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Vimentin is an endogenous ligand for the pattern recognition receptor Dectin-1

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Aims Atherosclerosis is a chronic inflammatory disorder of cholesterol deposition in monocyte-derived macrophages (MDM) within the arterial wall leading to impingement on the lumen of the vessel. In atherosclerotic lesions, MDM are the primary source of NADPH oxidase-derived superoxide anion (O_2^-) inducing low-density lipoprotein (LDL) oxidation leading to their unregulated uptake of oxidized LDL and foam cell formation. We recently discovered that zymosan potently activates monocyte NADPH oxidase via the non-toll pattern recognition receptor (PRR), Dectin-1. Other PRRs bind endogenous human ligands, yet no such ligands have been identified for Dectin-1. Our hypothesis was that inflammation generates endogenous ligands for Dectin-1 that activate O_2^- production and thereby contributes to atherogenesis.

Methods and results Human: anti-zymosan antibodies were used to identify similar, cross-reactive epitopes in human atherosclerotic tissue extracts. Immunoblot analysis revealed consistent antibody reactive protein bands on one- and two-dimensional gel electrophoreses. Vimentin was identified by mass spectrometry in the immunoreactive bands across different tissue samples. Direct binding of vimentin to Dectin-1 was observed using BIACORE. Further data revealed that vimentin induces O_2^- production by human monocytes. Analysis of human atherosclerotic lesions revealed that vimentin was detected extracellularly in the necrotic core and in areas of active inflammation. Vimentin also co-localized with Dectin-1 in macrophage-rich regions where O_2^- is produced.

Conclusion We conclude that vimentin is an endogenous, activating ligand for Dectin-1. Its presence in areas of artery wall inflammation and O_2^- production suggests that vimentin activates Dectin-1 and contributes to the oxidation of lipids and cholesterol accumulation in atherosclerosis.

Keywords Dectin-1 • Extracellular vimentin • Atherosclerosis • Superoxide anion • Alarmins

1. Introduction

Atherosclerosis is a chronic inflammatory disease characterized by lipid accumulation in macrophage foam cells in highly susceptible areas of the artery wall. Lipoprotein oxidation contributes to unregulated lipid uptake by artery wall macrophages and this oxidation can be mediated by the generation of superoxide anion (O_2^-) by activated human monocyte/macrophages. In model systems, a very potent trigger for the generation of O_2^- in macrophages is the yeast cell wall preparation called zymosan.[1] We have recently identified that the innate immune receptor that recognizes zymosan and triggers profound production of O_2^- and low-density lipoprotein (LDL) oxidation is Dectin-1.[2] Other innate immune receptors, the toll-like receptors (TLRs), participate in atherogenesis by recognizing endogenous ligands created by cellular damage, but the role of Dectin-1 in atherogenesis remains to be explored.[3–6] Since Dectin-1 engagement triggers the O_2^- production by monocyte/macrophages, we hypothesized that there might be an endogenous ligand that can trigger O_2^- production through Dectin-1 that is driven by an endogenous danger signal generated during the atherogenic process.

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Innate immune receptors, or pattern recognition receptors (PRRs), have been implicated in atherogenesis, suggesting a link between atherosclerosis and defence against both endogenously generated inflammatory ligands and invading exogenous foreign pathogens.[7] Endogenous and exogenous ligands for these receptors are termed 'damage associated molecular patterns' and represent danger signals to the host innate defence system.[6,8] Interestingly, both infectious and sterile inflammatory aetiologies induce similar responses.[8] The microbe-derived exogenous ligands, like lipopolysaccharides, bind and activate their respective receptors. Endogenous ligands termed 'alarmins' also trigger PRRs. Alarmins studied to date have been shown to bind to the TLR class of PRRs.[9] These include oxLDL, HMGB-1 protein, AGE products, S100 proteins, hsp60, fibronectin, hyaluronan fragments, and β-amyloid, all of which contribute to chronic inflammation.[10–14] These traditionally intracellular molecules are released or secreted into the extracellular compartment in non-classical ways under stressful conditions, including cell damage, infection, and inflammation. Once released, they become immunostimulatory by engaging PRRs.

Membrane-associated surface glycoproteins, C-type lectin-like receptors (CLR), belong to a major family of PRRs.[15] The most thoroughly characterized and archetypical of the CLRs is the Dectin-1 receptor.[16] Dectin-1 is a glycosylated type II transmembrane receptor with a single extracellular C-type lectin-like domain which recognizes β1,3- and/or β1,6-linked glucans, a short stalk region and a 40AA cytoplasmic N-terminal domain containing a single immunoreceptor tyrosine-based (ITAM-like) activation motif.[17] Dectin-1 is implicated in the innate immune recognition of yeasts and fungal pathogens.[18]

The involvement of two non-Toll CLR PRRs, Mincle and CLEC9A, in recognizing alarmins have been explored by very few studies. Though Mincle and CLEC9A have been speculated to sense immunogenic necrotic cell death by binding to SAP130 and an unknown ligand, respectively, this still requires further elucidation. Importantly, the studies with Mincle support the hypothesis that CLRs can bind to non-carbohydrate ligands.[19] An emerging concept is that alarmin release and detection by the innate immune system significantly contributes to the failure to resolve inflammation in chronic inflammatory diseases. Thus, identifying the innate immune receptors and their ligands is critical for intervening in chronic inflammatory responses.

