Functional properties of galectin-3. Beyond the sugar binding.

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Functional properties of galectin-3

Beyond the sugar binding

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In the first paper galectin-3 endocytosis was studied in a system of differently activated macrophages. We found that inflammatory, M1, and alternatively activated, M2, macrophages have a large capacity to internalize externally added galectin-3, besides expressing and secreting galectin-3. Additionally, we found that galectin-3 does not require the functional carbohydrate recognition domain (CRD) for its endocytosis.

In the second paper we used M2 macrophages to study how galectin-1 directs the endocytosis of hemoglobin-haptoglobin complex. Our results indicated that galectin-1 bound fraction of the complex takes a recycling intracellular route instead of the pathway for degradation.

The third paper shows how certain complex carbohydrate ligands can induce galectin-3 self-association that involves CRD to CRD binding. This “type-C” self-association engages more galectin-3 molecules than there are available glycan ligands and can explain certain biological events that require fast galectin-3 mobilization.

In the fourth paper we tested the inhibitory potential of a few plant products for several galectins. We found that their activity was very weak, hence the search for more potent anti-galectin, perhaps also anti-inflammatory remedies continues.

Key words: galectin-3, galectin-1, M1 macrophage, M2 macrophage, self-association, fluorescence anisotropy, citrus pectin, galactomannane

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Drugi rad proučava mehanizam kojim galektin-1 usmjerava endocitozu hemoglobinsko-haptoglobinskog kompleksa u M2 makrofagima. Rezultati ukazuju da se galektin-1 dijelom veže na kompleks te ga usmjerva unutar stanice, zaobilazeći put razgradnje, možda u korist recikliranja.

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Key words: galektin-3, galektin-1, M1 makrofag, M2 makrofag, samozdruživanje, fluorescentna anizotropija, citrusni pektin, galaktomanan	
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Abbreviations

Ag	antigen
AGEs	advanced glycation end products
ASF	asialofetuin
CDGs	congenital disorders of glycosylation
C/CRD	carbohydrate recognition domain
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FPP	Fractionated Pectin Powder
Fuc	Fucose
GAGs	glycosaminoglycans
Gal	Galactose
GalNAc	N-acetylgalactosamine
GBPs	glycan-binding proteins
GM-CSF	granulocyte-macrophage colony-stimulating factor
KO	knock-out
LacNAc	N-acetyllactosamine
LDL	low density lipoprotein
LPS	lipopolysaccharide
MDCK	Madin-Darby Canine Kidney
MPS	mononuclear phagocytic system
PMA	phorbol-myristate-acetate
PRRs	pattern recognition receptors
PSA	prostate specific antigen
ROS	reactive oxygen species
Sias	sialic acids
Siglecs	sialic-acid-binding immunoglobulin-like lectins
TCR	T cell receptor
WT	wild type

G=Glycine=Gly, P=Proline=Pro, A=Alanine=Ala, V=Valine=Val, L=Leucine=Leu, I=Isoleucine=Ile, M=Methionine=Met, C=Cysteine=Cys, F=Phenylalanine=Phe, Y=Tyrosine=Tyr, W=Tryptophan=Trp, H=Histidine=His, K=Lysine=Lys, R=Arginine=Arg, Q=Glutamine=Gln, N=Asparagine=Asn, E=Glutamic Acid=Glu, D=Aspartic Acid=Asp, S=Serine=Ser, T=Threonine=Thr

Summary

Contemporary diseases like diabetes, asthma, atherosclerosis etc. are marked by chronic inflammation, often supported by the activity of different macrophages. Proteins from galectin family were found to contribute to disease pathophysiology. Therefore, there is an intensive interest in understanding different galectin functions. The aim of this thesis was to pin-point specific galectin-3 roles in differently activated macrophages. Additional aim was to understand the mechanism and consequences of galectin-3 ligand binding.

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Sažetak

Kronična upala je u podlozi mnogih bolesti modernog čovjeka kao što su djiabetes, astma, ateroskleroza itd. Upalu predvode različiti tkivni makrofagi. Proteini iz porodice galektina, proizvedeni u makrofagima, dodatno pridonose razvoju bolesti, stoga je od velikog interesa odgonetnuti različite funkcije galektina. Ovaj rad nastoji identificirati specifične uloge galektina-3 u različito aktiviranim makrofagima te razumjeti mehanizam i posljedice vezanja galektina-3 za ligand.

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Introduction

Glycobiology and glycans

One possible exception from the rule that there are no strict rules in biology could be that all cells in nature are covered with a dense coating of glycans, called glycocalyx. Glycans are also contained within the cells, in different vesicular compartments, as well as in extracellular matrices of multicellular organisms. Sugar coating is not only important for the biological processes of the host cell, but it also carries the information that will define the relationship of that particular cell with other host cells, invading pathogens, chemicals, and the rest of the surrounding world. In principle, glycan coating allows cells to sample each other and their surroundings before making any long-term commitments and before defining the nature of the interaction with their environment. Glycans, carbohydrates, sugars or saccharides (from Greek *σάκχαρον*, sugar), as they are interchangeably referred to, were defined in 1893 [1] as aldehyde or ketone alcohols of the general formula $C_nH_{2n}O_n$ that are sweet to taste and optically active. Today we know just a bit more; first of all, not all sugars are sweet and, even though formaldehydes follow the same general formula, they are not considered sugars.

Nature holds remarkable structural variation of glycans which contribute to biological diversity and perhaps even to formation of new species [2]. Corresponding to genome and proteome, the collection of all glycan structures is called glycome. Despite the genetic conservation of organisms, glycan variations can serve to bridge the gap between genotype and vast phenotypic variety we see with every gaze upon the surrounding nature [3, 4]. Complex information written in glycan structures mostly remains a mystery, although specialized glycan-binding proteins (GBPs) can provide a useful tool and aid in deciphering sweet riddles of a glycocode. Distinct families of GBPs are capable of translating specific information written in certain parts of the glycocode, and in turn orchestrate a multitude of physiological processes in a given organism.

Glycosylation

Glycosylation, a process of adding glycans to proteins and lipids, can produce important functional diversity when the typical genome provides only a limited set of gene products [5]. It is considered that about 5 % of human genes encodes for proteins involved in glycan processing and recognition [6]. Our limited genome is further enlarged by the genome of the bacteria in the gut, that unlike us, produce enzymes for processing complex plant glycans like cellulose and pectin. Evidently, glycosylation is a highly regulated process that creates a plethora of diverse glycans, extremely valuable for every living cell. The primary source of glycan diversity is in their basic building blocks, monosaccharides. Vertebrates use 9 different monosaccharides (Figure 1) to synthetize their glycans. That does not seem much compared to 20 amino acids used in protein synthesis, but the spatial configuration of a monosaccharide (D and L), and its possibility to form bonds in 2 different directions (α and β) with each of its several hydroxyl groups, makes just 3 monosaccharides potentially arrange in over 27000 distinct trisaccharides. Unlike DNA or protein synthesis, glycosylation is not template driven; another reason for a vast array of glycan structures [7].

At least 250 unique enzymes, called glycosyltransferases, are used to assemble various glycans. An additional explanation for their variability is the fact that cell surface glycans are the first barrier and a recognition site for invading pathogens. Namely, genes for glycosyltransferases had to quickly respond to rapid pathogen evolution, since a typical pathogen has a very short generation time. Conversely, Vogt and colleagues found that gene mutations resulting in gains of glycosylation are often pathologic for humans, exactly because they involve gains of glycosylation [8].

Among other posttranslational modifications, glycosylation is currently considered the most extensive and complex one. There are two main types of glycan decorations found on proteins in eukaryotic cells: N-glycans and O-glycans.

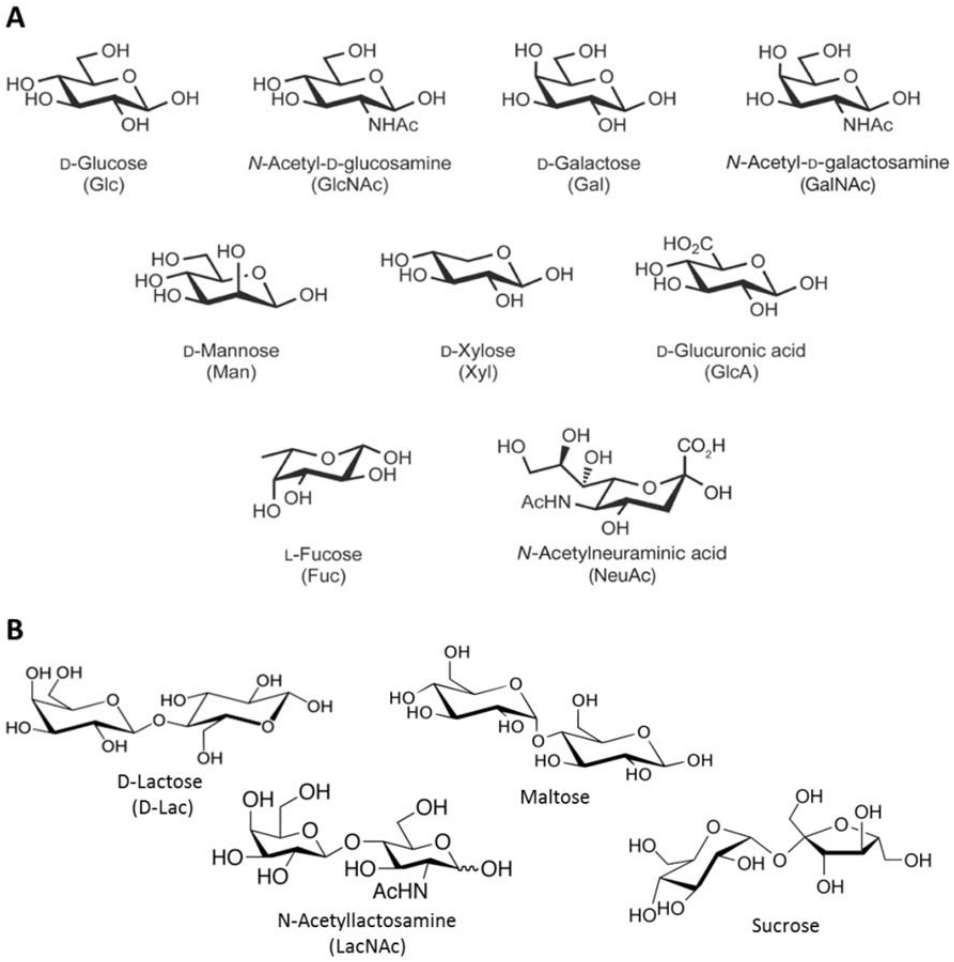

Figure 1 A. Common monosaccharides found in vertebrates. N-Acetylneuraminic acid is the most common form of sialic acid (Taken from *Essentials of Glycobiology*, Second edition, Chapter 2, Figure 4). **B. Some typical disaccharides.** Lactose is found in the milk of mammals. Maltose is formed after breakdown of starch. LacNAc is a typical component of complex glycans. Sucrose is found in flowering plants and usually extracted from sugar cane.

N-glycans

N-linked glycosylation is found in all 3 domains of life, but archaea, bacteria and eukaryotes all carry different glycans, assembled by very complex biosynthetic machineries. The unique and conserved acceptor for N-glycans is an asparagine residue present within the consensus sequence N-X-S/T, where X can

be any amino acid except proline [9]. N-glycans affect many properties of glycoproteins including their conformation, solubility, antigenicity, and recognition by glycan-binding proteins. The heterogeneity of glycoproteins is extended by their glycans; not only that glycoproteins usually contain more than one N-X-S/T glycosylation site, but also the same N-X-S/T sequence can carry glycan structures that differ from one glycoprotein molecule to another. The biosynthesis of N-glycans (Figure 2) begins in the cytosol as saccharides are added to a lipid acceptor (dolichol), and the sugar chain is transferred as a whole to the glycoprotein while it enters the endoplasmic reticulum (ER).

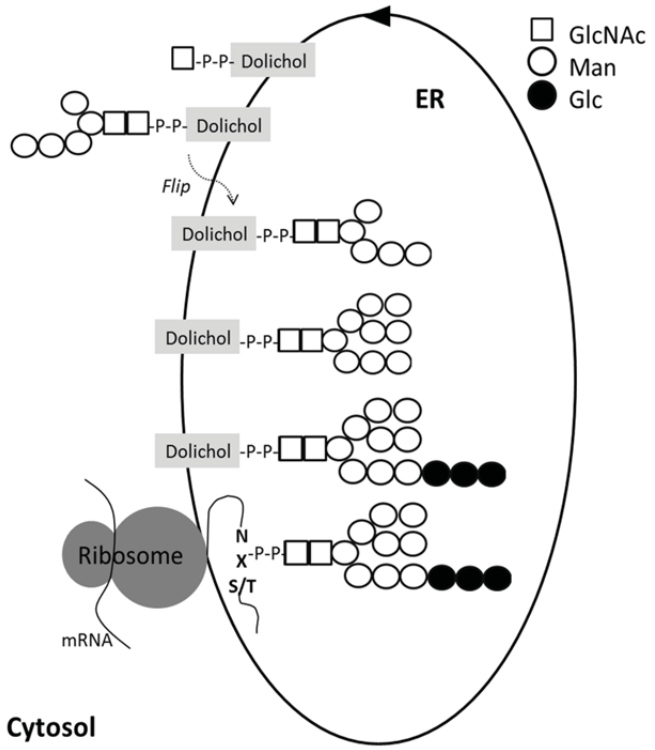

Figure 2. N-glycosylation pathway, simplified. Dolichol phosphate (Dol-P) located on the cytoplasmic face of the ER membrane receives GlcNAc-P from UDP-GlcNAc in the cytoplasm to generate Dol-P-P-GlcNAc. Dol-P-P-GlcNAc is extended to Dol-P-P-$GlcNAc_2Man_5$ before being "flipped" across the ER membrane to the luminal side. On the luminal face of the ER membrane, four mannose residues are added from Dol-P-Man and three glucose residues from Dol-P-Glc. Dol-P-Man and Dol-P-Glc are also made on the cytoplasmic face of the ER and "flipped" onto the luminal face.

Each of the sugar additions is catalyzed by a specific glycosyltransferase and the protein-bound N-glycan is further remodeled in the ER and Golgi by a complex

series of reactions. Subsequent sugar additions in the trans-Golgi network create an extensive array of mature, complex N-glycans. High mannose structures are added early in the synthetic pathway and are conserved among many species; they take part in proof reading in ER and early transport through Golgi (and back). The more complex structures (e.g. containing galactose) are added later in the cell and they developed later in the evolution and may have more detailed role in vesicular sorting coming after the Golgi. The immense importance of N-glycosylation is evident from mutations in N-glycan processing enzymes; they are either fatal or lead to sever pathological conditions [10].

O-glycans

O-glycosylation machinery decorates serine and threonine residues of mammalian glycoproteins with several types of sugars. The synthesis of O-glycans begins by addition of N-acetylgalactosamine (GalNAc) to S/T residues of the protein backbone, creating the so called Tn antigen. There are at least 20 genes plus their splicing variants that code for the family of enzymes that all catalyze this crucial reaction, albeit with slightly different specificity regarding on what peptide sequence it works and the neighboring O-glycans. It is still a mystery why nature would make such an investment in this one reaction, yet it points out the importance of correct O-glycosylation. Extraordinary diversity of O-glycans begins with the elongation of the Tn structure, creating eight distinct core structures. These are further modified in the Golgi by many different glycosyltransferases [11]. Mucins are the most represented O-glycosylated glycoproteins, rich in serine or threonine acceptor sites, carrying clustered O-glycans that may comprise 80% of the molecule by weight. Within the mucus layer, they shield and hydrate the epithelial surfaces of the body. Furthermore, O-glycans can provide recognition sites for pathogens but they can also mask underlying antigens and receptors. Attached O-glycans significantly affect the protein conformation and protect them from protease degradation [12].

There are also other forms of protein O-glycosylation like O-GlcNAc or glycans linked through O-xylose, O-mannose, O-fucose and O-GlcNAc. Additionally, glycosylation occurs on other bio-molecules, e.g. sphingolipids and protein-phospholipid anchors. There are also unattached glycan structures with no protein/lipid aglycone part like human milk saccharides, or glycosaminoglycans (GAGs) including hyaluronan and heparin, while their sulfated counterparts as chondroitin/dermatan sulfates and heparan sulfates exist O-linked to a protein.

Glycan determinants

A glycan motive contained within a carbohydrate molecule that is specifically recognized by a glycan-binding protein (GBP) is a glycan determinant. These are often glycan units at "outer" positions of a glycan molecule, which determine the function or recognition properties of a glycoconjugate. While the core glycan synthesis is constitutive in most cell types, the addition of branching and terminal sugars is often regulated in a tissue or cell specific manner. Well known is the ABO blood group system formed by alpha linked GalNAc, Gal and/or Fuc respectively; often attached to LacNAc or polyLacNAc chains, but the latter are not part of the determinants. Even though Karl Landsteiner discovered ABO blood groups in early twentieth century [13], it took 50 more years to discover that they were made of sugars.

Estimated number of glycan determinants is around 7000 [3] and they are widely presented in biological systems. The abundance of presented glycans enables a multivalent glycan ligand presentation, which in turn can enhance physiological apparent affinity (avidity) for GBPs. This multivalent presentation is often required to achieve the desired biological effect. GBP include lectins, antibodies, toxins and adhesins, which share the ability to recognize a glycan determinant in a non-enzymatic way. Three major families of mammalian GBPs include C-type lectins (calcium dependent), siglecs (sialic-acid-binding immunoglobulin-like lectins) and galectins, β-galactoside binding lectins. All three families have a predominant role in immunity [14]. However, galectins are emerging as particularly powerful and adaptable regulators of both innate an adaptive immune response.

