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Galectin-3 binds highly galactosylated IgG1 and is crucial for the IgG1 complex mediated inhibition of C5aReceptor induced immune responses



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ABSTRACT

Changes in the glycosylation of immunoglobulins have been shown to modulate immune homeostasis and disease pathology. In this sense it has been shown that highly galactosylated but not agalactosylated IgG1 immune complexes (ICs) inhibit C5aR-mediated pro-inflammatory immune responses via the assembly of FcγRIIB-Dectin-1 receptor complexes. In this study we demonstrated that Galectin-3, a galactose-binding lectin that is known to cross-link proteins on cell-surfaces via binding their N-glycans, bound to highly-galactosylated, but not agalactosylated IgG1. Further, Galectin-3 was essential for the IC-mediated inhibition of C5a-induced neutrophil chemotaxis *in vitro*. Taken together our results indicate that Galectin-3 mediates the interaction of ICs with the FcγRIIB-Dectin-1 receptor complex for delivering immunoregulatory signals to inhibit C5aR-mediated immune responses.

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1. Introduction

The complement system, as an ancient danger-sensing system is playing an important role for the immune surveillance and homeostasis by orchestrating inflammatory processes and by the cross-talk with other immune pathways [1]. Activation of the complement system initiates a proteolytic cascade with C3 and C5 as central molecules, leading subsequently to the formation of the membrane attack complex, but also to the formation of the smaller cleavage products C3a and C5a. C3a and C5a bind distinct surface receptors and induce various effector functions [2]. C5a is a pro-inflammatory mediator and a potent chemoattractant for immune cells. The interaction of C5a with its receptor C5aR1 (CD88) is involved in the disease pathology of various infectious and non-infectious inflammatory diseases such as sepsis, asthma and autoimmune disorders, e.g. rheumatoid arthritis [3,4]. Investigating the regulation of C5a-induced inflammation has become particularly important for the development of new therapeutic strategies. A promising negative feedback mechanism recently has been described by Karsten et al. [5]. They demonstrated in the murine

system that highly galactosylated IgG1 immune complexes (HiGal-IgG1-ICs) had an inhibitory capacity on C5a-induced inflammation by triggering the assembly of an FcγRIIB and Dectin-1 receptor complex. Stimulation with galactosylated ICs induced Dectin-1-dependent Syk-phosphorylation that was found to be linked to the phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) of FcγRIIB. Downstream phosphorylation of SH2-containing 5'-inositol phosphatase (SHIP) resulted in inhibition of C5aR-signaling and thereby suppressed C5a-triggered pro-inflammatory functions. Importantly, this inhibitory potential of ICs was associated with the abundance of terminal galactose residues in the N-linked Fc-glycosylation.

Galectin-3, a galactose residues binding soluble protein of the Galectin family, is expressed by a wide range of immune and non-immune cells and is described to regulate inflammatory immune responses [6,7]. It has a chimeric structure with a C-terminal Carbohydrate recognition domain (CRD) and an N-terminal non CRD with homologue repeats. In presence of glycan ligands Galectin-3 oligomerizes via its N-terminal domain [8]. This allows Galectin-3 oligomers with multivalent CRDs to cross-link glycosylated structures on cell surfaces [6,7]. Consequently, Galectin-3 serves as a promising candidate for mediating the interaction between galactosylated ICs, FcγRIIB and Dectin-1. Indeed, Shan et al. demonstrated the importance of Galectin-3 to facilitate the interaction of

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MUC2, a highly glycosylated mucosal protein covering the intestinal mucosa, with the FcγRIIB-Dectin-1 receptor complex in the context of human Dendritic cells [9]. They further demonstrated that the assembly of the Galectin-3-FcγRIIB-Dectin-1 receptor complex interfered with the activation of nuclear factor κB (NF-κB) dependent gene expression. Subsequently, LPS-induced expression of inflammatory cytokines was suppressed.

In the study presented we aimed to investigate if binding of Galectin-3 to highly galactosylated IgG1 ICs compared to reduced binding to agalactosylated IgG1 ICs might be the missing link to explain the ability or inability of ICs to suppress C5aR-induced effector functions, depending on IgG1-galactosylation.

