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Chapter 6

The major GalNAcβ1-4GlcNAc-containing glycoprotein of *Schistosoma mansoni* eggs, kappa-5, induces type 2-polarized granulomas in a pulmonary mouse model

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Abstract

The main pathology of schistosomiasis is caused by a granulomatous response which develops around eggs that get trapped in host tissue. Previously, granuloma formation has been explored in pulmonary and hepatic mouse models using antigen-coated Sepharose beads as artificial eggs. Using the hepatic model, it has been shown that the glycan portion of soluble egg antigens (SEA) can induce granuloma formation similar to SEA. Moreover, from a group of synthetic schistosome-related glycoconjugates, only those terminating in GalNAcβ1-4GlcNAc (LDN) and Galβ1-3/4GlcNAc (LN) were able to induce the same granulomatous responses. So far, the native *S. mansoni* egg glycoproteins expressing these granuloma-inducing glycans have remained elusive. Recently, we have identified kappa-5 as the major glycoprotein in SEA that expresses LDN-motifs. In this report, we show in a pulmonary mouse model for granuloma formation that kappa-5 coated to Sepharose beads is able to induce type 2-polarized granulomatous responses. Furthermore, we show that the capacity of kappa-5 to induce granulomas involves its LDN motifs, as selective removal of LDN significantly diminished granuloma formation.

Introduction

Schistosomes are parasitic helminths that infect over 200 million people in (sub-) tropical areas around the world. The main pathology of *S. mansoni* infection is initiated by the large proportion of parasite eggs that instead of being excreted with the feces, get trapped in various organs such as the liver. Here, egg antigens induce a type 2 inflammatory response, leading to the formation of periovular granulomas composed of mainly CD4+ T cells, macrophages and eosinophils, and collagen fibers [1]. The development of the egg granuloma is shown to be highly dependent on Th2-polarized CD4+ T cells [2] and alternatively activated macrophages [3].

The nature of *S. mansoni* egg molecules involved in the induction and modulation of granulomas has been explored in established experimental mouse models by injection of antigen-coated Sepharose beads as artificial eggs into the liver [4-6] and lungs [7-9]. In the liver model, beads coated with *S. mansoni* soluble egg antigens (SEA) give rise to granulomas comparable to those around schistosome eggs in terms of cellular composition and expression of adhesion molecules and extracellular matrix components [4;6]. Destruction of the integrity of SEA glycans by meta-periodate abolished the inflammatory properties of the SEA beads, demonstrating a major role for glycosylation in the initiation of the granulomatous reaction [6]. To explore the type of glycans involved, beads were injected coated with a selected set of synthetic model glycoconjugates containing glycan elements representative for schistosome eggs, such as GalNAcβ1-4GlcNAc (LacdiNAc, LDN), Galβ1-4(Fucα1-3)GlcNAc (Lewis X), Fucα1-2Fucα1-3GlcNAc (DF-Gn) and Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F) [10]. Interestingly, from the tested set, only beads coated with a synthetic LDN-conjugate and beads coated with asialofetuin, a glycoprotein with terminal Galβ1-3/4GlcNAc (LacNAc, LN) groups, were able to induce granulomas in this model. All other glycoconjugates tested elicited only a monolayer of macrophages, similar to uncoated and albumin-coated beads.

Recently, we have described kappa-5 as one of the major antigens in *S. mansoni* SEA [11;12]. Glycosylation analysis demonstrated that kappa-5 contains four N-glycosylation sites which for a large part carry triantennary LDN motifs. Moreover, kappa-5 was found to be the major LDN-containing glycoprotein in SEA [11], in terms of abundance as well as reactivity with the GalNAc-specific lectin soybean agglutinin and an LDN-binding antibody. These characteristics prompted us to test the immunological properties of kappa-5 in the pulmonary mouse model. Although kappa-5 is presumably not present during the early stages of egg development, it is definitely presented to the immune system later on, as it gives rise to antibody responses in infected hosts [13]. Therefore we set out to investigate whether kappa-5 might have a role in granuloma formation and/or modulation. Using the pulmonary model, we demonstrate that kappa-5 coated beads are able to induce granulomas of a similar nature as egg- and SEA-induced granuloma. Furthermore, beads containing kappa-5 of which the LDN motifs were enzymatically removed induce significantly less and smaller sized granulomas opposed to untreated kappa-5 coated beads. Conclusively, here we describe the first native *S. mansoni* egg glycoprotein with glycan-dependent granuloma-inducing properties.