Sialic acids

If one would picture different glycan structures as sentences, read by lectins, sialic acids (Sias) can be seen as the punctuation at the end, vital to disambiguate the meaning of the sentence [15]. Such prominent role of Sias is justified by their remarkable diversity resulting from the different α-linkages that may be formed between the C-2 of Sias and C-3 or C-6 positions of galactose, with N-acetyl or hydroxyl groups as additionally added modifications [16]. Sias can act either as masks or recognition sites for binding of various proteins. Siglecs are proteins that specifically bind Sias, whereas α-2, 6 sialylation tend to block the binding of galectins, and α-2, 3 sialylation may enhance, be tolerated or decrease binding. Sialylation is subject to rapid alterations and because of that it is often believed that it serves as an "on and off" switch for galectin binding [17]. In particular, α-2,

6 sialylation is considered to block galectin-3 binding, while α-2, 3 sialylation is generally tolerated [18]. However, the story gets more complicated; namely, α-2, 6 sialylation blocks galectin-3 binding to a glycan with single terminal LacNAc, but if α-2, 6 sialylation is on the terminal LacNAc of a poly(LacNAc) chain, galectin-3 still binds well to internal LacNAc units [19]. Obviously the mode of glycan presentation and glycan conformation can alter lectin binding specificity. Glycan-lectin interactions may also be altered through lateral association with other membrane glycoproteins and glycolipids [20].

Considering all the possible modes of interaction and their consequences, there is a great need to study glycan-lectin binding within different contexts to grasp their true biological relevance.

Glycosylation changes and disease

The glycome of an organism resembles a symphonic masterpiece, played by proteins and conducted by both genes and environment. When well-orchestrated, it allows a smooth flow of multicellular life. If the clef, notes or the players fail, once masterpiece becomes a sad tune of disease. Serum protein glycomes show some variability between individuals, but within a single healthy individual the glycome seems to be stable [21] with a certain degree of environmental influence [22]. It is well established that the epigenome (chemical DNA or histone modifications) is particularly sensitive to environmental influences, often connected to cancer through silencing of tumor-suppressors or activation of oncogenes [23]. The emerging view links epigenetics and glycosylation since the promoter region of certain glyco-genes undergoes DNA methylation [24]. These genes are often related to cancer and involve both genes for glycan processing and glycan recognition. There are indications that galectin-1 and galectin-3, lectins involved in immune system physiology, undergo epigenetic regulation in a human monocyte cell line (unpublished work). Defects of the glycosylation machinery can lead to loss or shortening of oligosaccharide moieties linked to glycoproteins and are termed congenital disorders of glycosylation (CDGs). Clinical manifestations of CDGs involve mental retardation, seizures, hypotonia, cerebellar hypoplasia and ataxia, liver disease, coagulopathy, and dysmorphia [25]. There are sixteen diseases connected to protein N-glycosylation: 14 assembly defects (CDG-I group) and 2 processing defects (CDG-II group) [26]. Furthermore, common phenotypic change of cancer cells is the modification of cellular glycosylation. Increased β-1,6-branching and general increase in sialylation of N-linked and O-

linked glycans are hallmarks of carcinoma cells and are associated with grade, invasion, metastasis and poor prognosis [27].

The immune-cell glycome is particularly complex since it changes during cell differentiation, activation and apoptosis. These alterations are important for organism homeostasis but they are also connected to immune-cell disease mechanisms, including immune-cell trafficking and differentiation, activation of antigen and cytokine receptors, and autoimmunity [28-30].

Additionally, a non-enzymatic addition of glucose, termed glycation, leads to formation of irreversible protein or lipid modifications called advanced glycation end products (AGEs). They occur mainly in diabetes and are used as a measure of long term increase in plasma glucose levels. The role of AGEs is still poorly understood, but they are thought to contribute to the various complications seen in diabetes and are also regularly found in post mortem examination of patients with neurodegenerative disorders like Alzheimer's and Parkinson's [31].

Many more acquired human diseases involve changes in glycosylation or the glycan recognition, including atherosclerosis, diabetes, bacterial and viral infections, asthma, rheumatoid arthritis etc. [32]. Considering high morbidity and mortality caused by those diseases, glycobiology could serve as a magic toolbox for novel diagnostic and therapeutic approaches.

Glycan biomarkers

The growing and aging population, committed to a sedentary lifestyle with an abundance of high calorie foods led to increased prevalence of diseases like diabetes, atherosclerosis, allergies and cancer. The development of diagnostic methods and treatment strategies have not been as fast, thus the search for suitable biomarkers, medication and treatments continues. Altered glycosylation is often connected to disease and can mirror the processes involved in disease origin, occurrence, progression and occasionally resolution. Glycans have pivotal clinical relevance, since they may be used as specific biomarkers for several disorders. In diabetes, for example, AGEs may be used as markers of tissue damage and may predict long-term complications, as mentioned in previous section [31]. Overexpression and aberrant localization of mucins, densely O-glycosylated proteins, is a typical feature found in breast adenocarcinomas [27]. Besides focusing solely on glycan structures, several glycan binding proteins have been used as prominent biomarkers and several members of the galectin family show promising potential. A study from 2012 identified elevated serum levels of

galectin-3 as a potential tumor marker for pancreatic carcinoma. It was found that combined determination of serum galectin-3 with previously used markers greatly aids the diagnosis [33]. Besides total galectin level, the amount bound to its specific ligand [34] can serve as a sensitive biomarker. Galectins can also be used to detect specific glycosylation forms of their ligands, with diagnostic and prognostic potential if these ligand glycoforms are connected to a certain disease [35].

Galectins

An expanding body of studies is focusing on biochemical properties and physiological roles of galectins. However, the knowledge about how their biochemical and structural aspects influence their biological activities, both in health and disease, remains limited.

Various historical names of galectins further indicate their involvement in many diverse processes, attracting the interest of different research groups over the years. Electrolectin was the first isolated galectin from the electric organ of the electric eel (*Electrophorus electricus*) in 1974 [36]. The term 'S-type' lectin was proposed in 1988 [37] when it was thought that thiol groups are required for galectin activity, thus 'S' stood for 'sulphydryl'. Even though only five mammalian galectins were discovered by 1993, the family was about to grow, considering the complexity and evolution of their glycoconjugate binding partners [38]. By 1994 it was well established that galectins prefer β-galactosides [39], and they were, for example, called galaptins by some, lactose lectins by other and various other names [40, 41], before the name galectin was coined. At the same time galectins were known to be important in macrophage biology [42-44] and they were connected to tumor development and metastasis [45]. The name galectins was elegantly crafted by a group of scientists in the galectin field [46] and introduced in a mini review about the β-galactoside-binding lectin family in 1994 [47]. Since then, galectin research provides new insights in different areas where their roles were documented, ranging from development and immunology to inflammation and cancer.

Galectins can be found in virtually all organisms, with 15 mammalian members discovered so far [48]. Today, galectins are defined as an evolutionary conserved family of endogenous lectins regarding the structure of their carbohydrate recognition domain (CRD) and preference for β-galactoside

containing ligands [49]. Their ability to differentially regulate numerous biological responses is tightly related to their structure and exquisite binding specificity. The carbohydrate recognition domain of galectins (Figure 3) has approximately 130 amino acids, although only about 7 residues within the CRD make up the canonical galactose binding site [50]. The CRD is a globular region containing ligand binding groove that defines its specificity [51].

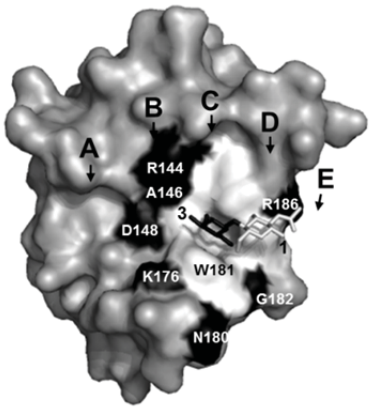

Figure 3. The galectin CRD with bound sugar. Folded into two slightly bent anti-parallel β-sheets, with the concave surface forming a groove that is long enough to accommodate a tetrasaccharide and therefore may be divided into sites A, B, C, and D. Site C is the galectin-defining β-galactose-binding site (white). Neighboring site C are key residues that determine the specificity (black). Taken from [52].

Based on the structure and presentation of the CRD, galectins are often, although old fashioned, divided into 3 subgroups: proto-type, tandem-repeat type and chimera-type (Figure 4) [53, 54].

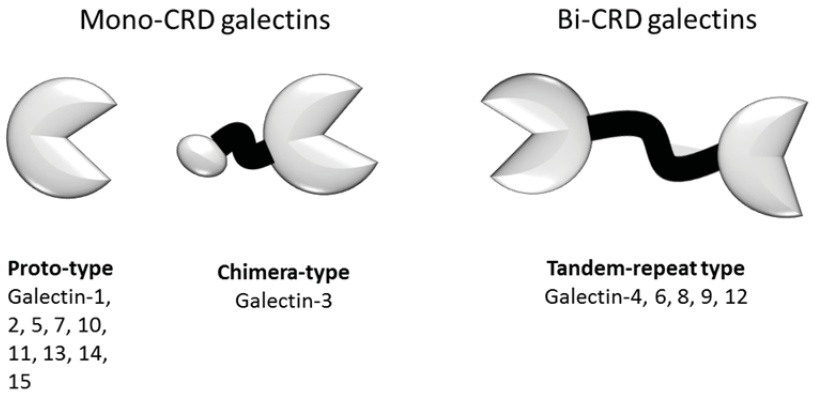

Figure 4. Classification of galectins.

This classification was established even before the use of the name galectins [38]. Since the proto-type group is not homogenous regarding sequence, specificity or mode of oligomerization, and galectin-3 is the only galectin in the chimera-type group, a better classification could separate galectins with one CRD and galectins with two CRDs [55]. Mono-CRD galectins, like galectin-1 and galectin-3, can occur as monomers, dimers or higher order oligomers. Bi-CRD galectins have two distinct CRDs connected by a linker region. Bi-CRD galectins might also associate into higher oligomers. Oligomerisation of galectins depends on their concentration and the presence, concentration and chemical properties of the ligand, and it appears to be essential for a set of galectin functions. Oligomerisation can provide selectivity and precise positioning of glycan ligands and in turn increase binding affinity [56]. Galectins evolved to accommodate different functions by adjusting their binding orientation and oligomerisation, perhaps even faster than changing their binding specificity and affinity [57].

Carbohydrate binding

The ligand binding groove within the galectin CRD (Figure 3) is long enough to hold a tetrasaccharide and can consequently be divided into subsites A, B, C and D [55]. The most conserved is site C that interacts with the β-galactose (β-Gal) [18]. Even though β-Gal binding in site C is galectin-defining, it is very weak for all galectins. Additional binding in sites A, B and D may improve affinity and it fine tunes galectin specificity, determining different biological properties of each individual galectin [52, 58]. LacNAc, a common glycan determinant for galectin binding, is found in many N- and O-linked glycans and may be presented as multiple poly(LacNAc) units [59]. This arrangement of LacNAc strongly influences the binding of galectin-3 since it binds internal units of linear poly(LacNAc) structures while galectin-1 prefers the terminal LacNAc-residues in branched structures [18, 19]. Thermodynamic studies by Di Lella and colleagues [60] showed how ligand binding to galectin-1, with additional galectin dimerization, provided thermal stabilization of the protein without altering its structure. This shows how the stability and functional properties of galectins involve different events, from ligand presentation and binding to possible galectin self-association, which all have to be in an appropriate order and magnitude.

A broad spectrum of possible glycan ligands and their affinities and specificities for different galectins (dictated by the galectin CRD and monomer/multimer form) by far exceeds the number of processes currently

connected to galectin-ligand interaction; hence, many more are waiting to be discovered.

Expression and cellular localization

Galectins can be found in any given organism and in almost all cell types, though different cells usually express different sets of galectins [61]. Synthesized on cytoplasmic ribosomes as typical cytosolic proteins, galectins lack a signal peptide to be targeted for classical ER/Golgi secretion pathway [55]. Nevertheless, galectins are also found outside the cell, at the cell surface or in the extracellular matrix (ECM) [62]. In a, so called, non-classical secretion, galectin-3 and galectin-1 accumulate below the plasma membrane, followed by an export mechanism that appears to involve the formation of membrane-bound vesicles [63]. In this way galectins can remain segregated from their glycoprotein ligands that traverse membranes in a classical way, until they are secreted outside the cell [64]. Cells can have a wide range of capacities to secrete galectins, tightly controlled during development, also depending on the cell type, polarization and, as often observed, malignant transformation. The dynamics of galectin intracellular transport and integration into the extracellular space is one of the ways to control galectin behavior. Interestingly, galectins can have opposing effects on the opposite sides of the cell membrane. For example, galectin-3 can either protect cells from apoptosis when functioning intracellulary, or promote cell death when functioning extracellulary. T and B cells overexpressing galectin-3 are protected from apoptosis [65, 66], while extracellulary added galectin-3 induces T cell apoptosis [67].

Galectin-3

Structurally and functionally unique, galectin-3 was first discovered in 1982 as a specific macrophage marker, named Mac-2 [68]. In parallel, galectin-3 was isolated from mouse fibroblasts by binding to asialofetuin (ASF) [69], and named CBP35 (carbohydrate binding protein with molecular weight of 35 kilo Daltons). Studies by Wang and others provided early knowledge about galectin-3 expression in various cells and cell compartments [70-74] as well as carbohydrate binding specificity [69, 75]. Soon after its initial characterization, it was found that the

expression of galectin-3 varies between functionally different macrophages [76], moreover, it was associated with a certain subtype of macrophages with cytotoxic, antitumor effect [77]. Around the same time Liu worked on a specific IgE binding protein, that he called εBP, which had all the characteristics of galectin-3. Not much later, εBP was proved to be identical to CPB35 [78], Mac-2 [79, 80], RL-29, HL-29 [81] and L-30 [82].

cDNA sequencing showed that albeit galectin-3 was a secreted protein, it lacked the usual signal peptide [79], a characteristic typical for all galectins. Even with reported connection to CBP35 and Mac-2, Liu committed to the name εBP in a review from 1990 that focuses on galectin-3 molecular structure and relationship with other lectins, providing some important clues about its possible functional role [83]. The aptness of the name S-type lectins used for galectins at the time, came in question when it was found that galectin-3 maintained its carbohydrate binding activity also in non-reducing conditions [84]. The name S-type lectins was later occasionally used to describe galectins as soluble proteins [85]. Furthermore, in an attempt to explain apparently disparate functions connected with galecitin-3, Woo suggested that besides forming heterodimers with other proteins, galectin-3 could also from disulfide-linked homodimers, i.e. self-associate [84]. This confirmed earlier speculations on both, unnecessary reducing conditions and self-association [86].

Galectin-3 research continued to grow ever since, yet its functional properties are still mostly unknown and in need of further exploration.

Galectin-3 from N to C

Even before it was officially called galectin-3 [46] its two domain structure was defined. The *LGALS3* gene, on chromosome 14, codes for a 29-35 kDa single galectin-3 polypeptide chain [87]. One CRD on the C-terminus has both hydrophobic and hydrophilic regions typical of globular proteins, and one distinct N-terminal domain composed of repeats of a proline and glycine rich sequence, but without lectin activity [85]. Nevertheless, the N-terminal domain participates together with the CRD in glycan binding [88, 89] and it is responsible for, at least one type of, galectin-3 multimerisation [90]. Although probably all mono-CRD galectins form dimers and higher oligomers, galectin-3 is particularly interesting in this regard. Massa at al. suggested that oligomerisation results in positively cooperative binding of galectin-3 to immobilized ligand [91], independent of the structural properties of the ligand. Cooperative binding was inhibited by lactose,

suggesting that lectin activity also contributed to the oligomerisation trough N-terminus. Physiological significance of coopeartivity was soon recognized and motivated further research which could reveal additional details of such binding. One highly relevant example of cooperativity is modulation of the immune response by galectin-3. Oligomerisation of galectin-3 bound to T cell receptor (TCR) results in formation of galectin–glycoprotein lattice that restricts TCR recruitment to the site of antigen presentation. In the opposite scenario, when galectin-3 is unable to induce lattice formation, T cells may become over-reactive through TCR signaling, increasing the susceptibility to, e.g. autoimmune diseases [92, 93].

Besides enabling oligomerisation, the N-terminus of galectin-3 has at least two more reported functions. It governs galectin-3 secretion by interacting with a number of cytoplasmic or membrane proteins along the secretory pathway, and facilitates galectin-3 aggregation at the cytoplasmic side of plasma membrane during secretion [94-96]. N-terminus is also suggested to direct anti-apoptotic properties of galectin-3 through phosphorylation of Ser^{6} [97]. The anti-apoptotic effect of galectin-3 was also connected to its conserved ^{180}NWGR^{183} motive near the C-terminus, since the mutation at position 182 (G182A) abrogated galectin-3 anti-apoptotic activity [52]. Moreover, the NWGR sequence of galectin-3 is homologous to the anti-apoptotic protein Bcl-2 [98]. It is possible that the N-terminus and CRD act in concert to support the full anti-apoptotic function of galectin-3, as they do during galectin-3 glycan binding and oligomerisation.

Galectin-3 C-terminus contains the whole CRD folded into two slightly bent anti-parallel β-sheets, with the concave surface forming a glycan binding groove with sites A, B, C, and D [52, 99]. Binding of a monosaccharide in the galectin-defining site C is normally weak, with a Kd in the low mM range, but other sites can enhance or decrease affinity depending on what saccharides are linked to the galactose moiety bound in site C (Figure 3) [52]. The galectin-3 CRD is fairly flexible and can get preorganized to recognize a ligand. In the absence of a ligand, water molecules acquire ligand-like shape of oxygens within the CRD [100, 101]. Besides being important for ligand binding, water can form an adhesive hydrogen-bond network between the hydrophilic protein interfaces, thus enabling protein self-association and perhaps cooperative binding [102, 103]. In this way water could contribute to galectin-3 self-association, e.g. through its CRDs since the ligand binding groove contains mostly hydrophilic amino acids. Yang [104] suggested that galectin-3 self-associates through CRDs facing each other (Figure 5), while the ligand binding to CRD prevents CRD to CRD self-association. Other types of self-association could also be helped by water molecules.

