

Results

Induction of TNF- α and MIP-2 in Macrophages by BGN is Specific for BGN and Requires the Intact and Soluble BGN Molecule.

We investigated whether induction of TNF- α and MIP-2 in macrophages incubated with BGN was in fact specific for BGN and whether it was dependent on its core protein or its glycosaminoglycan (GAG) chains. Preincubation of BGN with a blocking antibody (1), but not with non-immune serum (Supplemental Figure 1A and not shown), markedly reduced the induction of TNF- α in macrophages, indicating that this effect was in fact BGN-mediated. Neither BGN-core protein nor BGN-derived GAG chains (obtained by β -elimination) alone did affect TNF- α - and MIP-2-levels (Supplemental Figure 1B and 1C), suggesting that both the core protein and the GAG chains are necessary for the stimulation of macrophages. Moreover, whereas the keratan sulfate chain-carrying SLRP family member fibromodulin did not affect TNF- α and MIP-2 levels in macrophages, the structurally closest relative of BGN, decorin (1), partially mimicked the effects of BGN (Supplemental Figure 1B and 1C).

Finally, we examined whether soluble BGN and BGN immobilized on plastic, type I collagen, and fibronectin, were equally able to stimulate TNF- α in macrophages. As the efficacy of coating with 20 μ g/ml BGN (examined with [35 S]sulfate labeled BGN) was 10% for uncoated (plastic) and 15% for coated wells (fibronectin or type I collagen), 2 μ g/ml of soluble BGN was used as control. Soluble, but not immobilized BGN, increased TNF- α levels in culture media from *Bgn*⁻⁰ macrophages after 24 h (Supplemental Figure 1D). Effects of coating on adherence of macrophages was excluded by an adhesion assay and normalization of the results by the protein content of adherent macrophages. These data suggest that BGN has to be released from the ECM to convey stimulatory effects on macrophages.

Biglycan Interacts with TLR4 and TLR2 and Forms a Complex Additionally Containing MD-2 and CD14

In order to demonstrate physical interaction between BGN and the receptor proteins TLR4 and TLR2, *Bgn*⁺⁰ macrophages were challenged with BGN (4 µg/ml) for 2 h at 4°C in the presence of [Bis(sulfosuccinimidyl)suberate] as crosslinker. BGN-containing complexes were isolated by immunoprecipitation using an anti-BGN antibody and subjected to gel filtration under dissociating conditions as described in the Materials section. As a control, *Bgn*⁺⁰ macrophages were similarly incubated with BGN in the absence of a crosslinker and the cell lysate was analyzed by gel filtration without prior immunoprecipitation. Elution of all components of the suggested receptor complexes involving either TLR4 and/or TLR2 occurred in a broad range from fraction 23 to 56 (corresponding to K_{av} -values from 0.08 to 0.7) (Supplemental Figure 2). Importantly, coelution of these components cannot be observed in the early fractions (23 to 33, corresponding to K_{av} -values from 0.08 to 0.3) when the components have not been crosslinked prior to gel filtration. These data indicate that BGN physically interacts with a complex being composed of TLR4 and/or TLR2, MD-2, and CD14, whose molecular weight can roughly be estimated to be in the 600 – 700 kD range.

Contaminations do not contribute to BGN-mediated Stimulation of Macrophages

Endotoxin contaminants of BGN (80 µg/ml), measured with the LAL kinetic chromogenic assay in three different preparations of BGN were 23 EU/mg, 29 EU/mg, and 31 EU/mg, respectively. Results were reported in endotoxin units (EU) with reference to EC6 standard endotoxin (European Pharmacopoeia, Inc.; 1 ng EC6 = 10 EU) (2). Therefore, 1 µg of BGN should approximately correspond to 3 pg of LPS. Since concentrations of BGN used in our experiments were 1-10 µg/ml, we compared the effect of 4 µg/ml of BGN on TNF- α secretion by *Bgn*⁺⁰ macrophages into the culture medium (6 h of incubation) with the effects of 12-500 pg/ml LPS (Supplemental Figure 3A). LPS at concentrations of 12-50 pg/ml did

