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N-carboxyanhydride polymerization of glycopolypeptides that activate antigen presenting cells through Dectin-1 and -2

Matthew N. Zhou[+], Corleone S. Delaveris[+], Jessica R. Kramer, Justin A. Kenkel, Edgar G. Engleman, and Carolyn R. Bertozzi*

Abstract: The C-type lectins Dectin-1 and Dectin-2 contribute to innate immunity against microbial pathogens by recognizing their foreign glycan structures. These receptors are promising targets for vaccine development and cancer immune therapy. However, currently available agonists are heterogeneous glycoconjugates and polysaccharides from natural sources. Here, we designed and synthesized the first chemically-defined ligands for Dectin-1 and -2. They comprised glycopolypeptides bearing mono-, di-, and trisaccharides and were built via polymerization of glycosylated N-carboxyanhydrides. Through this approach, we achieved glycopolypeptides with high molecular weights and low dispersities. We identified structures that elicit a proinflammatory response through Dectin-1 or -2 in antigen presenting cells. With their native proteinaceous backbones and natural glycosidic linkages, these agonists are attractive for translational applications.

Microbial pathogens display a variety of glycans that differ from human host structures. The innate immune system employs glycan-binding proteins, including C-type lectin receptors (CLRs), to recognize pathogen-associated motifs and trigger immune cell activation.[1] Many CLRs are expressed on antigen-presenting cells, and thus have been investigated as targets for vaccine adjuvants and for antigen delivery.[2] These studies have primarily focused on dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)[3] and mannose receptor CD206[4], although interest in other CLRs is growing as they become better characterized.[5] CLRs may also be attractive targets for provoking an anticancer immune response in the typically immunosuppressive tumor microenvironment.[5] This strategy is currently under clinical investigation with other classes of innate immune receptors[6], with the aim of complementing medicines that enhance adaptive immune mechanisms such as checkpoint inhibitors[7] and adoptive cell therapies.[8]

[*] M. N. Zhou,[+] C. S. Delaveris,[+] Prof. C. R. Bertozzi
Department of Chemistry
Stanford University
Stanford, Ca 94305
E-mail: bertozzi@stanford.edu
Prof. J. R. Kramer
Department of Bioengineering
University of Utah
Salt Lake City, UT 84112
Dr. J. A. Kenkel, Prof. E. G. Engleman
Department of Pathology and Medicine
Stanford University
Stanford, CA 94305
Prof. C. R. Bertozzi
Howard Hughes Medical Institute
Stanford University
Stanford, CA 94305

[+] These authors contributed equally to this work.

Dectin-1 (Dec-1) and Dectin-2 (Dec-2) are CLRs expressed on antigen presenting cells such as dendritic cells and macrophages that are best known for their roles in anti-fungal and -bacterial immunity. Dec-1 binds to microbial β-1,3-glucans, and is a key player in immunity against various pathogenic fungi in mouse models (Fig. 1a).[9] Dec-1 signals through phosphorylation of its immune-receptor tyrosine activation motif (ITAM), leading to activation of NF-κB and AP-1. This drives production of the proinflammatory cytokines, including TNFα and IL-6.[10] Engagement of Dec-1 also promotes phagocytosis of the pathogenic cell.[10] Interestingly, administration of particulate fungal β-1,3-glucans has been shown to improve anti-tumor T cell responses in mice, and Dec-1 was recently implicated in innate immune recognition of tumor-associated glycans and anti-tumor cytotoxicity.[11,12] Notably, smaller soluble glucans bind the receptor but do not result in immune cell activation, which is thought to require receptor clustering.[13] Dec-2 also plays an important role in anti-fungal immunity, and might be similarly involved in anti-tumor innate immunity.[14,15] It selectively binds to high-mannose glycans in a microarray format[16] as well as fungal and mycobacterial structures that are rich in mannosides.[17] Like Dec-1, Dec-2 activates immune cells through NF-κB and AP-1 upon receptor clustering, but signals through the ITAM domain of an associated FcRγ subunit (Fig. 1a).[17c]

Previous work on Dec-1 and -2 has exclusively relied on heterogeneous microbial extracts as sources of activating ligands. However, the investigation of Dec-1 and Dec-2 as translational targets for innate immune activation will require chemically-defined agonists. Herein we report the development of fully synthetic glycopolypeptide-based agonists of Dec-1 and -2. Our design was inspired by a recent study of the fungal pathogen *Malassezia furfur* that identified heavily Ser-O-α-1,2-mannobiosylated glycopeptides as potent agonists of Dec-2 signaling.[17] Accordingly, we synthesized densely glycosylated polypeptides based on this motif (Figure 1b). Natural Dec-1 ligands are rich in β-1,3-glucan structures, which we also integrated into glycopolypeptides.

