.[79] In addition, CLC/Gal-10 was found not to be actively secreted to the extracellular space in basophils,[80] but instead becomes rapidly associated with small cytosolic vesicles involved in granule protein and cytokine transport and secretion in the process of eosinophil PMD, with re-cycling of CLC/Gal-10 during basophil recovery from stimulation with physiologically relevant agonists.[69, 70, 76, 77, 80] Ultrastructural TEM studies showed changing distributions of CLC/Gal-10 in human basophils upon activation, documenting the capability of basophils to undergo complex release and recovery reactions that may be pertinent to the functions of CLC protein.[81] A similar recycling process in human eosinophils is supported by our finding that CLC/Gal-10 returns to its original cytosolic localization 60 min after IFN-γ activation. However, there is also evidence that CLC/Gal-10 may be secreted during eosinophil differentiation or during eosinophil-associated inflammatory reactions.[3, 6, 82-85]

The effect of CLC/Gal-10 deficiency on the activation and degranulation of in vitro differentiated eosinophils was assessed by measuring the secretion of EDN and EPX following stimulation of the cells with PAF. Platelet activating factor is a well-studied eosinophil secretagogue shown to induce eosinophil degranulation via both exocytosis[73] and piecemeal degranulation,[86] although the exact mechanism of its action on eosinophils is unknown.[87] Stimulation with PAF resulted in a dose-dependent secretion of EPX and EDN, suggesting that CLC/Gal-10 deficient eosinophils do not have a generalized defect in degranulation. However, secretion of EDN by CLC/Gal-10 deficient eosinophils was significantly higher than that of controls, and EDN was found to “leak” into the media in the absence of a secretagogue, suggesting a deficiency in EDN packaging and/or transport not evident for other secondary granule constituents such as EPX.

Of interest, analysis of CLC/Gal-10 ShRNA knock-down cell lysates showed decreased levels of MBP-1, but not EDN, ECP or EPX. It is possible that EDN/ECP translation is unaffected, while the targeting of these proteins to the granules through the golgi during differentiation is defective. Although the presence of cationic ribonucleases in eosinophils with impaired granulogenesis would imply cellular cytotoxicity, this was not the case in our current study. Possible reasons could include an altered form of subcellular localization or even aggregation of these ribonucleases. Impaired granulogenesis could also drive expression of modified/inactive EDN and ECP. Unlike the other eosinophil-derived granule proteins (including MBP-1), EDN is a poor cationic

toxin with limited toxicity for helminth parasites and mammalian cells at high concentrations. As a ribonuclease, it is considerably more effective against single-stranded RNA viruses.[88] Native ECP purified from leukocytes shows considerable molecular heterogeneity, from multiple glycosylated isoforms to the non-glycosylated native protein; these glycoforms vary considerably in cytotoxic activity toward mammalian cells.[88, 89] Notably, we observed complete cessation of cell proliferation upon knock-down of CLC/Gal-10, an effect not seen in controls transfected with non-target shRNA, indicating CLC/Gal-10-deficiency does not lead to cytotoxicity, but significantly impairs packaging of both the ribonucleases and other granule cationic proteins during eosinophil development. The defective granulogenesis observed in developing CLC/Gal-10-deficient eosinophils is also likely responsible for decreased gene expression and/or protein synthesis of MBP-1, but further studies are needed to reach more definitive conclusions.

Recognizing the crucial role of CLC/Gal-10 in eosinophil granulogenesis, and the identification of eosinophil ribonucleases as intracellular ligands for CLC/Gal-10, opens a window to understanding the physiologic roles of this unique member of the galectin family in eosinophil (and basophil) biology, and its potential functions in innate and adaptive immunity.

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FIGURE LEGENDS

Figure 1. CLC/Gal-10 interacts with eosinophil granule cationic ribonucleases. (A, B) CLC/Gal-10 was used as a probe to “ligand blot” blood eosinophil lysate, followed by detection with anti-CLC/Gal-10 antibody. CLC/Gal-10 binds to an ~21kD band in blood eosinophil lysate that is reduced to ~18 kD upon digestion of the lysates with PNGase F. **(C)** EDN co-purifies with CLC/Gal-10 from lysates of AML14.3D10 eosinophils or purified blood eosinophils. Samples affinity purified over an anti-CLC/Gal-10 antibody column were blotted with either anti-CLC/Gal-10 or anti-EDN/ECP (cross-reactive) antibodies. Purified MBP-1 and anti-MBP-1 antibodies were used as a control. **(D)** EDN is co-immunoprecipitated by anti-CLC/Gal-l0 antibody (left panel) and CLC/Gal-10 is co-immunoprecipitated by anti-EDN antibody (right panel) from AML14.3D10 eosinophil lysate. The initial AML14.3D10 lysate (input) was included as a positive control. The immunoprecipitations were performed using rabbit non-immune (NI) IgG, anti-CLC/Gal-10, or anti-EDN antibodies.

Figure 2. Interaction of CLC/Gal-10 with the cationic endoribonucleases is not glycan-dependent. (A) Purified human (EDN, ECP, MBP-1) and murine (EARS) eosinophil granule proteins (2.5 µg/ each) bind CLC/Gal-10 in ligand blot, with or without prior PNGase digestion. Electrotransferred samples were detected by Coomassie Blue stain (top), anti-CLC/Gal-10 antibody (middle) or glycoprotein stain (PAS) (bottom). **(B)** Native (glycosylated) human EDN binds CLC/Gal-10 even after being subjected to sequential digestions with PNGase F, Sialidase A, O-Glycanase, β-(1-4) Galactosidase, and β-N-Acetylglucosaminidase. The samples were stained for protein by Coomassie Blue (top), ligand blotted using CLC/Gal-10 followed by anti-CLC/Gal-10 (middle), and glycoprotein by PAS (bottom).
(C) Purified native EDN and recombinant EDN have similar affinities for CLC/Gal-10 binding, as demonstrated by ligand blotting increasing amounts of EDN (1, 2, 4, and 8

μg) with crystal-derived CLC/Gal-10, followed by detection of bound CLC/Gal-10 with anti-CLC/Gal-10 antibodies. Bacterially expressed non-glycosylated rEDN was included for comparison.

Figure 3. CLC/Gal-10 does not inhibit the ribonuclease activity of EDN. (A) 80 pg of purified native EDN was analyzed for RNase activity in the absence or presence of increasing amounts of CLC/Gal-10 protein, or placental RNase inhibitor. The relative fluorescence units reflect the amount of fluorescence emitted by the cleavable fluorescent-labeled RNase substrate. The amount of crystal-derived CLC/Gal-10 protein ranged from 0 to 1600 pg, and human placental RNase inhibitor ranged from 0 to 5 units. **(B)** Increasing amounts of EDN were incubated with a constant amount of either CLC/Gal-10 (400 pg) or placental RNase inhibitor (5 units). Results are representative of 3 independent experiments with three different preparations of CLC/Gal-10 protein purified by crystallization from blood eosinophils. ns = not significant

Figure 4. Activation of blood eosinophils with IFN-γ induces the intracellular co-localization of CLC/Gal-10 with EDN and CD63.
(A) Representative confocal images of blood eosinophils cultured without (control) or with IFN-γ (500 U/ml) for periods of 10 to 30 min. Upon activation with IFN-γ, the merged images clearly display yellow regions indicative of co-localization of CLC/Gal-10 and EDN. Co-localization reaches maximum levels 30 min after activation and dissipates after 60 min (not shown). **(B)** Purified eosinophils stimulated with IFN-γ (500 U/ml) for periods of 2, 10, 30, or 60 minutes show CLC/Gal-10 and CD63 co-localization at discrete punctate sites in the cytosol consistent in size with eosinophil secondary granules. Maximum colocalization is visible 30 min after activation. DIC shows the appearance of typical eosinophil secondary granules in the cytosol. Results include

representative images from 4 independent experiments. Arrows highlight pockets of co-localization. White size bars in lower right corner indicate 5µm.