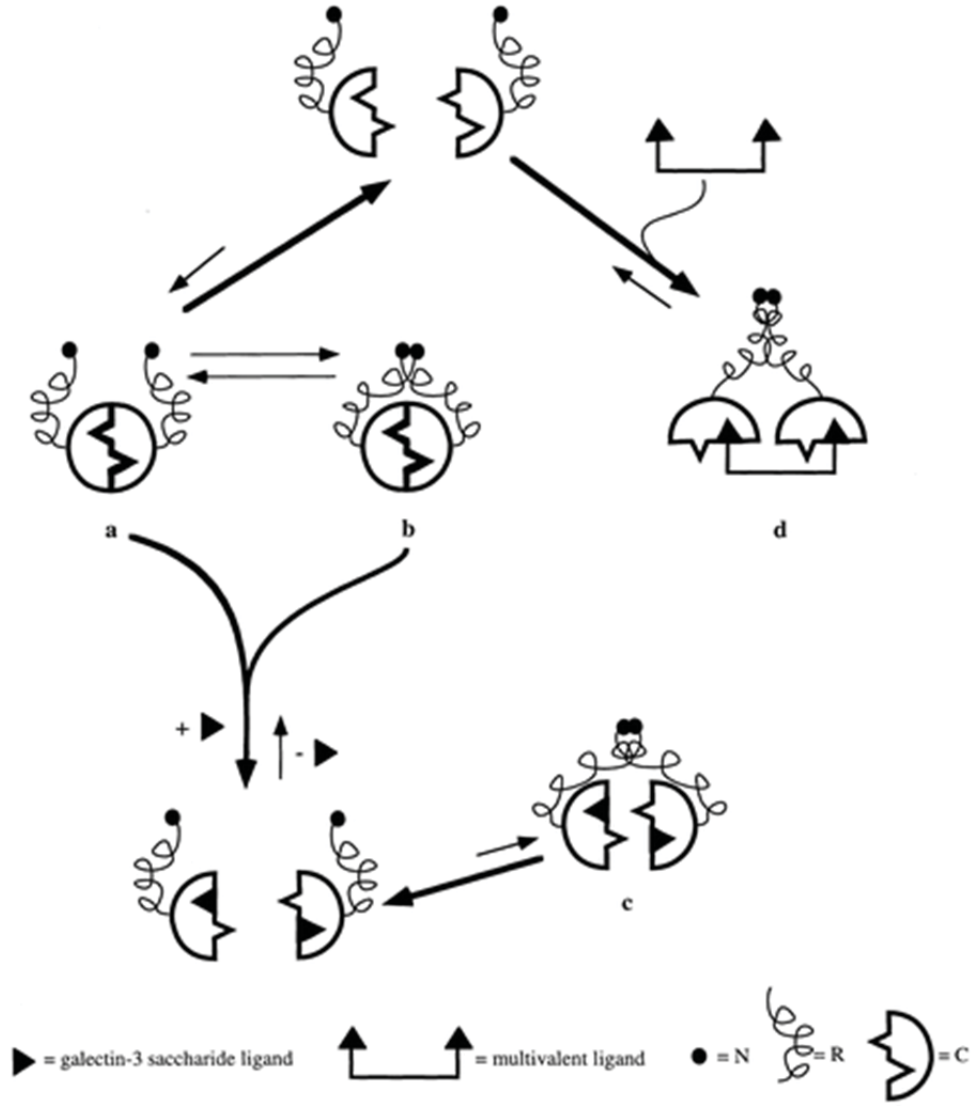

Figure 5. Galectin-3 self-association as suggested by Yang [104]. Galectin-3 can self-associate through its CRD (a) and N-terminus, or both, simultaneously (b). After binding to a monovalent (c) or a multivalent (d) ligand, CRD to CRD self-association is prevented, thus only self-association through N-terminus can occur (c and d). Reprinted with permission from [104] Copyright 1998, American Chemical Society.

Galectin-3 ligands

Galectin-3 lectin activity is thought to be a driving force for a majority of its physiological functions. The galectin-3 binding to poly(LacNAc) components of many N-glycans has been connected to cell adhesion [105, 106], receptor turnover [107], interaction with pathogens [108, 109] and intracellular trafficking, of both galectin-3 [110] and other proteins, generally glycoproteins [111, 112]. Availability of galectin-3 ligands depends on the time and place of their expression, but also on the expression and the activity of β-1,6-N-acetylglucosaminyl transferase (*Mgat5* or *GnT-V*), an enzyme that creates galectin-3 preferred sugar moieties on its protein ligands [87, 92]. Mutational studies of galectin-3 CRD (Figure 3) revealed its multifaceted specificity defined by ligand structure and CRD properties [52]. Only a certain match will, e.g. steer the intracellular sorting, as seen for galectin-8 [113], activate immune cells [114], and even decide in a life and death interplay of cancer and other cells [115]. Apart from fine-tuned specificity, certain physiological effects require complete block of galectin-3 ligand binding. As discussed under ***Sialic acids***, α-2, 6 sialylation can switch off galectin-3 binding [17], thus preventing galectin-3 induced apoptosis of some cells, mostly cancer cells [116, 117]. Another proposed "on and off" switch for galectin-ligand binding is phosphorylation of galectin-3 N-terminal Ser^{6}, also suggested to prevent galectin-3 multivalent interactions necessary for its biological functions [118]. Furthermore, phosphorylation at Tyr^{107} renders galectin-3 resistant to cleavage by prostate specific antigen (PSA) [119]. Since PSA is a glycoprotein, galectin-3 binding to PSA glycans could be an essential step in galectin-3 cleavage by PSA, and phosphorylation abrogates galectin-3-glycan binding. PSA-resistant, intact galectin-3 may contribute to the progression of prostate cancer through multivalent interactions resulting in downstream oncogenic signaling.

In addition to its lectin activity, galectin-3 can interact with several unglycosylated molecules through protein–protein interactions. It is generally assumed that this type of galectin-3 interaction occurs where no galactoside-bearing ligands are present, mainly in the nucleus and cytoplasm [120]. An intriguing finding was that the galectin-3 interaction with the cytosolic, anti-apoptotic Bcl-2 could be inhibited by lactose, even though Bcl-2 is not a glycoprotein [121]. Binding probably occurs through shared NWGR motif and the carbohydrate-binding site of galectin-3 is closely involved in this molecular interaction, responsible for the anti-apoptotic effect of galectin-3. Conversely, galectin-3 protein-protein interaction with Nucling, another cytosolic unglycosylated protein, was shown to induce apoptosis. Nucling also inhibited

NF-κB mediated galectin-3 expression [122]. Furthermore, galectin-3 can interact with the spliceosome in the nucleus, acting as a pre-mRNA splicing factor. This function is managed by both galectin-3 and galectin-1, but their activity in the nucleus is mutually exclusive [123].

Deeper understanding of the mechanisms of galectin-3-ligand binding is necessary for exploring versatile galectin-3 functions. They could in part be explained by its unique structure, coordinated activity of its CRD and N-terminus and a multitude of its glycan and other ligands.

Galectin-1 and 3 in the immune system

The circumstances of its discovery (as a marker of inflammatory macrophages, Mac-2, and IgE binding protein) instantly revealed the intimate relationship of galectin-3 with the functions of the immune system. One is destined to bump into galectin-3 when studying immunity related genes, receptors or the regulation of the immune response. This is also true for many other galectins. Even though it was hard to define their precise role, a more general characterization emerged for, e.g. galectin-3, as the regulator of immune response, whether positive or negative [87]. Ligand binding is an obvious modifier of galectins' functions, but the search for their physiological ligands was not that straightforward. Since β-galactosyl residues could not be found in the cytosolic compartments, it was believed that galectins are stored in the cytosol but play a functional role at another location [124]. Indeed, once cytosolic galectin-3 exits the cell it can bind extracellular matrix proteins, e.g. laminin, promoting the adherence or migration of the immune cells along basement membrane [125]. Besides controlling the inflammatory response, galectin-3 can mediate cell attraction and adhesion, thus supporting the progression and metastasis of various cancer cells, including melanoma [106], breast [126] and prostate [127].

Host-pathogen interaction

Pathogenesis of many infections involves interaction of pathogen's surface glycans with host lectins. Galectins have been discovered to bind glycans on the surface of viruses, bacteria, protista and fungi, potentially acting as pattern recognition receptors (PRRs) [128]. There is evidence that galectin-1 can promote HIV-1 infection through cross-linking HIV-1 protein gp120 and CD4 on T cells

[129]. Galectin-3 was also shown to bind a pathogen component, i.e. bacterial lipopolysaccharides (LPSs). With its CRD galectin-3 binds to lactosyl moieties on LPS, and with its N-terminus to the lipid A part of the LPS molecule [130]. In 2008 Li reported that galectin-3 knock-out (KO) mice experience LPS induced shock associated with excessive induction of inflammatory cytokines and nitric oxide production, because galectin-3 was not available to bind and sequester this endotoxin [131]. A newer, 2011 finding suggested that LPS binding can induce galectin-3 oligomerisation trough N-terminus, consequently decreasing LPS concentration threshold for neutrophil activation ten times [132]; the sooner neutrophils are activated, the better chance for pathogen eradication. Once again galectins showed their duality; they can be used by sugar-coated pathogens to promote infection or conversely, they help to kill the invader by inducing a host immune response.

Galectins can potentially act as opsonins, cross-linking two or more cells by binding to their surface carbohydrates. This interaction is important for the phagocytosis of bacteria at sites where typical opsonins, as antibodies and complement, are scarce, for example in the urinary tract [133]. Indeed, galectin-3 can bind lipooligosaccharides of certain gram negative bacteria [108] (*N. gonorrhoeae* and *H. influenzae*) and cross-link them to neutrophils [134]. Similarly, galectin-4 and galectin-8 have been shown to specifically recognize and kill strains of E. coli that express the blood group B–related antigen [135].

Modulation of the immune response

Galectins are often found to modulate the functions of immune cells by inducing functional changes and by promoting or preventing apoptosis of certain immune cell sub-populations. Galectin-3 cross-linking ability could lead to the activation of mast cells; besides IgE, galectin-3 binds the IgE receptor. It is thus possible that galectin-3 acts through cross-linking receptor-bound IgE, receptor alone, and both [136].

Given that some galectins can be co-expressed in a certain microenvironment, the cross-talk between them is another key aspect to be considered. For example, galectin-1 was found to provide a co-stimulatory signal for naive and antigen (Ag) specific T cells [137], similar to galectin-8 [138], through abundant and heavily glycosylated cell surface mucin CD45 [139]. Galectin-3 was shown to prevent galectin-1 and galectin-8 co-stimulation of T cells by negative regulation of TCR [137]. Binding to TCR can lead to galectin-3 oligomerisation, preventing TCR lateral mobility after surface lattice formation

[93]. Galectin-1 was also shown to selectively induce apoptosis of pro-inflammatory Th1 and Th17 cells, but not naive Th2 cells [140], hence it induced the synthesis of anti-inflammatory IL-10 and other Th2 cytokines [141]. Moreover, Th1 cells secreted galectin-1 that further sustained Th2 skewing [142]. Galectin-1 was suggested to be of key importance in sustaining pregnancy and preventing fetal loss by balancing Th1 and Th2 response and by restoring T cell homeostasis [143]. On the other hand, added galectin-3 was found to induce phosphatidylserine exposure, an early event in apoptosis, in activated T cells, even though not necessarily followed by cell death [144]. Induction of apoptosis was found to be unselective for Th1 and Th2 cells [140]. In cancer, galectin-3 can indirectly promote tumor growth by inducing apoptosis of tumor-reactive T cells [145], or impairing their function by preventing TCR and CD4/CD8 co-localization [146]. Similarly, galectin-1 contributes to tumor immune escape by its overall immunosuppressive activity [147].

Galectin-3 location is recurring as an important functional modifier. As mentioned earlier, while extracellular galectin-3 induces cell death or at least phosphatidylserine exposure, ectopic galectin-3 expression in human T cells was shown to prevent apoptosis induced by staurosporine and anti-Fas receptor antibody [121, 148]. Furthermore, galectin-3 was connected to development of diabetes. Namely, galectin-3 KO mice were more resistant to induced diabetes than wild type (WT) mice [149]. Still, there could be benefits from galectin-3 in diabetes; by binding to AGE, typically found in diabetes, galectin-3 can prevent AGE induced tissue injury [150]. Galectin-3 was also suggested to be beneficial in a mouse model of asthma. Intranasal administration of galectin-3 cDNA reduced airway inflammation after antigen challenge [151, 152]. In another study, galectin-3 KO mice presented less severe airway inflammation and hyper-responsiveness compared to WT mice [153], again strengthening the possibility that the galectin-3 location strategically alters its biological function. Galectin-3 can also contribute to atherosclerosis by sustaining inflammation in the atherosclerotic plaque [154].

Both galectin-1 and galectin-3 are able to activate oxidative burst of human neutrophils [155, 156], and galectin-3 activity is dependent on both the CRD and the N-terminus [157]. Both domains are also necessary for galectin-3 specific chemotactic capacity for monocytes and macrophages [158].

Obviously, galectins demonstrate a plethora of functions and it is probably safe to state that some responses will be induced when any recombinant galectin is added to any of the immune cells [159]. In general, it appears that galectin-1 has a broad spectrum of anti-inflammatory activity, while galectin-3 has an overall pro-inflammatory activity. Galectin-1 has thus been an attractive immunosuppressive

agent in autoimmune diseases or other conditions underlined by chronic inflammation [160].

The broadness of galectin-3 immune-regulatory potential in various T cell mediated inflammatory processes, autoimmune diseases and tumor progression, justifies the continuous search for galectin-3 based therapies, mainly focusing on galectin-3 specific inhibitors. The search was often relying on plant products as safe and easily available. Various biologically active pectin preparations and plant galactomannans have been repeatedly suggested to prevent cancer metastasis and inflammation, by inhibiting galectin-3 and perhaps also other galectins [127, 158, 161]. Even if galectin-inhibitor interaction was a focal point of many studies, most of them did not directly measure the binding of galectin to an inhibitor, creating a need for more in depth biochemical characterization of such interactions.

Galectin-3 in macrophages

Nowadays, galectin-3 can hardly be used as a marker of activated macrophages as when it was initially discovered [68], considering its wide expression in a variety of other cells and tissues [162]. Galectin-3 was found in almost all cells of the immune system; in neutrophils [163], eosinophils [164], basophils and mast cells [136, 165], Langerhans cells [166, 167] and dendritic cells [168]; but also in a number of other cell types like fibroblasts [71], different skeletal tissue cells [169-171], keratinocytes [172], Schwann cells [173] and gastro-intestinal cells [174], as well as in epithelial [175-179] and endothelial [180] cells from tissues and organs all over the body. Still, macrophages by far exceed any other cell type in the amount of produced galectin-3. Actually, galectin-3 is the fifth most expressed protein of all proteins in macrophages (F.O. Martinez, Sir William Dunn School of Pathology, University of Oxford, Oxford, UK, personal communication). Even if not specific for macrophages, its fundamental role in macrophage function is undoubted.

Macrophages' polarization

In a 2012 review simply called ***The Macrophage*** [181], these immune cells were defined as professional phagocytes involved in maintaining basic tissue homeostasis by the clearance of erythrocytes, apoptotic cells and cellular debris, and tissue remodeling. Furthermore, macrophages are the key players in the host response to pathogens, resolution and repair after infection [182]. Multiple

functional properties of macrophages are made possible by their remarkable plasticity. The activation phenotype of a particular macrophage depends largely on the surrounding microenvironment [183]. The simplest classification divides a spectrum of activated macrophages in the two opposing phenotypes: classically activated, M1 and alternatively activated, M2, macrophages [184, 185]. Even if the opposing phenotypes can simplify the understanding of macrophage function, all the subtypes in between should still be considered as unique functional entities. Dividing all the possible macrophage phenotypes to either M1 or M2 is like seeing the rainbow only in yellow and purple, missing out on all the colors in between.

In tissues, macrophages respond to environmental cues like microbial products, cell damage or activated lymphocytes, by the acquisition of distinct functional phenotypes. Stimulation of macrophages by TLR ligands (e.g. LPS) and IFN-γ pushes the activation towards inflammatory, M1 phenotype, while alternative, M2 activation is stimulated by IL-4/IL-13 (Figure 6).

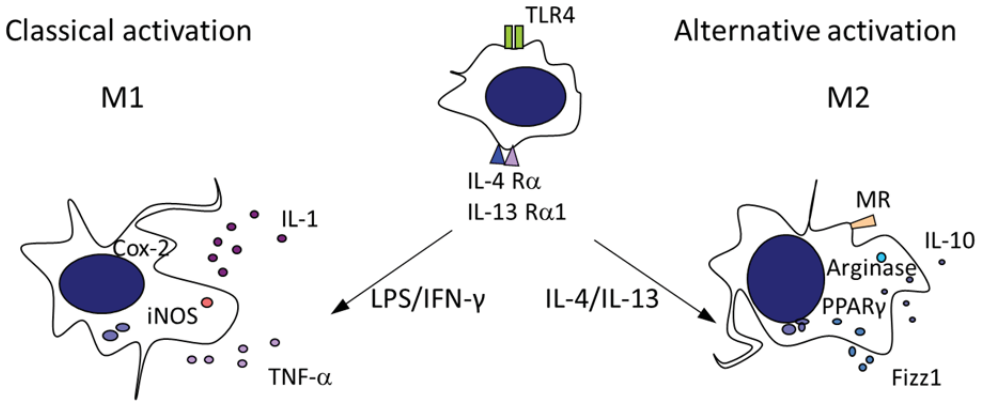

Figure 6. Classical and alternative macrophage activation. Resident tissue macrophages respond to, e.g. bacterial LPS (that binds surface TLR4 receptor) and produce inflammatory cytokines like TNF-α, IL-1 etc. On the other hand, IL-4 and IL-13 activate resident macrophages through corresponding receptors and initiate alternative macrophage activation, marked by elevated arginase activity and a production of anti-inflammatory IL-10.

These states resemble the Th1–Th2 polarization of T cells [186-188]. Once polarized into either M1 or M2 direction, macrophages can, to some extent, be reversed in vitro and in vivo [189, 190]. The changes in macrophage activation dynamically follow the processes of initiating and sustaining inflammation towards resolution and tissue repair [191]. The distortion of this dynamic is frequently associated with pathology, leading to either inadequate immune

response or chronic inflammation. There have been many suggested markers for different macrophage subtypes, mostly trying to distinguish between far ends of activation phenotypes. Typically reported M1 markers are TNFα, Cox-2, iNOS and IL-1, while arginase, Fizz1, PPARγ, IL-10 and mannose receptor (MR) have been connected to M2 phenotype (Figure 6) [192-195]. Given the spectrum of macrophage activation states, these markers are hardly reliable, but rather offer an orientation point while exploring the busy macrophage world.

Galectin-3, a specific marker for macrophage phenotypes?