No endogenous ligands have been identified for Dectin-1, yet our recent studies show that in primary human monocytes, zymosan induces NADPH oxidase activity to produce O_2^- exclusively in a Dectin-1-dependent manner, without collaboration with the TLRs.[2] We hypothesized that Dectin-1 may serve as an innate immune receptor mediating O_2^- production in chronic inflammatory sites in response to alarmins/endogenously generated danger signals. To test this hypothesis, we used anti-zymosan antibodies to identify similar, cross-reactive epitopes on molecules in extracts from human atherosclerotic plaque tissue. These antibodies were previously shown to block the binding of zymosan to Dectin-1 and the production of O_2^-. We predicted they would recognize other potential ligands for Dectin-1 that could potentiate macrophage activation, O_2^- production, and lipid oxidation and contribute to atherogenesis. Our studies identified vimentin as a specific antigen recognized by anti-zymosan antibody and showed direct binding between vimentin and dectin-1.

Vimentin is primarily known as an intermediate filament protein. Interestingly, we found it to additionally exist in an extracellular form within areas of inflammation and necrosis in atherosclerotic lesions. Extracellular vimentin was shown to be an activating ligand for O_2^- production through direct binding to Dectin-1. Its proximity to Dectin-1-expressing macrophages in the inflammatory shoulders of atherosclerotic plaques highlight its availability to interact with Dectin-1, induce O_2^- production and LDL oxidation, and contribute to the pathogenesis of this devastating chronic inflammatory disease.

2. Methods

2.1 Materials

Human atherosclerotic tissue samples were obtained from the Co-operative Human Tissue Network (NIH), the Department of Vascular Surgery at the Cleveland Clinic and Biochain (Hayward, CA, USA). The study was reviewed and approved by the Institutional Review Board and our study conforms to the Declaration of Helsinki. Antibodies used in these studies were anti-zymosan antibody from Invitrogen, mouse anti-human Dectin-1 antibody from R&D Systems, anti-vimentin V9 monoclonal antibody from Sigma (Lot number: 027K4773; 1:50-1:1000), CD68 antibody from Santa Cruz (Lot number GR15767-1; 1:50), and Alexa Fluor-488 and -568 conjugated secondary antibodies from Invitrogen. The cyanogen bromide sepharose 4B column (CnBr) was ordered from GE Healthcare. Vimentin from American Research Products was isolated from bovine lens and claimed to be >98% pure by SDS–PAGE. Recombinant human Dectin-1 was from R&D systems (Lot number: IKT0509091). Recombinant human CD36 proteins, with and without a His-tag, were generous gifts from Dr Roy Silverstein's laboratory. Laminarin, superoxide dismutase, and cytochrome C were ordered from Sigma.

2.2 Immunoblotting of human carotid atherosclerotic tissue samples

Human carotid atherosclerotic tissue samples were homogenized in a 1% SDS solution and diluted to 0.1% SDS. Protein concentration of the tissue lysates was measured using the Bio-Rad DC (detergent compatible) assay (Hercules, CA, USA). Homogenized tissue lysates were subjected to immunoblot analysis. Corresponding tissue extracts were subjected to two-dimensional gel electrophoresis (Bio-Rad, CA, USA), stained with Coomassie Blue or transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA) and probed with anti-zymosan antibody as noted.[20] The bands that were observed on staining were selected for LC-MS sequencing.

2.3 Immunoaffinity purification of proteins that bind to anti-zymosan antibody

To detect proteins in human carotid atherosclerotic tissue samples that bind to anti-zymosan antibody, the antibody was immobilized on CnBr sepharose 4B as per instructions from the manufacturer in 0.8 cm × 4 cm columns. Five milligrams of anti-zymosan antibody was coupled to the column overnight at 4°C. After coupling, the column was washed and blocked for 4 h at 4°C. Tissue extraction was performed in phosphate-buffered saline containing 1% Triton X-100 overnight at 4°C. Ten-fold diluted tissue extracts were passed through the column continuously overnight at 4°C using a peristaltic pump. After extensive washing, elution was performed using 0.1 M glycine pH 2.5 and the eluate was subjected to SDS–PAGE and stained with Coomassie Blue.[20] The observed protein band was sequenced using mass spectrometry. To detect vimentin secretion, immunoblotting was performed on supernatants derived from monocytes incubated for 3 days in the presence or absence of MCP-1 (see detailed protocol in Supplementary material online, Methods).