Figure 5. shRNA knock-down of CLC/Gal-10 in cord-blood derived eosinophil progenitor cells leads to impaired eosinophil differentiation/ granulogenesis. (A) Purified human CD34+ cord blood-derived progenitor cells were transduced with specific CLC/Gal-10 shRNA or Non-Target control shRNA and then differentiated toward the eosinophil lineage with IL-5. Immunofluorescence staining of cells at day 14 shows an almost complete loss of CLC/Gal-10 expression in cells treated with CLC/Gal-10 specific shRNA (Fig. 5A, top panel). Fast Green/Neutral Red staining of cells shows characteristic features of mature eosinophils, including red nuclei, pink cytoplasm and turquoise green granules, confirming that the cells were successfully differentiated toward the eosinophil lineage (middle panel). After 14 days, the CLC/Gal-10 knock-down cells display a significant reduction in the number of Fast Green stained secondary granules (middle panel), and cells stained on day 21 show increasing differences in cellular morphology as compared to control cells (bottom panel), with large empty granules (arrow). **(B)** Representative images of Fast Green/ Neutral Red stained cells 21 days post transduction. Cells transduced with CLC/Gal-10 specific shRNA manifest predominantly with large, empty granules. Images are representative of 2 independent experiments performed in triplicate. Size bars represent 5 µm.

Figure 6. CLC/Gal-10 deficient (knock-down) eosinophils have fewer secondary granules, a non-proliferative phenotype, decreased MBP-1 expression, and increased secretion of EDN in response to PAF stimulation. (A) CLC/Gal-10 knock-

down cells display an ~42% decrease in average number of secondary granules as compared to non-target shRNA transduced cells. **(B)** Cell populations transduced with CLC/Gal-10 shRNA show a dramatic increase of cells with no detectable granules and a strikingly smaller number of cells with more than 15 granules. **(C**) CLC-deficient cells display a non-proliferative phenotype, in contrast to non-target shRNA treated cells that continued to proliferate throughout the 21 days, although at a slower pace than untreated cells (expected being under Puromycin selection). **(D)** CLC/Gal-10 knock-down causes a significant decrease in ELISA detected levels of CLC/Gal-10 and MBP-1 in cell lysates, but no significant change in EDN, ECP, and EPX expression. CLC-deficient eosinophils still exhibit dose-dependent secretion of EDN **(E)** and EPX **(F)** when activated with the secretagogue PAF. The secretion of EPX **(F)** by CLC-deficient eosinophils was not significantly different from untreated or non-target shRNA control cells; however, amounts of secreted EDN **(E)** was significantly higher in CLC-deficient eosinophils. Results represent mean (200 counted cells per treatment group) ± SEM from 2 independent experiments. (ns = not significant, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$).