Even though galectin-3 is not exclusively expressed in macrophages, recent studies indicate that it acts at a decision point during macrophage activation, particularly in M2 direction [196]. More than 20 years ago it was observed that certain immunosuppressive macrophage phenotype is marked by an increase in surface galectin-3 expression [197]. These macrophages were found in tumor sites, as a response to granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by cancer cells [198]. Galectin-3 on the surface of cancer cells was suspected to be responsible for their suppressive properties by inhibiting T and B cell mitogenic reactions [199]. Not long after, it was found that the amount of surface galectin-3 does not directly translate to an overall galectin-3 expression in different macrophage subtypes [200]. Namely, inflammatory thioglycolate-elicited peritoneal macrophages also increased surface galectin-3 expression. However, after additional LPS treatment, surface galectin-3 noticeably decreased, while overall galectin-3 expression showed only minor changes. Surface expression of galectin-3 most likely correlates with the appearance of preferred galactosyl residues on cell surface glycoproteins, a process regulated during development and cell activation [201, 202]. Gathered information by mid ‘90s led Sato and Hughes argue against any specific role of galectin-3 in macrophages, despite its constitutive synthesis and secretion by both resident and variously stimulated macrophages [200]. Nevertheless, new evidence connecting galectin-3 to macrophage specific functions continued to appear. First, it was confirmed that the transition from circulating monocytes to differentiated macrophages is marked by a dramatic increase of total cellular galectin-3 level [203]. This dramatic shift was again seen during induced differentiation of a monocyte cell line, THP-1, to adherent macrophages [204]. Next, it was seen that endogenous secreted galectin-3 could, by autocrine or paracrine lectin activity, sustain macrophage activation [203].

In studies with KO mice it was observed that galectin-3 null macrophages had decreased areas of adherence in culture, unlike highly spread wild type macrophages [205]. Since cell surface restricts phagocytosis, one of the basic macrophage functions, galectin-3 again emerged as the key player in macrophage biology. Galectin-3 KO mice also showed an overall attenuated peritoneal inflammatory response after antigen challenge.

More than a decade ago it was observed that macrophages exposed to modified lipoproteins [206] or AGE [207] overexpress galectin-3. They resembled classically activated macrophages, as we see them today, even though classical activation was repeatedly coupled to decreased overall galectin-3 expression [196, 200]. These macrophages were suspected to participate in the development of atherosclerotic lesions [204], a notion confirmed on several occasions for inflammatory macrophages [208].

There is an apparent lack of agreement in published data on the amount and the role of galectin-3 expressed in different macrophages. Recent studies on macrophage cell models, using either primary cells or cell lines, showed that both M1- and M2-like macrophages increase galectin-3 production, with varying amounts of surface bound and secreted galectin-3 [209, 210].

Great diversity of macrophages and their markers depend on the macrophage source, experimental design and many other factors. Thus, it has been difficult to strictly characterize M1 and M2 markers, specific functions of each subtype and their dependence on galectin-3. Less fixed standpoint could better explain the dynamic interplay between macrophage differentiation and the expression of different markers. Galectin-3 should perhaps be considered as macrophage type-sensitive rather than macrophage type-specific marker.

Specific roles of galectin-3 in macrophages

Galectin-3 might not be specific for a certain macrophage subtype, nevertheless, several basic macrophage functions were shown to be dependent on galectin-3.

Galectin-3 plays a critical role in the phagocytosis of apoptotic cells and IgG-opsonized material by macrophages [211]. Sano and colleagues assumed that the formation of phagocytic cups and phagosomes was controlled by cytosolic galectin-3, since the phagocytosis was not inhibited by added lactose. This finding was in agreement with a conventional view that all (and only) extracellular functions of galectin-3 are mediated by glycan binding.

Galectin-3 can serve as a macrophage pattern recognition receptor. This role has been assigned primarily to the extracellular, secreted galectin-3, conveyed by its CRD binding to glycan moieties on invading pathogens [109]. Pathogen recognition and binding could be an initial step leading to either pathogen cell death [212] or phagocytosis and neutralization of the pathogen inside the macrophage. Similar to pattern recognition activity of galectin-3, connected mostly to xenoproteins or other pathogen related structures, galectin-3 can also serve as a sensor for physiological components, like LDL (low density lipoprotein) or AGE. Interaction of galectin-3 with lipoproteins or advanced glycation/lipoxidation endproducts can result in their removal [213] or it can provide an instruction signal for a functional change of macrophage phenotype [204, 206].

Galectin-3 produced by macrophages can also act in a cytokine-like fashion. The provided signal can be used either by the macrophages themselves, to sustain galectin-3 dependent activation [214], or it can provoke various downstream effects in the surrounding cells. For example, it has been suggested that galectin-3 may act in the regulatory loops of the immune response by inhibiting IL-12 and the expression of other Th1 cytokines [215]. Galectin-3 was also shown to induce myofibroblast activation and collagen production by fibroblasts [214]. As a consequence, galectin-3 can promote fibrosis in lungs [216], liver [217], kidney [218], heart [219] and other organs.

Besides regulating inflammation and tissue remodeling through its roles in phagocytosis, pattern recognition and signaling, an emerging view connects galectin-3 to intracellular sorting and trafficking of glycoproteins. Related publications hitherto used mostly polarized cells or cell systems other than macrophages, yet it is plausible that galectin-3 would also direct trafficking of glycoproteins related to macrophages' functions.

Galectin-3 trafficking

The early observations that galectin-3 can reside in the cytoplasm, the nucleus, on the cell membrane, as a part of the extracellular matrix or soluble in human plasma, indicated that galectin-3 undergoes extensive trafficking and shuttling between different cellular compartments [220]. Galectins have an intriguing ability to be secreted without a conventional secretion signal, avoiding classical ER-Golgi pathway [221], a feature shared by only a few other proteins, as certain cytokines, like IL-1 [222] and several growth factors [63]. Another early

finding was that nuclear localization of galectin-3 is highly regulated, rather than due to the passive diffusion through nuclear pores [223], despite the lack of a specific nuclear transport sequence. Complete galectin-3 CRD seemed absolutely essential for nuclear transport, while the N-terminus was not necessary but it promoted nuclear entrance of galectin-3 [224]. Studies also showed that galectin-3 secretion and intracellular translocation use two different pathways, with an apparent cross-talk between them, since disruption of intracellular galectin-3 localization indirectly promoted the secretion of galectin-3 [225]. With the use of polarized epithelial cells, it was shown that galectin-3 can distinguish between the apical and the basolateral membrane, directing most of its exocytosis towards the apical cell membrane [110]. Prior to its exocytosis galectin-3 was found to be localized in specific endosomal domains involved in cargo recycling.

Only a few studies have investigated the endocytosis of galectin-3. Galectin-3 vesicular import through apical membrane of polarized epithelial cells was observed to be very rapid (1 min), with subsequent delivery into subapical recycling endosomes (within 10-20 min). This process was entirely dependent on the galectin-3 CRD since it was inhibited by lactose and a point mutation in the galectin-3 CRD (R186S) that strongly reduced galectin-3 sugar binding. Furthermore, all available studies suggested that sugar binding of externally added galectin-3 is absolutely required for its attachment to the cell surface and a prerequisite for endocytic uptake. This has been shown in breast carcinoma cells [226], polarized epithelial cells mentioned above and fibroblast like cells [110].

Besides different mechanisms used for galectin-3 trafficking, some glycoproteins use galectin-3 as a part of their own trafficking mechanism. The first indication that galectin-3 was involved in glycoprotein sorting was obtained using polarized epithelial cells, MDCK (Madin-Darby Canine Kidney). Galectin-3 was absolutely necessary for apical sorting of the non-raft-associated proteins, whereas there was no effect on the polarized targeting of raft-associated proteins. Galectin-3 knockdown caused missorting of apical glycoproteins to the basolateral membrane.

It was observed that galectin-3 accumulates in post-Golgi carriers and acts as an apical sorting receptor for certain set of glycoproteins. Delacour and colleagues [227] suggested an interesting mechanistic sequence for the observed sorting events: galectin-3 binding to a glycoprotein initiates a galectin-3 oligomerization process and as a result, galectin-3 forms clusters with bound glycoprotein, which can directly serve as a starting point for vesicle formation, an ideal vehicle for protein trafficking. As it was confirmed that galectin-3 driven oligomerization into large cross-linked clusters is essential to initiate correct

sorting, additional mechanisms were suggested; one involved the formation of increased membrane curvature and the other the assembly of a cytosolic coat, both relying on galectin-3 self-association and subsequent glycoprotein clustering [228]. Allowing for a conventional view that galectin-3 forms oligomers of up to 5 molecules by binding through their N-terminus [90], it is hard to envision how a cluster of galectin-3 pentamers, with flanking glycoproteins, could assist in vesicle generation, membrane curvature formation or cytosolic coat assembly. If a glycoprotein would induce rapid accumulation of more than 5 galectin-3 molecules, it could better support the suggested mechanism.

Galectin-3 can, not only direct sorting of glycoproteins based on their preference towards apical or basolateral surfaces, but also cause the trafficking divergence if sorted protein exists in two or more distinct glycoforms. Transferrin, for example, exists in at least 2 glycoforms and only one is bound by galectin-3. Compared to the transferrin fraction that does not bind galectin-3, the bound fraction undergoes a faster intracellular recycling route [15]. Transferrin is an extensively studied glycoprotein with an important function in iron delivery after cellular uptake [229], yet, up until recently there was no notion on the functional role of transferrin glycans.

Given the high diversity of complex N-glycan structures, it could be expected that galectin-3 facilitates many more sorting events, and that other galectins also share this function, each directing the traffic of a particular set of glycoproteins based on their glycan determinants [230]. It has been shown that galectin-1 regulates the endocytosis of the hemoglobin-haptoglobin complex by a subset of alternatively activated macrophages, based on haptoglobin glycoforms [35]. The usefulness of such a distinction between haptoglobin glycoforms might not be directly evident, but considering the cytotoxicity of the free hemoglobin, it becomes clear that the removal of hemoglobin-haptoglobin complex by macrophages strongly contributes to tissue homeostasis.

Galectin-3 trafficking could also be connected to its function as an opsonin. It was demonstrated that galectin-3 rapidly accumulated around vacuoles containing viable *M. tuberculosis* [231]. It is possible that galectin-3 was incorporated into a phagosome during phagocytosis of the mycobacterium. However, the rapidly increasing accumulation over time points to delivery of galectin-3 to the phagosome via the sorting/recycling endocytic pathway, possibly after high-order galectin-3 multimerisation. Galectin-3 was also found to be intersecting with the endocytic route of the phagocytic vacuoles lysed by the invading bacteria. It was suggested that lysed vacuoles exposed a glycan galectin-3 ligand that triggered fast galectin-3 recruitment and lattice formation [232]. As

soluble receptor, galectin-3 could also be involved in the recognition, host cell interaction and the trafficking of *M. tuberculosis* mycolic acids [233].

Endocytosis has been recognized as a mean for coordinating the type, duration, and amplitude of signals transduced by surface receptors. The initial signal can either be blocked or amplified by receptor surface retention. It was suggested that the galectin-3-glycan lattice negatively regulates TCR signaling by, either reducing lateral mobility of the receptor or by inducing TCR internalization and inhibiting its recycling back to the T cell surface [92, 93]. Furthermore, there is evidence that galectin-3 can inhibit the execution of the apoptosis signal by forming a cell surface glycan lattice, thus preventing the endocytosis of a certain death receptor [112]. Conversely, maintaining the epidermal growth factor receptor (EGFR) at the cell surface has an opposite effect. Tumor cells overexpress Mgat5 modified N-glycans on EGFR. Binding of galectin-3 to those N-glycans leads to lattice formation that causes tumor proliferation through prolonged EGFR signaling [234, 235]. In addition, regulation of EGFR trafficking and expression by galectin-3 can influence the mobility of a whole cell. Impaired EGFR trafficking by intracellular, but not surface galectin-3, was shown to reduce keratinocyte migration [236]. Bearing in mind that galectin-3 is a chemotractant for monocytes and macrophages [158] and that it can facilitate mobility of other immune cells by binding ECM proteins [125], the possibility arises that galectin-3 could control trafficking of not only itself and specific glycoproteins, but of whole cells and perhaps even cell groups.

Protein trafficking through endocytic/exocytic compartments, with a help of sorting proteins or modifying enzymes, is important for selective regulation of their function, activity and release. Cargo segregation into distinct pathways additionally enables selective cellular responses to various internal or external stimuli [237].

Present investigation

Study aims

Despite the extensive research in the glyco-field concerning structure and function of glycans, lectins and glycosylation processes, many areas are still left unexplored. The aim of this thesis was to pave an extra bit of path through that dense field, predominantly focusing on various aspects of galectin-3 function.

The general aim was to further clarify functional properties of galectin-3, its role in the immune system linked to macrophages, glycoprotein and other ligand binding specificities. Several specific aims were set from the beginning of the experimental work for this thesis:

- to study the mechanism of galectin-3 endocytosis in macrophages.
- to investigate the role of galectin-3 in intracellular trafficking in different macrophages.
- to study the interaction of galectin-3 with glycoprotein ligands in solution.

Pursuing the aims above we obtained a set of puzzling results that generated a few additional questions:

- what are the structural requirements for galectin-3 endocytosis in different cells?
- what are the specific roles of alternative, M2, macrophages in relation to galectins?
- what is the potency of small synthetic glycan ligands to inhibit galectin-3 endocytosis?
- what is the mechanism and biological significance of galectin-3 self-association?
- what is the inhibitory potential of citrus pectins and plant galactomannans for galectin-3 and other family members?

A broad stretch of posed questions required a multiple level approach with different experimental settings. An adequate cell system was necessary to answer the questions concerning the biological aspects of galectin-3 function, while different biochemical techniques were needed to further explain the galectin-3-ligand interaction.

Cell based approach

Since the day of its discovery galectin-3 was closely linked to macrophage biology [68]. Therefore, a logical decision was to employ a monocyte-macrophage cell system for studying galectin-3 biological functions. We decided to use a human leukemic cell line (THP-1) cultured from the blood of a boy with acute monocytic leukemia [238]. Motivated by accumulating evidence showing how macrophage polarization has a deep impact on many biological functions [182], we also wanted to develop an *in vitro* system that resembles macrophage polarization *in vivo*. As discussed in detail in Paper I (Appendices), THP-1 cells have been used as a macrophage model in many studies. They can develop macrophage like functions following the treatment with phorbol-myristate-acetate (PMA) [239] or other stimulators, while the subsequent addition of cytokines can produce a homogenous group of cells similar to different in vivo stages of maturation and activation depending on the cytokine(s) used. We developed a system of classical, so called M1, and alternative, M2 macrophages using PMA differentiated THP-1 cells with the addition of *E. coli* LPS or Th2 cytokine, IL-4, respectively. As an intermediate, M0 macrophage phenotype, we used PMA differentiated THP-1 cells without subsequent activation in either M1 or M2 direction. This M0, M1, M2 cell system was used for most of galectin-3 endocytosis and trafficking studies, but also in an attempt to study the involvement of galectin-3 in trafficking of other glycoproteins like asialofetuin (ASF) and LDL. Furthermore, M2 macrophages were used to study the role of galectin-1 in the hemoglobin-haptoglobin complex endocytosis (Paper II).

Several key findings concerning galectin-3 endocytosis were additionally confirmed in an *ex vivo* model, using monocytes isolated from human peripheral blood, differentiated to resemble M1 and M2 polarized cells.

Other *in vitro* cell models served mostly as a part of comparative studies between the cells of the mononuclear phagocytic system (MPS), represented by THP-1 derived macrophages, and different non-MPS cells. These were lung

fibroblast cells HFL1, derived from a 16-18 week old human fetus with a finite lifespan of around 57 divisions. Moreover, HFL1 fibroblasts were used in an attempt to build an asthma model in a co-culture study with M1 and M2 polarized THP-1 cells. Alternative macrophages were shown to have an active role in exacerbating Th2 responses in asthma, including tissue remodeling and fibrosis [240].

Another non-MPS cell line used was SKBR3, human mammary gland epithelial cells isolated from a breast cancer patient in 1970. SKBR3 cells additionally served as a galectin-3 non-expressing control, since THP-1, derived macrophages, and HFL1 cells constitutively express galectin-3.

Biochemical approach

The study of galectin-ligand binding, with the addition of other induced processes, like galectin-3 self-association, becomes particularly complex in a cellular system. Usually cells contain multiple galectin-3 ligands, other galectins and perhaps even their inhibitors. In order to enable more precise investigation of the mechanism, affinity and specificity of galectin-3-ligand binding, it was necessary to step out from the busy cell model. For this thesis we mostly used the fluorescence anisotropy assay to study galectin-3 interactions in solution. This method has been extensively used by our group to test interactions of different galectins, sugars, inhibitors, probes, complex glycoconjugates and multi-component biological samples, like human serum. The method does not require physical separation of galectin and the ligand, i.e. the presence of unreacted components does not interfere with the measurement. Therefore, it is a quick, reproducible and very sensitive method, based on the movement of fluorescent molecules in the solution [241]. A fluorescently tagged molecule (probe) is freely moving in the solution and it gets excited by plane polarized light. Excited molecule now rotates very fast and by relaxation emits the light that is partially depolarized. If a fluorescent molecule is bound to a protein, the now larger complex will have a slower rotation after excitation, thus emitted light will be less depolarized (more polarizes). The emitted light is measured in two channels through a vertical (I_p) and horizontal (I_h) polarization filter respectively, and the polarization (P) signals are converted to anisotropy (A) that simplifies further calculations.

$$P = (I_p - I_h) / (I_p + I_h) \qquad A = (I_p - I_h) / (I_p + 2I_h)$$

Fluorescence anisotropy can be used to measure ligand binding affinity either directly, or indirectly, by a competitive assay. In direct binding, increasing galectin concentrations are added to a fixed concentration of the fluorescently labeled ligand in solution. In competitive inhibition studies, galectin and the fluorescent probe are mixed in fixed concentrations, while the added inhibitor in increasing concentrations competes with the probe for galectin binding. Obtained anisotropy values can be used to calculate binding constants as explained in [241], but also to estimate the mode of galectin-ligand interaction, number of binding sites and possibly other occurring interactions [52, 242]. Fluorescent polarization was the basic method used for an extensive analysis of ligand-induced galectin-3 self-association (Paper III) and for testing citrus pectin and galactomannan as potential inhibitors of galectin-3 and other galectins (Paper IV).