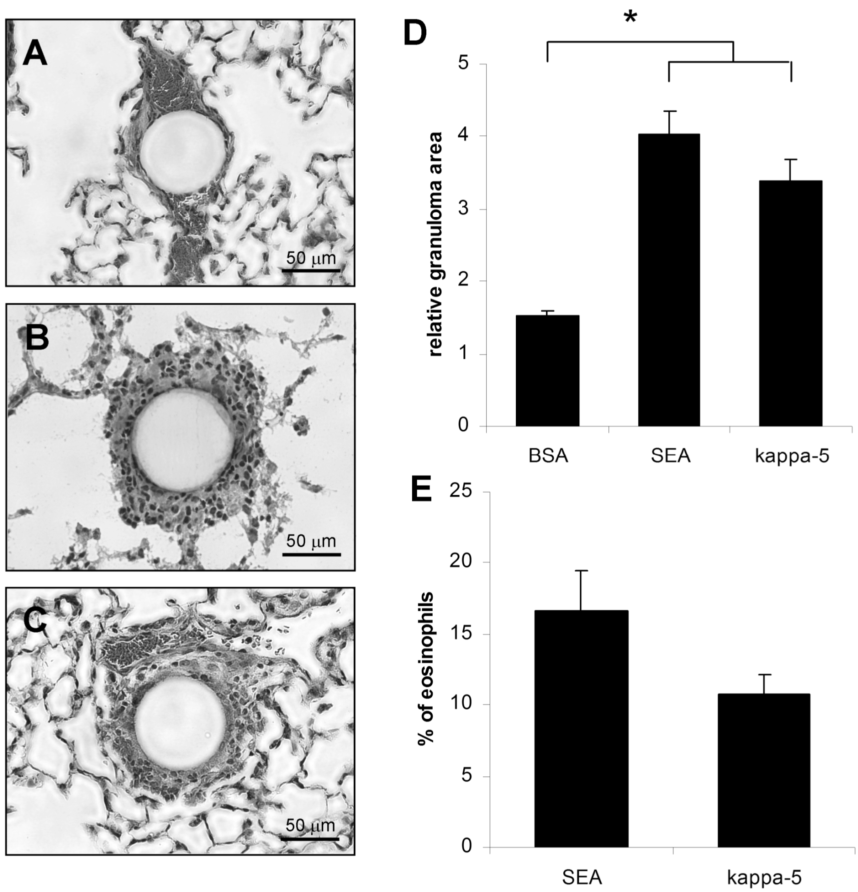

Figure 1. Induction of pulmonary granuloma formation by kappa-5 in an experimental mouse model. Antigen-coated beads were injected into the lungs of mice and at 14 days post-injection, granuloma formation was assessed. (A-C) Representative examples of BSA- (A), SEA- (B) and kappa-5- (C) induced granuloma are shown for every experimental group. Lung slides are stained with H&E. (D) The relative granuloma area (granuloma area divided by bead area) was assessed for each experimental group. (E) The percentage of eosinophils was calculated for SEA- and kappa-5-induced granulomas Graphs show mean values with SEM (Mann-Whitney: * indicates $p < 0.001$).

Results

The S. mansoni egg glycoprotein kappa-5 induces Th2-polarized granuloma formation

Sepharose beads were coated with SEA or the SEA component kappa-5, and tested in an established mouse model for pulmonary granuloma formation [7]. Fourteen days after bead injection, control beads coated with bovine serum albumin (BSA) showed no cellular reaction except for a monolayer of cells (Figure 1A), while granuloma formation was observed around beads coated with SEA (Figure 1B). Importantly, the majority of beads (93 %) coated with isolated, native kappa-5 were able to induce granulomatous responses (Figure 1C). As the size of the commercially obtained beads varied significantly, the granuloma area relative to the bead area was used as a measure for the granuloma size (relative granuloma area; Figure 1D). We found that there was no significant difference between the granuloma sizes induced by SEA- and kappa-5-coated beads.

To investigate the nature of the granulomas induced by the antigen-coated beads, granulomas within H&E-stained lung slides were screened for the presence of eosinophils, which is a hallmark for type 2 granulomas [3;6]. 16.6 ± 2.9 % of the cells within the SEA-induced granulomas were eosinophils (Figure 1E). For the kappa-5-induced granulomas, the majority of inspected granulomas also contained eosinophils (10.7 ± 1.4 %; Figure 1E).