2.4 Binding studies using surface plasmon resonance and solution affinity kinetics

The direct binding between Dectin-1 and vimentin was measured using label-free surface plasmon resonance using the BIACORE 3000 (BIACORE, Uppsala, Sweden). CM5 sensor chips (GE Healthcare, Piscataway, NJ, USA) were used for all binding studies. Indirect immobilization of vimentin

was performed using a capture molecule (see detailed protocol in Supplementary material online, Methods).

2.5 Superoxide anion assay

Superoxide anion production by human monocytes was measured using the Cytochrome C reduction assay as described before.[21] This method is a modification of an assay previously published by Pick and Mizel.[22] Human monocytes were isolated from fresh donor blood as previously described.[23] Statistical differences between experimental groups were evaluated using the Student's *t*-test. *P*-values of $P < 0.05$ were considered significantly different (See detailed protocol in Supplementary material online, Methods).

2.6 Immunofluorescent histochemistry

Single- and double-label immunofluorescence staining was performed on frozen 7 μm OCT-embedded human coronary atherosclerotic plaque tissue sections (Type IV-V).[24] Five different human tissue specimens were used for staining studies. We could procure only specimens belonging to advanced stages of atherosclerosis (see detailed protocol in Supplementary material online, Methods).

3. Results

3.1 Detection of proteins that react with anti-zymosan antibody in human carotid atherosclerotic tissue samples

Based on our prior studies, zymosan induces O_2^- production through Dectin-1 in human monocytes.[2] Detection of proteins with similar epitopes to zymosan that may serve as endogenous ligands for Dectin-1 in atherosclerotic tissue samples, thereby promoting inflammation was our goal. Tissue extracts were analysed by performing western blots probed with anti-zymosan antibody. Prominent bands of ~50 kDa were observed consistently (*Figure 1A*). The blot was stripped and reprobed with the secondary antibody to ensure that the bands were specific, e.g. not the heavy chain of human IgG. No immunoreactivity was detected (*Figure 1B*). This blot was then stripped and reprobed with anti-zymosan antibody to ensure that antigen was retained on the blot and a similar result to *Figure 1A* was observed. Similar protein bands were consistently observed across five different human tissue specimens, two of which are shown in *Figure 1C*.

3.2 Mass spectrometry analysis of proteins that react with anti-zymosan antibody detected by two-dimensional gel electrophoresis of human carotid atherosclerotic tissue samples

Upon confirming the consistency of the immunoreactive proteins in human atherosclerotic tissue samples, LC-MS was performed to identify the proteins.[25] For this experiment, 100 μg of a processed human carotid atherosclerotic tissue sample was loaded on duplicate 2D gels.[26] Immunoblotting was performed on one blot using anti-zymosan antibody (*Figure 1D*) and the other gel was stained with Coomassie Blue (*Figure 1E*). Anti-zymosan antibody reacted with a few well separated proteins and the hits were circled. The circled spots on the immunoblot were correlated and cut out from the Coomassie stained gel and identified. Almost all of the spots were positively identified. Vimentin was the major component identified in three of the six spots (spots 2, 3, and 6) by the presence of 25–31 peptides covering 53–63% of the protein sequences (*Table 1*). Alternative phosphorylation likely accounts for different mobilities.

3.3 Detection of vimentin as a protein that binds to anti-zymosan antibody by immunoaffinity purification

A complementary and specific approach to identify potential Dectin-1 ligands in atherosclerotic tissue samples was performed. Immunoaffinity interacting proteins were purified and analysed by SDS–PAGE. After staining with Coomassie Blue, a major band just above the 50 kDa marker was detected (*Figure 1F*).[20] The band was analysed by LC-MS and the sequence indicated that the protein was vimentin. No non-specific protein bands were detected when a mock column without the immobilized anti-zymosan antibody was used as a control. For vimentin to serve as a ligand for dectin-1, it would need to be present in the extracellular space. Mor-Vaknin *et al.*[27] published that macrophages activated by proinflammatory cytokines are induced to secrete vimentin. We therefore explored whether MCP-1, an important proinflammatory cytokine in atherosclerosis, induced vimentin secretion by primary human monocytes. We found a substantial increase in supernatant vimentin upon monocyte activation (*Figure 1G*), thus confirming these prior published studies. Recombinant vimentin was shown to co-migrate with this immunoreactive band (data not shown).