While trying to round up a story of galectin-3, many other methods had to be used. Some of them will be mentioned as a support to **General discussion**; however all of them are thoroughly explained in appended manuscripts (Papers I-IV).

General discussion

Past 30 years of research still haven't brought us closer to precisely define the function of galectin-3 in macrophages. Nevertheless, by now we know that the code written in glycans, read by galectin-3, translates to specific macrophage functions. Glycan structures carry a set of different information, essential for a macrophage to fulfill its intended purpose. Understanding galectin-3 recognition, fine specificity of its CRD and N-terminus, and cooperative binding, could finally link galectin-3 to relevant macrophage, thus also, other biological functions. Our initial findings on the cellular level urged us to narrow the focus down to galectin-3 molecular interactions in solution. As a result, our understanding of functional properties of galectin-3 was greatly extended, from big to small, and back again.

Galectin-3 from big to small

Initial studies for this thesis focused on galectin-3 trafficking in the established system of M0, M1 and M2 polarized macrophages (Paper I). As a reference point, we first studied the endocytosis of galectin-3 in non-macrophage cell types, HFL1 and SKBR3. Both cell lines could internalize added galectin-3, but also galectin-3 mutant, which contained only the CRD without the N-terminus. Apparently, activity of the N-terminus, like cross-linking, was not necessary for endocytosis. Endocytosis was completely prevented by CRD binding inhibitors, including lactose, a potent synthetic inhibitor (TD139) and a mutation within the galectin-3 CRD (R186S) that made it deficient in binding to LacNAc, common galectin-3 ligand determinant. Another mutant (K176LN180T) with enhanced galectin-3 affinity for poly(LacNAc) residues but decreased for 2, 3 sialylated galactosides was endocytosed equally well as galectin-3 WT. All this was in agreement with previous findings in various cell types showing that galectin-3 endocytosis was mediated by its lectin activity.

To our great surprise this was not the case for differently activated macrophages within our THP-1 derived system. Endocytosis of galectin-3 in M1

macrophages appeared to be completely carbohydrate independent, possibly conveyed by its N-terminus, as we initially assumed. To our even greater surprise, we could observe endocytosis of the galectin-3 mutant that lacked N-terminus, plus remaining CRD had deficient glycan binding (R186S C). This revealed a very unique mechanism of, apparently, receptor mediated endocytosis in M1 polarized cells, without involving the galectin-3 N-terminus and tolerating very weak to none carbohydrate binding. Later, while studying ligand induced galectin-3 self-association (Paper III), we came closer to a possible explanation for the R186S C mutant endocytosis, also bearing in mind previous findings that galectin-3 might take part in the assembly of trafficking vesicles by forming large glycoprotein-galectin-3 clusters [228]. We found that a certain glycoprotein can induce galectin-3 self-association, thus cluster formation, without involving the galectin-3 N-terminus and the CRD affinity for the encountered glycoprotein can be very weak, just enough to initiate the nucleation process. This mode of galectin-3 self-association was named type-C, to distinguish it from the previously proposed modes mediated mainly by the N-terminal domain, which we referred to as type-N.

Type-C self-association could also explain the observed unsaturable uptake of R186S mutant in M1 and M2 cells, even in concentrations higher than found physiologically. Activated macrophages greatly increase the expression of surface galectin-3 ligands, which can mediate rapid internalization of galectin-3 WT. When more and more galectin-3 was added, we could observe the saturation of receptor mediated endocytosis. The R186S mutant instead did not bind to macrophage surface ligands, or bound just enough to initiate (nucleation) type-C self-association. After oligomerisation was initiated, the formed R186S clusters could help the formation of vesicles that would in turn get internalized without signs of saturation.

While studying galectin-3 endocytosis in different macrophages we also observed a difference between classically, M1, and alternatively, M2, polarized macrophages. We found that M2 cells are similar to non-macrophage cells since galectin-3 uptake could be inhibited by carbohydrates, but similar to other macrophage-like cells since R186S mutant was also internalized, but only if it contained its N-terminus. So far we could not connect our findings to any specific macrophage function, but some could be easily suspected. Macrophage phagocytosis was repeatedly connected to galectin-3. Phagocytosis of pathogens and other material is the basic function of inflammatory macrophages and one of the basic mechanisms to sustain tissue homeostasis. If M1 macrophages can utilize

carbohydrate independent galectin-3 endocytosis, it could be the major internalization route for various toxic components recognized by the galectin-3 CRD, like bacterial LPS or LDL. To address this we tested galectin-3 involvement in LDL trafficking within our established macrophage model. The LDL particle contains a single apolipoprotein B-100 and a highly hydrophobic core consisting of polyunsaturated fatty acid and cholesterol molecules. LDL enables transport of cholesterol within the bloodstream and to the cells through LDL surface receptor [243]. For our study we used both native and oxidized LDL, as the latter is involved in the pathogenesis of atherosclerosis. Oxidized LDL undergoes receptor mediated endocytosis and accumulates inside monocytes, forming M2 like, foam cells. Small oxidized lipid components of LDL are potent inducers of reactive oxygen species (ROS) that can trigger cell apoptosis. The death of monocyte-derived foam cells has been shown to promote coagulation and plaque rupture, worsening the prognosis of atherosclerosis [244]. As a part of our ongoing, unpublished work we separated native and oxidized LDL samples in to galectin-3 binding and non-binding fractions using a galectin-3 affinity column (Figure 7) (also explained in detail for haptoglobin separation on a galectin-1 column in Paper II). We found that around 30% of oxidized and 40% of native LDL bound to galectin-3.

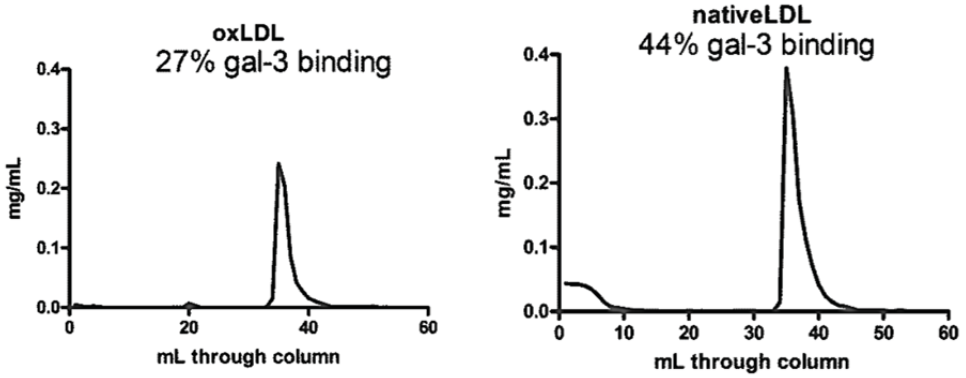

Figure 7. Affinity chromatography of LDL on immobilized galectin-3. Galectin-3 was coupled to a NHS-activated Hi-Trap affinity column in a bicarbonate coupling buffer. LDL (native and oxidized) samples were circulated on the column for 30 min in cold and sealed for additional 30 min to enhance binding. The column was washed with PBS and fractions of the unbound LDL (flow through) were saved. Galectin-3 binding LDL was eluted with 150 mM lactose. Protein concentrations of collected fractions were determined by protein assay and plotted as chromatogram in GraphPad Prism software.

After separation, LDL was labeled with a red lipid dye and added to different macrophages for endocytosis (Figure 8). All cells in our system, M0, M1 and M2,

internalized both oxidized and native LDL, but we could not see the difference in the amount or localization between galectin-3 binding and non-binding LDL.

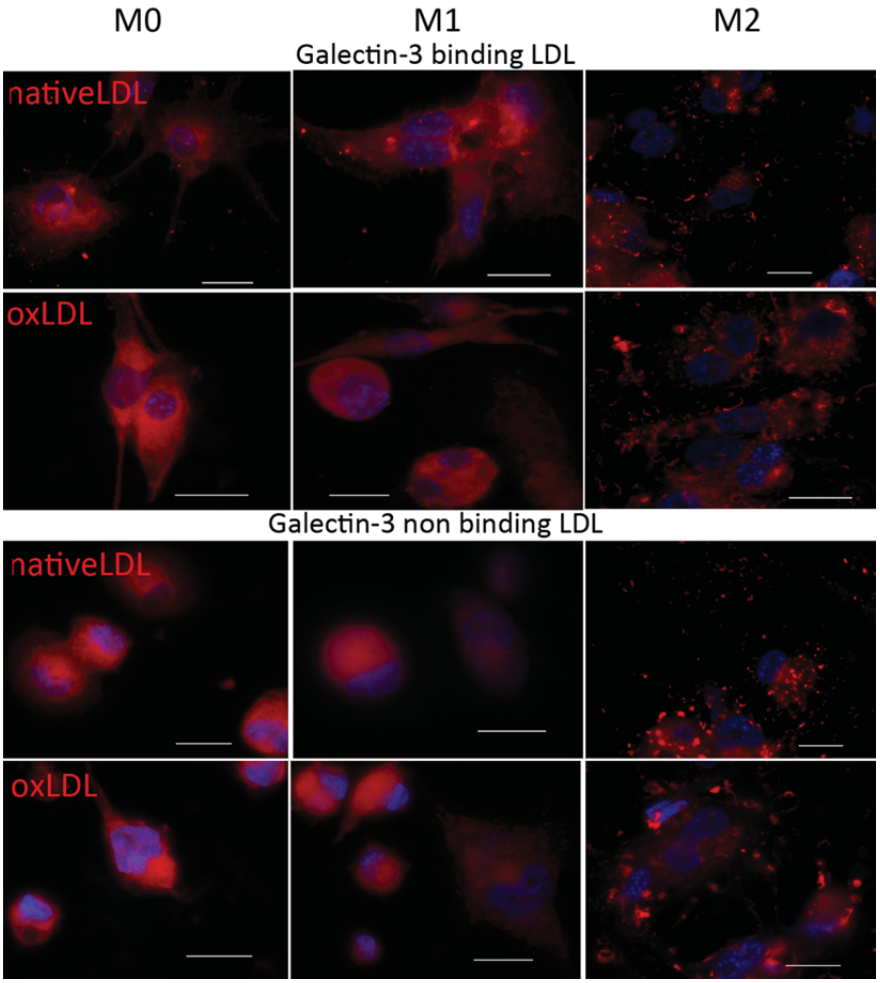

Figure 8. Endocytosis of galctin-3 affinity column separated LDL in differently activated macrophages. After separating both native and oxidized LDL into fractions that bind or do not bind galectin-3, as explained in *Figure 7*, all 4 different LDL samples were labeled with fluorescent red lipid dye (Nile red). Unreacted dye was separated by size exclusion chromatography. Labeled LDL samples were independently added to M0, M1 and M2 macrophages (differentiated as explained in *Paper I*). After 20 min of endocytosis, cells were washed with cold PBS, fixed with paraformaldehyde and nuclei were stained with blue DNA dye (Hoechst). Endocytosed LDL particles were observed and the images captured using Nikon eclipse TE2000-U fluorescence microscope equipped with a digital still camera. Bar is 10 μm.

Perhaps galectin-3 regulation of LDL trafficking is much more subtle than the detection limit of our current methods. By using LDL we wanted to see if there was a difference in endocytosis between the galectin-3 binding and non-binding fraction, while using a model glycoprotein, asialofetuin (ASF), we wanted to see if galectin-3 inhibitor could alter the endocytosis of unfractionated ASF. Macrophages can easily internalize ASF and galectin-3 was shown to bind to at least some of possible binding sites on ASF. Assuming that galectin-3 directs ASF trafficking through its lectin activity, in our ongoing studies we tested galectin-3 glycan binding inhibitors, lactose and TD139. The results have not yet been published. ASF internalization in M1 and M2 macrophages remained uninterrupted despite the galectin-3 inhibitors treatment. Allowing for our novel finding that not all extracellular functions of galectin-3 are mediated by its CRD, but N-terminus could also be involved, and the fact that there might be an additional, non-glycan macrophage receptor for galectin-3, it becomes easier to reconcile uninhibited internalization of ASF, even if it was directed by galectin-3.

Nevertheless, we observed a great importance of M2 macrophages in clearing toxic components from the cellular environment coupled with another galectin family member, galectin-1 (Paper II). After hemolysis, circulating free hemoglobin poses a toxic threat to cells. Haptoglobin forms a tight complex with hemoglobin that allows binding to the scavenger receptor CD163 on M2 macrophages, leading to endocytosis of the complex and its neutralization [245]. We showed that galectin-1 can bind a certain haptoglobin glycoform, that is approximately 30% of the hemoglobin-haptoglobin complex. After endocytosis, the galectin-1 bound fraction is diverted from degradation to another, still poorly defined pathway. In cancer, almost 80% of hemoglobin takes this pathway of scavenging by haptoglobin [35]. Since M2 skewed macrophages are often related to cancer, increased binding of haptoglobin-hemoglobin to galectin-1 could steer the complex from degradation to, perhaps, a recycling pathway, allowing for the angiogenic activity of haemoglobin, thus promoting cancer growth and metastasis.

Another major role of M2 macrophages is in tissue rebuilding after inflammation, or otherwise caused injury, by sending a repair signal to the surrounding cells. Fibroblasts around a macrophage-infiltrated healing wound or fibrotic tissue secrete higher levels of ECM components and proliferate more than fibroblasts from healthy tissues [246]. If the tissue repair balance gets distorted, the consequence might be tissue scaring, like it is often seen in asthma. There are numerous findings of M2 macrophages in vicinity of lung fibroblast, and it is known that both cell types are involved in lung fibrosis [247]. Finding a link

between the two could provide a therapeutic target for diseases presented with fibrosis and excessive scaring. This again puts galectin-3 in the focus, since it was shown that galectin-3 can stimulate the proliferation of fibroblasts through several mechanisms; by attachment to fibroblast cell surface glycoconjugates, binding to intracellular receptors [248] or by inducing the secretion of inflammatory cytokines from macrophages [214]. We previously reported increased galectin-3 expression in activated macrophages, coupled with galectin-3 secretion. Now, we wanted to investigate macrophage-fibroblasts interplay using THP-1 derived macrophages and HFL1 fibroblasts. In our preliminary, unpublished observations, fibroblasts growing in the M2 macrophage conditioned media increased their galectin-3 content by approximately 40% compared to the control fibroblasts (Figure 9). This increase could be due to galectin-3 endocytosis from the conditioned media, or *de novo* synthesis by HFL1 fibroblasts.

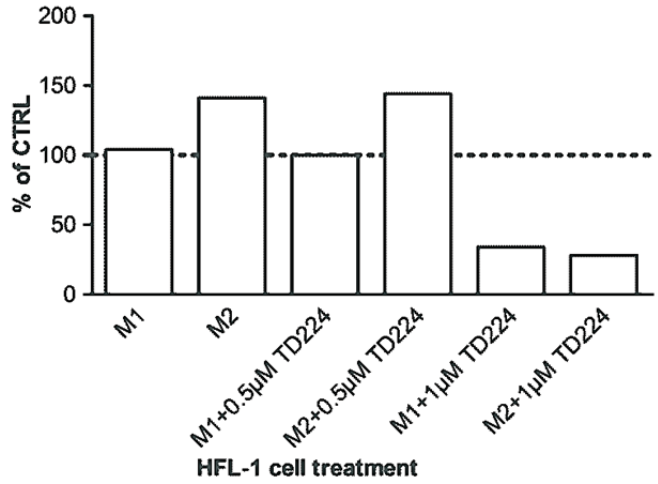

Figure 9. Galectin-3 content in HFL1 fibroblasts. HFL1 cells were grown to 80% confluence and treated for 96 h with different macrophages' supernatants, supplemented or not with galectin-3 inhibitor, TD139. Treated fibroblasts were collected for SDS-PAGE protein separation, followed by Western blot detection of galectin-3. The density of galectin-3 protein bands was measured using ImageJ freeware, then related to house-keeping protein, GAPDH and compared between treated and control fibroblasts. The results are plotted as the % of the control (fibroblasts in cell medium for 96h).

Moreover, in our ongoing work we detected changes in the formation of stress actin fibers, collagen1 deposition (Figure 10) and metabolic activity (Figure 11) of fibroblasts, as a response to M1 and M2 macrophages. Several changes could be reversed if the conditioned media was supplemented with galectin-3 inhibitor, TD139.

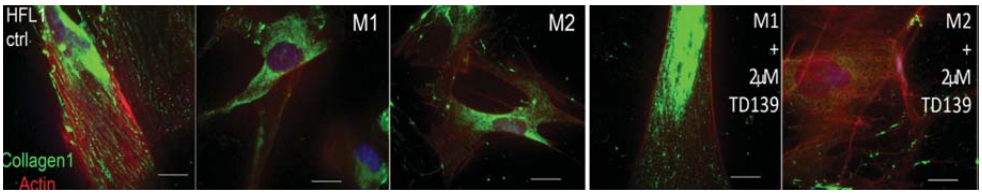

Figure 10. Collagen1 and actin immunostaining in HFL1 fibroblasts. HFL1 cells were grown and treated as explained in *Figure 9.* After treatment, the cells were washed, fixed and permeabilised to allow staining with anti-collagen antibodies (green fluorescence) and actin staining with red fluorescent Phalloidin. Nuclei were stained with Hoechst (blue). Fluorescence in 3 different channels was observed using different microscope filters, and captured images of all the channels (green, red and blue) were merged into a single image using NIS-elementAR software. Bar is 10 μm.

Galectin-3 or TD139 alone had a less prominent effect on measured fibroblast features.

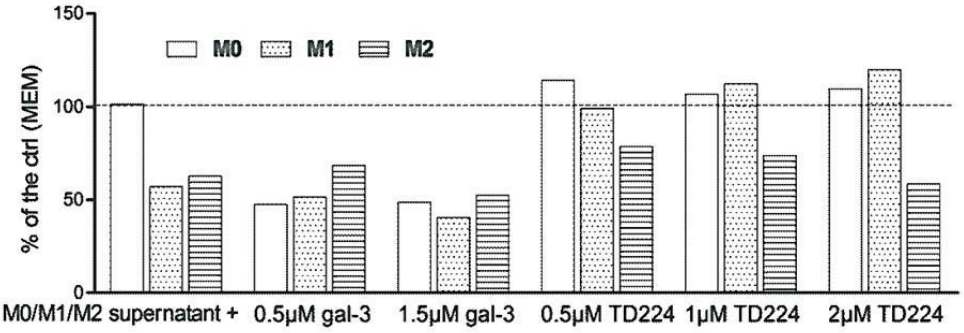

Figure 11. Metabolic activity of HFL1 fibroblasts. Cell viability was determined by MTT assay after the cells were treated for 3 days with supernatants alone or supplemented with galectin-3 or TD139. An MTT solution was added to cells and incubated at 37˚C for 1 h. Formed formazan crystals were dissolved and absorbance was read at 570 nm. Viability was expressed as a percentage of the control (untreated HFL1 cells).