2. Materials and methods

2.1. Antibodies and recombinant proteins

The TNP-specific mouse IgG1, proven before to be highly galactosylated (Karsten et al., 2012 [5], clone 107.3, HiGal-IgG) was from BD Pharmingen and preparation of TNP-OVA anti TNF-OVA IgG1 immune complexes (IC) was performed as described [10]. For degalactosylation of the HiGal-IgG recombinant Glyko® β(1,3,6)-Galactosidase/GALase II (ProZyme) was used. Recombinant human C5a was purchased from Sigma-Aldrich. HRP-labeled antibodies against mouse or rabbit IgG were from Dianova. Genes encoding for human Galectin-3 were cloned into pET-3c vector (Novagen, Merck KGaA, Darmstadt, Germany), recombinant Galectin-3 (rGal3) was expressed in *E. coli* and purified via affinity chromatography to lactosyl-sepharose as described [11]. Mutated recombinant Galectin-3 (rGal3-R186S) with arginine186 replaced by a serine was produced as described [12].

2.2. Animals

Bone marrow-derived cells were isolated from Femur and Tibia of 8–11 week old wild-type (C57BL/6J) and Galectin-3 knockout (C57BL/6J B6.Cg-Lgals3tm1Poi/J [13]) mice. Galectin-3 knockout mice were purchased from The Jackson Laboratory (Maine, USA). All experiments were approved by institutional and governmental authorities (Thüringer Landesamt für Verbraucherschutz (TVL), Bad Langensalza).

2.3. Fractionized lysis of bone marrow-derived neutrophils (BMN)

Bone marrow-derived cells, flushed out of femur and tibia of 8–11 week old mice, were subjected to erythrocyte lysis using hypotonic buffer, and washed with PBS. Subsequently, neutrophils were isolated by negative selection via MACS® cell sorting using the mouse neutrophil isolation Kit (Miltenyi Biotec) and counted on a hemocytometer. For fractionized lysis 3×10^6 BMN were homogenized in 100 μl hypoosmolar buffer (10 mM HEPES, 2 mM KCl, 1.5 mM MgCl₂, 1 mM PMSF, pH 7.2). After incubation for 1 h on ice the suspension were homogenized through a 0.4×25 -gauge needle and NP-40 to a final concentration of 0.1% was added. For whole cell lysate 15 μl were removed from the sample and mixed with reducing sample buffer ($4 \times$ NuPage LDS sample buffer, Life technologies, supplemented with 1 mM DTT) containing DNase. Centrifugation of the remaining sample to remove the nuclei was followed by a second centrifugation of the supernatant at 11000 rcf for 10 min in order to remove cellular debris. Next, plasma membrane fragments and cytosol were separated by centrifugation of the supernatant at 15000 rcf for 25 min. The supernatant containing the cytosol was removed and reducing sample buffer was added. The pellet containing plasma membrane fragments was washed with the hypoosmolar buffer. After centrifugation the

pellet was diluted in $1 \times$ reducing sample buffer. All samples were denatured at 95 °C for 5 min and analyzed for Galectin-3 expression by Western Blot, using polyclonal rabbit anti-Galectin-3 antibody (H-160, Santa Cruz Biotechnologies, 1:2000). Success of fractionized lysis was evaluated via re-blot with rabbit monoclonal anti CD11b antibody (1:1000, clone EPR1344, Abcam) and polyclonal rabbit anti Actin (20–33) (1:4000, Sigma-Aldrich).

2.4. Immunoprecipitation

High-galactosylated IgG1 (HiGal-IgG) or degalactosylated IgG1 (LoGal-IgG), bound to Protein-G Agarose, was incubated with recombinant human Galectin-3 (rGal3) for 2 h at 4 °C. After intensive washing the IgG1 was eluted from the Agarose with 8 M urea. Binding of rGal3 to the IgG was tested via Western Blot, using rat monoclonal anti Galectin-3 (1:1000, clone ebioM3/38, eBioscience).

2.5. Surface plasmon resonance assay

Experiments were performed on a Biocore® X100 instrument (GE Healthcare, UK) with HBS-P (0.05% P20) used as flow buffer. The mouse IgG1 was immobilized on the surface of a CM5 biosensor microchip by the amine coupling method using *N*-ethyl-*N*-dimethylaminopropyl-carbodiimide (EDC) and *N*-hydroxy-succinimide (NHS).