3.4 Direct, high-affinity binding of vimentin to Dectin-1 was observed using surface plasmon resonance

To explore whether vimentin directly binds to Dectin-1 or Dectin-2, binding studies were performed using label-free surface plasmon resonance using a BIACORE system. Proteins were immobilized using amine coupling to a CM5 sensor chip. Indirect immobilization of vimentin was performed by using a monoclonal anti-vimentin antibody on the chip onto which vimentin was captured. To assess binding, we performed a single injection kinetic titration method. This method involves injection of an analyte in serial concentrations in a sequential manner with no regeneration between injections.[28] Serial concentrations of Dectin-1 or Dectin-2 were passed through the flow cell over the immobilized vimentin. Dose-dependent binding was observed with relatively strong binding between Dectin-1 and vimentin (*Figure 1H*) and 12-fold weaker binding between Dectin-2 and vimentin (*Figure 1I*). The dissociation constant for interactions between vimentin and Dectin-1 was $K_d = 4.03 \times 10^{-7}$ M while that between vimentin and Dectin-2 was K_d of 3.41×10^{-6} M.

A control experiment was performed to determine whether there was non-specific binding of Dectin-1 to the anti-vimentin antibody. The anti-vimentin antibody (16 μM) was passed through a flow channel over Dectin-1 immobilized on a CM5 sensor chip. No non-specific binding between the two was observed (data not shown). As an additional control, we also examined the binding of another unrelated scavenger receptor, CD36, with and without a His-tag, (10 μM) to indirectly immobilized vimentin. No binding was observed between CD36 and vimentin, with or without the His tag (data not shown).

3.5 Vimentin induces superoxide anion production in human monocytes via the Dectin-1 receptor

Once the binding between vimentin and Dectin-1 was confirmed, we examined whether vimentin induces O_2^- production via the Dectin-1

