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# Siglecs: A journey through the evolution of sialic acid-binding immunoglobulin-type lectins

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ABSTRACT

Siglecs (sialic acid-binding immunoglobulin-type lectins) are a family of immune regulatory receptors predominantly found on the cells of the hematopoietic system. A V-set Ig-like domain mediates the recognition of different sialylated glycoconjugates, which can lead to the activation or inhibition of the immune response, depending on the involved Siglecs. Siglecs are categorized into two subgroups: one including all CD33-related Siglecs and the other consisting of Siglec-1 (Sialoadhesin), Siglec-2 (CD22), Siglec-4 (myelin-associated glycoprotein, MAG) and Siglec-15. In contrast to the members of the CD33-related Siglecs, which share ~50–99% sequence identity, Siglecs of the other subgroup show quite low homology (approximately 25–30% sequence identity). Based on the published sequences and functions of Siglecs, we performed phylogenetic analyses and sequence alignments to reveal the conservation of Siglecs throughout evolution. Therefore, we focused on the presence of Siglecs in different classes of vertebrates (fishes, amphibians, birds, reptiles and mammals), offering a bridge between the presence of different Siglecs and the biological situations of the selected animals.



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## 1. Introduction

All eukaryotic cells are surrounded with proteoglycans, glycosphingolipids and glycoproteins that form an essential functional unit: the glycocalyx (Varki, 2017b). Indeed, no living cell can survive without its "sugar jacket" (Gagneux et al., 2015). These sugar residues are directly involved in the modulation of numerous crucial cellular processes, like cell-cell interaction and communication (Crocker et al., 2007; Green et al., 1995; Kelm and Schauer, 1997; Ohtsubo and Marth, 2006; Sato, 2004; Schauer, 2000; Varki, 2007, 2017b; Varki and Angata, 2006; Varki and Varki, 2007). In vertebrates, most of these glycans have sialic acid residues on their outermost positions.

Sialic acids consist of a 9-carbon backbone; the most common forms of these acidic sugars are *N*-acetylneuraminic acid (Neu5Ac), followed by *N*-glycolylneuraminic acid (Neu5Gc) (Angata and Varki, 2002; Schauer, 1996, 2004). The substitution of one or more hydroxyl groups of Neu5Ac, Neu5Gc or deaminated neuraminic acid (KDN) with, e.g., acetyl, methyl or sulfate residues results in high diversity and more than 50 derivatives were described until now (Angata and Varki, 2002; Schauer, 2004, 2009). Since sialic acids are located at the distal part of the non-reducing end of a glycan, these α-keto acids play a special role in physiological processes. Intriguingly, sialic acids belong to the self-associated-molecular patterns (SAMPs), which are molecules that are recognized by inhibitory receptors in order to dampen immune reactions (Varki, 2011b; Varki, 2017a; b). Such modulations can be mediated via the interaction of sialic acid-binding immunoglobulin-like lectins (Siglecs) with distinct sialylated glycoconjugates (Crocker, 2005; Crocker et al., 2007; Pillai et al., 2012; Varki and Angata, 2006; Varki and Gagneux, 2012). Already in 2009, 14 different human Siglecs and 9 murine Siglecs have been identified (O'Reilly and Paulson, 2009; Pillai et al., 2012); each type of Siglec prevalently recognizes a particular set of sialylated structures (Crocker et al., 2007; Macauley et al., 2014; Pillai et al., 2012), carrying different α2,3-, α2,6- or α2,8-linked sialic acid residues (Bakker et al., 2002; Blixt et al., 2003).

Siglecs are mostly located on the cell surface of hematopoietic cells (Crocker et al., 2007; Varki and Angata, 2006), but cells outside the immune system can also express Siglecs. For instance, Siglec-4 (myelin-associated glycoprotein, MAG) is predominantly expressed in Schwann cells, as well as oligodendrocytes (Quarles, 2007).

Based on sequence homologies of human Siglecs, members of the Siglec family can be subdivided in two groups: one group comprises CD33 (Siglec-3)-related Siglecs, showing low gene conservation across orthologues but a high degree of sequence identity throughout the subfamily members; the other group includes Siglec-1 (Sialoadhesin), Siglec-2 (CD22), Siglec-4 and Siglec-15, which show low sequence identity between each other (Angata et al., 2004; Crocker et al., 2007). Both groups include both activating and inhibitory receptors. The inhibitory Siglecs contain the so-called 'immune receptor tyrosine-based inhibition motifs' (ITIM) antagonizing the initiation of an immune reaction, which is mediated via the 'immune receptor tyrosine-based activation motif' (ITAM) (Crocker et al., 2007; Ravetch and Lanier, 2000). Such an ITAM-dependent activation can be mediated by Siglec-14, Siglec-15 and Siglec-16. They support activation via the interaction partner DAP10/12 (DNAX-activation protein of 10/12 kDa), which includes an ITAM domain required for the production of proinflammatory cytokines (Crocker et al., 2007).

In addition, there are two Siglecs in humans without a direct connection to the ITIM and ITAM systems. Both Siglec-1 and Siglec-4 lack activating and inhibiting motifs. In the case of Siglec-1, it has been suggested that it is mainly responsible for cell-cell interactions and/or the phagocytosis of sialylated pathogens (Chang and Nizet, 2014; Crocker et al., 2007), whereas Siglec-4 is prevalently involved in myelination processes and the stabilization of cell-cell contacts (Owens and Bunge, 1989; Quarles, 2007).

Sialylated glycoconjugates have been present on the cell surfaces and/or been released to modulate physiological processes for more than 500 million years (Gagneux et al., 2015; Varki, 2011a, 2017a; b). Since not only eukaryotic cells but also distinct prokaryotic cells are able to build differently sialylated glycans, immune-competent cells use sialylated glycans as recognition molecules for endogenous cells and to detect pathogens forming a complex network of several identification, activation and inhibition processes (Varki, 2006, 2009; 2017a; b). As already outlined by Varki and colleagues, the co-presence of distinct sialylated structures on endogenous cells and on pathogens might represent one of the most important triggers for the evolution of both inhibitory and activating Siglec family members (Varki, 2006, 2009; 2017a; b). However, whereas there is already quite lot knowledge about the Siglec expression in mice and primates including humans, the knowledge about Siglec expression in other vertebrates like fishes, amphibians, reptiles and birds is still fragmentary, leading to an insuperable gap within evolutionary explanations. With the aim to contribute to the organization of the ever-growing number of complex immunoregulatory receptor families, we summarized the presence of Siglecs in several species by the performance of Blast searches and discuss the findings in the context of physiological changes throughout evolution (placenta types, lactation, and ecological niche/habitat).