Central to our approach was the use of N-carboxyanhydride (NCA) polymerization, a technique we previously employed to synthesize monosaccharide-bearing glycopolypeptides with low dispersities and tunable glycosylation densities.[18] The structural modularity of this method also allows for conjugation of the glycopolypeptides to carriers and probes. Some other glycopolymer platforms share these attributes, including ring-opening metathesis polymerization (ROMP)-based materials[19,20] and reversible addition fragmentation chain transfer (RAFT)-based glycopolymers that we[21] and others[22] have reported. However, NCA-derived glycopolymers possess a native proteinaceous backbone and comprise all natural glycans, glycosyl linkages, and amino acid building blocks. Thus, they can be biodegraded to natural components, which we feel is important for translational potential. Notably, NCA-derived polypeptides are an established pharmaceutical treatment:

glatiramer acetate was approved by the FDA in 1996 as a multiple sclerosis therapy.[23]

As building blocks for polymerization, we synthesized O-glycosylated Ser NCAs bearing the β-1,3-glucan fragments β-laminaribiose (Glc2) and β-laminaritriose (Glc3) to engage Dec-1, as well as α-mannose (Man) and α-1,2-mannobiose (Man2) to target Dec-2. We also prepared control glycopolymers adorned with α-N-acetylgalactosamine-O-Ser (GalNAc) and β-lactose-O-Ser (Lac), which should not bind either receptor. Glycosyl amino acids **1a-f** were prepared via the corresponding glycosyl bromides, and the glycosylated amino acids were converted to NCA monomers **2a-f** via the Fuchs-Farthing method (Scheme 1, S1).[24] Glycosylated NCAs (glyco-NCAs) were isolated via cold aqueous workup followed by anhydrous silica gel chromatography to give highly pure monomers in 58-95% yield.[25,26]

We next sought to polymerize the NCA monomers using $(PMe_3)_4Co$, which is known to induce controlled polymerization of other NCAs (Scheme S2).[27] Although we had previously polymerized α-GalNAc-O-Ser NCA, it was not clear that higher-order glycans would be tolerated, as previous attempts to polymerize disaccharide-conjugated NCAs had failed.[28] To our delight, highly purified NCAs **2a-f** formed glycopolymers in the presence of $(PMe_3)_4Co$, with molecular weights as high as 250 kDa and molar mass dispersities under 1.15 (Tables S1-S6). Notably, this was the first demonstrated high-molecular weight polymerization of NCAs bearing di- and trisaccharides.

We further characterized the polymerization parameters for various NCA monomers. Variation of monomer:initiator (M:I) ratios for each glyco-NCA gave low-dispersity glycopolypeptides whose lengths increased linearly with stoichiometry (Fig. 2a, Tables S1,3-6). Polymer molecular weights were higher than would be predicted assuming perfect initiator efficiency, which was consistent with previous NCA literature.[29] Remarkably, even disaccharide NCAs could be polymerized to well over 300 residues. We compared polymerization kinetics of Ser(Man) NCA **2c** and Ser(Man2) NCA **2d** to a non-glycosylated, protected Lys NCA and found that rates were comparable, suggesting that our glyco-NCAs would be incorporated stochastically during copolymerizations (Fig. S1).

Notably, Ser(Glc3) NCA **2b** could only be fully polymerized at M:I ratios lower than ca. 30:1, likely due to trace impurities that impeded polymerization at higher ratios. Polymerization with $(PMe_3)_4Co$ in THF at an M:I ratio of 20:1 gave fully glycosylated chains over 100 residues in length, indicating that steric hindrance is not a limitation in this reaction. Promisingly, **2b** could be efficiently copolymerized at higher M:I ratios as a mixture of 20% Ser(Glc3) NCA with 80% Glu(O*t*Bu) NCA, yielding copolypeptides up to 365 residues (Table S5).