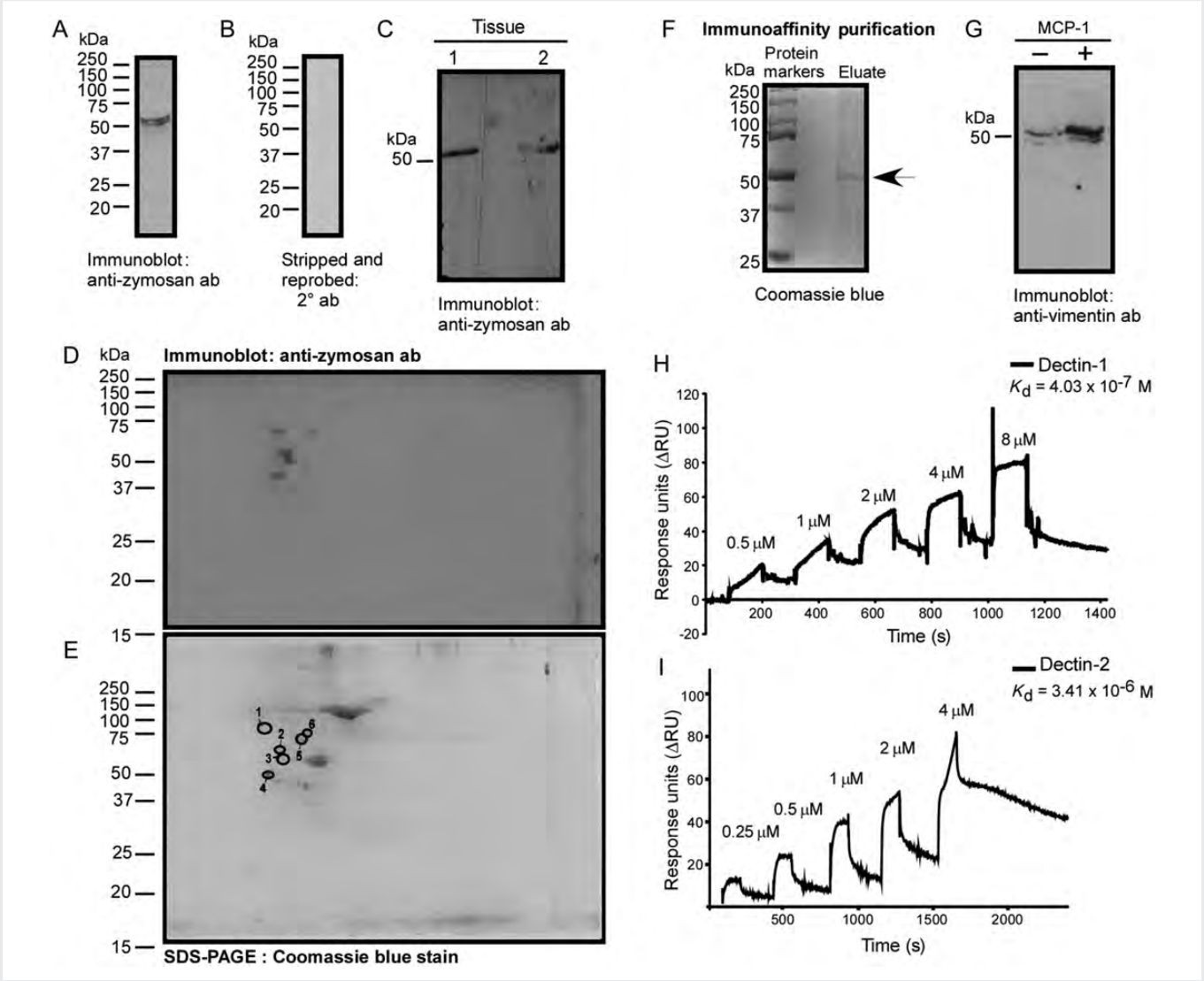

Figure 1 Immunoreactive protein bands were detected in extracts of human carotid atherosclerotic tissue extracts upon immunoblotting with anti-zymosan antibody. Homogenized carotid atherosclerotic tissue samples were subjected to immunoblotting with anti-zymosan antibody. (*A*) Protein bands ~50 kDa were immunoreactive. (*B*) No bands were detected upon stripping and reprobing with the secondary antibody. (*C*) Two tissue specimens from different donors showed similar immunoreactive bands. (*D*) 2D gel electrophoresis was performed on two gels simultaneously, one was probed with anti-zymosan antibody (*D*) and the other was stained with Coomassie Blue (*E*). The circled spots in (*E*) were selected for sequencing by superimposing them on the spots observed on immunoblotting with anti-zymosan antibody. Vimentin was identified on 2D gels as a predominant component sharing epitopes with zymosan. (*F*) Atherosclerotic tissue extracts were passed through an activated CnBr column and the proteins that bound to anti-zymosan antibody were eluted. This eluate was subjected to SDS–PAGE and Coomassie Blue staining. A major band was detected just above 50 kDa (see arrow) and upon sequencing was identified as vimentin. This extraction was performed using combined tissue specimens from five human tissue specimens in order to provide sufficient material for detection. (*A*)–(*E*) are representations of five experiments performed using different human tissue specimens. (*G*) Vimentin secretion by primary human monocytes was assessed as described in Methods in the presence or absence of MCP-1. The immunoblot is a representative result of three similar experiments. (*H* and *I*) Direct binding was assessed between vimentin and Dectin-1 or Dectin-2 by surface plasmon resonance using BIACORE. Vimentin was captured on a CM5 sensor chip using a monoclonal anti-vimentin antibody. Serial concentrations of Dectin-1 were passed through the flow cell over the immobilized vimentin without regeneration after each injection. The black line indicates the actual curve calculated after correction for the buffer effect. The binding between Vimentin and Dectin-1 was performed three times while the binding between vimentin and Dectin-2 represents a single experiment.

receptor. Zymosan, as predicted, significantly induced O_2^- production. Vimentin also proved to be a potent and significant stimulus of O_2^- production. Laminarin is a soluble β-glucan that is well known to bind Dectin-1 with very high affinity and block ligand interaction with Dectin-1. Anti-zymosan antibody was also employed as a blocking reagent as well as an anti-Dectin blocking antibody and their controls, rabbit non-immune IgG and mouse non-immune IgG. Upon addition of laminarin, the Dectin-1 blocking antibody or anti-zymosan antibody, there was significant inhibition of O_2^- production (*Figure 2*). The vimentin was tested for endotoxin contamination using the LAL Endotoxin Assay kit (Genscript) and found to be $<$0.002 EU/5 μg. These results clearly indicate that vimentin induces NADPH oxidase activity to produce

Table 1 Identification of proteins in human carotid atherosclerotic tissue samples by two-dimensional electrophoresis

Spot	Identification: protein name, NCBI database index number, calculated MW	Coverage[a]	Mascot Score[b]
1	Unidentified	–	–
2	Vimentin (62414289, 53 kDa, 5.0)	30 (62%)	1646
3	Vimentin (62414289, 53 kDa, 5.0) Alpha 2 actin (4501883, 42 kDa, 5.2)	31 (53%) 4 (14%)	1326 216
4	Tropomyosin 2 (beta) isoform 2 (47519616, 33 kDa, 4.6)	25 (66%)	1367
5	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member (50363217, 46 kDa, 5.3) ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor (32189394, 56 kDa, 5.2) Tubulin, beta, 2 (5174735, 50 kDa, 4.7)	16 (52%) 7 (22%) 5 (13%)	1210 456 256
6	Vimentin (62414289, 53 kDa, 5.0)	25 (63%)	1391

[a]The number of peptides discovered.
[b]A probability-based Ion Score for each peptide match.