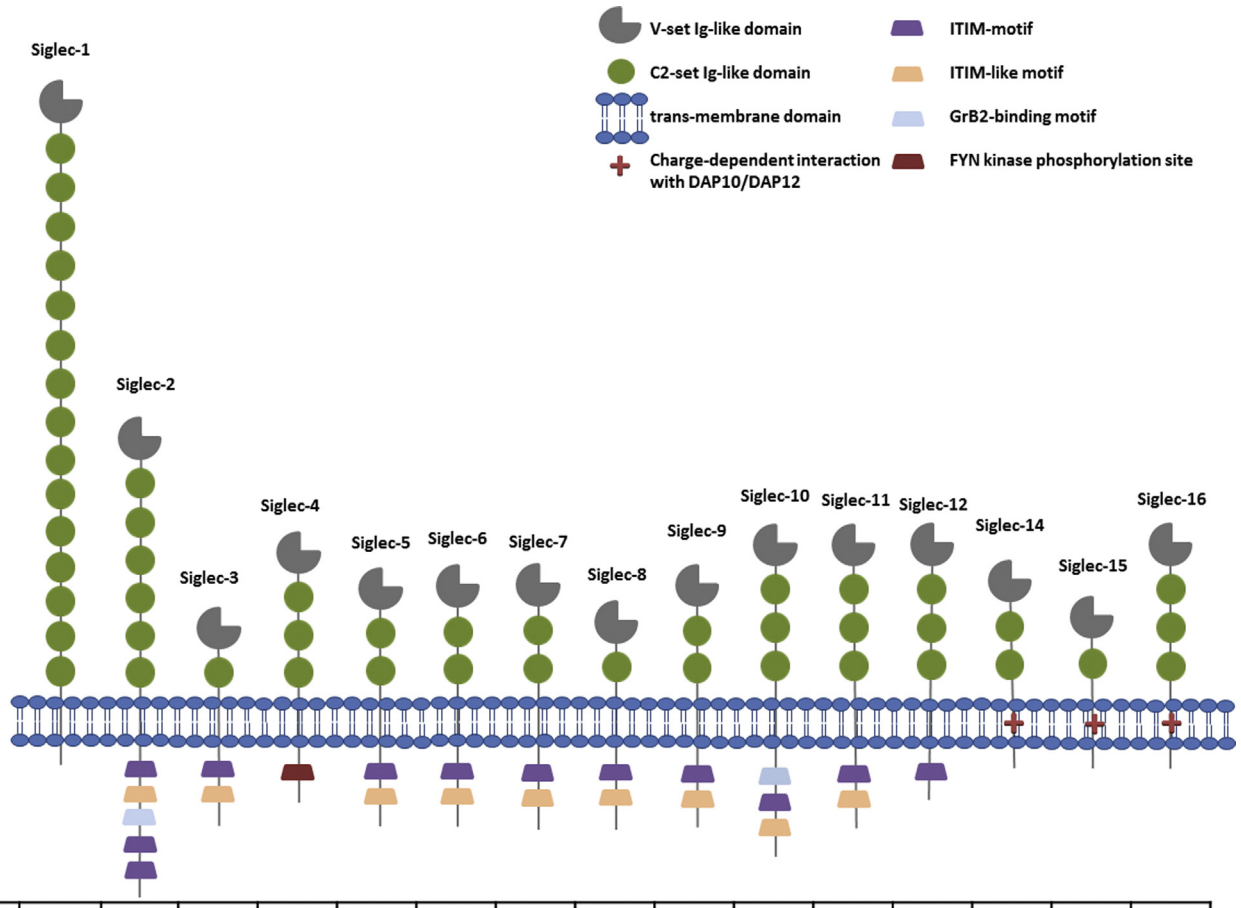
## 2. Structure of Siglecs

Siglecs are cell-surface receptors containing protein domains belonging to the Immunoglobulin (Ig) superfamily. Their extracellular part consists of a variable number of so-called 'C2-set' Ig-like domains with high sequence and folding similarities to the constant region of immunoglobulins (Fig. 1) (Jandus et al., 2011), and an amino-terminal Ig-like domain with high similarities to the variable domain of antibodies, abbreviated as the V-set domain. This domain contains the sialic acid binding domain (Angata et al., 2004; Crocker et al., 2007; O'Reilly and Paulson, 2009). Depending on the number of C2-set Ig-like domains, Siglecs tend to bind sialic acid residues on the same cell surface in cis-mode or on adjacent cells in trans-mode. For example, Siglec-1 (15 Ig-domains) commonly binds glycans in trans-mode, whereas Siglec-3, Siglec-8 and Siglec-15 (one Ig-domain) are believed to favor the cis binding of sialylated sugar structures (Angata et al., 2004; Crocker et al., 2007; Hartnell et al., 2001).

### 2.1. Sialic acid binding by Siglecs

In general, it seems that one salt bridge, mediated by an arginine residue, is particularly important for the interactions (Supplement 1A). Regarding human Siglec-1 (hSiglec-1), it is known that the sialic acid binding domain is located at the N-terminus of the protein (May et al., 1998). The guanidine group of R116 of hSiglec-1 forms a salt bridge with the carboxyl group of Neu5Ac. The acetyl group of Neu5Ac is in van der Waals contact with the indole ring of W21 and the C9 of the glycerol residue of sialic acid interact with the aromatic chain of W125.

The interaction of hSiglec-5 and the sialylated structures involves a salt bridge between arginine (R124) and the carboxyl group of Neu5Ac (Zhuravleva et al., 2008), similar to Siglec-1 (Supplement 1B). In addition, K132 and S134 interact via hydrogen bonds with the secondary amine and the hydroxyl group of C8 of sialic acid, respectively. Moreover, van der Waals




| hSiglec | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ITIM | - | +++ | + | - | + | + | + | + | + | + | + | + | - | - | - |
| ITIM-like | - | + | + | - | + | + | + | + | + | + | + | - | - | - | - |
| ITAM | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + |

**Fig. 1. Structure of the 15 Siglecs known to be expressed in humans**. The illustrations were plotted using the SMART (Simple Molecular Architecture Research Tool, Heidelberg) tool. For ITIM and ITIM-like motifs positive signs indicate the availability of signaling motifs in the cytoplasmic area, whereas for ITAM motifs positive signs indicate the charge-dependent association with DAP10/12, ITAM bearing proteins. Negative signs show the absence of ITIMs, ITIM-likes and ITAMs. Based on Jandus et al. (2011).

interactions take place between the aromatic part of Y133 and position C9 of sialic acid (Zhuravleva et al., 2008). Thus, distinct structural changes affect the character of individual Siglec binding pockets and modulate their affinity to a particular panel of ligands.

### 2.2. The signaling motifs of Siglecs

As mentioned above, some members of the Siglec family—Siglec-14, Siglec-15 and Siglec-16—are able to promote immune responses by interacting with DAP10/12 (Fig. 1) (Angata et al., 2007; Arase and Lanier, 2004). DAP10/12 contains ITAM domains (Angata et al., 2006, 2007; Ishida-Kitagawa et al., 2012) with the signature sequence Y-X-X-L/I-$X_{6-8}$Y-X-X-X-L/I (with X representing any amino acid) (Lanier and Bakker, 2000). This interaction with DAP10/12 is initiated by positive residues in the transmembrane region of these lectins (Ishida-Kitagawa et al., 2012; Pillai et al., 2012).

However, Fig. 1 illustrates that most Siglecs contain ITIMs in the intracellular domain (Ando et al., 2008). ITIMs share the signature (I/V/L/S)-X-Y-X-X-(L/V) (Crocker et al., 2007), which allows counteracting the activation of the immune system via the inhibition of ITAM-mediated signaling (Crocker et al., 2007; Ravetch and Lanier, 2000). Additionally, most of the known Siglecs possess not only an ITIM, but also ITIM-like motifs. Mutagenesis experiments with CD33-related Siglecs showed that ITIM functionally dominates over the ITIM-like motif, although both are relevant for recruiting the Scr-homology-2 domain containing protein tyrosine phosphatases SHP1 and SHP2 (Avril et al., 2005; Paul et al., 2000; Taylor et al., 1999; Ulyanova et al., 2001; Yu et al., 2001). The binding of SHP1 and SHP2 requires the phosphorylation of the tyrosine residues of both the ITIM and the ITIM-like motif (Avril et al., 2005; Crocker et al., 2007). One exception is Siglec-5, which can weakly bind SHP1, although it cannot be phosphorylated since the required tyrosine residue is replaced by alanine (Avril et al., 2005).