not lead to concentrations of TNF- α significantly different to the non-stimulated controls (Supplemental Figure 3A). In contrast, corresponding BGN markedly enhanced the TNF- α content in culture media from macrophages. Furthermore, co-incubation with Polymyxin B (25 and 50 $\mu\text{g/ml}$) did not reduce the stimulatory effects of BGN (4 $\mu\text{g/ml}$) on macrophages, whereas the effects of LPS (0.5 ng/ml) were entirely abolished (Supplemental Figure 3B). Similar results were obtained by treatment of BGN with Detoxi-Gel Endotoxin Removing Gel (data not shown). Finally, the effects of BGN on TNF- α and MIP-2 were completely eliminated by boiling and by digestion of BGN with trypsin, whereas LPS proved to be resistant to both treatments (data not shown).

In order to demonstrate the purity of the BGN preparation, we performed 1D SDS-PAGE of intact and chondroitinase ABC-digested BGN, followed by silver staining. The typical staining pattern for BGN and components of the degradation reaction could be observed, without any additional bands (Supplemental Figure 3C). Contamination of BGN with IL-6, IL-1 β , TGF- β 1, TNF- α , and MIP-2 was excluded by ELISA techniques. Furthermore, we were unable to detect selective TLR2- and TLR4-ligands (3), namely the heat shock proteins 60 and 70, fibrinogen, and fibronectin-extra domain A in our BGN preparation using specific antibodies. The final proof that the activation of macrophages was in fact due to BGN itself and not to potential other contaminants like HS or HA, was based on the finding that only intact BGN was able to affect macrophages, whereas the core protein or the GAG chains of BGN were not active (Supplemental Figure 1B and 1C). Therefore, we first proved that chondroitinase ABC specifically digested only CS/DS, but not HA and HS (Supplemental Figure 3D). Next, chondroitinase ABC digestion converted the BGN to lower molecular weight forms, as demonstrated by Western blotting (Supplemental Figure 3E), confirming the efficacy of the digestion shown in Supplemental Figure 3D. However, BGN core protein remained intact (Supplemental Figure 3F). Since BGN core protein prepared in this manner was not able to stimulate TNF- α and MIP-2 in macrophages (Supplemental

Figure 1B and 1C), we conclude that the pro-inflammatory effects truly were due to BGN and that they were not caused by any contaminations with LPS, TLR-ligands, other proinflammatory factors, or HA- or HS-chains.

Macrophage Activation by BGN Does not Depend on Interactions of BGN with the SR-AI and Endocytic Mannose Receptor

Since in *TLR2^{-/-}/TLR4-M* macrophages an inflammatory response to BGN was completely abolished, a role of alternative receptors mediating the BGN effect is hardly conceivable. However, as interactions of BGN with SR-AI and the endocytic mannose receptor have been reported (11, 12), we tested whether these receptors would be important for BGN signaling. To explore whether BGN may act as a candidate ligand for SR-AI, CHO-SR-AI and control CHO cells were incubated with radioactively labeled BGN; for control experiments, acLDL, a known and high-affinity ligand for SR-AI, was used. In comparison to acLDL, BGN binds only weakly to CHO-SR-AI cells (Supplemental Figure 4A and 4B, experiments performed under serum-free conditions). Similar results were obtained in the presence of 5% FCS (data not shown). Next, we tested whether BGN may induce intracellular MAPK cascades via SR-AI. No induction of p-Erk (Supplemental Figure 4C and 4D) and p-p38 (Supplemental Figure 3E and 3F) could be observed in CHO-SR-AI and control cells with any of the BGN concentrations tested at none of the time points we looked at. By testing the mannose-receptor-negative murine macrophage cell line J774 A (Supplemental Figure 4G and 4H) and by lack of blocking effects of D(+) mannose on the BGN-mediated induction of TNF- α (Supplemental Figure 4I) and MIP-2 (not shown), we could also exclude a role of mannose receptor in BGN-dependent signaling in macrophages.