Figure 1

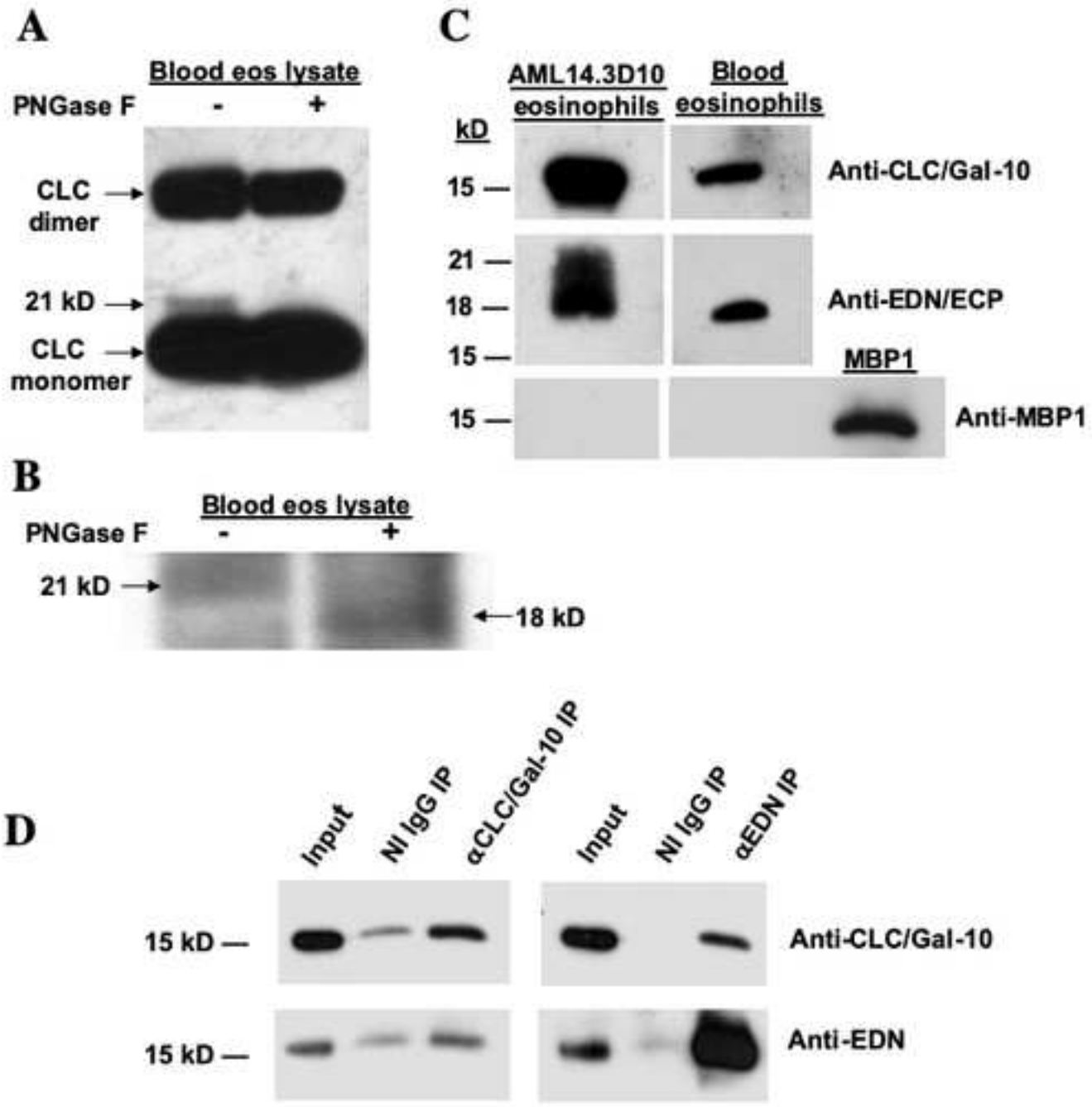


Figure 2

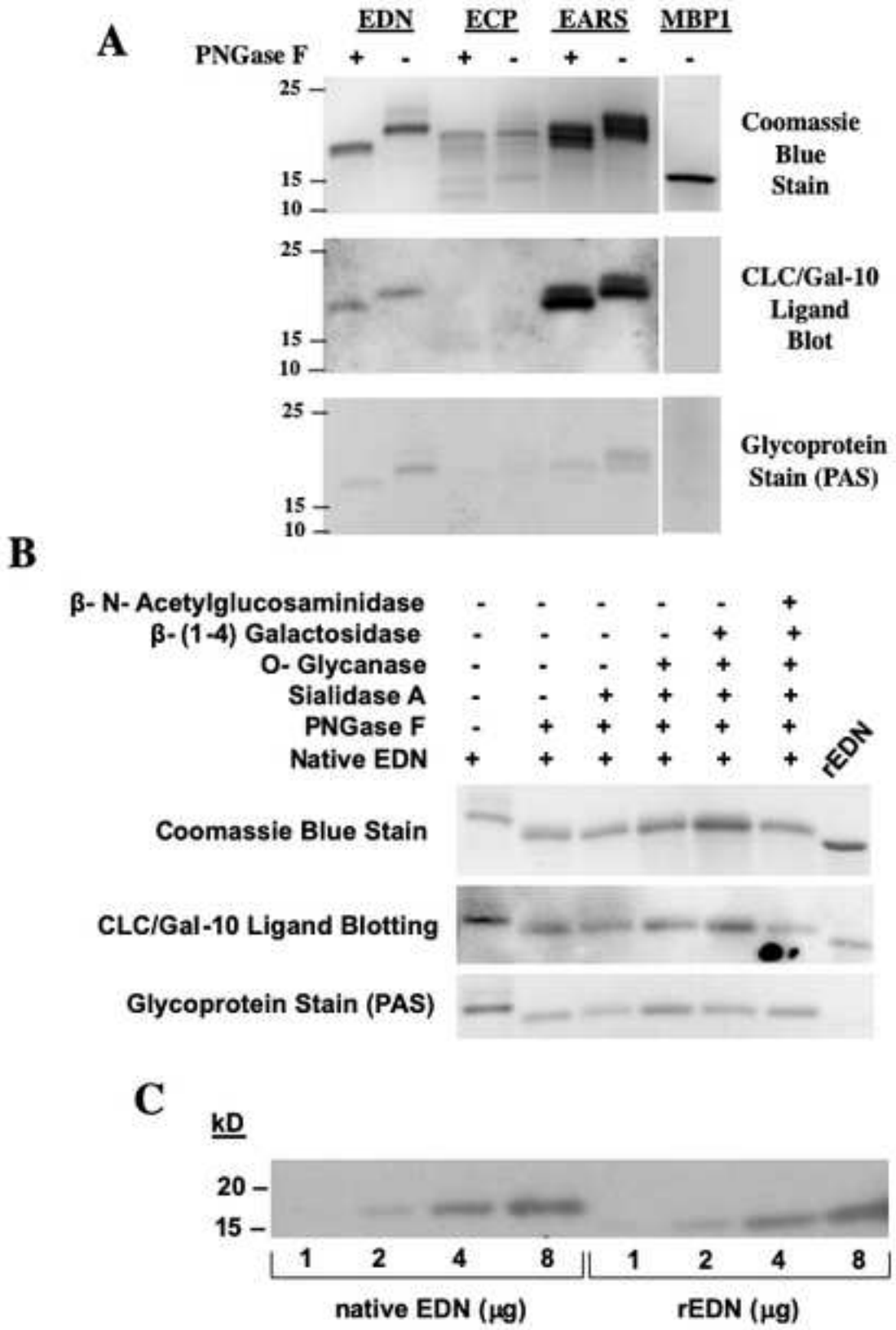


Figure 3

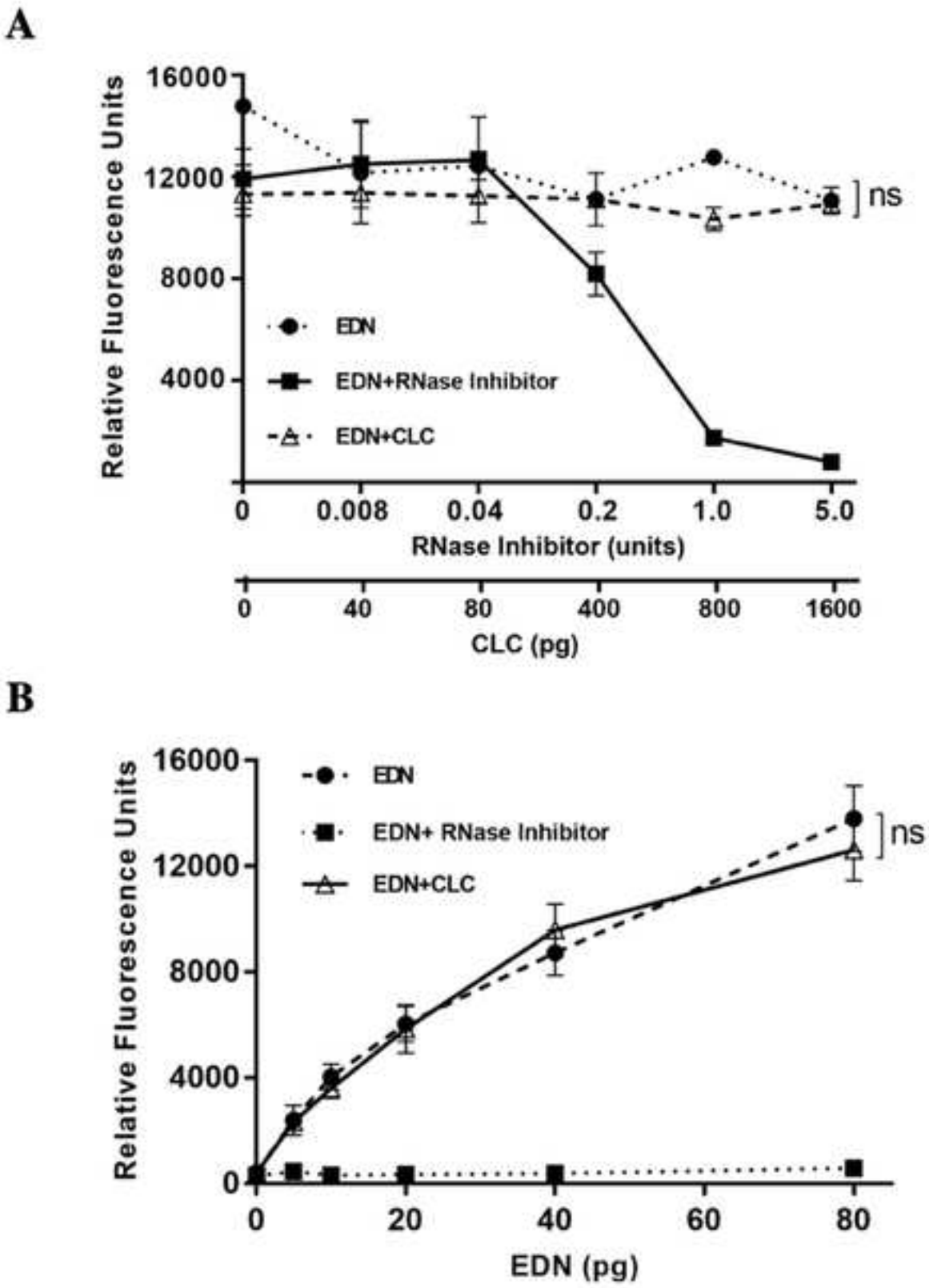