O_2^- via Dectin-1 in primary human monocytes and extends our findings to indicate that vimentin, similar to zymosan, induces O_2^- production in a Dectin-1-dependent manner. Although we were limited by the number of monocytes for inclusion of the rabbit non-immune IgG control group, we have repeatedly tested this control in other experiments and never observed inhibition of O_2^- production.

3.6 Detection of vimentin and Dectin-1 in human coronary atherosclerotic plaque samples

Based on our findings, we next examined human atherosclerotic plaques for vimentin immunoreactivity. The human coronary atherosclerotic plaque (*Figure 3*) contains a well-defined lipid-rich, necrotic core covered by a fibrous cap. Vimentin was localized in intact cells within the fibrous cap and in smooth muscle cells in the non-lesion areas. In addition, strong vimentin staining in the inflammatory shoulder areas proximal to the necrotic core of the atherosclerotic plaque was also detected extracellularly as determined by its presence in areas devoid of DAPI staining as shown in the z-stack confocal staining (*Figure 3B*). In control experiments, using 10-fold excess of the vimentin peptide antigen, the immunoreactivity with the anti-vimentin antibody was blocked with the exception of some minor autofluorescence that was also present in controls (Supplementary material online, *Figures S1* and *S2*).

Double staining using anti-dectin-1 and anti-CD68 antibodies showed significant co-localization especially around the shoulder and fibrous cap

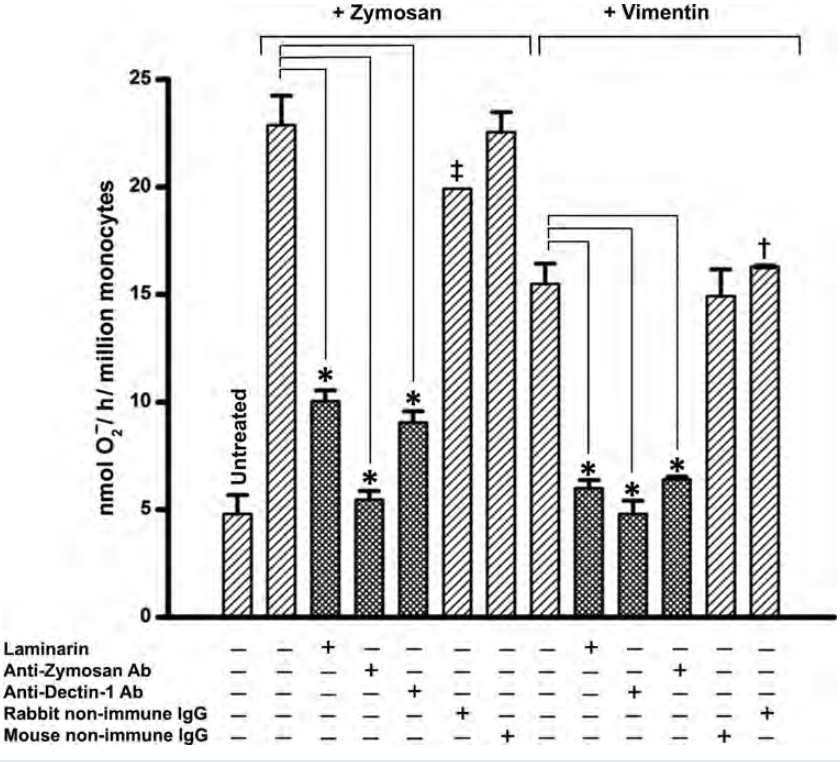

Figure 2 Vimentin induces superoxide anion production in primary human monocytes via the Dectin-1 receptor. Vimentin was assessed for its ability to activate monocytes to produce NADPH oxidase dependent O_2^- production when compared with zymosan (the positive control). The Dectin-1 dependence of this activation was evaluated by either anti-zymosan antibody or a Dectin-1 blocking antibody. Corresponding non-immune IgG were used as negative controls. A single asterisk refers to $P < 0.01$. The graph represents the mean ± SEM of three different experiments except for *n*-values for ‡ and † groups where $n = 1$ and $n = 2$, respectively, for experiments where monocyte numbers limited the number of experimental groups.