We previously reported a rigid extended conformation for α-GalNAc-O-Ser glycopolypeptides, which we and others proposed is due to hydrogen bonding between the peptide backbone and the glycosyl acetamide.[30] This was supported by the unusual circular dichroism (CD) absorbances we observed. In contrast, Glc2- and Man2-functionalized polypeptides **3a** and **3d** gave aqueous CD absorption spectra corresponding to disordered, random coil conformations, while Man-bearing **3c** showed absorptions consistent with some helical character (Fig. 2b).[31] The larger steric bulk and hydration sphere of the disaccharide sidechains may inhibit the formation of organized structures.

We next investigated immunostimulatory capabilities of glucosylated and mannosylated glycopolypeptides through Dec-1 and Dec-2, respectively. To create a particulate architecture analogous to natural ligands on microbial cells, we covalently attached Glc2- or Man2-glycopolypeptides to fluorescent, 0.8 μm diameter polystyrene beads (Fig. 3a). Poly(ethylene glycol) (PEG) and Lac-glycopolypeptide conjugated beads were used as controls. Polymerizations were performed using Ni(II) catalyst **4**, which installs a C-terminal azide group for post-polymerization functionalization that is chemically orthogonal to the N-terminal amino group (Scheme 1).[18] Amino- functionalized beads were treated with a bicyclo[6,1,0]non-4-yn-9-yl (BCN) *N*-hydroxysuccinimidyl ester and then conjugated to the azide-terminated polypeptides or azido-PEG. Because steric crowding could inhibit receptor binding to fully glycosylated homopolymers, we used 50% and 65% glycosylated polypeptides for Ser(Glc2/Glc3) and Ser(Man2/Lac), respectively. Glycopolypeptides were copolymerized with 1:1 Ala:Glu, all to approximately 100 residues. Ala was chosen as an inert amino acid spacer, while Glu was used to improve solubility. Successful loading of glycopolypeptides onto beads was measured via fluorescence intensity of polypeptides labeled with fluorophores at the N-terminus (Fig S2).

We used a commercial reporter cell line, RAW-Blue, to study immune cell activation through Dec-1. Derived from the RAW264.7 macrophage line, RAW-Blue cells possess an NF-κB-dependent secreted alkaline phosphatase (SEAP), the expression of which can be detected using colorimetric substrates (Fig 3b). The parent RAW macrophages do not express Dec-2, which is expected as expression is typically associated with dendritic cells, not macrophages (Fig. S3).[17] Because we did not have access to a reporter line for Dec-2, we used the Dec-2-expressing murine immature dendritic cell line JAWS II (Fig. S3) and murine monocyte-derived dendritic cells to probe this receptor. Proinflammatory cytokines TNFα and IL-6 were detected by ELISA, and microbead phagocytosis was studied using flow cytometry and fluorescence microscopy (Fig 3b).

Treatment of RAW-Blue cells with Glc2- or Glc3-glycopolypeptide-conjugated beads induced increased expression of the SEAP reporter protein in a Dec-1-dependent manner, indicating activation of the NF-κB transcription factor (Fig. 4a,b, S4). Promisingly, beads displaying Glc2- and Glc3-glycopolymers elicited a comparable response to the bacterially-derived Dec-1 agonist curdlan.[32] Likewise, when JAWSII cells were incubated with Man2-glycopolymer-coated beads, we observed dose-dependent TNFα secretion (Fig. 4c). The cells also responded to Man2- but not Lac-glycopolymer beads with a modest increase in Dec-2-dependent phagocytosis (Figs. S5 and S6). However, the anti-Dec-2 blocking antibody has been reported as a partial agonist, and indeed we observed low-level TNFα expression in JAWSII cells, complicating analysis (Fig. S7a).[33] As an alternative model system, we assayed murine monocyte-derived dendritic cells in plates that had been coated with Lac- or Man2-glycopolypeptides through passive adsorption.

With these primary cells, we observed an exceptional cytokine response to a Man2-glycopolymer with near-quantitative knockdown using the anti-Dec-2 blocking antibody when compared Lac-glycopolymer controls (Fig. 4d, S7b). Remarkably, although other mannose-binding CLRs are known, the cytokine response was highly Dec-2 dependent; however, future studies of how cell type and glycan structure affect the CLR activation profile are of interest to us. Regardless, these results demonstrate that the microbe-inspired glycopolypeptides effectively mimic canonical agonists and induce expected immunological outcomes.