## 3. Functions of Siglecs

### 3.1. DAP-associated Siglecs

Siglec-14, Siglec-15 and Siglec-16 have been reported to interact with DNAX-activation proteins. However, Siglec-15 is the most investigated DAP12- and DAP10-associated Siglec. Due to the aspartic acid residue in its transmembrane area, DAP12 can interact in a charge-dependent mechanism with lysine located in the transmembrane domain of Siglec-15 (Angata et al., 2007). When Siglec-15 interacts with a sialylated binding partner, the tyrosine residues of ITAM are phosphorylated by the kinases of the Src family. The phosphorylated ITAM domains of DAP12 serve as docking sites for the SH2 domains of the ZAP70 and the Syk kinases, which in turn activates the immune system (Angata et al., 2007; Lanier and Bakker, 2000).

#### 3.1.1. Biological role of DAP-associated Siglecs

Besides a possible immunological role, Hiruma et al. described the critical role of Siglec-15 in the differentiation of osteoclasts (hematopoietic-origin) (Hiruma et al., 2011), which is important for bone resorption (Teitelbaum, 2000). Mice lacking Siglec-15 show mild osteopetrosis characterized by dense bones (Hiruma et al., 2013). *In vitro* studies have demonstrated that the interaction of Siglec-15 with DAP12 after sialic acid binding leads to a signal-activating osteoclast differentiation into their multinucleated states, which is responsible for bone resorption (Hiruma et al., 2011). This differentiation step can be inhibited using anti-Siglec-15 antibodies, causing dimerization and the subsequent internalization and degradation of Siglec-15 dimers (Stuible et al., 2014). Thus, Tremblay and co-workers recommended an administration of antibodies against Siglec-15 to counteract bone loss (Stuible et al., 2014).

### 3.2. ITIM-bearing Siglecs

In contrast to DAP-associated Siglecs, ITIM-containing Siglecs are able to silence ITAM-dependent immune responses. The binding of different sialylated glycans induces the phosphorylation of the intracellular tyrosine residues of the receptor by Src-family members. Phosphorylation initiates the recruitment of SHP1 and SHP2, which bind to the receptor and are able to inhibit kinase-dependent pathways (Avril et al., 2005; Crocker et al., 2007).

One prominent example of a Siglec-dependent inhibition-mechanism is the modulation of antibody production. When its B-cell receptor (BCR) recognizes a specific antigen, the B-cells differentiate into antibody-producing plasma cells. In the case that the recognized antigen is co-localized with sialylated structures on endogenous cells, these sialylated glycans are bound by the Siglec-2 present on B-cells (Nitschke, 2005; Tedder et al., 2005). The resulting clustering of BCR, together with the Siglec-2 molecules, recruits SHP1 and SHP2, followed by the inhibition of the kinase-dependent signaling pathway. Thus, reduced antibody production against the autoantigen is initiated (Nitschke, 2005; Tedder et al., 2005). However, Siglec-2 is also known to recruit GRB2 (growth-factor-receptor-bound protein 2), SHC (SH2-domain-containing transforming protein C), PI3K (phosphoinositide 3-kinase) and PLC$\gamma$2 (phospholipase C$\gamma$2), which are effectors of cellular activation, suggesting that Siglec-2 related tasks depend on the respective activated B-cells (Tedder et al., 2005).

#### 3.2.1. Biological role of ITIM-bearing Siglecs

Another example of Siglec-mediated suppression of immune cell function was recently described by Varki and colleagues (Lizcano et al., 2017). They demonstrated that Siglec-9 inhibits neutrophil activation via binding to sialylated glycoproteins on erythrocytes. The authors suggested that this might explain the phenomenon that isolated neutrophils can be very easily stimulated to form neutrophil extracellular traps (NETs), for example. Thus, a sialylated structure may act as a SAMP on erythrocytes to counteract the activation of neutrophils in the blood stream.

Interestingly, cancer cells also seem to exploit Siglec-mediated mechanisms. Leukemic cells from patients suffering from acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) produce, for instance, a much higher amount of ligands for Siglec-7 and Siglec-9. These Siglecs are present on the cell surfaces of NK cells and the detection of their ligands on tumor cells inhibits their activation, thus promoting tumor growth (Hudak et al., 2014; Jandus et al., 2014; Macauley et al., 2014).

Furthermore, an imbalance in the Siglec-mediated system can provoke pathophysiological situations and might be associated with neurodegenerative diseases (Angata et al., 2002; Bradshaw et al., 2013; Cao et al., 2008; Griciuc et al., 2013; Linnartz-Gerlach et al., 2014; Malik et al., 2013). Siglec-3, for instance, is known as a high potential risk factor for Alzheimer, since an increased expression of Siglec-3 on microglia, tissue macrophages of the neuronal system, leads to an insufficient uptake of toxic plaques of amyloid β (Bertram et al., 2008; Hollingworth et al., 2011; Naj et al., 2011).

In addition, polymorphisms of the gene encoding Siglec-8 are associated with the outbreak of allergic asthma (Gao et al., 2010). Siglec-8 is mainly present on human eosinophils. Current research has revealed that ligand binding to Siglec-8 on eosinophils regulates the levels of eosinophils in the tissue by promoting the apoptosis of eosinophils. In mouse models of ovalbumin-induced airway inflammation, epithelia cells and mucins showed an increased amount of ligands for Siglec-F (homologue of hSiglec-8), followed by a decreased number of invading eosinophils (Hudson et al., 2009; Kiwamoto et al., 2013; Macauley et al., 2014).

These five examples (of many) demonstrate from a medical point of view that ITIM-bearing Siglecs play an essential role in controlling the immune response and might represent a powerful therapeutic target during pathophysiological conditions. However, pathogens also seem to make use of the Siglec functions. Pathogens like *Neisseria meningitides, Haemophilus influenza, Campylobacter jejuni, Pseudomonas aeruginosa* and *group B Streptococcus* generate sialylated binding partners of Siglecs to influence the immune response (Chang and Nizet, 2014). *Group B Streptococcus*, for example, produces Sia$\alpha$2,3-Gal$\beta$1,4-GlcNAc, a ligand recognized by Siglec-9, on its capsule. The interaction decreases neutrophil oxidative burst, thus reducing NET formation and promoting the survival of pathogens (Carlin et al., 2009).

## 4. Vertebrates and highly-conserved Siglecs

The development of the first vertebrates dates back to the Paleozoic era more than 500 million years ago. Distinct Siglecs—Siglec-1 (Cao et al., 2009), Siglec-2 (Cao et al., 2009), Siglec-4 (Lehmann et al., 2004) and Siglec-15 (Angata et al., 2007)—are conserved in several branches of vertebrates including fish. In order to prove and summarize the presence of Siglecs in invertebrates as well as vertebrates, we performed BLAST analyses (NCBI Blastn (Altschul et al., 1990)) based on published human and mice sequences. Most likely, the first vertebrates possessed Siglec-2 and Siglec-15 and, accordingly, one pro- and one anti-inflammatory receptor, both of which are still present in all extant species of the five vertebrate classes: fishes, amphibians, reptiles, birds and mammals (Supplements 2, 3 and Fig. 2). It is also likely that Siglec-1 and Siglec-4 are ancient receptors, having been expressed together with Siglec-2 and Siglec-15 in the common ancestor of modern vertebrates.