Methods

Cell Culture and Stimulation

For neutralization of BGN-activity, 4 µg/ml of BGN were incubated for 1 h at 37°C with a BGN-blocking antibody (0.1-10 IgG µg/ml) (1) or, as control, with non-immune rabbit serum and subsequently applied to thioglycollate-elicited macrophages for 6 h. To obtain BGN core protein, the proteoglycan was digested with chondroitinase ABC (Seikagaku Corporation) and purified as described (6). BGN-derived GAG chains were obtained by β-elimination followed by dialysis against RPMI 1640 medium (7). For quantification, protein and hexuronic acid content in GAG chains and intact BGN were measured (8). Human decorin from stably transfected 293 HEK cells (1-10 µg/ml), fibromodulin from calf cartilage, chondroitin sulfate B from porcine intestinal mucosa (both Sigma-Aldrich, 1-10µg/ml) and chondroitin sulfate C from shark cartilage (Seikagaku Corporation, 1-10 µg/ml) were used for assessing specificity of BGN effects. In some experiments BGN (20 µg/ml) was immobilized on 6-well culture plates uncoated or coated with fibronectin or type I collagen (BD Biosciences) (6). Efficacy of coating with BGN, examined with [³⁵S]sulfate labeled BGN (6), was 10% for uncoated and 15% for fibronectin- or type I collagen-coated wells. Adhesion of macrophages was quantified by CytoMatrix Cell Adhesion Strips (Chemicon International Inc.). When required, macrophages were preincubated for 30 minutes with 10-100 mM of D(+) mannose (Sigma-Aldrich).

Stably transfected CHO cells expressing murine scavenger receptor class A type I (CHO[mSR-AI]) were maintained in Ham's-F12K medium supplemented with 3 % lipoprotein-deficient serum (LPDS), 250 µM mevalonate, 40 µM mevinoлин and 3 µg/ml acetylated low-density lipoprotein acLDL, 2 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen) (9). Vector-transfected CHO and HEK cells used as controls were cultured as described (10). To obtain quiescent cells, the cells were maintained in serum-free

F12K supplemented with 0.1 mg/ml of fatty acid-free BSA for 24 h prior to the addition of BGN or LDL.

The mannose-receptor-negative murine macrophage cell line J774A was kindly provided by Dr. S.O. Kolset (University of Oslo, Norway) and cultured in DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen). To obtain quiescent cells, J774A were maintained in serum-free DMEM supplemented with 0.1 mg/ml of fatty acid-free BSA for 24 h prior to the addition of BGN.

Gel Filtration

Thioglycollate-elicited *Bgn*⁺⁰ macrophages (10^7 cells) or HEK-Blue-4 cells were incubated with 8 µg of intact human BGN for 2 h at 4°C followed (where indicated) by incubation with 1mM [Bis(sulfosuccinimidyl)suberate] (BS³, Pierce Biotechnology Inc.) for cross-linking BGN to binding proteins. Immunoprecipitation of human BGN was performed as described (1). Immunoprecipitates were desorbed with 10 mM HEPES (pH 7.5), 4 M guanidinium chloride (J.T. Baker), 1% (w/v) CHAPS, 1 mM EDTA, 0.2 mM PMSF, additionally containing antipain, aprotinin, chymostatin, leupeptin and pepstatin (5 µg/ml each, all from Sigma-Aldrich) by boiling for 5 min. Samples (100 µl) were cleared by centrifugation at 14.000 rpm for 15 min and applied to a Superose 6 HR 10/30 column (Amersham Biosciences) operated with 10 mM HEPES (pH 7.5), 4 M guanidinium chloride, 1% (w/v) CHAPS, 1 mM EDTA, 0.2 mM PMSF at a constant flow rate of 15 ml/h. The High Molecular Weight Gel Filtration Calibration Kit (Amersham Biosciences) was used to calibrate the column. Fractions (300 µl) were collected and analyzed for BGN, TLR4, TLR2, CD14, and MD-2 after dotting aliquots (50 µl) to a PVDF membrane (11). The following antibodies were used: rabbit anti-BGN antibody (1), rabbit anti-human TLR-4 cross-reacting

with mouse TLR4 (Santa Cruz Biotechnology, Inc.), rabbit anti-mouse TLR-2 and mouse anti human MD-2 (both from Imgenex), mouse anti-human CD14-PE (Beckman Coulter).