In summary, we developed a new class of synthetic glycopolypeptide innate immune cell activators that exploit the anti-microbial first-responders Dec-1 and -2. The use of NCA polymerization was critical in this effort, as polypeptides with the size and complexity of glycopolymers **3a-f** cannot be synthesized by conventional peptide or glycosylation chemistry. Our observation that plate- and bead-immobilized glycopolymers are potent activators of Dec-1 and -2 signaling is consistent with models for these receptors as components of a phagocytic synapse wherein mechanical resistance is important for activation.[13] Notably, TLR agonists on polymeric scaffolds have shown enhanced potency, and liposomal nanostructure formulations are undergoing preclinical evaluation.[34,,35] With this novel platform, we intend to explore how Dec-1 and -2 agonists perform in related supramolecular structures.

Acknowledgements

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Keywords: glycoconjugates • ring-opening polymerization • glycopeptides • immunology • glycopolymers

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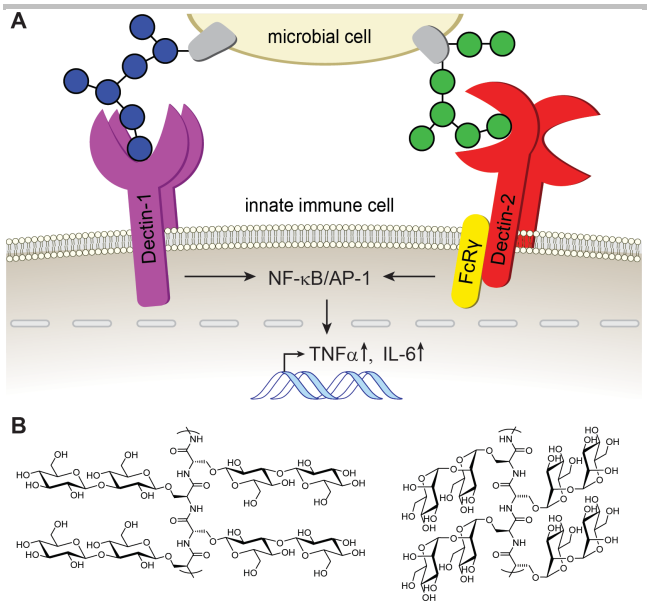
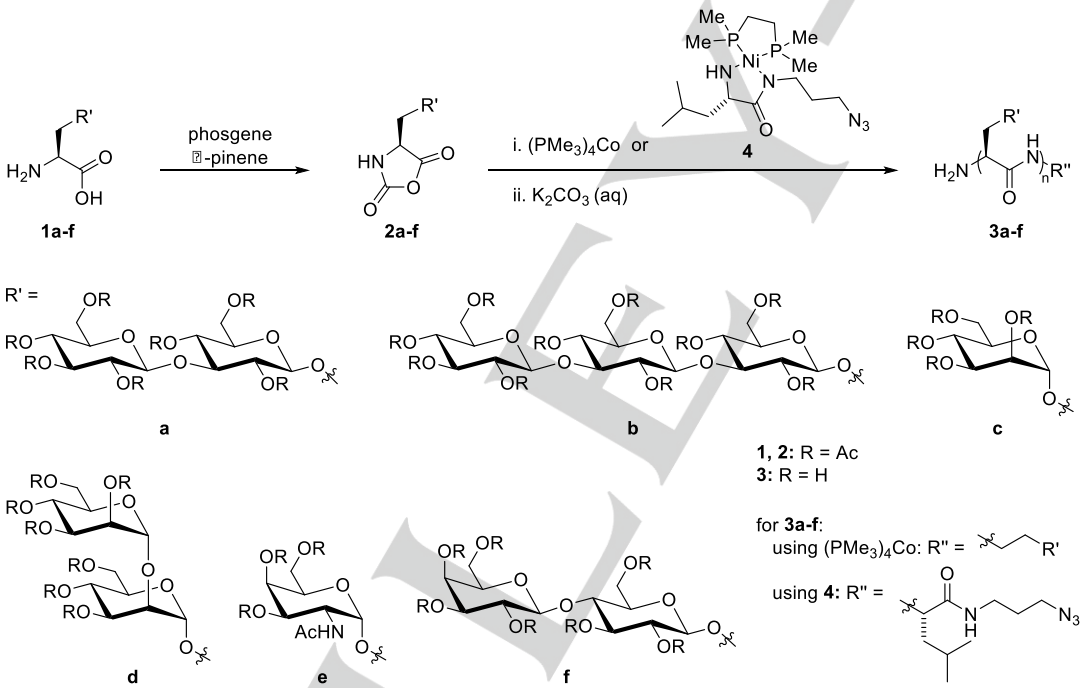


Figure 1. A) C-Type lectin receptors Dec-1 and Dec-2 recognize β-glucans and α-mannans, respectively, and induce expression of cytokines such as TNFα and IL-6 through activation of NF-κB and AP-1. Dec-1 signals through a cytoplasmic signaling domain, while Dec-2 requires an associated Fc receptor γ (FcRγ) subunit. B) Representative target glycopolypeptide structures based on natural β-1,3-glucan agonists of Dec-1 (left) and α-1,2-mannobiosylated agonists of Dec-2 (right).