### 4.1. Siglec-4

Jawless fishes are obviously an exception, as this most primitive form of vertebrates seems to be Siglec-negative (Supplement 2). It might be reasonable to assume that striking events took place, including the emergence of the first Siglec molecules linked to particular cellular immune function, after the separation of bony fishes from jawless fishes. Jawless fishes are the only vertebrates without myelin and these fishes seem to lack Siglec-4 (Knowles, 2017; Zalc, 2006), which is mainly responsible for the stabilization of the myelin sheath in vertebrates. The same applies to invertebrates. In invertebrates, as well as jawless fishes, glial cells surround the axons, while in higher animals Schwann cells or oligodendrocytes are myelin-forming cells (Knowles, 2017; Zalc, 2006). Our alignment of the amino acid sequences of the sialic acid binding domains exhibited a low degree of structural variance between humans, amphibians, reptiles and birds (Supplement 4A) and likewise, hSiglec-4 was similar to its counterpart in different myelin positive fishes (Supplement 4B). Besides sharks, belonging to the cartilaginous fishes and thus representing the “oldest” fish after jawless fishes, only fugu showed a higher degree of sequence differences. Nevertheless, manifest key nucleotide positions were preserved across different vertebrate species. Consequently, our comparison strongly suggests that the development of myelination evolved together with the emergence of Siglec-4. However, when we examined the published intracellular amino acid sequences of Siglec-4 for immunomodulatory elements, we observed that in three of four analyzed bony fishes, ITIM and ITIM-like sequences were present (Fig. 3). This was already observed by Dietz and colleagues and an involvement of Siglec-4 in signal transduction was suggested in fish by the authors (Lehmann et al., 2004). In contrast, in nearly all examined terrestrial vertebrates, these two motifs were absent, except for anole, gecko and koala. This pattern indicates that Siglec-4 might modulate more physiological functions in fish than in terrestrial vertebrates (Lehmann et al., 2004). This interesting aspect is now under investigation in our laboratories. Nonetheless, it is also possible that these sequences are adventitiously present. In this context, reference should be made to a very recent work showing that rock breams challenged with the relevant pathogens *E. tarda*/ *S. iniae*/ red seabream iridovirus (RSIV) respectively, showed significant changes in the expression levels of a Siglec in several organs that has been assigned to Siglec-3 (Jeswin et al., 2018). We hypothesize, however, that the described Siglec was Siglec-4. The sialic acid binding domain has characteristic motifs that are highly conserved in Siglec-4 orthologs (Supplement 5). For instance, the amino acids of the sequence motif **RAIW** in rock bream have chemical characteristics shared with the Siglec-4 sequences of medaka, zebrafish and coelacanth (Fig. 4). This is also the case in higher vertebrates (Supplement 4A). In Siglec-4, a nonpolar amino acid is always attached to the highly conserved tryptophan. In contrast, in Siglec-3, amino acid residues containing a hydroxyl group occupy the same position. Another interesting sequence motif of Siglec-4 is **GRT** (Fig. 4). This motif is highly conserved in medaka, zebrafish, coelacanth, rock beam (Fig. 4) and other vertebrates. In Siglec-3, however, the arginine of this motif is followed by the aromatic and highly hydrophobic amino acid phenylalanine (F), instead of the hydrophilic amino acids serine or threonine (S or T). In sum, the distinctive parallels in the sequences between the Siglec-4 of the analyzed fishes suggest that the described Siglec-3 in rock bream (Jeswin et al., 2018) belongs to Siglec-4 and that Siglec-4 has also immunomodulatory functions in fish. However, it has to be noted here that Cao and colleagues found evidence that the first ancestral regions for CD33-related Siglecs might have already emerged in at least some fish species (Cao et al., 2009).

### 4.2. Siglec-2

Looking at Siglec-2 during evolution, there is another Siglec-dependent system that differs between jawless fishes and higher vertebrates and directly affects the adaptive arm of the immune system. In contrast to higher vertebrates, jawless vertebrates and

| | Mammalians | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ornitho-rhynchidae | Diproto-dontia | Afrotheria | Laurasiatheria | | | | | | | | Glires | | | Human |
| Siglec | Platypus | Koala | Elephant | European shrew | Cat | Horse | Pig | Killer whale | Cow | Sheep | Goat | Guinea pig | Beaver | Mouse | |
| 1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 3 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 4 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 5 | + | - | + | + | + | + | + | + | + | + | + | + | + | - | + |
| 6 | - | - | + | - | - | + | - | - | + | - | - | - | - | - | + |
| 7 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 8 | - | - | + | - | - | + | - | - | + | - | + | + | - | + | + |
| 9 | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + |
| 10 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 11 | - | - | + | - | + | - | - | - | - | - | - | - | + | - | + |
| 12 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | + |
| 14 | - | + | + | - | + | + | + | - | + | + | + | + | + | - | + |
| 15 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 16 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |

**Fig. 2. Presence of Siglec family members in selected mammalian species**. Based on NCBI protein-protein BLASTs against the respective human and mouse sequences, the presence of Siglecs were investigated. Siglec-1, Siglec-2, Siglec-4 and Siglec-15 are labeled in green. Dark blue indicates CD33-related Siglecs, which are presumably expressed by more than 73% of the analyzed species, whereas fields labelled in light blue denote Siglecs expressed only by certain mammalian species. The positive sign indicates the availability of the referring gene sequence; the negative sign marks its absence. For the accession number of a respective sequence see Supplement 17. The sources of all photos are listed in Supplement 18. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

| Species | ITIM | ITIM-like |
|---|---|---|
| Shark | - | - |
| Medaka | + | + |
| Zebrafish | + | + |
| Fugu | - | - |
| Coelacanth | + | + |
| Xenopus | - | - |
| Tibetan frog | - | - |
| Phyton | - | - |
| Alligator | - | - |
| Turtle | - | - |
| Anole | - | + |
| Gekko | + | - |
| Chicken | - | - |
| Goose | - | - |
| Platypus | - | - |
| Koala | + | - |
| Mouse | - | - |
| European Shrew | - | - |
| Guinea Pig | - | - |
| Beaver | - | - |
| Pig | - | - |
| Cow | - | - |
| Sheep | - | - |
| Goat | - | - |
| Horse | - | - |
| Killer whale | - | - |
| Dog | - | - |
| Cat | - | - |
| Elephant | - | - |
| Human | - | - |

**Fig. 3. The presence of ITIM and ITIM-like motifs in Siglec-4 during evolution.** Positive signs indicate the presence of ITIM and ITIM-like motifs in different species based on published cDNA sequences in the NCBI library, negative signs the absence. For the accession number of a respective sequence see Supplement 17.

invertebrates do not express BCR. Invertebrates have no corresponding antigen receptors and jawless vertebrates use variable lymphocyte receptors (VLRBs). As described for Siglec-4, the common ancestors of sharks and bony fishes were apparently the first organisms on earth to express Siglec-2, a regulatory partner of BCR. Thus, in a timely manner a mechanism to counteract the production of autoimmune antibodies had possibly developed. In contrast to the amino acid sequence of Siglec-4, the sequence of Siglec-2 seems to be more variable (Supplement 4A: Siglec-4: identity: 33.34%; Supplement 6B: Siglec-2: identity: 4.9%). Whereas mammalian Siglec-2 sequences are comparably well conserved (Supplement 6A), a large majority of amino acids were exchanged in amphibians, reptiles and birds (Supplement 6B). At first glance, hSiglec-2 seems to share no homology with its orthologs in fish (Supplement 7A), but a pairwise comparison reveals that certain key residues of the sialic acid binding domain of Siglec-2 in fishes and humans are conserved (Supplement 7B). We observed the same when the orthologous sequences from amphibians, reptiles and birds were separately aligned (Supplement 8).