Procedures Ruling out Contamination of Biglycan

The purity of intact BGN and its core protein was verified by silver staining after SDS gel electrophoresis. Endotoxin contamination of BGN (80 µg/ml) at dilutions of 1:10-1:1000 was tested with the LAL kinetic chromogenic assay (The Endotoxin Testing Service, Cambrex Corporation) in the presence of a positive product control of 0.5 EU/ml (2). It was additionally controlled by boiling and trypsin digestion of BGN, by Detoxi-Gel Endotoxin Removing Gel (Pierce Biotechnology Inc.), and by co-incubation with Polymyxin B (Sigma-Aldrich, 25-50 µg/ml). Contaminations of BGN with IL-6, IL-1 β , TGF- β 1, TNF- α , and MIP-2 were tested with respective ELISAs (R&D Systems). The presence of selective TLR2 and TLR4 ligands was excluded by dot blots using 5-10 µg BGN followed by immunodetection using mouse anti-fibronectin extra domain A (Harlan Sera-Lab), mouse anti-human heat shock proteins 60 and 70 (StressGen Biotechnologies), and rabbit anti-human fibrinogen (Dako) as described (11). Finally, 1 µg of BGN, chondroitin sulfate (CS), heparan sulfate (HS), and hyaluronan (HA, kindly provided by Dr. P. Prehm, University of Muenster, Germany), respectively, were digested with protease-free chondroitinase ABC (Seikagaku Corporation). Progress of digestion was controlled by measuring the increase of absorbance at 232 nm. Efficiency of digestion of BGN with chondroitinase ABC and the persistence of its core protein were additionally tested by Western blotting using a monoclonal anti-proteoglycan Di-4S antibody (clone 2-B-6, Seikagaku Corporation) and rabbit anti-human BGN (1), respectively. Activation of macrophages by chondroitinase ABC-digested BGN was tested in mouse TNF- α ELISA (R&D Systems).

Binding of BGN to SR-AI

Iodination of BGN was performed with [^{125}I]NaI using N-Br-succinimide as the coupling agent (10). Routinely, 100 μCi of Na^{125}I was used to label 100 μg of BGN. This procedure resulted in specific activities between 300 and 500 dpm/ng of protein. Radiochemicals were from DuPont/NEN. Fucoidin, organic solvents and KBr were from Sigma-Aldrich. All other chemicals were obtained from Merck & Co, Inc..

The specificity of BGN binding to SR-AI was examined by competition experiments. LDL ($d = 1.035$ to 1.065 g/ml) was isolated by ultracentrifugation as described (12). LDL was labeled with [cholesteryl-1,2,6,7- ^3H]-palmitate ($[^3\text{H}]\text{CE}$) by cholesteryl ester transfer protein-catalyzed transfer from donor liposomes as described (10). Acetylation of $[^3\text{H}]\text{CE}$ -LDL was performed as described (12). The association of [^{125}I] BGN or $[^3\text{H}]\text{CE}$ -acLDL was competed for by the indicated concentrations of unlabeled competitors (acLDL or fucoidin). Briefly, CHO-SR-AI and control CHO cells were incubated at 37°C for 5 h with $1\mu\text{g}$ [^{125}I]BGN or with $10\mu\text{g/ml}$ $[^3\text{H}]\text{CE}$ -acLDL in the absence or presence of competitors and in the absence or presence of 5% FCS. Following this incubation, the medium was aspirated and the cells were rinsed two times with TBS (containing 5 % [w/v] BSA) followed by two washes with TBS only prior to lysis with 0.3 N NaOH. Radioactivity and protein content of cell lysates were measured in the same aliquot.