The granuloma-inducing properties of kappa-5 are partly mediated by its LDN motifs

To investigate whether the LDN motifs on kappa-5 are involved in the granuloma-inducing properties of this glycoprotein, kappa-5-coated beads were treated with β-*N*-acetylhexosaminidase to remove LDN motifs (kappa-5Δhexnac beads). Nano-LC-MS analysis verified the nearly complete removal of unsubstituted GalNAc and GlcNAc residues from kappa-5, as shown for site N251 in Supplementary Figure 1. When tested in the pulmonary mouse model, the overall relative granuloma area of kappa-5Δhexnac beads was significantly lower as compared to that of untreated kappa-5 beads (Figure 2A). To further explore this difference, the potency of coated beads to induce granulomas, as well as the distribution of the different sizes of the granulomas induced, was studied. Interestingly, 33 % of the kappa-5Δhexnac beads was no longer able to induce granulomas but instead induced monolayer reactions similar to the BSA control, compared to only 7 % of kappa-5 coated beads (Figures 2B and 2C). Moreover, when evaluating granuloma formation around individual beads, it became evident that the kappa5Δhexnac bead population contained more small-sized and less large-sized granulomas as compared to untreated beads (Figure 2D and Supplementary Figure 2). The reduction in granuloma size upon LDN removal was due to a decrease in cell numbers, as a

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strong correlation was found between granuloma area and cell numbers (r=0.468, p<0.001). To summarize, removal of LDN from kappa-5-coated beads resulted in a higher percentage of beads unable to induce granulomas and a reduction in granuloma cell numbers around the remaining, granuloma-inducing beads.

Notably, kappa-5Δhexnac beads initiated similar, Th2-type granulomas as untreated kappa-5 beads, as was demonstrated by a comparable percentage of eosinophils within kappa-5Δhexnac- and kappa-5-induced granulomas (10.3 ± 0.9 % and 10.7 ± 1.4 %, respectively).