### 4.3. Siglec 15

Siglec-15 appeared during the evolution from cartilaginous fishes to bony fishes (Arctic lamprey: Siglec-15 negative and shark: Siglec-15 positive). The amino acid sequence of the sialic acid binding domain is highly conserved in Siglec-15 positive vertebrates (Supplement 9). Since the deletion of Siglec-15 in mice leads to osteopetrosis (Hiruma et al., 2013), it seems likely that Siglec-15 of osteoclasts plays an important role during bone development. It seems that chondroclasts are mature osteoclasts (Knowles et al., 2012). Thus, in sharks, belonging to the cartilaginous fishes, Siglec-15 might be involved in the regulation of mechanical strength of cartilages. However, until now it is unknown, if Siglec-15 can be also expressed by chondroclasts.

### 4.4. Siglec-1

Siglec-1 is another highly conserved Siglec. This Siglec receptor lacks tyrosine-based motifs in its cytoplasmic tail (Crocker et al., 1995). Instead of implementing inhibitory or activating functions, Siglec-1 is tasked with phagocytic functions (Chang and Nizet, 2014; Chang et al., 2014; Crocker et al., 2007). Its V-set domain is highly conserved in all analyzed species (Supplement 10). Like the above Siglec-2 alignments, evolutionarily conserved residues became obvious in pairwise sequence comparisons (Supplement 11).

Taken together, the genomes of the analyzed bony fishes contain a comparably low number of Siglec types: Siglec-1, Siglec-2, Siglec-4 and Siglec-15. Remarkably, no dramatic changes have occurred during the evolution of the first terrestrial vertebrates, amphibians, reptiles and birds—still encoding species-specific variants of Siglec-1, Siglec-2, Siglec-4 and Siglec-15— although a complete change of the environment took place (Supplements 2, 3 and Fig. 2). However, we do not exclude that further possibly Siglec types may be present, which might also be species specific, besides the confidently identified Siglec-1, Siglec-2, Siglec-4 and Siglec-15 sequences, in the analyzed vertebrates.

Interestingly, in turtles as well as in anoles Siglec-variants with a high sequence homology with hSiglec-14 were detected (Supplement 3). When we compared the V-set domain of hSiglec-14 with these sequences, the obtained alignments showed a high similarity (Supplement 12). As mentioned above, Siglec-14 is an activating receptor. Angata et al. suggested that an arginine residue (R) in the transmembrane domain of hSiglec-14 interacts with DNAX-activation proteins (Angata et al., 2006). Consequently, this domain also was compared in detail (Fig. 5A). The transmembrane domains in turtle and in anole exhibit lysine residue (K) instead of arginine residue (R), which can also mediate the binding of DNAX-activation proteins. The similarity of the compared regions is approximately 50% and 70% in anole and turtle, respectively. Consequently, these two Siglecs may indeed represent orthologues of Siglec-14. However, since these orthologues could only be detected in two reptiles by database search, no evidence had been provided that a common ancestor of mammals and reptiles already expressed a variant of Siglec-14. In addition, in mammals, the Siglec-14 transmembrane domains are almost identical in all analyzed species (Fig. 5B), indicating that the structure of the transmembrane domain is very important for the functionality of Siglec-14. The very strong similarity of the transmembrane domain

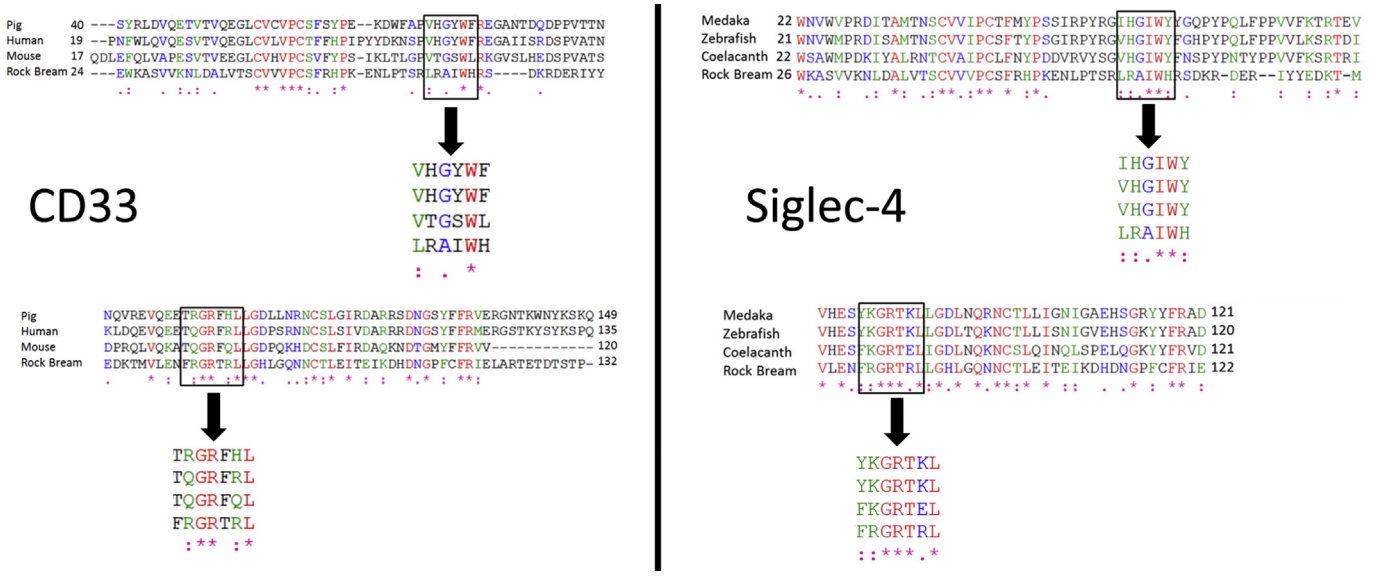



**Fig. 4. Multiple alignments of the V-set domain of CD33 and Siglec-4 sequences from selected species.** Multiple sequence alignments were performed using the Clustal Omega tool of EMBL-EBI using the currently published CD33 sequence in rock bream (GenBank accession number MF377634), as well as the CD33 sequences and Siglec-4 of several fishes. For the accession number of a respective sequence see Supplement 17. (*) equal amino acids; (:) highly similar amino acids; (.) similar amino acids.