So far, the results of our uncompleted studies indicate that certain roles of galectin-3 could be displayed only within a cellular context, possibly relying on some other cell products. Again, our later findings about galectin-3 interactions in solution could explain the former, since we observed that galectin-3 alone behaves differently than when it encounters a ligand. Moreover, only a certain type of ligand can induce specific galectin-3 actions. Galectin-3 in solution was found to be monomeric, even in very high concentrations. However, a certain type of multivalent ligand, e.g. the glycoprotein ASF, induced galectin-3 self-association within seconds (Paper III). Since galectin-3 self-association can occur by several different mechanisms it can fit in different biological settings, each carrying

unique functions. Previous reports suggested galectin-3 pentamer formation trough the N-terminus, and subsequent glycan cross-linking through available CRDs [90]. Another former study suggested galectin-3 dimer formation via 2 facing CRDs [104], thus making them unavailable for further glycan binding. In the model that we propose, galectin-3 self-association occurs through its CRD, but just after the initial glycan binding, additional CRD to CRD interaction may occur outside the glycan binding groove. Moreover, the N-terminus remains available for cross-linking since it is not necessary for this type of galectin-3 oligomerisation. Different available ligands in macrophages, fibroblasts or other cells can induce different types of self-association, or simultaneously, their combination, underlying various processes, e.g. carbohydrate independent endocytosis of galectin-3 in M2 cells, or carbohydrate dependent effects on fibroblasts.

Other observations made while studying galectin-3 interactions in solution and self-association (Paper III) could be used for further interpretation of galectin-3 functions, within a novel framework of type-C self-association. We found that only a certain type of complex ligand can induce CRD dependent, type-C self-association. It can thus be assumed that cells would present different glycan determinants if the desired effect requires galectin-3 type-C self-association, or type-N self-association and possible surface receptor cross-linking or simple one to one binding. Small saccharides, even the branched, polyvalent ones, could not induce type-C self-association, unlike glycoprotein ligand, ASF. It must be that some particular feature of ASF compared to its component galectin-3 binding sites is prerequisite for type-C self-association. Nevertheless, galectin-3 binding to branched saccharides could induce type-N self-association, leading to ligand cross-linking and complex precipitation.

Another intriguing observation was that the R186S mutant, with > 30-fold reduced affinity for ASF, still showed strong type-C self-association. Thus, ASF-induced nucleation of galectin-3 type-C self-association occurs even with low or no binding to glycans, but is much more efficient and occurs at much lower galectin concentration if higher affinity glycan binding also occurs, as with galectin-3 WT. This may be particularly favored in the cytosol and nucleus, where no galactoside ligands have been found. Perhaps some other nucleating ligand resides there instead. A good candidate for cytosolic nucleating agent could be anti-apoptotic Bcl-2. Even though Bcl-2 is not a glycoprotein, its interaction with galectin-3 could be inhibited by lactose [121]. It could be that this interaction involves type-C galectin-3 oligomers clustered with Bcl-2.

Coming back to galectin-3 controlled phagocytosis in macrophages; it could be assumed that it is, at least in part, conveyed through type-C galectin-3 self-

association. Namely, it was often reported how phagocytosis could not be inhibited by added lactose, possibly because the nucleating agent was residing hidden in some cytosolic compartment, out of reach of externally added lactose. This would allow for uninterrupted galectin-3 self-association and subsequent formation of phagocytic cups and phagosomes. Reported rapid accumulation of cytosolic galectin-3 around phagosomes lysed by bacteria may also be due to type-C self-association nucleated by glycoproteins normally inside the phagosome [231]. This process could serve as a defense and/or membrane repair signal. Bacterial LPS might also induce galectin-3 self-association since both galectin-3 CRD and the N-terminus interact with LPS. Different bacteria have a particular repertoire of glycan and lipid structures within their LPS that not all bind galectin-3; hence, LPS may or may not support some types of galectin-3 self-association. On one hand, LPS induced type-C oligomerisation may lead to LPS encapsulation, phagocytosis and neutralization in macrophages, as reported for galectin-3 KO mice that experienced septic shock when galectin-3 was not present to sequester LPS [131]. On the other hand, LPS binding trough N-terminus leaves CRDs free to interact with surface neutrophil receptors, which can decrease the LPS concentration threshold for neutrophil activation [132]. Nevertheless, in our preliminary experiments, LPS was unable to induce galectin-3 type-C self-association, even in very high concentration. Since we could not characterize LPS structure in detail, it is possible that the LPS from our *E. coli* source lacked required determinants to induce the necessary nucleation process.

Particular features of type-C galectin-3 self-association may favor galectin-3 trafficking. We already showed that galectin-3 can enhance its own uptake in macrophages (Paper I and III), probably after induced type-C self-association upon cell surface binding. This way large amounts of associated galectin-3 molecules can be transported over the cell membrane, compared to the limited amount transported by one to one ligand binding or type-N oligomerisation with restricted number of associated galectin-3 molecules. Galectin-3 involvement in the sorting of glycoproteins was also connected to its property to form oligomers [228]. In our view, type-C self-association could easily engage enough galectin-3 molecules to support the proposed mechanism involving high molecular weight clusters. The clusters should contain sufficient number of galetin-3 molecules with hydrophobic tails encapsulating the protein that is sorted, to enable the formation of functional trafficking vesicles. The suggested mechanism could hardly be supported by galectin-3 pentamers with occupied N-terminal parts, or even galectin-3 dimers where two CRDs face each other and do not allow initial, or subsequent lectin-type interactions.

Finally, considering all its possible functions in health and disease, galectin-3 is an attractive drug/therapy candidate. Galectin-3 based therapies could target its CRD activity, its expression, or they could use soluble galectin-3 protein or galectin-3 cDNA. In this work we focused primarily on CRD inhibitors. Lactose, TD139 or anti-galectin-3 antibody directed against N-terminus, served mainly in control experiments while studying galectin-3 dependent cellular effects. On the other hand, Pectasol-C, Fractionated Pectin Powder (FPP) and Davanat were tested as potential therapeutic agents, commonly used as dietary supplements (Paper IV). These plant products were often claimed to be galectin-3 CRD inhibitors with anti-cancer or anti-inflammatory properties [127, 161]. This was not unreasonable from a structural point of view, as pectin and galactomannan (Davanat) are complex polysaccharides that usually contain β-galactose side chains as potential galectin-3 ligands. However, the actual interaction between galectin-3 and the biologically active pectin or galactomannan has been analysed biochemically to only a limited extent. To test their inhibiting potential, we applied the fluorescence anisotropy assay and measured their interaction with galectin-3 and other galectins in solution. Unlike many reports so far, we found that Davanat, Pectasol-C and FPP are very weak inhibitors of galectins at physiological concentrations in solution. Therefore, the connection of their biological effects to inhibiting galectin carbohydrate-binding activity is questionable. It is still possible that Pectasol-C and other similar plant products suppress cancer [161] or kidney injury [249] as reported, perhaps by interfering with some regulatory loop or signaling system, rather than by inhibiting galectin-3 itself.

Even though it is of great interest to find reliable, potent and easily available galectin-3 inhibitors, the quest has been mostly unsuccessful. Often, very potent inhibitors as TD139 have to go a long way before any clinical use, while commonly used plant products turn out to be very poor inhibitors. Nevertheless, finding biological targets for these dietary supplements is of interest, but the search could benefit from looking past galectin-3.

In conclusion

The story about the galectin family and their journey through the enigmatic glycan forest is told in a very complex language. Each new translated page unlocks a bit of knowledge about each family member and their role in biology and pathophysiology. At the end of the story we might understand their central

physiological function, if there is one, but the pages before could hold a key for novel disease treatment strategies, diagnosis tools, drug targets, and to my great hope, a key for disease prevention.

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References

1. Stone, W.E., *The Use of the Term "Carbohydrates."* Science, 1893. **21**(528): p. 149-50.
2. Varki, A., Nothing in glycobiology makes sense, except in the light of evolution. Cell, 2006. **126**(5): p. 841-5.
3. Cummings, R.D., The repertoire of glycan determinants in the human glycome. Mol Biosyst, 2009. **5**(10): p. 1087-104.
4. Nagai, Y., Glycobiology in the 21st century: coming developments in glycobiology. Glycoconj J, 2002. **19**(3): p. 161-3.
5. Varki A, L.J., *Biological Roles of Glycans.*, in *Essentials of Glycobiology*, C.R. Varki A, Esko JD, et al, Editor. 2009, Cold Spring Harbor Laboratory Press: Cold Spring Harbor (NY).
6. Henrissat B, S.A., Stanley P., *A Genomic View of Glycobiology.*, in *Essentials of Glycobiology*, C.R. Varki A, Esko JD, et al., Editor. 2009, Cold Spring Harbor Laboratory Press: Cold Spring Harbor (NY).
7. Freeze, H.H., *Genetic defects in the human glycome.* Nat Rev Genet, 2006. **7**(7): p. 537-51.
8. Vogt, G., et al., Gains of glycosylation comprise an unexpectedly large group of pathogenic mutations. Nat Genet, 2005. **37**(7): p. 692-700.
9. Schwarz, F. and M. Aebi, *Mechanisms and principles of N-linked protein glycosylation.* Curr Opin Struct Biol, 2011. **21**(5): p. 576-82.
10. Stanley P, S.H., Taniguchi N., *N-Glycans*, in *Essentials of Glycobiology*, C.R. Varki A, Esko JD, et al., Editor. 2009, Cold Spring Harbor Laboratory Press: Cold Spring Harbor (NY).
11. Tabak, L.A., *The role of mucin-type O-glycans in eukaryotic development.* Semin Cell Dev Biol, 2010. **21**(6): p. 616-21.
12. Brockhausen I, S.H., Stanley P., *O-GalNAc Glycans.*, in *Essentials of Glycobiology*, E.J. Varki A, et al., Editor. 2009, Cold Spring Harbor Laboratory Press.: NY.
13. Stanley P, C.R., *Structures Common to Different Glycans*, in *Essentials of Glycobiology*, C.R. Varki A, Esko JD, et al., Editor. 2009, Cold Spring Harbor Laboratory Press: Cold Spring Harbor (NY).
14. Paulson, J.C., O. Blixt, and B.E. Collins, *Sweet spots in functional glycomics.* Nat Chem Biol, 2006. **2**(5): p. 238-48.
15. Carlsson, M.C., From serum to tissue: Galectin-binding glycoforms of serum glycoproteins as functional biomarkers, in Laboratory Medicine. 2012, Lund University: Lund.
16. Varki A, S.R., *Sialic Acids*, in *Essentials of Glycobiology*, C.R. Varki A, Esko JD, et al., Editor. 2009, Cold Spring Harbor Laboratory Press: Cold Sprin Harbor (NY).

17. de Oliveira, J.T., et al., Sialylation regulates galectin-3/ligand interplay during mammary tumour progression--a case of targeted uncloaking. Int J Dev Biol, 2011. **55**(7-9): p. 823-34.
18. Hirabayashi, J., et al., Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim Biophys Acta, 2002. **1572**(2-3): p. 232-54.
19. Stowell, S.R., et al., Galectin-1, -2, and -3 exhibit differential recognition of sialylated glycans and blood group antigens. J Biol Chem, 2008. **283**(15): p. 10109-23.
20. Zhuo, Y. and S.L. Bellis, Emerging role of alpha2,6-sialic acid as a negative regulator of galectin binding and function. J Biol Chem, 2011. **286**(8): p. 5935-41.
21. Gornik, O., et al., *Stability of N-glycan profiles in human plasma.* Glycobiology, 2009. **19**(12): p. 1547-53.
22. Knezevic, A., et al., Effects of aging, body mass index, plasma lipid profiles, and smoking on human plasma N-glycans. Glycobiology, 2010. **20**(8): p. 959-69.
23. Jaenisch, R. and A. Bird, Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet, 2003. **33 Suppl**: p. 245-54.
24. Zoldos, V., et al., Genomics and epigenomics of the human glycome. Glycoconj J, 2012.
25. Thiel, C. and C. Korner, *Mouse models for congenital disorders of glycosylation.* J Inherit Metab Dis, 2011. **34**(4): p. 879-89.
26. Jaeken, J., *Congenital disorders of glycosylation.* Ann N Y Acad Sci, 2010. **1214**: p. 190-8.
27. Cazet, A., et al., *Tumour-associated carbohydrate antigens in breast cancer.* Breast Cancer Res, 2010. **12**(3): p. 204.
28. van Kooyk, Y. and G.A. Rabinovich, Protein-glycan interactions in the control of innate and adaptive immune responses. Nat Immunol, 2008. **9**(6): p. 593-601.
29. Lowe, J.B., *Glycosylation, immunity, and autoimmunity.* Cell, 2001. **104**(6): p. 809-12.
30. Marth, J.D. and P.K. Grewal, *Mammalian glycosylation in immunity.* Nat Rev Immunol, 2008. **8**(11): p. 874-87.
31. Vicente Miranda, H. and T.F. Outeiro, The sour side of neurodegenerative disorders: the effects of protein glycation. J Pathol, 2010. **221**(1): p. 13-25.
32. Varki A, F.H., *Glycans in Acquired Human Diseases.*, in *Essentials of Glycobiology.*, C.R. Varki A, Esko JD, et al., Editor. 2009, Cold Spring Harbor Laboratory Press: Cold Spring Harbor (NY).
33. Xie, L., et al., The expressions and clinical significances of tissue and serum galectin-3 in pancreatic carcinoma. J Cancer Res Clin Oncol, 2012. **138**(6): p. 1035-43.
34. Carlsson, M.C., et al., Different fractions of human serum glycoproteins bind galectin-1 or galectin-8, and their ratio may provide a refined biomarker for pathophysiological conditions in cancer and inflammatory disease. Biochim Biophys Acta, 2012. **1820**(9): p. 1366-72.
35. Carlsson, M.C., et al., Galectin-1-binding glycoforms of haptoglobin with altered intracellular trafficking, and increase in metastatic breast cancer patients. PLoS One, 2011. **6**(10): p. e26560.

36. Levi, G. and V.I. Teichberg, Isolation and physicochemical characterization of electrolectin, a beta-D-galactoside binding lectin from the electric organ of Electrophorus electricus. J Biol Chem, 1981. **256**(11): p. 5735-40.
37. Drickamer, K., Two distinct classes of carbohydrate-recognition domains in animal lectins. J Biol Chem, 1988. **263**(20): p. 9557-60.
38. Hirabayashi, J. and K. Kasai, The family of metazoan metal-independent beta-galactoside-binding lectins: structure, function and molecular evolution. Glycobiology, 1993. **3**(4): p. 297-304.
39. Bachar-Lustig, E., Y. Gan, and Y. Reisner, Purification in large amounts of beta-D-galactoside-binding lectins from a murine thymic epithelial cell line. Carbohydr Res, 1991. **213**: p. 345-52.
40. Harrison, F.L., Soluble vertebrate lectins: ubiquitous but inscrutable proteins. J Cell Sci, 1991. **100 (Pt 1)**: p. 9-14.
41. Allen, H.J., et al., Synthesis, isolation, and characterization of endogenous beta-galactoside-binding lectins in human leukocytes. Biochemistry, 1991. **30**(36): p. 8904-10.
42. Frigeri, L.G. and F.T. Liu, Surface expression of functional IgE binding protein, an endogenous lectin, on mast cells and macrophages. J Immunol, 1992. **148**(3): p. 861-7.
43. Liu, F.T., et al., Expression and function of an IgE-binding animal lectin (epsilon BP) in mast cells. Immunopharmacology, 1993. **26**(3): p. 187-95.
44. Nangia-Makker, P., et al., Regulation of the expression of galactoside-binding lectin during human monocytic differentiation. Cancer Res, 1993. **53**(20): p. 5033-7.
45. Hughes, R.C., Mac-2: a versatile galactose-binding protein of mammalian tissues. Glycobiology, 1994. **4**(1): p. 5-12.
46. Barondes, S.H., et al., Galectins: a family of animal beta-galactoside-binding lectins. Cell, 1994. **76**(4): p. 597-8.
47. Barondes, S.H., et al., Galectins. Structure and function of a large family of animal lectins. J Biol Chem, 1994. **269**(33): p. 20807-10.
48. Yang, R.Y., G.A. Rabinovich, and F.T. Liu, *Galectins: structure, function and therapeutic potential.* Expert Rev Mol Med, 2008. **10**: p. e17.
49. Di Lella, S., et al., When galectins recognize glycans: from biochemistry to physiology and back again. Biochemistry, 2011. **50**(37): p. 7842-57.
50. Cummings RD, L.F., *Galectins*, in *Essentials of Glycobiology*, C.R. Varki A, Esko JD, et al., Editor. 2009, Cold Spring Harbor Laboratory Press: Cold Spring Harbor (NY).
51. Liao, D.I., et al., Structure of S-lectin, a developmentally regulated vertebrate beta-galactoside-binding protein. Proc Natl Acad Sci U S A, 1994. **91**(4): p. 1428-32.
52. Salomonsson, E., et al., *Mutational tuning of galectin-3 specificity and biological function.* The Journal of biological chemistry, 2010. **285**(45): p. 35079-91.
53. Vasta, G.R., Galectins as pattern recognition receptors: structure, function, and evolution. Adv Exp Med Biol, 2012. **946**: p. 21-36.
54. Cerliani, J.P., et al., Expanding the universe of cytokines and pattern recognition receptors: galectins and glycans in innate immunity. J Clin Immunol, 2011. **31**(1): p. 10-21.