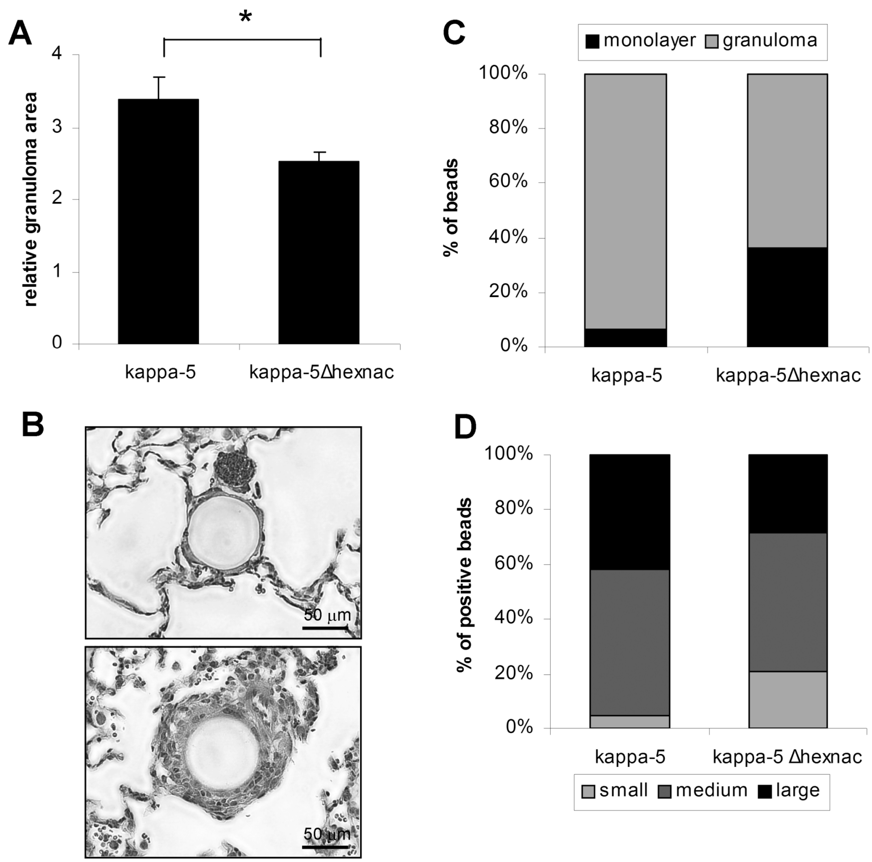

Figure 2. Enzymatic removal of LDN from kappa-5 leads to less and smaller sized granulomas. β-*N*-hexosaminidase-treated and untreated kappa-5 beads were injected into the lungs of mice and at 14 days post-injection, granuloma formation was assessed. (A) The relative granuloma area (granuloma area divided by bead area) was assessed for untreated and treated kappa-5 beads. Graphs show mean values with SEM (Mann-Whitney: * indicates $p < 0.05$). (B) Representative example of kappa-5 beads treated with β-*N*-acetylhexosaminidase inducing either a monolayer of cells or a multilayer granuloma. Lung slides are stained with H&E. (C) Percentage of beads surrounded by a monolayer of cells (monolayer) and beads surrounded by a granulomatous response (granuloma) for treated and untreated kappa-5 beads was calculated. (D) Untreated and treated kappa-5 beads surrounded by a granulomatous response (positive beads) were grouped on the basis of their relative granuloma area; small (<2), medium (2-4) and large (>4).

Discussion

S. mansoni egg glycoconjugates induce strong Th2 type inflammatory responses in the host, which include the formation of granulomas around tissue-lodged eggs. Previous reports using a hepatic mouse model have demonstrated that glycans from *S. mansoni* SEA play a major role in the initiation of this inflammatory response [6]. Also, in the same mouse model, LDN in the context of a synthetic glycoconjugate coated to beads was demonstrated to have similar granuloma-inducing properties [10]. However, the native *S. mansoni* glycoproteins that carry these immunogenic glycans have remained undetermined. In this report, we present for the first time a single, native *S. mansoni* egg glycoprotein, the SEA-component kappa-5, with granuloma-inducing properties. Moreover, we have found that the glycan motif LDN significantly contributes to this immunogenic effect.

The type 2 granulomatous response induced by *S. mansoni* eggs and SEA-coated beads is characterized by an influx of Th2-polarized CD4+ cells, alternatively activated macrophages as well as eosinophils [3;6]. As confirmed by the presence of eosinophils, we here show that granulomas induced by beads coated with kappa-5 and kappa-5Δhexnac share this type 2-polarized nature. This indicates that the granulomatous properties of LDN and other, yet unknown components of kappa-5, could be of relevance during natural *S. mansoni* infection. Notably, the percentage of eosinophils in our SEA-induced granulomas is comparable with granulomas elicited by SEA beads in a similar model of pulmonary granuloma induction [9]. Granulomas surrounding kappa-5 (Figure 1E) and kappa-5Δhexnac (data not shown) beads appear to contain lower percentages of eosinophils, although experiments need to be repeated to achieve statistical relevance. This might indicate that SEA contains other (glycosylated) proteins that have a positive effect on eosinophil infiltration.

The mechanisms through which kappa-5 exerts the immunomodulatory effects described in this report, still need to be elucidated. However, the involvement of the glycan motif LDN indicates that recognition by pattern recognition receptors (PRRs) on antigen-presenting cells (APCs) might play an important role. PRRs which were previously shown to recognize LDN and/or GalNAc are galectin-3, MGL and Dectin-2. MGL is a C-type lectin with a narrow specificity for unsubstituted, terminal α- and β-linked *N*-acetylgalactosamine [14]. Recognition of SEA by MGL has been demonstrated to be at least partially conferred by the GalNAc-containing LDN motifs [14]. Moreover, *in vitro* recognition of kappa-5 by dendritic cells (DCs) is in part dependent on MGL binding (Chapter 4 of this thesis). However, MGL does not seem to recognize LN [14], a motif structurally related to LDN which has been shown to exhibit comparable granuloma-inducing properties, suggesting that other receptors recognizing both LDN as well as

LN are more likely to mediate their immunomodulatory effects. Galectin-3 recognizes both LDN and LN motifs and has previously been proposed to be a key molecule in *S. mansoni* granuloma formation, as it is upregulated in egg granulomas of *S. mansoni*-infected hamsters and mice and colocalizes with LDN-glycans on eggshells [10;15]. However, galectin-3 $^{-/-}$ mice infected with *S. mansoni* only showed minor phenotypic changes compared to normal mice in terms of perioval granuloma formation [16-18]. Therefore, this receptor does not seem to play an important role in the initiation or modulation of granulomas during natural infection. Recently, Dectin-2 has been shown to bind terminal galactose and GalNAc residues [19], and thus could be a potential receptor for both LN and LDN motifs. SEA has been demonstrated to be recognized by and signal via Dectin-2, and mice deficient for ASC and Nlrp2, signal molecules involved in SEA-induced Dectin-2 signaling, developed smaller granulomas around *S. mansoni* eggs [20]. Hence, Dectin-2 signaling might pose a potential mechanism for kappa-5 to modulate perioval granuloma formation.