Figure 4

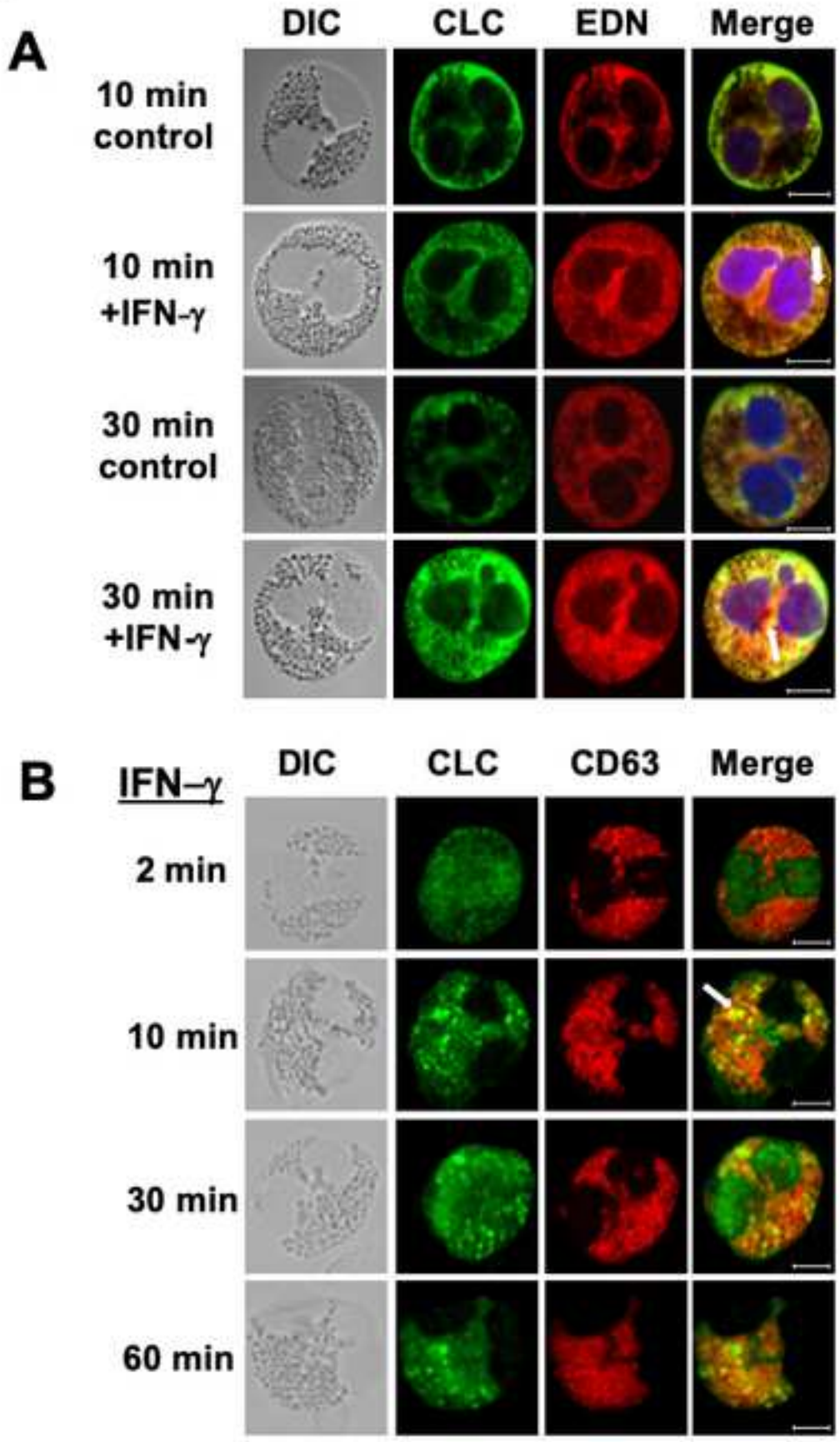


Figure 5

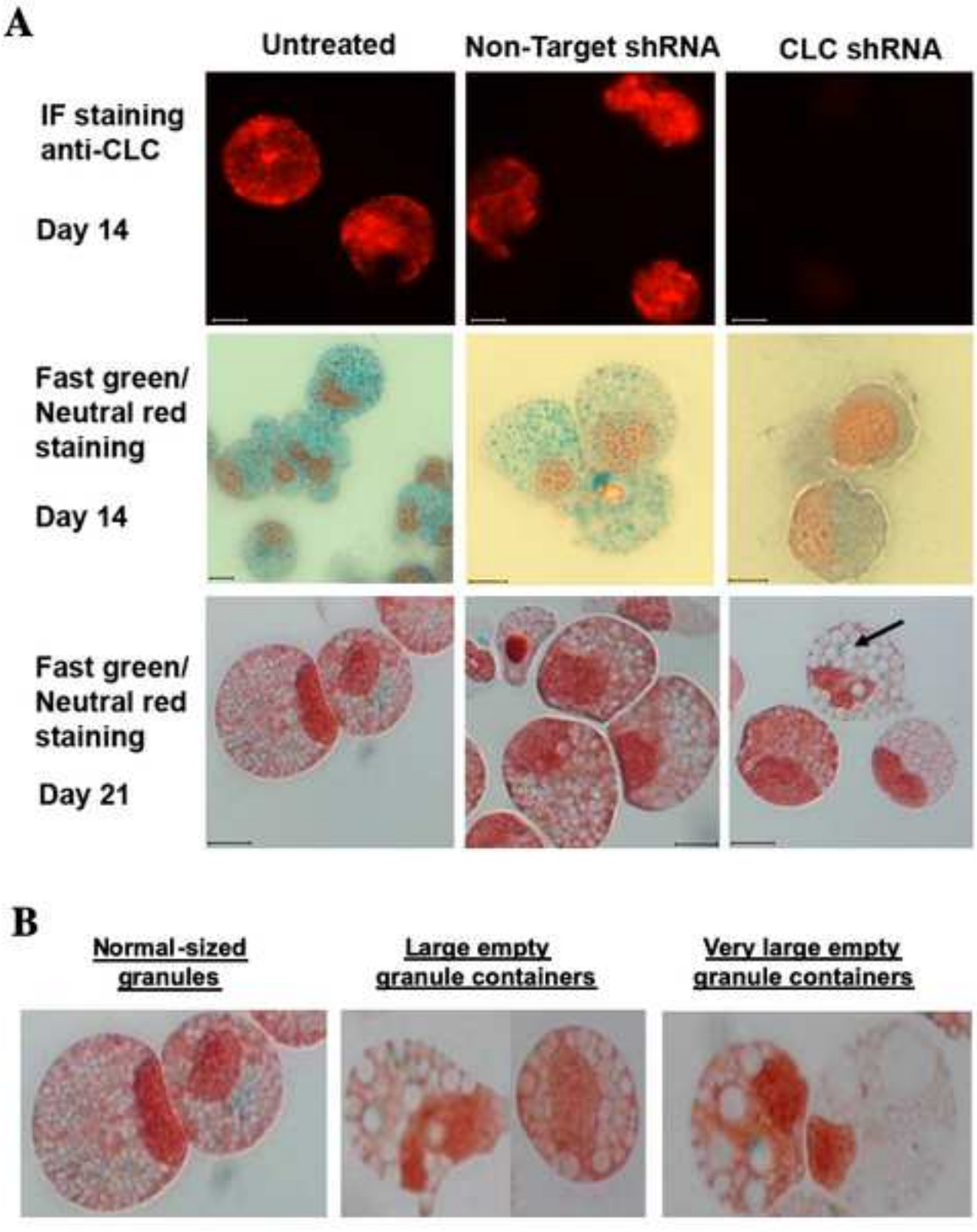
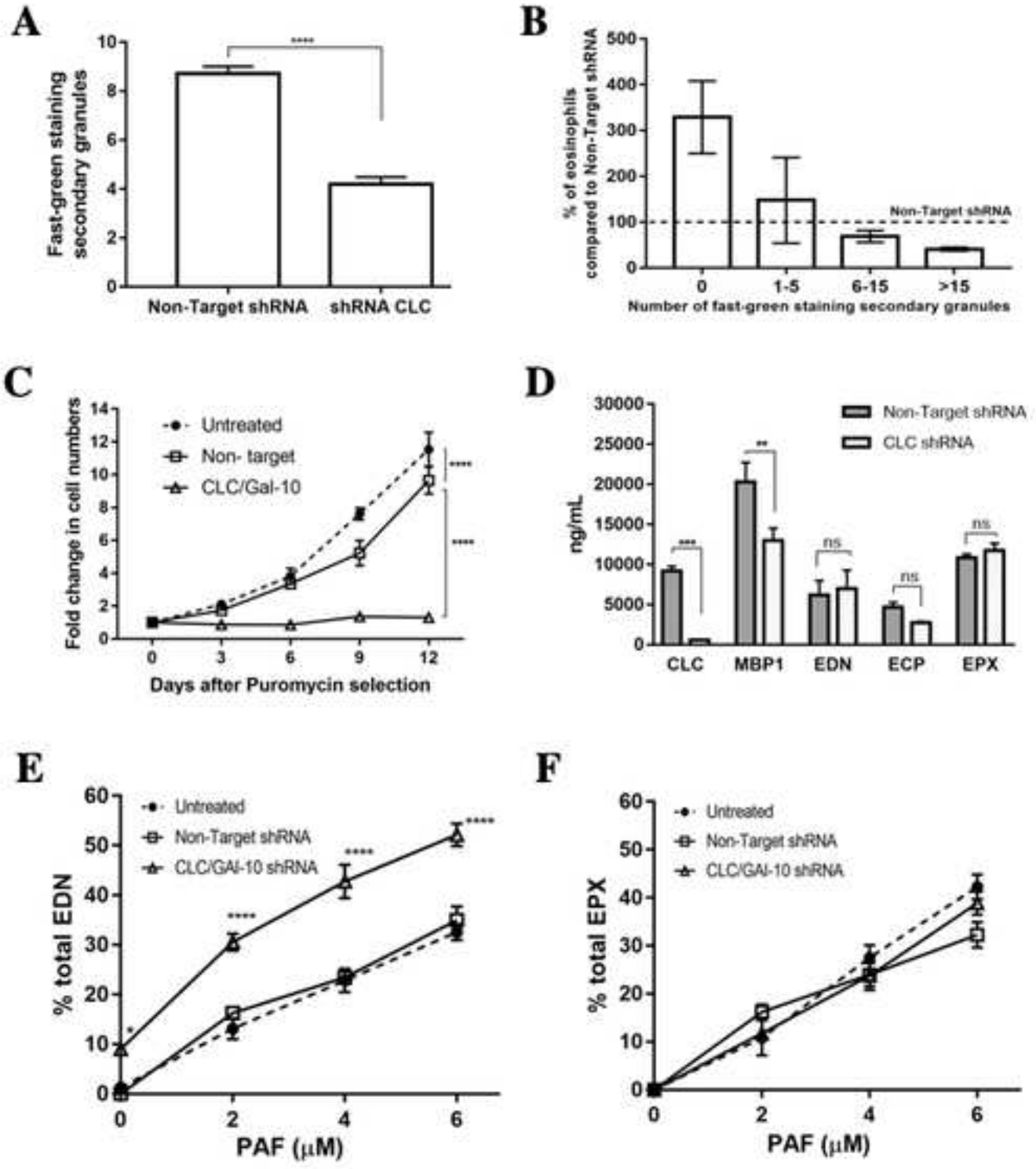