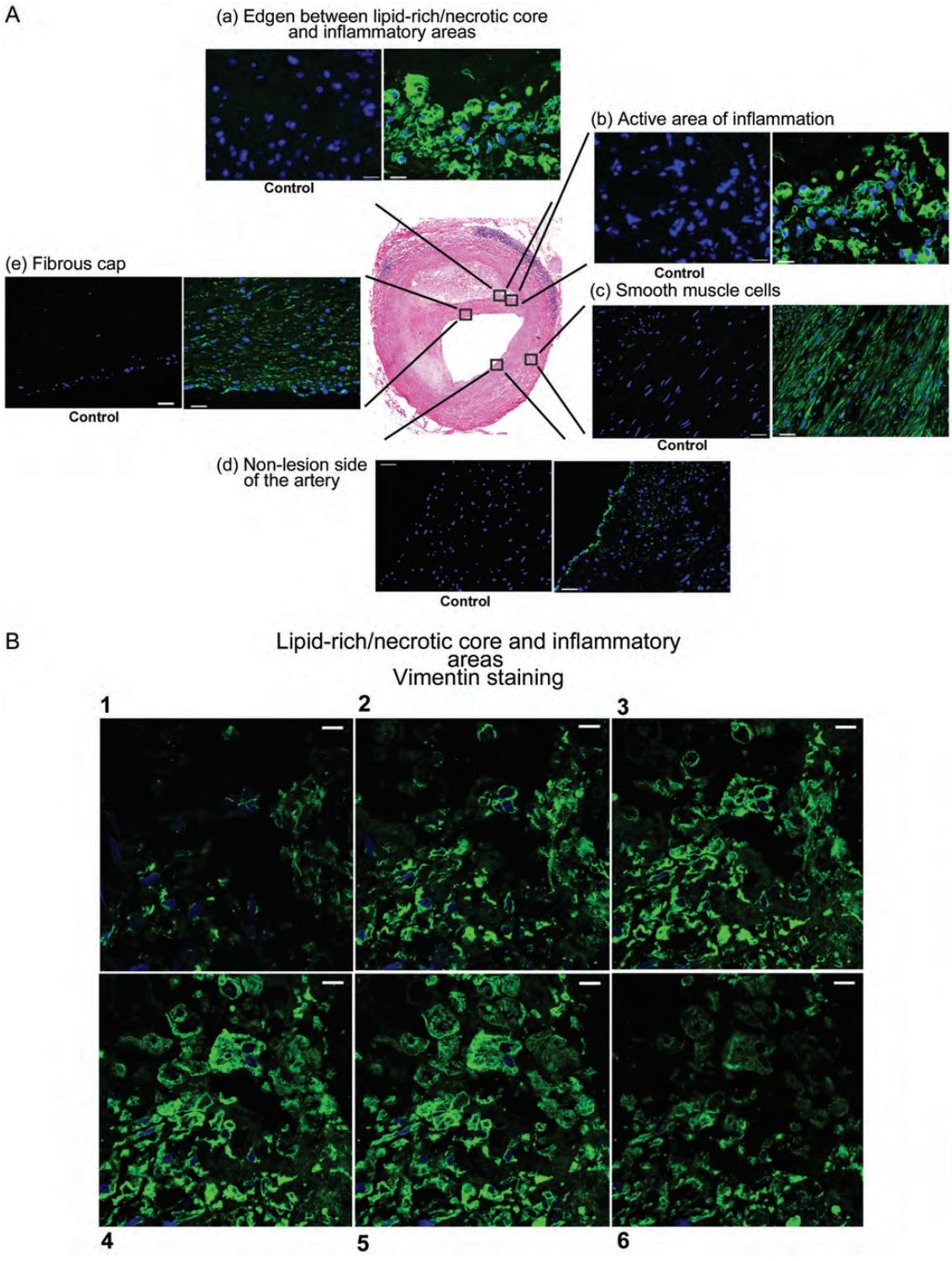

Figure 3 Identification of vimentin in active areas of inflammation. Human coronary atherosclerotic plaque tissue sections (types IV–V) were stained for vimentin. H&E staining was also performed as shown in the centre. Vimentin staining was most prominent in the active areas of inflammation at the edge of necrotic and inflammatory areas (*A*) and at the plaque shoulder (*B*). Vimentin was detected in other areas such as the fibrous cap, the intima, and the tunica media. Controls with no primary antibody are shown next to the figures. Scale bar (*a* and *b*) 20 μm; (*c*–e) 10 μm. *Figure 3B* shows the Z-stack confocal images of the vimentin staining also performed at the edge of necrotic and inflammatory areas. Every third section of the Z-stack series from the first to the last section and including both the first and the last sections are shown in panels 1–6. The panels show vimentin in these areas, especially in the brightly stained areas that were devoid of DAPI or nuclear association, suggesting the protein was extracellular. Scale bar = 20 μm. Figures are representative of staining of five different specimens.