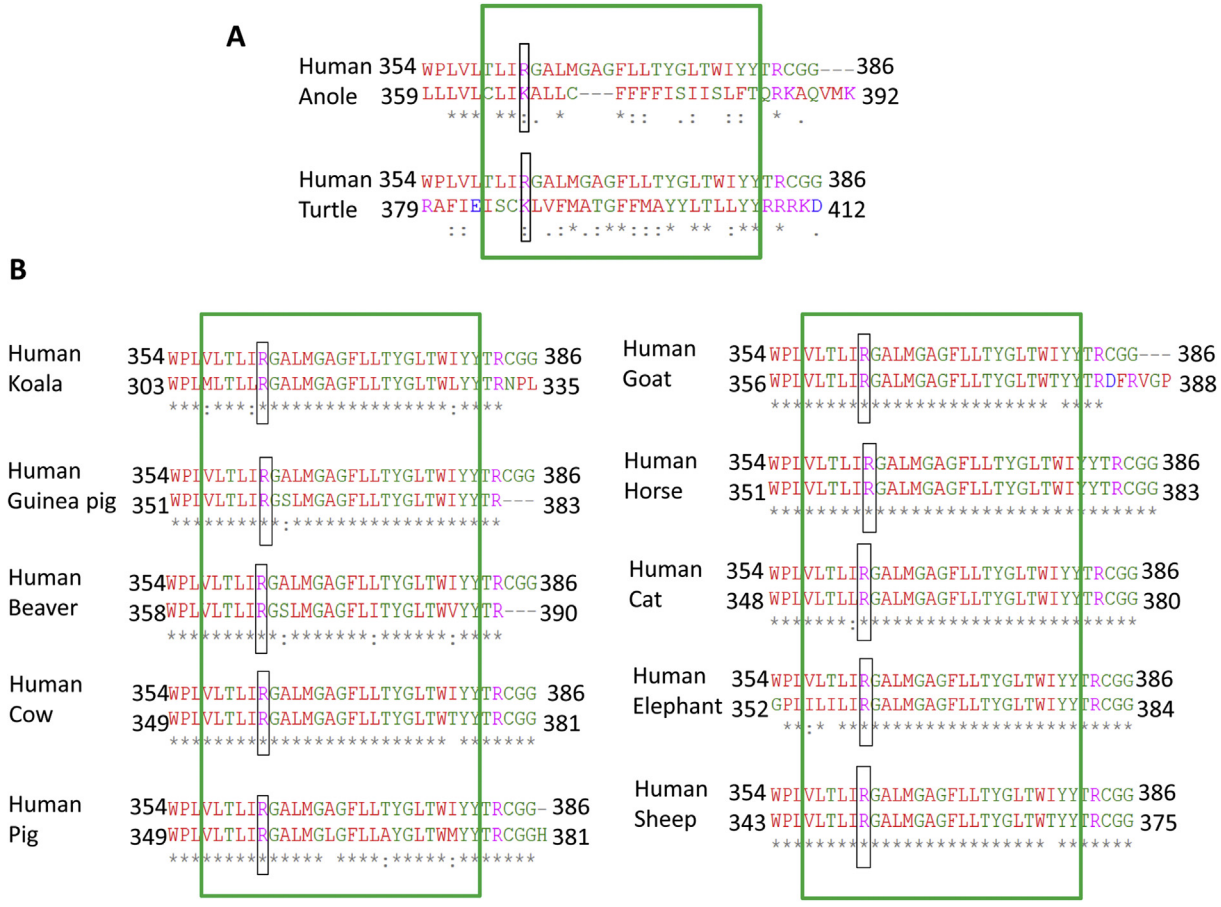



**Fig. 5. Multiple alignments of the transmembrane domain of Siglec-14 from selected species.** A) The transmembrane domain of human Siglec-14 was compared with potential membrane domains of the assigned Siglec-14 variants in turtle and anole. B) In addition alignments of the transmembrane domains of all human Siglecs were performed. Using the Clustal Omega tool of EMBL-EBI Multiple, sequence alignments were performed. For the accession number of a respective sequence see Supplement 17. (*) equal amino acids; (:) highly similar amino acids; (.) similar amino acids.

in mammals places the similarities between mammals and these two reptiles (turtles and anoles) in perspective. Actually, the similarity of the transmembrane domain of all different human Siglecs is comparable with the similarities between human and anole Siglec-14 (Supplement 13). Taken together, although noticeable similarities exist, at the moment one can only speculate whether these two proteins in anoles and turtles are really Siglec-14 variants and, if so, whether a common ancestor of reptiles and mammals already generated a Siglec-14-variant or whether parallel and independent developments took place. In addition, it should be noted in this context that this brief evolutionary outline refers basically to GenBank and Ensembl database entries, which might still be incomplete or incorrectly annotated, leading ultimately to inaccurate estimates.

Nevertheless, the outlined million-year lasting expansion of the Siglec family apparently mirrors well, to a degree, upward evolutionary development across vertebrate classes: humans contain at least 15 different Siglec types, whereas only up to four Siglecs are present in bony fishes. This particularity is somehow remarkable inasmuch as bony fish underwent several rounds of additional whole-genome duplications and simultaneous independent gene duplications (Hurley et al., 2007; Robinson-Rechavi et al., 2001), leading to the “more genes in fish” theory (Wittbrodt et al., 1998). The teleostean innate immune system has a much broader collection of complement proteins, including up to eight isoforms of the central complement component C3, in all fish species investigated so far (Forn-Cuni et al., 2014; Kobis et al., 2015; Lovoll et al., 2007; Nakao et al., 2000; Sunyer et al., 1997; Zarkadis et al., 2001). The family of Toll-like receptors (TLRs) represents a similarly well-investigated example of immune gene expansion in the teleostean lineage. TLRs recognize structurally-conserved components of microorganisms (Kawasaki and Kawai, 2014) and have moreover been demonstrated to interact with Siglecs to fine-tune the transduction of danger signals (Chen et al., 2014). While up to a dozen TLRs have been identified in mammals, fishes possess a significantly-expanded repertoire of TLRs (Ahn et al., 2014; Altmann et al., 2016; Gong et al., 2017; Quiniou et al., 2013; Solbakken et al., 2016), including counterparts to all human TLRs (except TLR6 and TLR10) plus ‘non-mammalian’ or even ’fish-specific’ TLRs (up to TLR28 (Wang et al., 2016), as well as multiple isoforms of certain TLR types (Lee et al., 2013; Sundaram et al., 2012). Several downstream molecules of the TLR signaling cascade have also multiplied in the teleostean lineage (Brietzke et al., 2014; Rebl et al., 2008). Compared with the mammalian repertoire of TLRs, amphibians are equipped with additional TLRs, too (Babik et al., 2015). These examples illustrate once more that gene expansion does not necessarily indicate an upward development during evolution.