Figure 6



Online Repository Materials

Materials and Methods

Affinity purification of Rabbit IgG antibodies to CLC/Gal-10

Rabbit IgG antibodies to crystal-derived eosinophil CLC protein were affinity-purified on a solid-phase CLC-Sepharose 4B column as previously described.[1] In brief, a solid-phased CLC column was first prepared using CLC protein isolated by crystallization from human eosinophil whole cell lysates; the CLC protein preparation contained a single homogeneous band on overloaded, silver-stained SDS-PAGE gels. The resolubilized, crystal-derived CLC protein was solid-phased to CNBr-activated Sepharose 4B resin using standard methodology.

Co-Immunoprecipitation of CLC/Gal-10 and EDN

Two different types of co-immunoprecipitations were performed to analyze the CLC/Gal-10-EDN/ECP interaction. First, blood eosinophil whole cell lysate was pre-cleared with 100μl (50% V/V) of protein A-Sepharose beads (Amersham Biosciences). The supernatant was then incubated with anti-CLC/Gal-10 rabbit IgG antibody for one hour on ice, followed by the addition of the protein A-Sepharose beads. After a second 1-hour incubation, the beads were boiled in SDS-PAGE sample loading buffer, and the supernatant analyzed by Western blotting using antibodies to CLC/Gal-10 and EDN. Second, AML14.3D10 cells were lysed on ice in RIPA buffer (Santa Cruz) containing protease inhibitors. The supernatant was used for immunoprecipitation following a pre-clearing step with a 50 μl (50% V/V) of protein G-Sepharose beads (Amersham

Biosciences) for two hours. The supernatant was incubated with 0.4 µg anti-CLC/gal-10 rabbit IgG antibody, 2 µg anti-EDN monoclonal antibody, or the appropriate non-immune rabbit IgG or mouse IgG control for 12 hours at 4°C. This was followed by the addition of protein G beads for 2 hours. The beads were collected and boiled in SDS-PAGE loading buffer for Western blotting.

Affinity co-purification of CLC/Gal-10 and EDN

Native CLC/Gal-10 was purified from eosinophils using a CLC/Gal-10 affinity-purified anti-CLC antibody affinity column as described previously.[1] For assessment of the effects of native CLC/Gal-10 protein on the RNase activity of purified EDN and ECP, CLC/Gal-10 was purified with or without mild reduction (5 mM β-mercaptoethanol) using the same affinity column.

Ribonuclease enzyme activity assay

For this assay, the green fluorescence emitted from a cleavable fluoro-labeled substrate (Ambion, Austin, TX) was measured as relative fluorescence units on a Fluorocount Microplate Fluorometer (PerkinElmer, Meriden, CT). Briefly, 80 pg of purified native EDN was mixed with different amounts of crystal-derived CLC/Gal-10 protein (0, 40, 80, 400, 800 and 1600 pg) or human placental ribonuclease inhibitor (New England Biolabs, Ipswich, MA) (0, 0.008, 0.04, 0.2, 1, or 5 units) in the presence of 5 mM DTT. After a 1-hr incubation at 4ºC, RNase substrate was added to each reaction mix and incubated for another 30 min at 37ºC before measuring relative fluorescence.

Confocal immunofluorescence microscopy

Cytocentrifuge slides were prepared using a Cytospin-2 cytocentrifuge (Shandon, UK) at 800 rpm for 2 minutes. Slides were fixed for 10 minutes in 0.4% parabenzoquinone (Sigma-Aldrich), and permeabilizated for 8 min with n-octyl-β-D-glucopyranoside (Sigma-Aldrich).[2] Slides were blocked with 1.5% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and probed with either monoclonal anti-CLC/Gal-10 IgG antibody (Diaclone, Stamford, CT), mouse anti-EDN monoclonal antibody (11 µg/ml), or CD63 monoclonal antibody (1 µg/ml) (BD Biosciences). Non-immune rabbit and mouse IgG (Jackson ImmunoResearch Labs) were tested at the same concentrations as the antibody isotype negative controls. Detection was performed with Alexa Fluor 488-labeled goat-anti-rabbit IgG (Invitrogen Molecular Probes), Texas Red-labeled goat-anti-mouse IgG (Jackson ImmunoResearch Labs), or Alexa Fluor 568 goat anti-mouse IgG (Invitrogen Molecular Probes). Images of immunofluorescent stained eosinophils were acquired using a Carl Zeiss 510 LSM equipped with a 63X oil immersion Plan-Apochromat objective.