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Figure Legends

Supplemental Figure 1: BGN-specific induction of TNF- α and MIP-2 in macrophages requires the intact and soluble BGN molecule.

(A) ELISA measurements of TNF- α in media from *Bgn*⁺⁰ macrophages stimulated for 6 h with BGN (4 μ g/ml) without and after preincubation for 1 h with a BGN-blocking antibody (10 μ g/ml) indicated that BGN is responsible for the enhanced expression of TNF- α in macrophages. ELISA of TNF- α (B) and MIP-2 (C) in media from *Bgn*⁻⁰ macrophages cultured for 24 h in the presence of intact BGN, BGN-core protein, BGN-derived GAG chains (β -elimination), chondroitin sulfate B (CS-B), intact decorin (DCN) and intact fibromodulin (FMOD) (each 10 μ g/ml). (D) ELISA of TNF- α in media from *Bgn*⁻⁰ macrophages after 24 h of culture in the presence of BGN (~ 2 μ g/well) immobilized on plastic (uncoated), type I collagen (Coll I) or fibronectin (FN) or 2 μ g/ml of soluble BGN. Immobilized BGN had no effects on TNF- α secretion from macrophages.

Supplemental Figure 2: Gel filtration demonstrates interaction of BGN with TLR4- and/or TLR2-containing receptor complexes

Dot blot analysis of eluate fractions from gel filtration of samples obtained after binding BGN to *Bgn*⁺⁰ macrophages, crosslinking and immunoprecipitation using an antibody specific for BGN (IP + crosslink) and of cell lysates obtained after binding of BGN to *Bgn*⁺⁰ macrophages in the absence of crosslinker (control) using antibodies recognizing BGN, TLR4, TLR2, MD-2, and C14, respectively, revealed that in the crosslinked sample all components of the suggested receptor complex coeluted together with BGN as higher molecular weight complex(es) (fractions 23 to 33 corresponding to K_{av} -values of 0.08 to 0.3), in addition to elution of the individual components (fractions 34 – 56, K_{av} -values 0.3 – 0.7) also seen in the non-crosslinked cell lysate. Reference proteins were eluted from the column with K_{av} -values

of 0.14 (thyroglobulin, 669 kD), 0.2 (ferritin, 440 kD), 0.4 (catalase, 232 kD), and 0.6 (aldolase, 158 kD), respectively.