## 5. Siglecs in mammals

Presumably in conjunction with the appearance of the earliest mammals, the family of CD33-related Siglecs has been growing for over 200 million years (Capuco and Akers, 2009). Our phylogenetic tree of mammalian Siglec sequences clearly clusters the sequences of Siglec-1, Siglec-2, Siglec-4 and Siglec-15 (Supplement 14), while the CD33-related Siglecs are less clearly separated (Fig. 6), especially with regard to Siglec-7, Siglec-8 and Siglec-9. The high degree of sequence similarity among CD33-related Siglecs might be driven by diverse selection pressures caused by pathogens using sialic acid-based molecular mimicry mechanisms, as well as pathogens utilizing host sialic acids as ligands during invasion (“Red Queen Effect”), already mentioned by Angata and Varki (Varki, 2006; Varki and Angata, 2006). With regard to Siglec-3, Siglec-5 and Siglec-6, it may be assumed that the high sequence identity of CD33-related Siglecs may lead to incorrect annotations (Supplement 15). Moreover, the amino acid sequences of Siglec-5 and Siglec-14 seem to be relatively similar, possibly complicating unambiguous separation (Supplement 15) (Angata et al., 2006). These two receptors interact as antagonists—one inhibits the immune response and the other one activates the immune system—suggesting that these two receptors evolved from each other (Angata et al., 2006). Interestingly, the activating Siglec-14 is absent in some humans and this circumstance allows group B *Streptococcus* to suppress the activation of neutrophils (Ali et al., 2014). Varki and colleagues suggested that the loss of Siglec-14 may increase the risk of prematurity (Ali et al., 2014), since amniotic epithelium—representing a part of the placenta system—expresses Siglec-14 and is a known target of invading group B *Streptococcus* (Vanderhoeven et al., 2014).

Fig. 2 gives a fair overview of the Siglec system in mammals. As expected, the highly conserved Siglec-1, Siglec-2, Siglec-4 as well as Siglec-15 are present in all analyzed species. Furthermore, in nearly all analyzed mammals hits for Siglec-3, Siglec-5, Siglec-10 and Siglec-14 were found. No ortholog sequences of hSiglec-7 have been published so far, while for all other Siglecs putative orthologue sequences were identified in one or more of the screened mammals. However, the reason for the presence of distinct CD33-related Siglecs in different mammals remains a mystery.

We wanted to investigate whether the different types of placentas in mammals could represent triggers for the development of different ITIM-containing CD33-related Siglecs. During evolution, different types of placentas developed. Human beings, primates and some rodents possess a hemochorial placenta that is characterized by direct exchange between the maternal part and the fetus, responsible for gas, metabolite, nutrition, hormone and antibody transfer between the embryo and the mother (Newman, 1960). Therefore, we expected that species with a hemochorial placenta would possess a bench of Siglecs in order to prevent immune reactions against the fetus. Human beings seem to be an exception within the species with hemochorial placentas (Supplement 16), as they possess the highest number of Siglec types. Animals like dogs, cats and elephants have an endotheliochorial placenta (missing blood contact between the fetus and the maternal blood vessels (Furukawa et al., 2014)) characterized by the maintenance of the blood vessel endothelia; these species bear comparable numbers of inhibitory Siglecs, like animals with a hemochorial placenta. In addition, mainly farm animals have epitheliochorial placentas (Furukawa et al., 2014), which are characterized by the maintenance of the maternal uterus epithelia, the maternal connective tissue and the maternal blood vessels (Furukawa et al., 2014). Consequently, no direct contact between the maternal blood—and thus the maternal immune system—and the fetus exists, leading to the prospect that these species might handle pregnancy without a high amount of transmembrane immune regulatory receptors like Siglecs. Altogether, we did not find any correlation between the number of Siglecs and the placenta type. However, we note that only the general presence of Siglec-encoding genes in an organism is retrievable from databases and information on the tissue-specific expression of Siglecs during pregnancy is limited.

Intriguingly, lactation apparently developed alongside with the growing of the mammalian CD33-related Siglec family and their modulation possibilities of the immune system (Cao and Crocker, 2011; Cao et al., 2009). Actually, egg-laying mammals—evolving for ~200 million years—seem to express at least one variant of inhibitory the mammalian CD33-related Siglec, since the platypus was positive for Siglec-5 (Figs. 2 and 7). Data from studies analyzing the milk oligosaccharides of platypus suggest that the milk of monotremes contains different α2,3- and α2,6-sialylated milk oligosaccharides (Urashima et al., 2015). Interestingly, koalas already