55. Leffler, H., et al., *Introduction to galectins.* Glycoconj J, 2004. **19**(7-9): p. 433-40.
56. Walser, P.J., et al., *Structure and functional analysis of the fungal galectin CGL2.* Structure, 2004. **12**(4): p. 689-702.
57. Dayhoff, J.E., et al., *Evolution of protein binding modes in homooligomers.* J Mol Biol, 2010. **395**(4): p. 860-70.
58. Carlsson, S., et al., Affinity of galectin-8 and its carbohydrate recognition domains for ligands in solution and at the cell surface. Glycobiology, 2007. **17**(6): p. 663-76.
59. Smith, D.F., X. Song, and R.D. Cummings, *Use of glycan microarrays to explore specificity of glycan-binding proteins.* Methods Enzymol, 2010. **480**: p. 417-44.
60. Di Lella, S., et al., Linking the structure and thermal stability of beta-galactoside-binding protein galectin-1 to ligand binding and dimerization equilibria. Biochemistry, 2010. **49**(35): p. 7652-8.
61. Liu, F.T., R.J. Patterson, and J.L. Wang, *Intracellular functions of galectins.* Biochim Biophys Acta, 2002. **1572**(2-3): p. 263-73.
62. Hughes, R.C., The galectin family of mammalian carbohydrate-binding molecules. Biochem Soc Trans, 1997. **25**(4): p. 1194-8.
63. Nickel, W., The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. Eur J Biochem, 2003. **270**(10): p. 2109-19.
64. Hughes, R.C., Secretion of the galectin family of mammalian carbohydrate-binding proteins. Biochim Biophys Acta, 1999. **1473**(1): p. 172-85.
65. Hsu, D.K., H.Y. Chen, and F.T. Liu, *Galectin-3 regulates T-cell functions.* Immunol Rev, 2009. **230**(1): p. 114-27.
66. Acosta-Rodriguez, E.V., et al., Galectin-3 mediates IL-4-induced survival and differentiation of B cells: functional cross-talk and implications during Trypanosoma cruzi infection. J Immunol, 2004. **172**(1): p. 493-502.
67. Stillman, B.N., et al., Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. J Immunol, 2006. **176**(2): p. 778-89.
68. Ho, M.K. and T.A. Springer, Mac-2, a novel 32,000 Mr mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies. J Immunol, 1982. **128**(3): p. 1221-8.
69. Roff, C.F. and J.L. Wang, Endogenous lectins from cultured cells. Isolation and characterization of carbohydrate-binding proteins from 3T3 fibroblasts. J Biol Chem, 1983. **258**(17): p. 10657-63.
70. Crittenden, S.L., C.F. Roff, and J.L. Wang, Carbohydrate-binding protein 35: identification of the galactose-specific lectin in various tissues of mice. Mol Cell Biol, 1984. **4**(7): p. 1252-9.
71. Moutsatsos, I.K., J.M. Davis, and J.L. Wang, Endogenous lectins from cultured cells: subcellular localization of carbohydrate-binding protein 35 in 3T3 fibroblasts. J Cell Biol, 1986. **102**(2): p. 477-83.
72. Moutsatsos, I.K., et al., Endogenous lectins from cultured cells: nuclear localization of carbohydrate-binding protein 35 in proliferating 3T3 fibroblasts. Proc Natl Acad Sci U S A, 1987. **84**(18): p. 6452-6.
73. Cowles, E.A., et al., Expression of carbohydrate binding protein 35 in human fibroblasts: comparisons between cells with different proliferative capacities. Exp Gerontol, 1989. **24**(5-6): p. 577-85.

74. Nabarra, B. and M. Papiernik, Phenotype of thymic stromal cells. An immunoelectron microscopic study with anti-IA, anti-MAC-1, and anti-MAC-2 antibodies. Lab Invest, 1988. **58**(5): p. 524-31.
75. Jia, S., et al., Carbohydrate-binding protein 35: molecular cloning and expression of a recombinant polypeptide with lectin activity in Escherichia coli. Gene, 1987. **60**(2-3): p. 197-204.
76. Yurochko, A.D., R.H. Pyle, and K.D. Elgert, Changes in macrophage populations: phenotypic differences between normal and tumor-bearing host macrophages. Immunobiology, 1989. **178**(4-5): p. 416-35.
77. Nanno, M., et al., Increased production of cytotoxic macrophage progenitors by Lactobacillus casei in mice. J Leukoc Biol, 1989. **46**(2): p. 89-95.
78. Laing, J.G., et al., Biochemical and immunological comparisons of carbohydrate-binding protein 35 and an IgE-binding protein. J Biol Chem, 1989. **264**(4): p. 1097-10.
79. Cherayil, B.J., S.J. Weiner, and S. Pillai, *The Mac-2 antigen is a galactose-specific lectin that binds IgE.* J Exp Med, 1989. **170**(6): p. 1959-72.
80. Woo, H.J., et al., The major non-integrin laminin binding protein of macrophages is identical to carbohydrate binding protein 35 (Mac-2). J Biol Chem, 1990. **265**(13): p. 7097-9.
81. Barondes, S.H., et al., *Multiple soluble vertebrate galactoside-binding lectins.* Biochimie, 1988. **70**(11): p. 1627-32.
82. Raz, A., G. Pazerini, and P. Carmi, Identification of the metastasis-associated, galactoside-binding lectin as a chimeric gene product with homology to an IgE-binding protein. Cancer Res, 1989. **49**(13): p. 3489-93.
83. Liu, F.T., Molecular biology of IgE-binding protein, IgE-binding factors, and IgE receptors. Crit Rev Immunol, 1990. **10**(3): p. 289-306.
84. Woo, H.J., et al., Carbohydrate-binding protein 35 (Mac-2), a laminin-binding lectin, forms functional dimers using cysteine 186. J Biol Chem, 1991. **266**(28): p. 18419-22.
85. Liu, F.T., *S-type mammalian lectins in allergic inflammation.* Immunol Today, 1993. **14**(10): p. 486-90.
86. Frigeri, L.G., M.W. Robertson, and F.T. Liu, Expression of biologically active recombinant rat IgE-binding protein in Escherichia coli. J Biol Chem, 1990. **265**(34): p. 20763-9.
87. Dumic, J., S. Dabelic, and M. Flogel, *Galectin-3: an open-ended story.* Biochim Biophys Acta, 2006. **1760**(4): p. 616-35.
88. Barboni, E.A., et al., Molecular modeling and mutagenesis studies of the N-terminal domains of galectin-3: evidence for participation with the C-terminal carbohydrate recognition domain in oligosaccharide binding. Glycobiology, 2000. **10**(11): p. 1201-8.
89. Birdsall, B., et al., NMR solution studies of hamster galectin-3 and electron microscopic visualization of surface-adsorbed complexes: evidence for interactions between the N- and C-terminal domains. Biochemistry, 2001. **40**(15): p. 4859-66.
90. Ahmad, N., et al., Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. The Journal of biological chemistry, 2004. **279**(12): p. 10841-7.

91. Massa, S.M., et al., L-29, an endogenous lectin, binds to glycoconjugate ligands with positive cooperativity. Biochemistry, 1993. **32**(1): p. 260-7.
92. Demetriou, M., et al., Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. Nature, 2001. **409**(6821): p. 733-9.
93. Chen, H.Y., et al., Galectin-3 negatively regulates TCR-mediated CD4+ T-cell activation at the immunological synapse. Proc Natl Acad Sci U S A, 2009. **106**(34): p. 14496-501.
94. Gong, H.C., et al., The NH2 terminus of galectin-3 governs cellular compartmentalization and functions in cancer cells. Cancer Res, 1999. **59**(24): p. 6239-45.
95. Mehul, B. and R.C. Hughes, Plasma membrane targetting, vesicular budding and release of galectin 3 from the cytoplasm of mammalian cells during secretion. J Cell Sci, 1997. **110 (Pt 10)**: p. 1169-78.
96. Menon, R.P. and R.C. Hughes, Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-Golgi complex. Eur J Biochem, 1999. **264**(2): p. 569-76.
97. Yoshii, T., et al., Galectin-3 phosphorylation is required for its anti-apoptotic function and cell cycle arrest. J Biol Chem, 2002. **277**(9): p. 6852-7.
98. Akahani, S., et al., Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. Cancer Res, 1997. **57**(23): p. 5272-6.
99. Seetharaman, J., et al., X-ray crystal structure of the human galectin-3 carbohydrate recognition domain at 2.1-A resolution. J Biol Chem, 1998. **273**(21): p. 13047-52.
100. Saraboji, K., et al., The carbohydrate-binding site in galectin-3 is preorganized to recognize a sugarlike framework of oxygens: ultra-high-resolution structures and water dynamics. Biochemistry, 2012. **51**(1): p. 296-306.
101. Gauto, D.F., et al., Carbohydrate-binding proteins: Dissecting ligand structures through solvent environment occupancy. J Phys Chem B, 2009. **113**(25): p. 8717-24.
102. Ahmad, M., et al., Adhesive water networks facilitate binding of protein interfaces. Nat Commun, 2011. **2**: p. 261.
103. Zhou, Y., H. Zhou, and M. Karplus, Cooperativity in Scapharca dimeric hemoglobin: simulation of binding intermediates and elucidation of the role of interfacial water. J Mol Biol, 2003. **326**(2): p. 593-606.
104. Yang, R.Y., et al., Role of the carboxyl-terminal lectin domain in self-association of galectin-3. Biochemistry, 1998. **37**(12): p. 4086-92.
105. Sato, S. and R.C. Hughes, Binding specificity of a baby hamster kidney lectin for H type I and II chains, polylactosamine glycans, and appropriately glycosylated forms of laminin and fibronectin. J Biol Chem, 1992. **267**(10): p. 6983-90.
106. Braeuer, R.R., et al., The Sweet and Bitter Sides of Galectins in Melanoma Progression. Pigment Cell Melanoma Res, 2012.
107. Lau, K.S. and J.W. Dennis, *N-Glycans in cancer progression.* Glycobiology, 2008. **18**(10): p. 750-60.
108. John, C.M., et al., Galectin-3 binds lactosaminylated lipooligosaccharides from Neisseria gonorrhoeae and is selectively expressed by mucosal epithelial cells that are infected. Cell Microbiol, 2002. **4**(10): p. 649-62.
109. van den Berg, T.K., et al., LacdiNAc-glycans constitute a parasite pattern for galectin-3-mediated immune recognition. J Immunol, 2004. **173**(3): p. 1902-7.

110. Schneider, D., et al., Trafficking of galectin-3 through endosomal organelles of polarized and non-polarized cells. Eur J Cell Biol, 2010. **89**(11): p. 788-98.
111. Delacour, D., A. Koch, and R. Jacob, *The role of galectins in protein trafficking.* Traffic, 2009. **10**(10): p. 1405-13.
112. Mazurek, N., et al., Cell-surface galectin-3 confers resistance to TRAIL by impeding trafficking of death receptors in metastatic colon adenocarcinoma cells. Cell Death Differ, 2012. **19**(3): p. 523-33.
113. Carlsson, S., M.C. Carlsson, and H. Leffler, *Intracellular sorting of galectin-8 based on carbohydrate fine specificity.* Glycobiology, 2007. **17**(9): p. 906-12.
114. Karlsson, A., et al., Galectin-3 activates the NADPH-oxidase in exudated but not peripheral blood neutrophils. Blood, 1998. **91**(9): p. 3430-8.
115. Lin, C.I., et al., Galectin-3 targeted therapy with a small molecule inhibitor activates apoptosis and enhances both chemosensitivity and radiosensitivity in papillary thyroid cancer. Mol Cancer Res, 2009. **7**(10): p. 1655-62.
116. Zhuo, Y., R. Chammas, and S.L. Bellis, Sialylation of beta1 integrins blocks cell adhesion to galectin-3 and protects cells against galectin-3-induced apoptosis. J Biol Chem, 2008. **283**(32): p. 22177-85.
117. Suzuki, O. and M. Abe, Cell surface N-glycosylation and sialylation regulate galectin-3-induced apoptosis in human diffuse large B cell lymphoma. Oncol Rep, 2008. **19**(3): p. 743-8.
118. Mazurek, N., et al., Phosphorylation of the beta-galactoside-binding protein galectin-3 modulates binding to its ligands. J Biol Chem, 2000. **275**(46): p. 36311-5.
119. Balan, V., et al., Tyrosine-phosphorylated galectin-3 protein is resistant to prostate-specific antigen (PSA) cleavage. J Biol Chem, 2012. **287**(8): p. 5192-8.
120. Haudek, K.C., et al., *Dynamics of galectin-3 in the nucleus and cytoplasm.* Biochim Biophys Acta, 2010. **1800**(2): p. 181-9.
121. Yang, R.Y., D.K. Hsu, and F.T. Liu, *Expression of galectin-3 modulates T-cell growth and apoptosis.* Proc Natl Acad Sci U S A, 1996. **93**(13): p. 6737-42.
122. Liu, L., et al., Nucling mediates apoptosis by inhibiting expression of galectin-3 through interference with nuclear factor kappaB signalling. Biochem J, 2004. **380**(Pt 1): p. 31-41.
123. Wang, W., et al., Immunoprecipitation of spliceosomal RNAs by antisera to galectin-1 and galectin-3. Nucleic Acids Res, 2006. **34**(18): p. 5166-74.
124. Rosenberg, I., et al., Mac-2-binding glycoproteins. Putative ligands for a cytosolic beta-galactoside lectin. J Biol Chem, 1991. **266**(28): p. 18731-6.
125. Kuwabara, I. and F.T. Liu, *Galectin-3 promotes adhesion of human neutrophils to laminin.* J Immunol, 1996. **156**(10): p. 3939-44.
126. Noma, N., et al., Involvement of NF-kappaB-mediated expression of galectin-3-binding protein in TNF-alpha-induced breast cancer cell adhesion. Oncol Rep, 2012. **27**(6): p. 2080-4.
127. Jiang, J., I. Eliaz, and D. Sliva, Synergistic and Additive Effects of Modified Citrus Pectin With Two Polybotanical Compounds, in the Suppression of Invasive Behavior of Human Breast and Prostate Cancer Cells. Integr Cancer Ther, 2012.
128. Vasta, G.R., *Roles of galectins in infection.* Nat Rev Microbiol, 2009. **7**(6): p. 424-38.

129. Sato, S., et al., *Glycans, galectins, and HIV-1 infection.* Ann N Y Acad Sci, 2012. **1253**: p. 133-48.
130. Mey, A., et al., The animal lectin galectin-3 interacts with bacterial lipopolysaccharides via two independent sites. J Immunol, 1996. **156**(4): p. 1572-7.
131. Li, Y., et al., Galectin-3 is a negative regulator of lipopolysaccharide-mediated inflammation. J Immunol, 2008. **181**(4): p. 2781-9.
132. Fermino, M.L., et al., LPS-induced galectin-3 oligomerization results in enhancement of neutrophil activation. PLoS One, 2011. **6**(10): p. e26004.
133. Ofek, I., et al., *Nonopsonic phagocytosis of microorganisms.* Annu Rev Microbiol, 1995. **49**: p. 239-76.
134. Mandrell, R.E., et al., Possible interaction between animal lectins and bacterial carbohydrates. Methods Enzymol, 1994. **236**: p. 231-54.
135. Stowell, S.R., et al., Innate immune lectins kill bacteria expressing blood group antigen. Nat Med, 2010. **16**(3): p. 295-301.
136. Frigeri, L.G., R.I. Zuberi, and F.T. Liu, Epsilon BP, a beta-galactoside-binding animal lectin, recognizes IgE receptor (Fc epsilon RI) and activates mast cells. Biochemistry, 1993. **32**(30): p. 7644-9.
137. Tribulatti, M.V., et al., Redundant and antagonistic functions of galectin-1, -3, and -8 in the elicitation of T cell responses. J Immunol, 2012. **188**(7): p. 2991-9.
138. Tribulatti, M.V., et al., Galectin-8 provides costimulatory and proliferative signals to T lymphocytes. J Leukoc Biol, 2009. **86**(2): p. 371-80.
139. Chen, I.J., H.L. Chen, and M. Demetriou, Lateral compartmentalization of T cell receptor versus CD45 by galectin-N-glycan binding and microfilaments coordinate basal and activation signaling. J Biol Chem, 2007. **282**(48): p. 35361-72.
140. Toscano, M.A., et al., Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. Nat Immunol, 2007. **8**(8): p. 825-34.
141. Cedeno-Laurent, F. and C.J. Dimitroff, *Galectin-1 research in T cell immunity: past, present and future.* Clin Immunol, 2012. **142**(2): p. 107-16.
142. Motran, C.C., et al., Galectin-1 functions as a Th2 cytokine that selectively induces Th1 apoptosis and promotes Th2 function. Eur J Immunol, 2008. **38**(11): p. 3015-27.
143. Blois, S.M., et al., *A pivotal role for galectin-1 in fetomaternal tolerance.* Nat Med, 2007. **13**(12): p. 1450-7.
144. Stowell, S.R., et al., Differential roles of galectin-1 and galectin-3 in regulating leukocyte viability and cytokine secretion. J Immunol, 2008. **180**(5): p. 3091-102.
145. Peng, W., et al., Tumor-associated galectin-3 modulates the function of tumor-reactive T cells. Cancer Res, 2008. **68**(17): p. 7228-36.
146. Demotte, N., et al., A galectin-3 ligand corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. Cancer Res, 2010. **70**(19): p. 7476-88.
147. Rubinstein, N., et al., Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. Cancer Cell, 2004. **5**(3): p. 241-51.
148. Matarrese, P., et al., Galectin-3 overexpression protects from cell damage and death by influencing mitochondrial homeostasis. FEBS Lett, 2000. **473**(3): p. 311-5.