2.6. Chemotaxis assay

Chemotaxis of Bone marrow-derived cells to the chemoattractant C5a was performed as described previously [14]. Briefly, bone marrow-derived neutrophils were suspended in PBS containing 2% BSA at a density of 5×10^6 cells per ml. Cells were then pre-incubated for 30 min with HiGal-IgG1 IC (1 μg/ml in PBS) or as a control only in PBS at 37 °C. C5a as a chemoattractant was used at a concentration of 10^{-8} M. The bottom wells of the chemotaxis chamber, containing the chemoattractant, were overlaid with a 3-μm polycarbonate membrane. Then 50 μl of the cell suspension was placed in the top well and incubated for 30 min at 37 °C. Subsequently, the membranes were removed and the cells on the bottom side of the membrane were stained with Diff-Quick. The numbers of migrated cells in five high-power fields were counted, and the number of cells per mm² was calculated by computer assisted light microscopy. Results are expressed as the mean value of triplicate samples.

3. Results

3.1. Galectin-3 is expressed by murine bone-marrow derived neutrophils

Galectin-3 is expressed in many tissues by hematopoietic and non-hematopoietic cells, and is secreted into the intercellular space during inflammation [6,7], but reports about the expression of endogenous Galectin-3 in neutrophils are contradictory. Consequently, in a first step the expression of Galectin-3 by the bone marrow derived neutrophils (BMN) used in this study had to be elucidated. For that reason, BMN were lysed by cellular fractions and analyzed for Galectin-3 expression via Western Blot analysis. As shown in Fig. 1 Galectin-3 protein was detected in the whole cell lysate, the cytosol and in the membrane fraction of BMN. Re-blots against CD11b and Actin were performed confirming the purity of the plasma-membrane and the cytosol fraction. In contrast to murine bone marrow derived macrophages, no Galectin-3 expression was detectable on the surface of BMN via flow

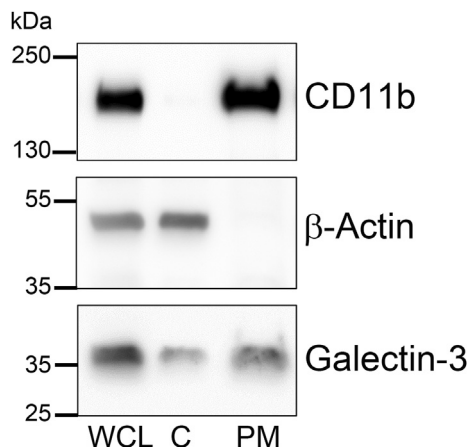


Fig. 1. Galectin-3 is expressed on mouse neutrophils. Western Blot analysis of different cell compartments of BMN against CD11b, β -Actin or Galectin-3, respectively. WCL – whole cell lysate; C – cytosol; PM – plasma membrane.

cytometry (data not shown), using a directly labeled antibody, representing a less sensitive method than Western Blot. Taken together this results indicate, that BMN express Galectin-3 intracellular and at their cell surface, but at lower levels than macrophages.

3.2. Binding of Galectin-3 with IgG1-ICs depends of Fc-galactosylation

The importance of protein glycosylation for the regulation of innate and adaptive immune responses is generally accepted [15,16]. Immunoglobulins are also glycosylated proteins and the composition of terminal sugars, e.g. terminal galactosylation, of Fc glycans is of significant importance for IgG effector functions [17–19]. Because terminal galactose residues are ligands for galectin-3, we hypothesized that Galectin-3 binds high galactosylated IgG1-ICs (HiGal-IgG1) with a much higher affinity than low galactosylated IgG1-ICs (LoGal-IgG1). This was confirmed by performing immunoprecipitation experiments of recombinant Galectin-3 (rGal3) with HiGal-IgG1 or LoGal-IgG1 (Fig. 2). Our data demonstrates the binding of rGal3 to HiGal-IgG1 but not to LoGal-

IgG1. For a further confirmation of this results a binding assay based on surface plasmon resonance using a Biacore instrument was performed (Fig. 3A–C). For this purpose, HiGal-IgG1 or LoGal-IgG1 was covalently bound to the chip surface. Interaction of wildtype rGal3 (rGal3-wt) with HiGal-IgG1 (Fig. 3A/C) resulted in higher relative Response Units (RU), than the interaction of rGal3 with LoGal-IgG1 (Fig. 3B/C). These data indicated, that rGal3 binds HiGal-IgG1 with a much higher affinity than LoGal-IgG1. Consequently, binding of Galectin-3 to IgG1 depended on the IgG1-galactosylation. Next, we tested the binding of a mutated Galectin-3 R186S (rGal3-R186S) protein in which arginine186 was replaced by a serine which is causing a decreased sugar binding affinity of the Carbohydrate recognition domain (CRD) [12]. As shown in Fig. 3A/B, binding of rGal3-R186S to HiGal-IgG1 was reduced, related to the wildtype Galectin-3 (rGal3-wt), but was still diminished when compared to LoGal-IgG1. Therefore, the reduced binding of rGal3-R186S, compared to rGal3wt, underlines the importance of the CRD for specific binding of Galectin-3 to galactosylated IgG1.