Quantitative colocalization analysis was performed on images acquired using a 100X oil immersion objective. Single cells were selected as regions of interest (n=40 per treatment group) and mean colocalization coefficients were calculated by Pearson's correlation method. Data is presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA) by two-way analysis of variance (ANOVA), followed by Tukey post hoc analysis.

Glycan microarray

High-throughput glycan microarray screening was performed in collaboration with the NIH-funded Consortium for Functional Glycomics (CFG) according to their standard protocol (Protein-Glycan Interaction Core (H), Emory University School of Medicine, Atlanta, GA). Purified protein and primary antibodies were provided to the CFG for screening. The primary antibodies used include the affinity-purified rabbit polyclonal anti-CLC/Gal-10 antibody (described in the manuscript), a monoclonal antibody to the FLAG-peptide (Sigma- Aldrich), and a monoclonal antibody to FLAG-FITC (Sigma-Aldrich). A number of screening strategies were used for three different attempts at CLC/Gal-10 ligand identification. These included: (1) Screening of crystal-derived CLC/Gal-10 purified from AML14.3D10 eosinophils an detection with purified polyclonal anti- CLC/Gal-10 antibody, (2) screening of recombinant CLC/Gal-10 with a C-terminal FLAG sequence (rCLC/Gal-10-FLAG) and detection with either anti-FLAG or anti-FLAG-FITC monoclonal antibodies, (3) screening of crystal-derived CLC/Gal-10 directly labeled with FITC, and (4) screening of the recombinant quintuple mutant of CLC/Gal-10 (rQM-CLC/Gal-10-FLAG) which contains a consensus galectin CRD (Q55N, C57R, R61T, Q75E, E77R) and detection with FITC- conjugated anti-FLAG antibody.

The first screening attempt utilized a microplate array (Plate version 3.0 containing 205 mammalian glycan targets), [3] while the subsequent samples were screen printed on microarrays (Printed microarray version 3.0 containing 285 mammalian glycan targets).[4]

Modeling molecular interaction of CLC/Gal-10 with EDN and ECP

The atomic coordinates of EDN (PDB: 2BZZ), ECP (PDB: 1H1H), and CLC (PDB: 1LCL) obtained from the Protein Data Bank were used for analysis. To account for CLC/Gal-10

dimers observed in solution, a CLC/Gal-10 possible dimer was modelled using Gal-1 as a template. A homology model was built for mEAR1 using EDN (PDB: 2BZZ) as a template. Structure based sequence alignments were carried out on FASTA format protein sequences of EDN (Uniprot reference number P10153), ECP (Uniprot reference number P12724), mEAR1 (GI number 32441901) and galectins- 1, 2, 3, 4, 7, 8, 9, 10 and 13 (GI numbers are: Gal1 – 56554348, Gal2 – 119580571, Gal3 – 157829667, Gal4 – 159163551, Gal7 – 3891480, Gal8 – 187609173, Gal9 – 219109219, Gal10 – 547870, Gal13 – 119577314). Only the mature polypeptide chain was taken for analysis.

The PyMOL Molecular Graphics System (Schrödinger, LLC) was used to visualize and produce figures and to calculate the surface charge potential on molecules. Swiss-Model (http://swissmodel.expasy.org) online server was used to build homology models and the ClustalW server from http://ebi.ac.uk was used to align the sequences.[5] Aline (http://crystal.bcs.uwa.edu.au/px/charlie/software/aline/) was used to edit the aligned sequences.[6] SHARP2 (http://www.bioinformatics.sussex.ac.uk/SHARP2/sharp2.html) was used to predict possible protein interaction sites on surface of protein structures.[7]

Results

Glycan microarray

The results of all glycan microarray screenings are summarized in Table S1.The use of either array format (Plate version 3.0 or Printed version 2.0) did not yield any high affinity binding partners for CLC/Gal-10. rCLC/Gal-10-FLAG showed some affinity for glycans 63 (6-Su-GalNAcα -Sp2 or α-N-acetyl-D-galactosamine-6-sulfate) and 147 (Neu5Acα2-3GalNeu5Aca2-3GalNAcα-PAA-Sp2 or GM4) on the version 3.0 plate array, but gave no results when tested on the version 2.0 printed array.

Table S1. Glycan microarray screens

Protein	Array	Results	Comment
Crystal-derived CLC/Gal-10 from AML14.3D10 cells	Plate version 3.0	No significant binding	Low signal
rCLC/Gal-10-FLAG	Plate version 3.0	(63) - 6-Su-GalNAcα-Sp2 (147) – Neu5Acα2-3GalNeu5Acα2-3GalNAcα-PAA-Sp2	Low signal Low affinity binding
Crystal-derived CLC/Gal-10 from AML14.3D10 cells Alexa 488 conjugate	Printed version 2.0	No significant binding	Highly variable
Crystal- derived CLC/Gal-10 FITC labeled	Printed version 2.0	No significant binding	Highly variable
rQM-CLC/Gal-10-FLAG	Printed version 2.0	(25) – GlcNAcβ1-3(GlcNAcβ1-4)(GlcNAcβ1-6)GlcNAc-Sp8 (27) -(3OSO_3) (6OSO_3)Galβ1-4GlcNAcβ-Sp0 (28) - (3OSO_3) Galβ1-4Glcβ-Sp8 (32) - (3OSO_3) Galβ1-3GalNAcα-Sp8 (35) - (3OSO_3) Galβ1-4(6OSO_3)GlcNAcβ-Sp8	High CVs

More rigorous washing of the plate resulted in dissociation of the bound CLC/Gal-10, suggesting non-specific and/or low-affinity interactions with these targets. Our collaborators at the CFG did not believe these interactions to be meaningful given the great variations between replicates (high coefficient of variation (CV). In addition, glycan 147 is a multivalent ligand that is not a typical galectin ligand. Similar low affinity and non-

specific interactions with atypical galectin ligands were observed for the recombinant quintuple mutant of CLC/Gal-10 (rQM-CLC/Gal-10-FLAG).

Molecular modelling of CLC/Gal-10 interactions with EDN and ECP

Based on the available 3D structures of EDN (PDB: 2BZZ) and ECP (PDB: 1H1H) their surface charges were determined to be very different, as seen in Figure S1 and S2. The homology model of mEAR1 exhibits similar surface charge distribution as EDN (Fig. S1).

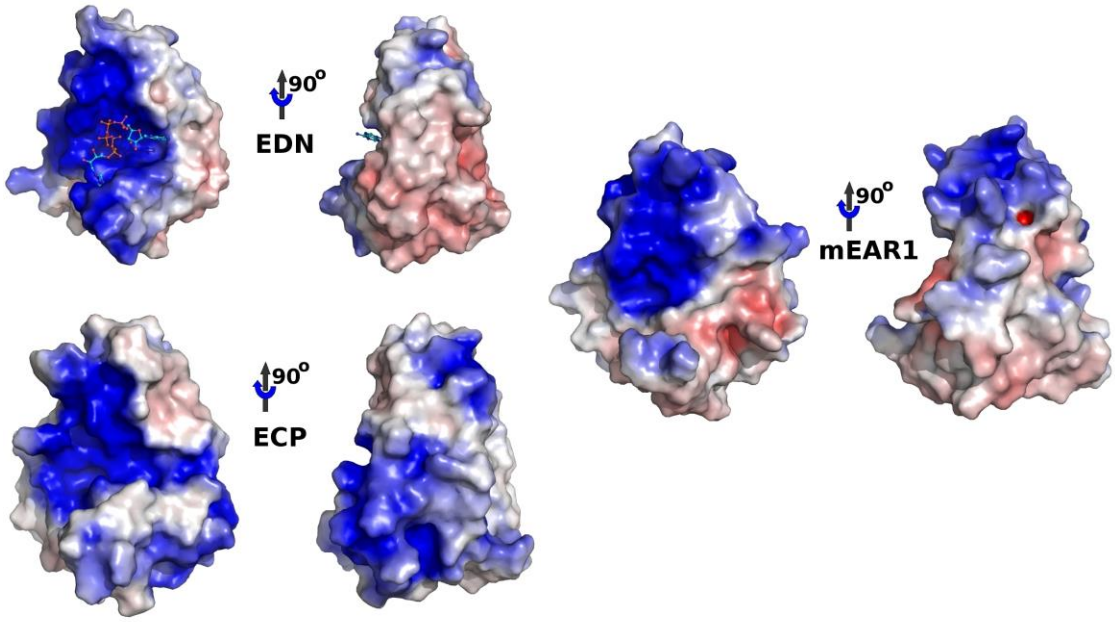


Figure S1. Surface charge potential representation of EDN, ECP and mEAR1. EDN and mEAR1 exhibit similar surface charge compared to ECP.