While we show that the LDN motifs on kappa-5 are involved in the granuloma-inducing properties of kappa-5, the glycoprotein must contain other granulomagenic features, as β-*N*-acetylhexosaminidase-treated kappa-5 still contains some activity (Figure 2). Upon LDN removal, the major fraction of kappa-5 glycans consist of an N-glycan core containing two core fucose and a xylose, while a small fraction of glycans additionally carry an α3-fucosylated GlcNAc (Supplementary Figure 1). The latter structural element was however previously shown to lack granuloma-inducing properties in the hepatic mouse model [10]. Also all other fucosylated structures tested were inactive in this model [10], suggesting that the di-fucosylated core is also not involved. A possible role for the core xylose residues on kappa-5 glycans in granuloma formation has so far not been investigated. In addition, involvement of the protein part of kappa-5 can also not be excluded, although the amino acid sequence of kappa-5 shares no similarities with any known functional motifs [11].

Granuloma formation is initiated when eggs lodge into the organs of the host. The egg-derived molecules involved in activation and maintenance of the granulomatous response may include soluble excretory/secretory (ES) glycoproteins as well as molecules associated with the egg shell. As kappa-5 is not considered to be secreted by eggs at the time point when granuloma formation sets in [11], it is unlikely that kappa-5 plays a dominant role in this stage of granulomagenesis. However, kappa-5 is effectively presented to the immune system as evident by the high antibody titers against kappa-5 that have been detected in *S. mansoni*-infected individuals [13]. Moreover, it is believed that kappa-5 is a constituent of hatching fluid [11], to which the host presumably is exposed when eggs die in the tissues. While kappa-5 is capable of inducing granulomas in our model, in the biological context it might therefore be involved in the maintenance or modulation of granulomas. Well-studied immunomodulatory molecules that are probably presented to the immune system at the earlier stages of granuloma development are the major ES glycoproteins IPSE/α1 and omega-1. The LeX-dominated glycosylation of these two ES glycoproteins is unlikely to be directly involved in the initiation of granuloma formation, as LeX motifs on a synthetic glycoconjugate did not show any granuloma-inducing properties in a hepatic mouse model [10;21]. IPSE/α1 has instead been reported to play a role in the down modulation of granuloma formation, as *S. mansoni*-infected mice that were treated with antibodies to block IPSE/α1 activity, developed significantly larger perioval granulomas compared to untreated infected mice [22]. Besides a possible role for other secreted egg glycoproteins, also the egg shell may have glycan-dependent granuloma-inducing properties. It has been recently demonstrated that the shell of *S. mansoni* eggs is glycosylated [23], but clear structural information is lacking so far.

To conclude, our data suggest that kappa-5 plays a role in the induction and modulation of periovular granulomas during schistosome infection, and that this property is partly dependent on the terminal LDN structures on the kappa-5 glycans. These data provide new insights in the glycan-dependent immunomodulatory properties of *S. mansoni* egg molecules.

Material and Methods

Animals

Six week old female BALB/c mice were purchased from Harlan. Mice were housed under SPF conditions and all animal experiments were approved by the animal experimental committee of the University of Leiden, The Netherlands.

Preparation of antigens and antigen-coated beads

S. mansoni soluble egg antigens (SEA) was prepared and isolated as described previously [24]. Kappa-5 was isolated by SBA affinity chromatography as described previously [12]. For bead coating, 1 mg of glycoprotein was coupled to 1 ml Cyanogen bromide-activated Sepharose 4B heads (Sigma, St. Louis, MO), ranging in diameter from 45 to 165 μm, as previously described [7]. 50.000 kappa-5 coated beads were treated with 62.5 mU β-*N*-acetylhexosaminidase from *Canavalia ensiformis* (Sigma) in 100 mM sodium phosphate buffer, pH 5.0 for 24 h at 37°C, after which the same amount of enzyme was added for another 24 h.

Induction of pulmonary granulomas in mice

BALB/c mice received 5000 antigen-coated beads suspended in sterile PBS i.v. in the lateral tail vein. Four mice were used for every set of beads. The animals were sacrificed 14 days after injection and lungs were removed.