3.3. IgG1-IC-induced inhibition of neutrophil migration towards C5a depends on Galectin-3 expression

Karsten et al. [5] has demonstrated that HiGal-IgG1 ICs but not LoGal-IgG1 ICs had an inhibitory effect on neutrophil migration towards C5a. In order to investigate the functional importance of Galectin-3 for the inhibitory capability of HiGal-IgG1 ICs, an *in vitro* neutrophil chemotaxis assay towards C5a was performed. As shown in Fig. 4, migration of untreated BMN towards C5a was unchanged in BMN out of wildtype (WT) mice (Fig. 4A) compared to Galectin-3 deficient (Gal3^{-/-}) BMN (Fig. 4B). Migration of WT BMN was significantly inhibited by HiGal-IgG1 IC (Fig. 4A), reaffirming the results of Karsten et al. [5]. In contrast to WT BMN, migration of Gal3^{-/-} BMN was not inhibited by HiGal-IgG1 ICs (Fig. 4B). This confirms that Galectin-3 is crucial for the inhibitory ability of HiGal-IgG1 ICs on C5a-induced chemotaxis.

4. Discussion

Galectin-3 is known to play an important role in modulating the immune response in the pathogenesis of numerous microbial infections [20–25]. It is expressed in many tissues by hematopoietic and non-hematopoietic cells, particularly activated macrophages, regulating the function of neutrophils, monocytes, macrophages, epithelial and endothelial cells [6,7]. During inflammation Galectin-3 is shown to be also secreted into extracellular spaces [7]. For example, Galectin-3 is released into the alveoli during infections with *Streptococcus pneumoniae* [26]. Extracellular Galectin-3 binds to neutrophils and modulates neutrophil functions (e.g. CD11b regulation and L-selectin-shedding) [21,27]. The published results about the expression of endogenous Galectin-3 by neutrophils are contrary. While some groups found no Galectin-3 expression in neutrophils [21,28], others reported that Galectin-3 was expressed intracellular and released by neutrophil activation [22]. Therefore, we first clarified the expression of Galectin-3 by the BMN (Fig. 1) used in this study and found that they express Galectin-3 intracellular and at their cell surface, although the surface expression of Galectin-3 on BMN was clearly lower than on murine bone marrow derived macrophages.

N-linked glycosylation of proteins plays a distinct role for the control of innate and adaptive immune responses [15,16]. Immunoglobulins are also glycosylated proteins, with changing installation in health and disease [17,19]. It has been demonstrated that the frequency of agalactosylated IgGs increases in chronic inflammatory conditions like rheumatoid arthritis [17]. Karsten et al.

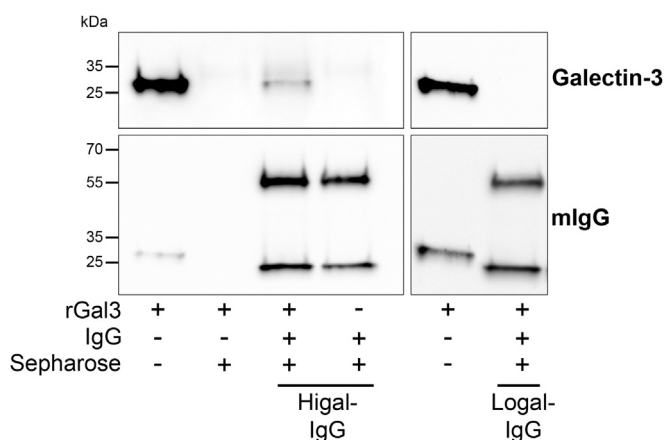


Fig. 2. Galectin-3 binds to glycosylated mouse IgG1. Galectin-3-mIgG1-immunoprecipitation: Western Blot analysis targeting Galectin-3 (upper blots) followed by a reblot targeting mouse IgG (lower blots). Recombinant Galectin-3 (rGal3) was co-incubated with high-galactosylated mouse IgG1 (HiGal-IgG) or degalactosylated mouse IgG1 (LoGal-IgG), respectively.