149. Mensah-Brown, E.P., et al., Targeted disruption of the galectin-3 gene results in decreased susceptibility to multiple low dose streptozotocin-induced diabetes in mice. Clin Immunol, 2009. **130**(1): p. 83-8.
150. Iacobini, C., et al., Galectin-3/AGE-receptor 3 knockout mice show accelerated AGE-induced glomerular injury: evidence for a protective role of galectin-3 as an AGE receptor. FASEB J, 2004. **18**(14): p. 1773-5.
151. del Pozo, V., et al., Gene therapy with galectin-3 inhibits bronchial obstruction and inflammation in antigen-challenged rats through interleukin-5 gene downregulation. Am J Respir Crit Care Med, 2002. **166**(5): p. 732-7.
152. Lopez, E., et al., Inhibition of chronic airway inflammation and remodeling by galectin-3 gene therapy in a murine model. J Immunol, 2006. **176**(3): p. 1943-50.
153. Zuberi, R.I., et al., Critical role for galectin-3 in airway inflammation and bronchial hyperresponsiveness in a murine model of asthma. Am J Pathol, 2004. **165**(6): p. 2045-53.
154. Nachtigal, M., A. Ghaffar, and E.P. Mayer, Galectin-3 gene inactivation reduces atherosclerotic lesions and adventitial inflammation in ApoE-deficient mice. Am J Pathol, 2008. **172**(1): p. 247-55.
155. Almkvist, J., et al., Activation of the neutrophil nicotinamide adenine dinucleotide phosphate oxidase by galectin-1. J Immunol, 2002. **168**(8): p. 4034-41.
156. Yamaoka, A., et al., A human lectin, galectin-3 (epsilon bp/Mac-2), stimulates superoxide production by neutrophils. J Immunol, 1995. **154**(7): p. 3479-87.
157. Liu, F.T., et al., Modulation of functional properties of galectin-3 by monoclonal antibodies binding to the non-lectin domains. Biochemistry, 1996. **35**(19): p. 6073-9.
158. Sano, H., et al., Human galectin-3 is a novel chemoattractant for monocytes and macrophages. J Immunol, 2000. **165**(4): p. 2156-64.
159. Liu, F.T. and G.A. Rabinovich, *Galectins: regulators of acute and chronic inflammation.* Ann N Y Acad Sci, 2010. **1183**: p. 158-82.
160. Rabinovich, G.A., et al., An emerging role for galectins in tuning the immune response: lessons from experimental models of inflammatory disease, autoimmunity and cancer. Scand J Immunol, 2007. **66**(2-3): p. 143-58.
161. Glinsky, V.V. and A. Raz, Modified citrus pectin anti-metastatic properties: one bullet, multiple targets. Carbohydr Res, 2009. **344**(14): p. 1788-91.
162. Van den Brule, F.A., et al., Differential expression of galectin-1 and galectin-3 during first trimester human embryogenesis. Dev Dyn, 1997. **209**(4): p. 399-405.
163. Truong, M.J., et al., Human neutrophils express immunoglobulin E (IgE)-binding proteins (Mac-2/epsilon BP) of the S-type lectin family: role in IgE-dependent activation. J Exp Med, 1993. **177**(1): p. 243-8.
164. Truong, M.J., et al., IgE-binding molecules (Mac-2/epsilon BP) expressed by human eosinophils. Implication in IgE-dependent eosinophil cytotoxicity. Eur J Immunol, 1993. **23**(12): p. 3230-5.
165. Craig, S.S., et al., Immunoelectron microscopic localization of galectin-3, an IgE binding protein, in human mast cells and basophils. Anat Rec, 1995. **242**(2): p. 211-9.
166. Wollenberg, A., et al., Human keratinocytes release the endogenous beta-galactoside-binding soluble lectin immunoglobulin E (IgE-binding protein) which

binds to Langerhans cells where it modulates their binding capacity for IgE glycoforms. J Exp Med, 1993. **178**(3): p. 777-85.
167. Smetana, K., et al., Coexpression of binding sites for A(B) histo-blood group trisaccharides with galectin-3 and Lag antigen in human Langerhans cells. J Leukoc Biol, 1999. **66**(4): p. 644-9.
168. Dietz, A.B., et al., Maturation of human monocyte-derived dendritic cells studied by microarray hybridization. Biochem Biophys Res Commun, 2000. **275**(3): p. 731-8.
169. Colnot, C., et al., Cellular and subcellular distribution of galectin-3 in the epiphyseal cartilage and bone of fetal and neonatal mice. Cell Mol Biol (Noisy-le-grand), 1999. **45**(8): p. 1191-202.
170. Stock, M., et al., Expression of galectin-3 in skeletal tissues is controlled by Runx2. J Biol Chem, 2003. **278**(19): p. 17360-7.
171. Niida, S., et al., Expression of Mac-2 antigen in the preosteoclast and osteoclast identified in the op/op mouse injected with macrophage colony-stimulating factor. J Bone Miner Res, 1994. **9**(6): p. 873-81.
172. Konstantinov, K.N., et al., Expression of epsilon BP, a beta-galactoside-binding soluble lectin, in normal and neoplastic epidermis. Exp Dermatol, 1994. **3**(1): p. 9-16.
173. Reichert, F., A. Saada, and S. Rotshenker, Peripheral nerve injury induces Schwann cells to express two macrophage phenotypes: phagocytosis and the galactose-specific lectin MAC-2. J Neurosci, 1994. **14**(5 Pt 2): p. 3231-45.
174. Lotan, R., et al., Expression of a 31-kDa lactoside-binding lectin in normal human gastric mucosa and in primary and metastatic gastric carcinomas. Int J Cancer, 1994. **56**(4): p. 474-80.
175. Hrdlickova-Cela, E., et al., Detection of galectin-3 in tear fluid at disease states and immunohistochemical and lectin histochemical analysis in human corneal and conjunctival epithelium. Br J Ophthalmol, 2001. **85**(11): p. 1336-40.
176. Cao, Z., et al., Galectins-3 and -7, but not galectin-1, play a role in re-epithelialization of wounds. J Biol Chem, 2002. **277**(44): p. 42299-305.
177. Bao, Q. and R.C. Hughes, Galectin-3 expression and effects on cyst enlargement and tubulogenesis in kidney epithelial MDCK cells cultured in three-dimensional matrices in vitro. J Cell Sci, 1995. **108 (Pt 8)**: p. 2791-800.
178. Kasper, M. and R.C. Hughes, Immunocytochemical evidence for a modulation of galectin 3 (Mac-2), a carbohydrate binding protein, in pulmonary fibrosis. J Pathol, 1996. **179**(3): p. 309-16.
179. Villa-Verde, D.M., et al., Galectin-3 modulates carbohydrate-dependent thymocyte interactions with the thymic microenvironment. Eur J Immunol, 2002. **32**(5): p. 1434-44.
180. Lotan, R., et al., Expression of galectins on microvessel endothelial cells and their involvement in tumour cell adhesion. Glycoconj J, 1994. **11**(5): p. 462-8.
181. Verschoor, C.P., A. Puchta, and D.M. Bowdish, *The macrophage.* Methods Mol Biol, 2012. **844**: p. 139-56.
182. Martinez, F.O., *Regulators of macrophage activation.* Eur J Immunol, 2011. **41**(6): p. 1531-4.
183. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.
184. Benoit, M., B. Desnues, and J.L. Mege, *Macrophage polarization in bacterial infections.* J Immunol, 2008. **181**(6): p. 3733-9.

185. Mantovani, A., et al., Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol, 2002. **23**(11): p. 549-55.
186. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* J Clin Invest, 2012. **122**(3): p. 787-95.
187. Biswas, S.K., et al., Macrophage polarization and plasticity in health and disease. Immunol Res, 2012.
188. Biswas, S.K. and A. Mantovani, Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol, 2010. **11**(10): p. 889-96.
189. Saccani, A., et al., p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. Cancer Res, 2006. **66**(23): p. 11432-40.
190. Guiducci, C., et al., Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res, 2005. **65**(8): p. 3437-46.
191. Martinez, F.O., L. Helming, and S. Gordon, *Alternative activation of macrophages: an immunologic functional perspective.* Annu Rev Immunol, 2009. **27**: p. 451-83.
192. Gordon, S., *Alternative activation of macrophages.* Nat Rev Immunol, 2003. **3**(1): p. 23-35.
193. Odegaard, J.I., et al., Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. Nature, 2007. **447**(7148): p. 1116-20.
194. Pello, O.M., et al., Role of c-MYC in alternative activation of human macrophages and tumor-associated macrophage biology. Blood, 2012. **119**(2): p. 411-21.
195. Szanto, A., et al., STAT6 transcription factor is a facilitator of the nuclear receptor PPARgamma-regulated gene expression in macrophages and dendritic cells. Immunity, 2010. **33**(5): p. 699-712.
196. MacKinnon, A.C., et al., *Regulation of alternative macrophage activation by galectin-3.* J Immunol, 2008. **180**(4): p. 2650-8.
197. Fu, Y.X., et al., The role of tumor-derived cytokines on the immune system of mice bearing a mammary adenocarcinoma. I. Induction of regulatory macrophages in normal mice by the in vivo administration of rGM-CSF. J Immunol, 1991. **146**(2): p. 783-9.
198. Mazzei, G.J., et al., Human granulocyte-macrophage colony-stimulating factor plus phorbol myristate acetate stimulate a promyelocytic cell line to produce an IL-1 inhibitor. J Immunol, 1990. **145**(2): p. 585-91.
199. Tsuchiya, Y., et al., Production of colony-stimulating factor by tumor cells and the factor-mediated induction of suppressor cells. J Immunol, 1988. **141**(2): p. 699-708.
200. Sato, S. and R.C. Hughes, Regulation of secretion and surface expression of Mac-2, a galactoside-binding protein of macrophages. J Biol Chem, 1994. **269**(6): p. 4424-30.
201. Sorokin, S.P., et al., Macrophage development: II. Early ontogeny of macrophage populations in brain, liver, and lungs of rat embryos as revealed by a lectin marker. Anat Rec, 1992. **232**(4): p. 527-50.
202. Petryniak, J., T.K. Huard, and I.J. Goldstein, alpha-D-galactose-bearing glycoproteins on the surface of stimulated murine peritoneal macrophages.

Biochemical and immunochemical characterization of purified glycoproteins. Eur J Biochem, 1992. **206**(1): p. 197-207.
203. Liu, F.T., et al., Expression and function of galectin-3, a beta-galactoside-binding lectin, in human monocytes and macrophages. Am J Pathol, 1995. **147**(4): p. 1016-28.
204. Kim, K., E.P. Mayer, and M. Nachtigal, Galectin-3 expression in macrophages is signaled by Ras/MAP kinase pathway and up-regulated by modified lipoproteins. Biochim Biophys Acta, 2003. **1641**(1): p. 13-23.
205. Hsu, D.K., et al., Targeted disruption of the galectin-3 gene results in attenuated peritoneal inflammatory responses. Am J Pathol, 2000. **156**(3): p. 1073-83.
206. Zhu, W., et al., The role of galectin-3 in endocytosis of advanced glycation end products and modified low density lipoproteins. Biochem Biophys Res Commun, 2001. **280**(4): p. 1183-8.
207. Pugliese, G., et al., The diabetic milieu modulates the advanced glycation end product-receptor complex in the mesangium by inducing or upregulating galectin-3 expression. Diabetes, 2000. **49**(7): p. 1249-57.
208. Butcher, M.J. and E.V. Galkina, Phenotypic and functional heterogeneity of macrophages and dendritic cell subsets in the healthy and atherosclerosis-prone aorta. Front Physiol, 2012. **3**: p. 44.
209. Novak, R., S. Dabelic, and J. Dumic, Galectin-1 and galectin-3 expression profiles in classically and alternatively activated human macrophages. Biochim Biophys Acta, 2012. **1820**(9): p. 1383-90.
210. Lepur, A., et al., Galectin-3 endocytosis by carbohydrate independent and dependent pathways in different macrophage like cell types. Biochim Biophys Acta, 2012. **1820**(7): p. 804-18.
211. Sano, H., et al., *Critical role of galectin-3 in phagocytosis by macrophages.* J Clin Invest, 2003. **112**(3): p. 389-97.
212. Kohatsu, L., et al., Galectin-3 induces death of Candida species expressing specific beta-1,2-linked mannans. J Immunol, 2006. **177**(7): p. 4718-26.
213. Iacobini, C., et al., Accelerated lipid-induced atherogenesis in galectin-3-deficient mice: role of lipoxidation via receptor-mediated mechanisms. Arterioscler Thromb Vasc Biol, 2009. **29**(6): p. 831-6.
214. Nishi, Y., et al., *Role of galectin-3 in human pulmonary fibrosis.* Allergol Int, 2007. **56**(1): p. 57-65.
215. Bernardes, E.S., et al., Toxoplasma gondii infection reveals a novel regulatory role for galectin-3 in the interface of innate and adaptive immunity. Am J Pathol, 2006. **168**(6): p. 1910-20.
216. Konigshoff, M. and M. Rojas, *Galectin-3: the bridge over troubled waters.* Am J Respir Crit Care Med, 2012. **185**(5): p. 473-5.
217. Jiang, J.X., et al., Galectin-3 modulates phagocytosis-induced stellate cell activation and liver fibrosis in vivo. Am J Physiol Gastrointest Liver Physiol, 2012. **302**(4): p. G439-46.
218. Henderson, N.C., et al., Galectin-3 expression and secretion links macrophages to the promotion of renal fibrosis. Am J Pathol, 2008. **172**(2): p. 288-98.
219. Lok, D.J., et al., Prognostic value of galectin-3, a novel marker of fibrosis, in patients with chronic heart failure: data from the DEAL-HF study. Clin Res Cardiol, 2010. **99**(5): p. 323-8.

220. Hubert, M., et al., Intranuclear distribution of galectin-3 in mouse 3T3 fibroblasts: comparative analyses by immunofluorescence and immunoelectron microscopy. Exp Cell Res, 1995. **220**(2): p. 397-406.
221. Sato, S., I. Burdett, and R.C. Hughes, Secretion of the baby hamster kidney 30-kDa galactose-binding lectin from polarized and nonpolarized cells: a pathway independent of the endoplasmic reticulum-Golgi complex. Exp Cell Res, 1993. **207**(1): p. 8-18.
222. Rubartelli, A., et al., A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. EMBO J, 1990. **9**(5): p. 1503-10.
223. Gaudin, J.C., B. Mehul, and R.C. Hughes, *Nuclear localisation of wild type and mutant galectin-3 in transfected cells.* Biol Cell, 2000. **92**(1): p. 49-58.
224. Nakahara, S. and A. Raz, Regulation of cancer-related gene expression by galectin-3 and the molecular mechanism of its nuclear import pathway. Cancer Metastasis Rev, 2007. **26**(3-4): p. 605-10.
225. Yu, F., et al., Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria. A role for synexin in galectin-3 translocation. J Biol Chem, 2002. **277**(18): p. 15819-27.
226. Baptiste, T.A., et al., Mechano-transduction mediated secretion and uptake of galectin-3 in breast carcinoma cells: implications in the extracellular functions of the lectin. Exp Cell Res, 2007. **313**(4): p. 652-64.
227. Delacour, D., et al., *Requirement for galectin-3 in apical protein sorting.* Curr Biol, 2006. **16**(4): p. 408-14.
228. Delacour, D., et al., Apical sorting by galectin-3-dependent glycoprotein clustering. Traffic, 2007. **8**(4): p. 379-88.
229. Richardson, D.R. and P. Ponka, The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. Biochim Biophys Acta, 1997. **1331**(1): p. 1-40.
230. Vagin, O., J.A. Kraut, and G. Sachs, *Role of N-glycosylation in trafficking of apical membrane proteins in epithelia.* Am J Physiol Renal Physiol, 2009. **296**(3): p. F459-69.
231. Beatty, W.L., et al., Association of a macrophage galactoside-binding protein with Mycobacterium-containing phagosomes. Cell Microbiol, 2002. **4**(3): p. 167-76.
232. Paz, I., et al., *Galectin-3, a marker for vacuole lysis by invasive pathogens.* Cell Microbiol, 2010. **12**(4): p. 530-44.
233. Barboni, E., S. Coade, and A. Fiori, The binding of mycolic acids to galectin-3: a novel interaction between a host soluble lectin and trafficking mycobacterial lipids? FEBS Lett, 2005. **579**(30): p. 6749-55.
234. Granovsky, M., et al., Suppression of tumor growth and metastasis in Mgat5-deficient mice. Nat Med, 2000. **6**(3): p. 306-12.
235. Lajoie, P., et al., Plasma membrane domain organization regulates EGFR signaling in tumor cells. J Cell Biol, 2007. **179**(2): p. 341-56.
236. Liu, W., et al., Galectin-3 Regulates Intracellular Trafficking of EGFR through Alix and Promotes Keratinocyte Migration. J Invest Dermatol, 2012.
237. Mattila, P.E., et al., Multiple biosynthetic trafficking routes for apically secreted proteins in MDCK cells. Traffic, 2012. **13**(3): p. 433-42.
238. Tsuchiya, S., et al., Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer, 1980. **26**(2): p. 171-6.

239. Pivoriunas, A., et al., PI 3-K signaling pathway suppresses PMA-induced expression of p21WAF1/Cip1 in human leukemia cells. Mol Cell Biochem, 2007. **302**(1-2): p. 9-18.
240. Dasgupta, P. and A.D. Keegan, Contribution of Alternatively Activated Macrophages to Allergic Lung Inflammation: A Tale of Mice and Men. J Innate Immun, 2012.
241. Sorme, P., et al., Fluorescence polarization as an analytical tool to evaluate galectin-ligand interactions. Anal Biochem, 2004. **334**(1): p. 36-47.
242. Salomonsson, E., et al., *Monovalent interactions of galectin-1.* Biochemistry, 2010. **49**(44): p. 9518-32.
243. Wikipedia-Contributors. *Low-density lipoprotein*. 2012 27 June 2012 12:46 UTC 25 July 2012 10:29 UTC]; Available from: http://en.wikipedia.org/w/index.php?title=Low-density_lipoprotein&oldid=499596668.
244. Oh, J., et al., Endoplasmic reticulum stress controls M2 macrophage differentiation and foam cell formation. J Biol Chem, 2012. **287**(15): p. 11629-41.
245. Madsen, M., J.H. Graversen, and S.K. Moestrup, *Haptoglobin and CD163: captor and receptor gating hemoglobin to macrophage lysosomes.* Redox Rep, 2001. **6**(6): p. 386-8.
246. Kalluri, R. and M. Zeisberg, *Fibroblasts in cancer.* Nature Reviews Cancer, 2006. **6**(5): p. 392-401.
247. Mora, A.L., et al., Activation of alveolar macrophages via the alternative pathway in herpesvirus-induced lung fibrosis. Am J Respir Cell Mol Biol, 2006. **35**(4): p. 466-73.
248. Sharma, U.C., et al., Galectin-3 marks activated macrophages in failure-prone hypertrophied hearts and contributes to cardiac dysfunction. Circulation, 2004. **110**(19): p. 3121-8.
249. Kolatsi-Joannou, M., et al., Modified citrus pectin reduces galectin-3 expression and disease severity in experimental acute kidney injury. PLoS One, 2011. **6**(4): p. e18683.