Supplemental Figure 3: Procedures ruling out contamination of BGN

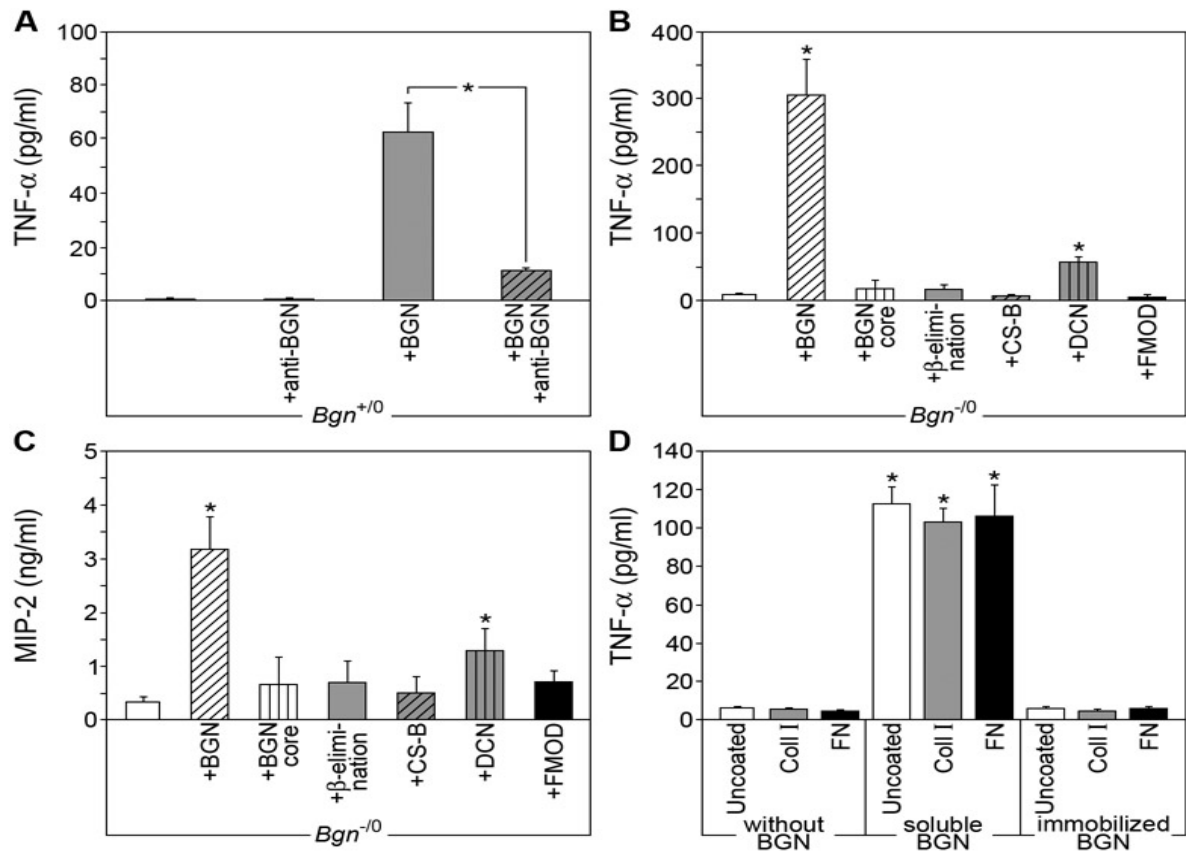
(A) ELISA measurements of TNF- α in culture media from *Bgn*⁺⁰ macrophages incubated for 6 h in the presence and absence of BGN or LPS at the indicated concentrations showed stimulation of TNF- α by BGN (4 μ g/ml), but no significant effects of LPS at concentrations of 12 and 50 pg/ml. Mean \pm SD, n=3, *p<0.05 for LPS- or BGN-stimulated macrophages versus control. (B) Polymyxin B (25 or 50 μ g/ml) inhibited LPS (500 pg/ml)-, but not BGN (4 μ g/ml)-mediated activation of TNF- α in *Bgn*⁺⁰ macrophages. Mean \pm SD, n=3, *p<0.05 for LPS + polymyxin B versus LPS without polymyxin B. (C) 1D SDS-PAGE of intact and chondroitinase ABC-digested BGN (1 μ g) followed by silver staining. (D) Time-dependent increase of absorbance (232 nm) during incubation of BGN, chondroitin sulfate (CS), heparan sulfate (HS), or hyaluronan (HA, 1 μ g each) with protease-free chondroitinase ABC. (E) Western blot of intact and chondroitinase ABC-digested BGN using an antibody specifically recognizing 4-O-sulfated chondroitin and dermatan sulfate following digestion with chondroitinase ABC. Detection of BGN-core protein with this antibody was due to remaining stubs. (F) Western blot of chondroitinase ABC-digested BGN using anti-BGN antibody.

Supplemental Figure 4: Interactions of BGN with the SR-AI and the endocytic mannose receptor