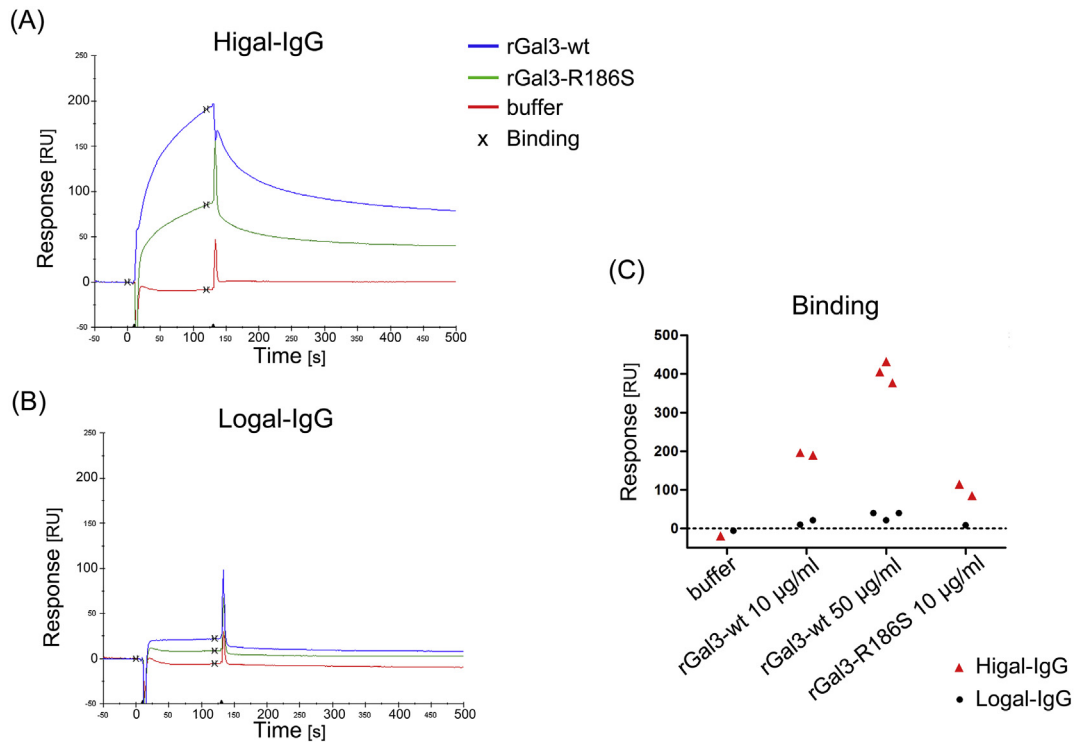


Fig. 3. Binding of Galectin-3 depends on its Carbohydrate recognition domain and IgG1-galactosylation. Surface plasmon resonance (Biacore) binding assay of recombinant Galectin-3 (analyte) to HiGal-IgG or LoGal-IgG (ligand), respectively. Binding of recombinant wildtype-Galectin-3 (rGal3-wt) was compared to a recombinant Galectin-3 with mutated Carbohydrate recognition domain (rGal3-R186S). (A) Adjusted sensogram of the binding of 10 µg/ml rGal3-wt (blue line) or rGal3-R186S (green line) to HiGal-IgG. (B) Adjusted sensogram of the binding of 10 µg/ml rGal3-wt (blue line) or rGal3-R186S (green line) to LoGal-IgG. (C) Binding (marked with a cross in the sensogram in (A) and (B)) of rGal3-wt or rGal3-R186S to HiGal-IgG (red triangles) or LoGal-IgG (black dots) during repeated measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