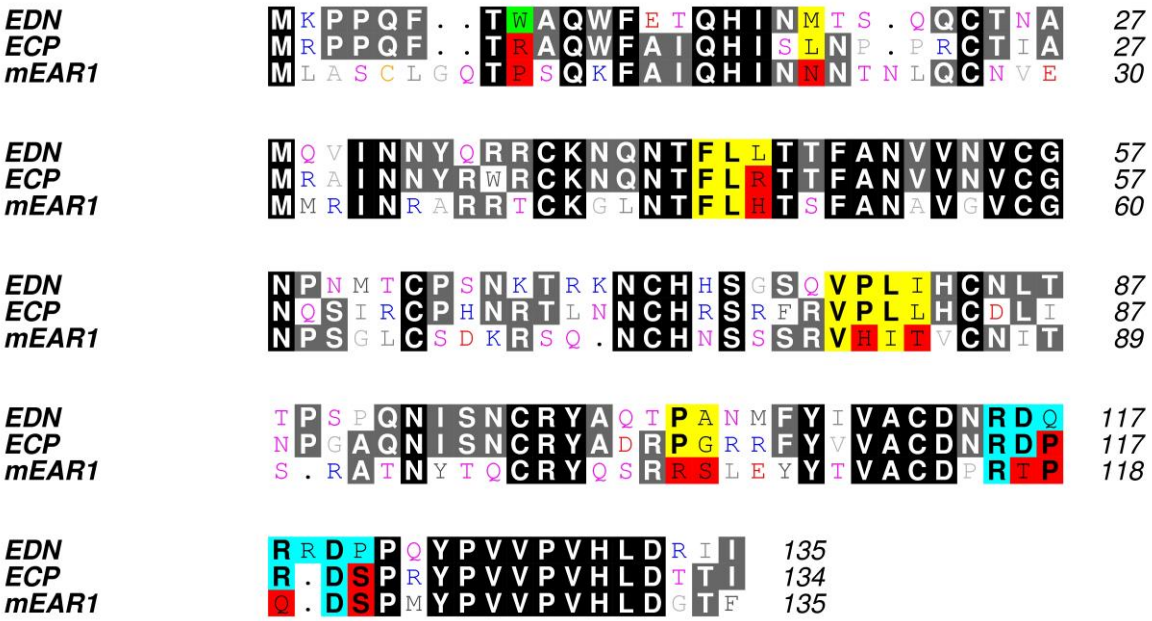


Figure S2. Multiple sequence alignment of EDN, ECP and mEAR1. Residues that belong to the hydrophobic patch (highlighted in yellow) and positively charged residues (highlighted in cyan) of EDN and corresponding residues in ECP and mEAR1 are shown. Residues highlighted in red are different in terms of their property when compared to EDN. The residue highlighted in green is the mannosylated tryptophan of EDN.

To confirm our observation, we used SHARP7 to identify possible residues that would be involved in protein-protein interactions between EDN and CLC/Gal-10. The results are shown as cartoon figures S3 (for EDN) and S4 (for CLC/Gal-10). Residues as identified by SHARP are listed below for EDN, CLC/Gal-10 and mEAR1:

EDN: L45, T46, S76, Q77, V78, P79, I81, H82, T101, P102, A103, N104, M105, F106, I133, I134

CLC/Gal-10: S2, L3, L4, P5, V6, P7, Y8, T9, P26, L27, V28, C29, L31, N32, E33, Y35, H53, Q55, R60, R61, Q125, W127, R128, D129, S131, F135

mEAR1: T46, H29, T50, N54, V58, C59, C66, H75, N76, S78, S79, R80, V81, H82, V85,

R104, S105, L106, E107, Y108, T110, L131, D132, G133, T134, F135

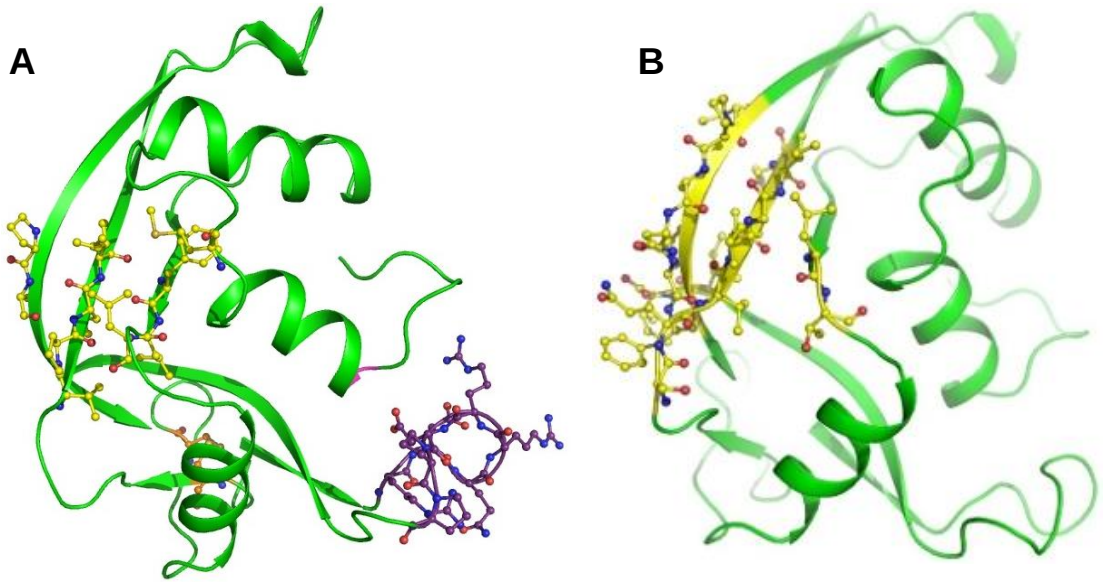


Figure S3. (A) Cartoon representation of EDN with hydrophobic residue rich region (yellow) (with some electro-negative charge) and positively charged residues (purple) shown as ball-and-stick model. **(B)** Hydrophobic residue rich region as calculated by SHARP[2] on the surface of EDN that might be involved in protein-protein interactions. The total surface area covered by this patch is ~39 $Å^3$ which is equivalent to the similar surface patch observed on CLC/Gal-10 (~39 $Å^3$).