Scheme 1. Synthesis of glycopolypeptides via metal-catalyzed ring-opening polymerization of N-carboxyanhydride (NCA) monomers derived from O-glycosylated serine. Polymerization using $(PMe_3)_4Co$ affords glycopolypeptides with functionalizable N-termini, whereas catalyst **4** additionally installs a C-terminal azide for dual end-functionalization.

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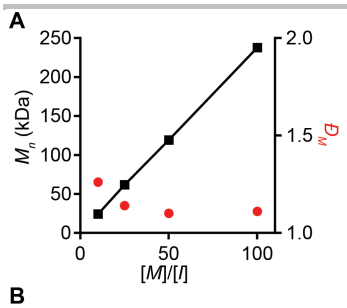

[M]/[I]	M_n (Da)	DP	$Đ_M$
10	24,127	34	1.26
25	61,738	87	1.14
50	119,218	168	1.10
100	237,726	335	1.11

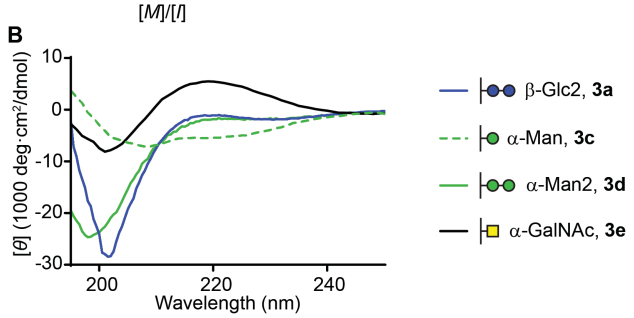

Figure 2. Polymerization parameters and circular dichroism analysis of glycopolypeptides. A) Representative polymerization data for Man2 NCA **2d** using $(PMe_3)_4Co$, illustrating the linear relationship between monomer to initiator ratio [*M*]/[*I*] and number-average molecular weight M_n, as well as molar-mass dispersities $Đ_M$. B) Circular dichroism spectra for glycopolypeptides.