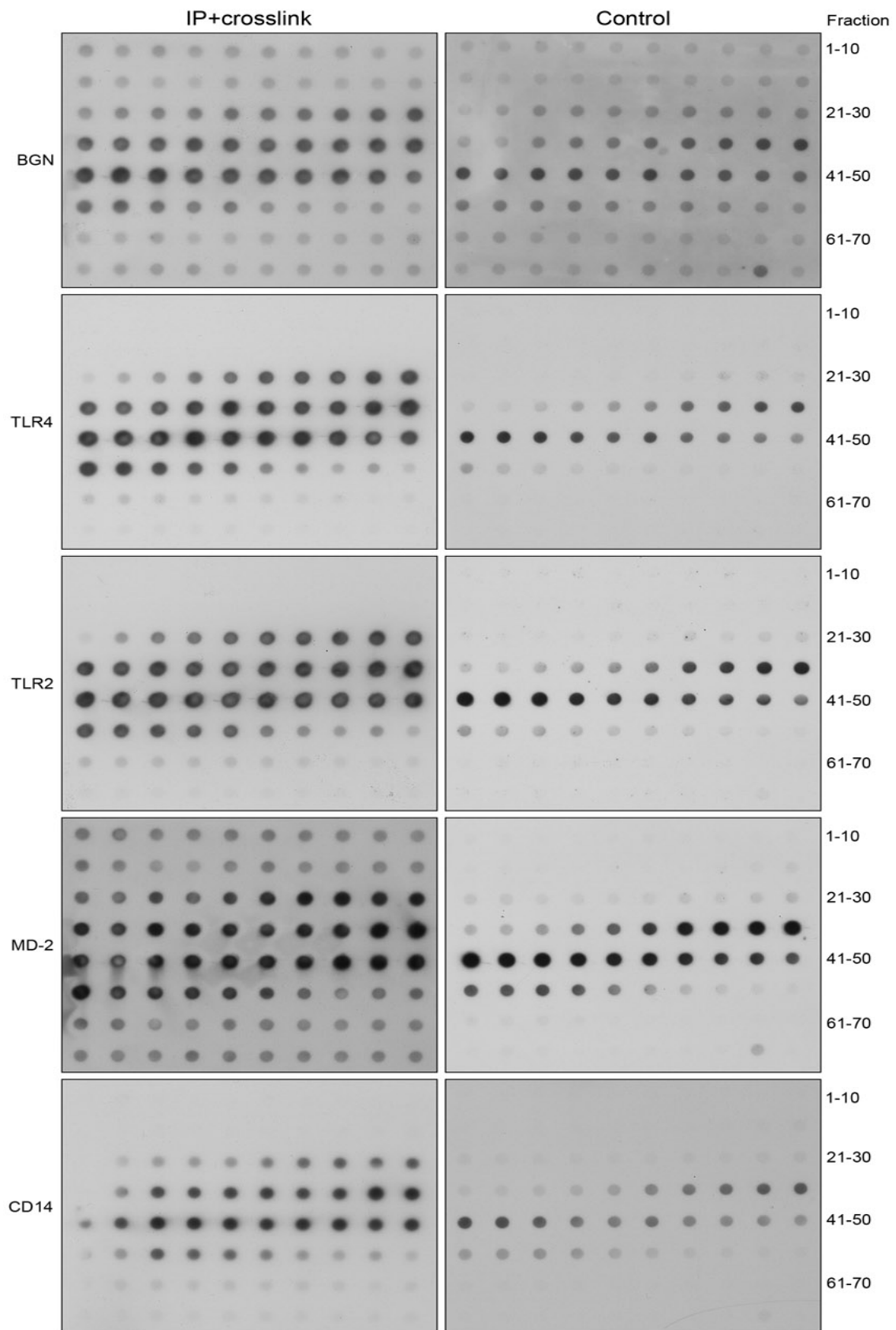
Confluent CHO-SR-AI and control CHO cells were incubated for 5 h at 37°C with 1 μ g/ml ¹²⁵I-BGN (A) or 5 μ g/ml [³H]CE-acLDL (B) in the absence or presence of indicated competitors (n=3) and cell-association was measured. Time- (C, E) and concentration-dependent (D, F) effects of BGN on the phosphorylation of Erk (C, D) or p38 (E, F). In C and E cells were incubated for indicated times with 250 μ g/ml LDL (positive control) or 5