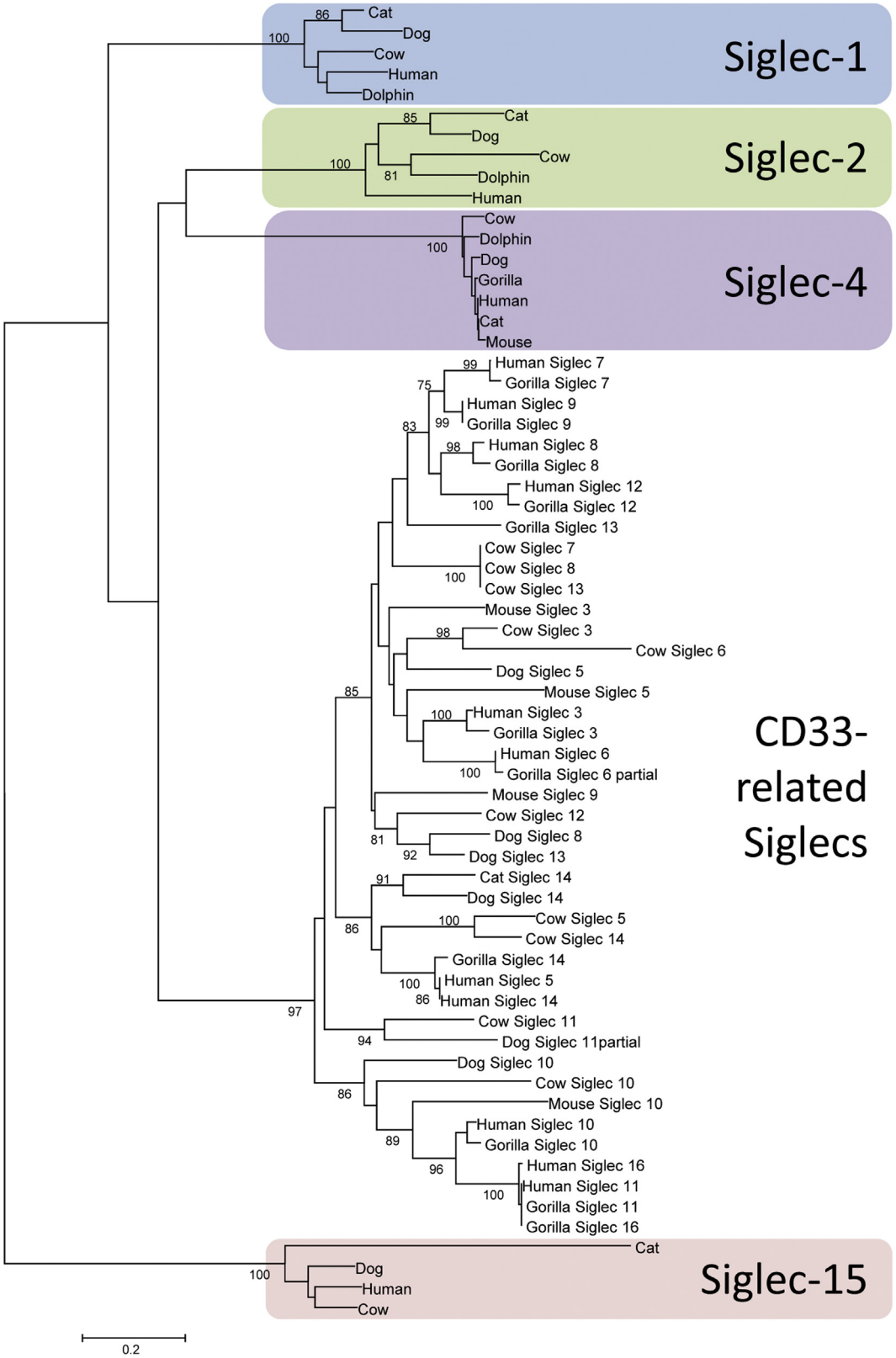



**Fig. 6. Phylogenetic relationship of Siglec proteins from representatives of selected mammalians**. This dendrogram was constructed using the Neighbor-Joining Method applying the Poisson correction distance model included in the Molecular Evolutionary Genetics Analysis package (MEGA, version 6.0) as conducted in our previous report (Rebl et al., 2011). Bootstrap confidence values represent the percent frequency of appearances of each clade in 1000 replicas. Labeling includes the species name. For the accession number of the respective sequence, see Supplement 17. Due to high sequence identities, the CD33-related Siglecs could not be separated clearly (Supplement 15). Used animals: Cat, Dog, Cow, Dolphin, Human, Mouse and Gorilla.

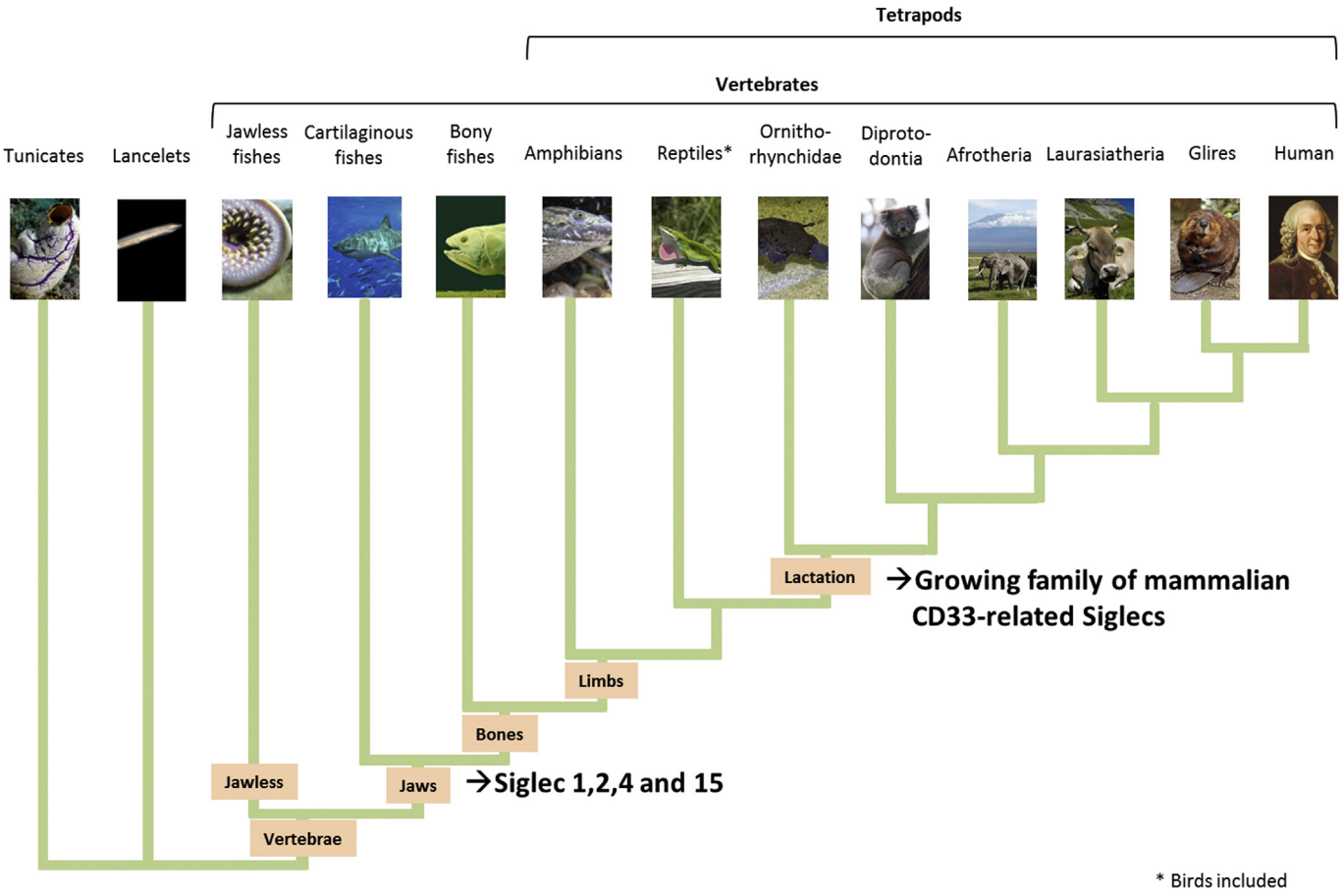



**Fig. 7. Milestones of Siglec evolution.** With the emergence of jaws, the highly conserved Siglec-1, -2, -4 and -15 occurred. Around 200 MY ago, with the emergence of lactation (Capuco and Akers, 2009), the family of CD33-related siglecs expanded. The sources of all photos are listed in Supplement 18.

express three different CD33-related Siglec variants, which bear strong sequence homology with Siglec-3, Siglec-10 and Siglec-14. Also, koala milk oligosaccharides contain α2,3- as well as α2,6-linked sialic acid residues.

Since the microbiome of milk directly influences the composition of the gut microbiome in offspring (Pannaraj et al., 2017), the interplay during lactation between sialylated milk oligosaccharides and the sialome of the microbiome of the mammary gland, as well as in the intestine of the offspring, might represent important triggers for the evolution of CD33-related Siglec mechanisms. This might explain the parallel gene expansion of the mammalian CD33-related Siglec family and the development of lactation.

## 6. Conclusion

Our analyses of Siglecs from numerous species from evolutionary distinct clades suggest, in agreement with previous studies, that Siglec-1, Siglec-2, Siglec-4 and Siglec-15 were present in the ancestral vertebrate before the separation of tetrapod's (e.g. humans) and teleost fishes (e.g. salmon) more than 400 million years ago (Angata et al., 2007; Cao and Crocker, 2011; Cao et al., 2009; Lehmann et al., 2004). Thus, an ecological niche/habitat-independent high evolutionary pressure on these four highly conserved receptors exists, whereas a significant expansion of the CD33-related Siglec genes took place during the evolution of mammals (Betancur et al., 2015). Siglec-2 might prevent autoimmunity (Poe and Tedder, 2012). Siglec-4, which is expressed on the innermost myelin wrap, may stabilize the myelin-axon interaction (Sun et al., 2004). The conservation of Siglec-15 might reflect its important function for bone resorption (Macauley et al., 2014). Thus, it seems very likely that during the evolution of first vertebrates, the emergence of Siglec-2, Siglec-4 and Siglec-15 is closely linked to the development of the BCR system, myelination and bones, respectively (Fig. 7). A further important key step concerning the evolution of Siglecs might be the development of lactation, which may initiate the progress of the mammalian CD33-related Siglec family (Fig. 7). Thus, sialic acid and Siglec machinery underwent remarkable changes during essential stages of vertebrate evolution.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2018.05.008.

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