Light microscopy and immunohistochemistry

To evaluate the granulomatous response, 4 μm-thick formalin-fixed paraffin-embedded lung sections as well as acetone-fixed cryostat sections were stained with H&E. Granuloma as well as bead areas were quantified using Bersoft Image Measurement Software v4.03 (Bersoft Inc., Puerto Plata, Dominican Republic) and the relative granuloma area was calculated, representing the average of the granuloma area divided by the average of the bead area. Only granulomas with visible central beads and bead area above 1600 μm^2 were included. Also, granuloma sizes were grouped in small, medium and large-sized granulomas, based on relative granuloma areas of under 2 (small), between 2 to 4 (medium) and over 4 (large). For each experimental group, at least 50 granulomas were assessed and the variables were compared and statistically analyzed with a Mann-Whitney test. P values of less than 0.05 were considered significant. In addition, at least 20 granulomas per experimental group were visually inspected for the total number of cells and number of eosinophils.

Mass spectrometry

Trypsin (Promega, Leiden, The Netherlands) was added to a sample of the β-*N*-acetylhexosaminidase and untreated kappa-5 beads at a 1:100 trypsin/antigen ratio and incubated overnight at 37 °C. Resulting (glyco)peptides were analyzed using nano-HPLC-ESI-ion trap-MS as described previously [21].

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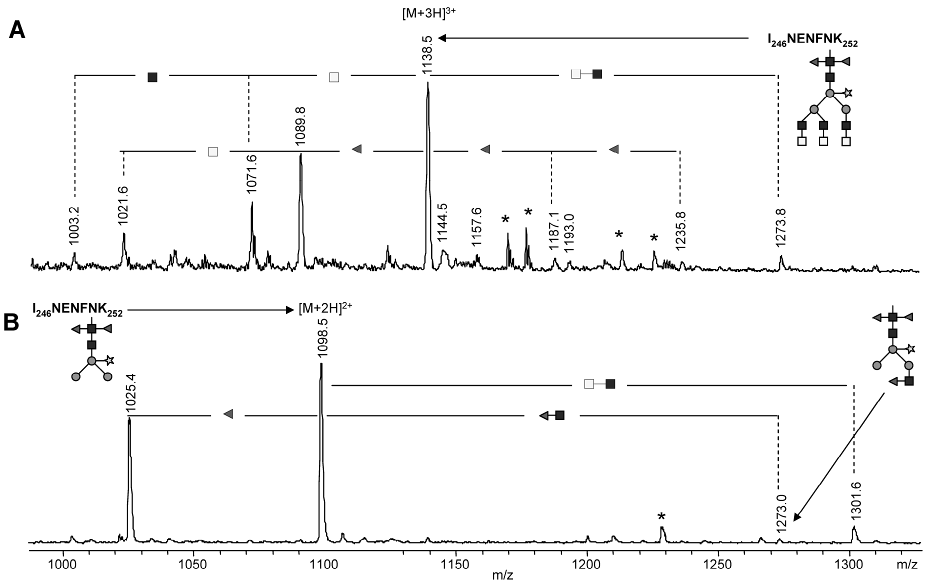
Supplementary Figures



Supplementary figure 1. β-*N*-acetylhexosaminidase treatment of kappa-5 beads. Beads coated with kappa-5 were either left untreated (A) or treated with β-*N*-acetylhexosaminidase (B) and a sample of beads was digested with trypsin. Resulting kappa-5 glycopeptides were analyzed by nano-LC-MS. The accumulative mass spectrum representing all glycoforms of the tryptic glycopeptide containing glycosylation site N251 is shown. Signals are labeled with monoisotopic masses. Differences in fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine content of the glycan moiety are indicated. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; light star, xylose.

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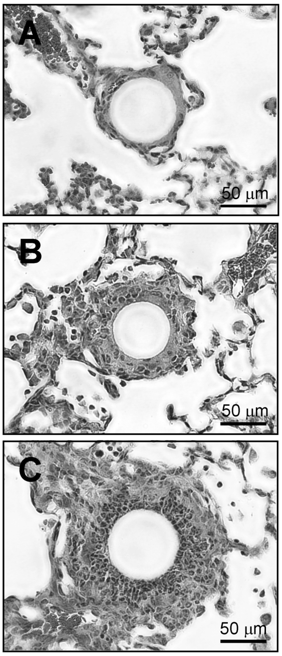

Supplementary figure 2. Representative examples of small (A), medium (B) and large (C) sized granulomas. Granulomas were grouped on the ba sis of their relative granuloma area; small (<2), medium (2-4) and large (>4).