$\mu\text{g/ml}$ BGN. In **D** and **F** cells were incubated with indicated concentrations of LDL (10 minutes) or BGN (30 minutes). HEK cells were used to show antibody specificity. BGN-mediated (10 $\mu\text{g/ml}$, 24 h) stimulation of TNF- α (**G**) and MIP-2 (**H**) in J774A cells, negative for mannose receptor. Mean \pm SD, n=3, *p<0.05 for J774A + BGN versus J774A cells without BGN. (**I**) BGN-mediated (10 $\mu\text{g/ml}$, 24 h) stimulation of TNF- α was not inhibited by D (+) Mannose (100 mM) in *Bgn*⁻⁰ macrophages. Mean \pm SD, n=3, *p<0.05 for *Bgn*⁻⁰ +BGN versus *Bgn*⁻⁰ or *Bgn*⁻⁰ + BGN+ D (+) Mannose versus *Bgn*⁻⁰ + D (+) Mannose.

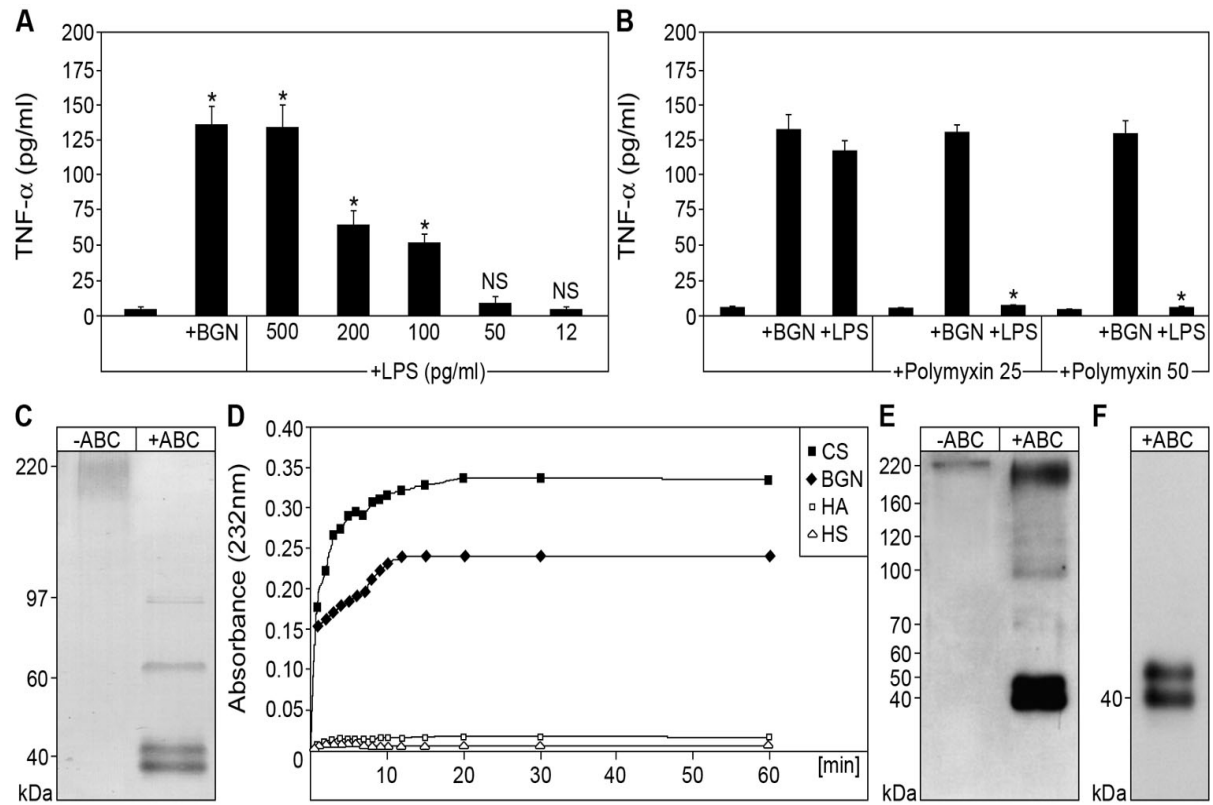
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4