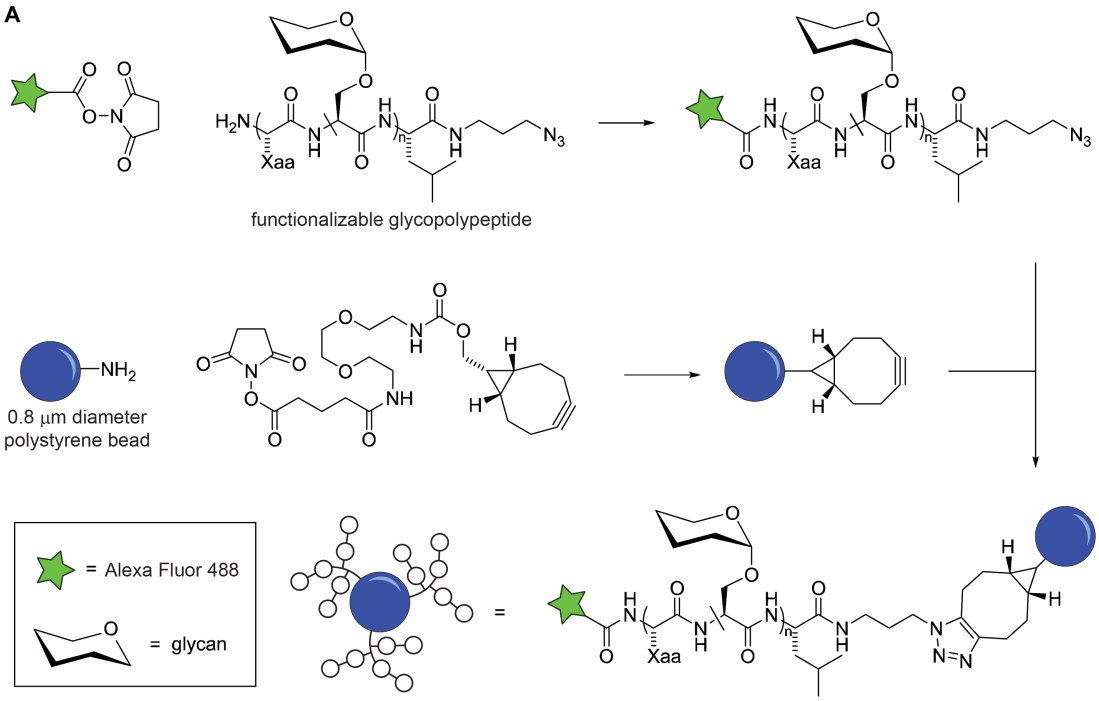

Figure 3. Covalent attachment of glycopolypeptides to fluorescent polystyrene beads for cell culture evaluation of immune activation and phagocytosis. A) Glycopolypeptides synthesized using azide-functionalized catalyst **4** were labeled with Alexa Fluor 488 NHS ester and then conjugated to fluorescent BCN-functionalized beads. B) Glycopolypeptide-bead conjugates induce a variety of cellular responses upon engagement with CLRs. Cytokine secretion was detected by ELISA, and phagocytosis could be observed by

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fluorescence microscopy and quantified by flow cytometry. Alternatively, NF-κB- and AP-1-induced expression of a secreted alkaline phosphatase (SEAP) in the macrophage reporter cell line RAW Blue can be detected using a commercial chromogenic substrate.

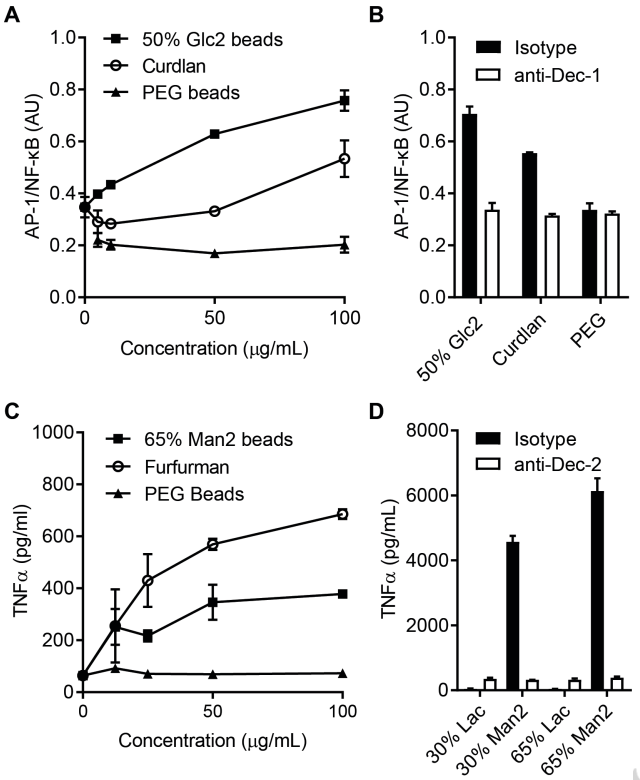

Figure 4. Glucosylated and mannosylated glycopolypeptides induce receptor-specific inflammatory responses in cell culture. A) Dose-response curve showing AP-1/NF-κB activation in RAW Blue cells when exposed to Glc2 glycopolypeptide-decorated beads. Cells were incubated with beads for 16 h, after which supernatant aliquots were mixed with the chromogenic substrate, incubated for four hours, and then absorbance measured at 620-655 nm. PEG-coated beads and the canonical agonist curdlan were used as negative and positive controls, respectively. B) The same beads and cells were tested using the same general protocol in the presence of an αDec-1 blocking antibody or an isotype control antibody to confirm that activation was Dec-1 dependent. C) JAWSII cells were similarly incubated with Man2 glycopolypeptide-decorated beads, Dec-2 agonist furfurman, or PEG-beads for 16 h, and dose-response relationships were measured via TNFα ELISA. D) Murine monocyte-derived dendritic cells were incubated for 20 hours with an aDec-2 blocking antibody or an isotype control antibody in plates that had been pretreated with Man2 or Lac polymers to confirm that TNFα release was Dec-2-dependent. Data in panels A-C were measured in triplicate, those in panel D represent duplicates; error bars indicate standard error of the mean.