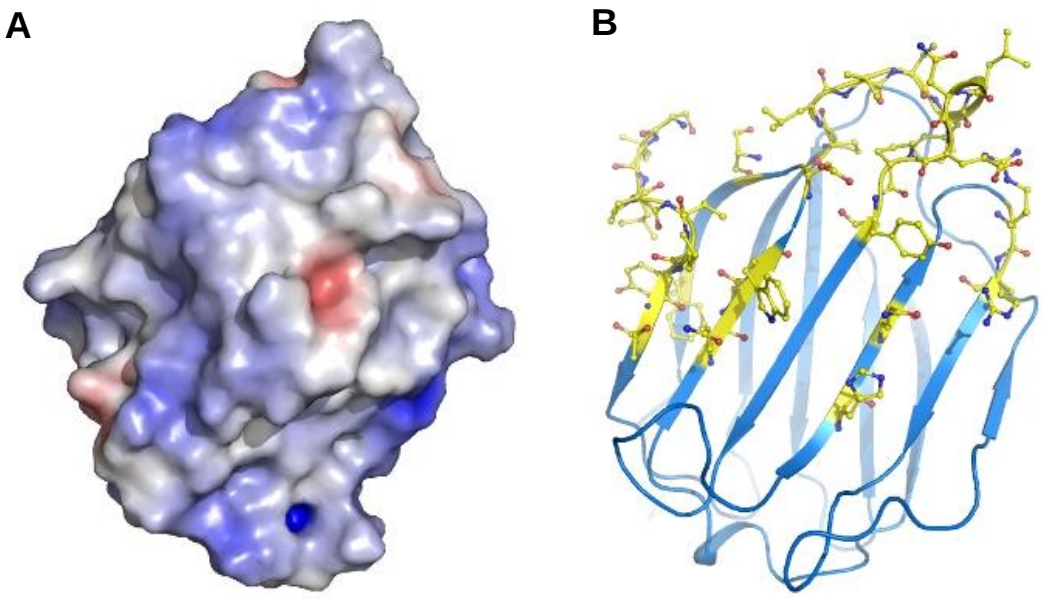


Figure S4. (A) Complementary surface patch with hydrophobic residues and electro-positive charge on CLC/Gal-10 that is predicted to exhibit interaction with EDN. **(B)** SHARP predicted residues are shown as ball-and-stick model.

Determination of protein purity by SDS-PAGE

In order to estimate the purity of protein preparations, we analyzed the purified proteins by SDS-PAGE (Figure S5). Both CLC/Gal-10, derived by crystal solubilization, and EDN were shown to be of high purity.

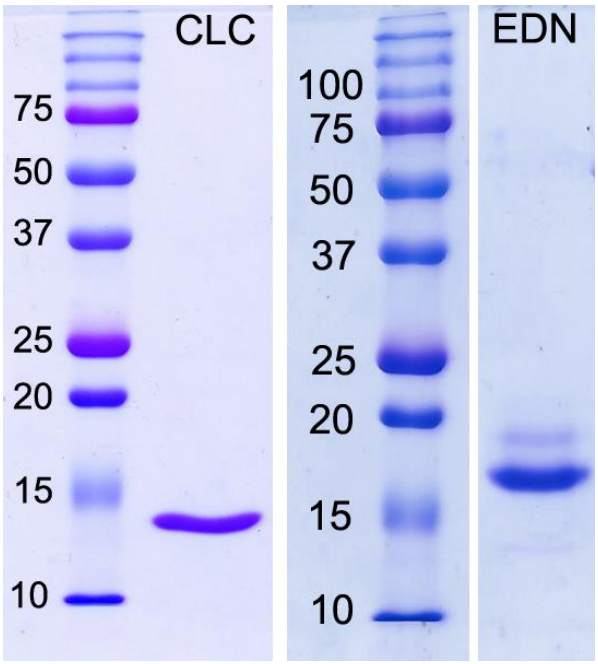


Figure S5. SDS-PAGE of CLC/Gal-10 and EDN shows purity of protein preparations.

CLC/Gal-10 shRNA knock-down in western blot

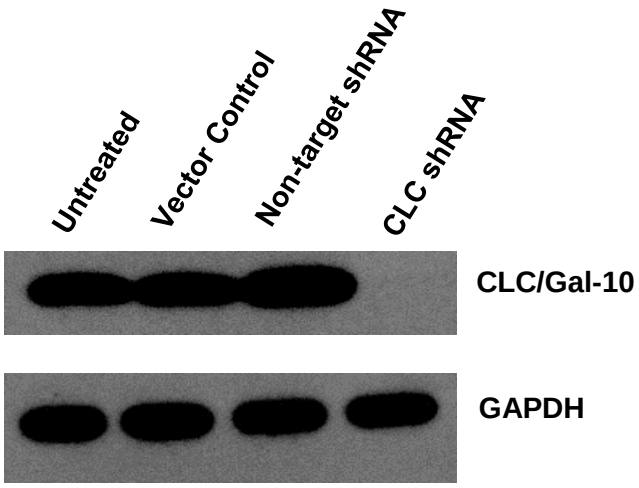


Figure S6. Analysis of cell lysates 14 days after CLC/Gal-10 shRNA knock-down in CD34+ hematopoietic progenitors induced to differentiate into eosinophils by IL-5 demonstrates their complete lack of CLC/Gal-10 expression by western blotting compared to untreated, vector and non-target shRNA control cells. Immunoblotting for GAPDH shows equivalence of protein loading.

Quantitation of colocalization between CLC/Gal-10 and EDN/CD63

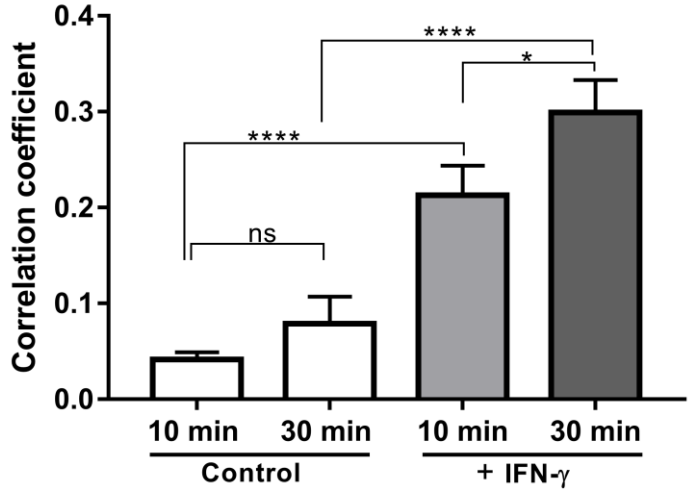


Figure S7A. Pearson's correlation coefficients for CLC/Gal-10 and EDN colocalization without (control) or with IFN-γ (500 U/ml) activation of human peripheral blood eosinophils at 10 and 30 minutes.

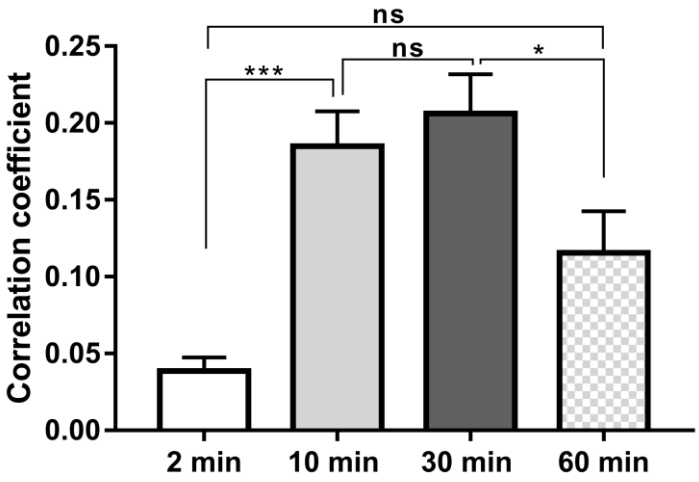


Figure S7B. Pearson's correlation coefficients for CLC/Gal-10 and CD63 colocalization following activation of human peripheral blood eosinophils with IFN-γ (500 U/ml) for 2, 10, 30, and 60 min.

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