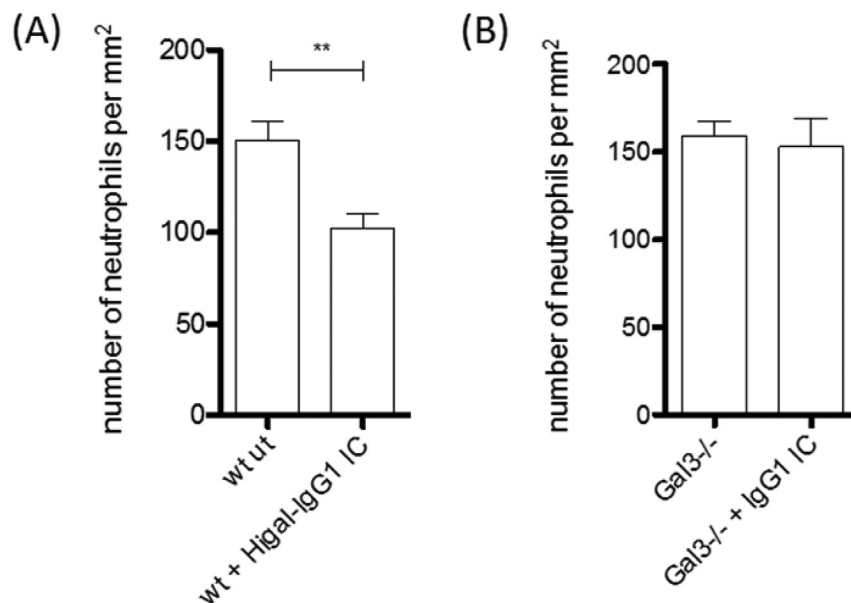


Fig. 4. Expression of Galectin-3 is essential for IgG1-mediated inhibition of neutrophil transmigration *in vitro*. *In vitro* chemotaxis of bone marrow derived neutrophils (BMN) towards recombinant C5a with or without addition of HiGal-IgG1 IC, performed in a Boyden chamber. (A) Chemotaxis of BMN derived from wildtype mice (wt). (B) Chemotaxis of BMN derived from Galectin-3 knockout mice (Gal3^{-/-}). n = 7, p < 0.01 **.

demonstrated, that highly galactosylated, but not agalactosylated IgG1-ICs were able to inhibit C5a-induced pro-inflammatory effector functions, such as the chemotaxis of neutrophils [5]. Thus, it is tempting to speculate that IgG1-IC mediated inhibition of

complement induced inflammation may serve as a regulation feedback loop that prevents exaggerated immune reactions and is invalid in hyperinflammatory diseases like rheumatoid arthritis due to a shift towards agalactosylated IgG1. Terminal galactose

residues are ligands for Galectin-3, that is also known to bind N-glycan of IgE [6,7,17,29]. In accordance with this our data demonstrated that rGal3 bound to HiGal-IgG1, but not to LoGal-IgG1 via the CRD of rGal3.

In a next step, the hypothesis that Galectin-3 can cross link HiGal-IgG1 IC, but not LoGal-IgG1 IC with the glycosylated receptors FcγRIIB and Dectin-1 was tested. Thereby, Galectin-3 hypothetically enables the assembly of the inhibitory FcγRIIB-Dectin-1 receptor complex, leading to inhibition of C5a-mediated effector functions of neutrophils like chemotaxis or CD11b up regulation. Indeed, HiGal-IgG1 ICs inhibited the migration of WT BMN but were not able to inhibit the chemotaxis of Gal3^{−/−} BMN towards C5a (Fig. 4).

We also found that C5a-induced migration of BMN was unchanged in WT BMN compared to Gal3^{−/−} BMN (Fig. 4). At the first view this seems to be contrary to the results of Baseras et al. [28], who demonstrated that migration of human neutrophils towards C5a was inhibited by Galectin-3. But a deeper look at the experimental setting reveals that the results are not comparable. In our study only the effect of the low abundant endogenous Galectin-3 was taken into account, while Baseras et al. added exogenous recombinant Galectin-3 to their assays, inducing an activation of the neutrophils by the recombinant Galectin-3 itself.

In summary, we could demonstrate that binding of IgG1 by Galectin-3 was dependent of the Fc-galactosylation and that HiGal-IgG1 ICs needed Galectin-3 to inhibit C5a-induced chemotaxis of neutrophils. These results support the assumption that Galectin-3 is responsible for the IC-induced formation of the FcγRIIB-Dectin-1 receptor complex. This is highly reinforced by the findings of Shan et al. [9], demonstrating that MUC2, a highly glycosylated protein of the gut mucus, enabled the formation of a Galectin-3-FcγRIIB-Dectin-1 receptor complex that inhibited the expression of pro-inflammatory cytokines in dendritic cells. Shan et al. revealed the importance of the FcγRIIB-Dectin-1 receptor complex and the additional importance of the presence of Galectin-3 on Dendritic cells in the context of LPS-induced inflammation in the gut. In contrast, Karsten et al. demonstrated the importance of the FcγRIIB-Dectin-1 receptor complex, on neutrophils in the context of immune complexes and C5a-induced inflammation during chronic inflammatory diseases. Our findings now suggest that Galectin-3 is also in this system important for the interaction of FcγRIIB-Dectin-1 receptor complex. Taken together, the Galectin-3-FcγRIIB-Dectin-1 receptor complex might play a global role for regulating inflammatory immune responses of various immune cells, which can be modulated by the binding of differently galactosylated structures to Galectin-3.

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