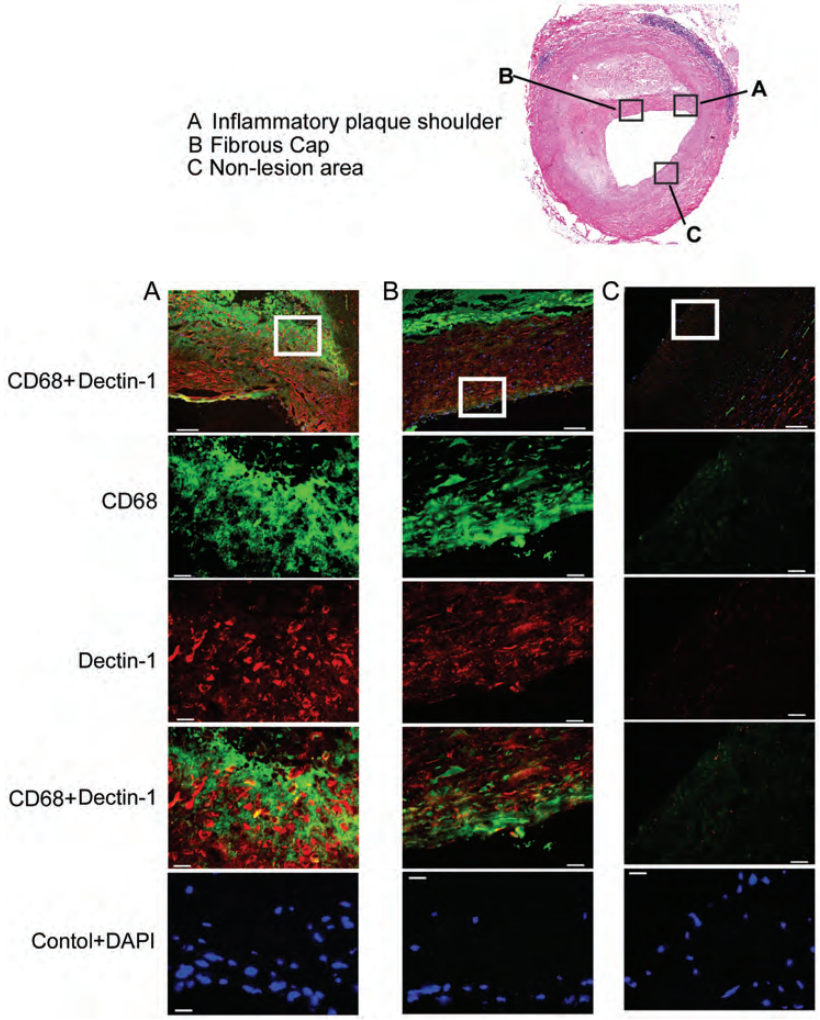

Figure 4 Double staining of Dectin-1 and CD68 in inflammatory areas of human coronary atherosclerotic plaque tissue sections. Double staining of atherosclerotic plaques of human carotid artery tissue sections shows that the immunoreactivity of Dectin-1 and CD68 colocalize in most macrophages, specifically in the active areas of inflammation within the coronary vessel wall. H&E staining was also performed as shown in the top section. The three panels show different areas of the vessel wall stained for Dectin-1 and CD68. Co-localized staining is predominantly observed in the plaque shoulder and the fibrous cap, whereas the non-lesion side of the intima shows less staining. Controls without primary antibodies are shown at the bottom of each panel. Scale bar—top panel (*A*), (*B*), (*C*) = 20 μm, other panels (*A*), (*B*), (*C*) = 10 μm. Figures are representative of staining of five different specimens.

regions of the atherosclerotic plaque (*Figure 4*). The inflammatory plaque shoulder contains numerous activated macrophages as evidenced by strong immunoreactivity with CD68. Colocalization in this area of the lesion was 56.9 ± 1.2%. The non-lesion side of the artery showed minimal immunoreactivity, thereby further validating the immunoreactivity observed in the plaque areas. Interestingly, we were able to observe the co-localization of vimentin and Dectin-1 in the plaque shoulder area where the components of NADPH oxidase were found by others to be abundant and also the active area of O_2^- production (*Figure 5*).[29] The mean percentage co-localization of vimentin and Dectin-1 staining was calculated using ImagePro and found to be 72% ± 3.2 in the inflammatory regions of the artery. Co-localization was determined in five tissue specimens (measuring five high power fields each). Staining in the different tissue sections is shown in Supplementary material online, *Figure S6*. We also quantified the co-localization of vimentin and CD68 in the inflammatory areas (*Figure 6*). The mean percentage co-localization of vimentin and CD68 staining was found to be 76.2 ± 2.4% in the inflammatory region. Minor autofluorescence was observed and was similar in non-specific binding controls where no primary antibodies were added and also with isotype control IgG antibodies (Supplementary material online, *Figures S1–S6*).

4. Discussion

As introduced earlier, alarmins are endogenous danger signals that interact with innate immune receptors and trigger inflammatory responses. These are diverse in structure and function and possess dual functions with respect to their intracellular or extracellular location. Alarmins have been discovered to become immunostimulatory upon cell release by necrosis or cell activation during tissue damage or host defence. Though several alarmins have been identified, none has been shown to engage and trigger Dectin-1 until this report.[6,30]

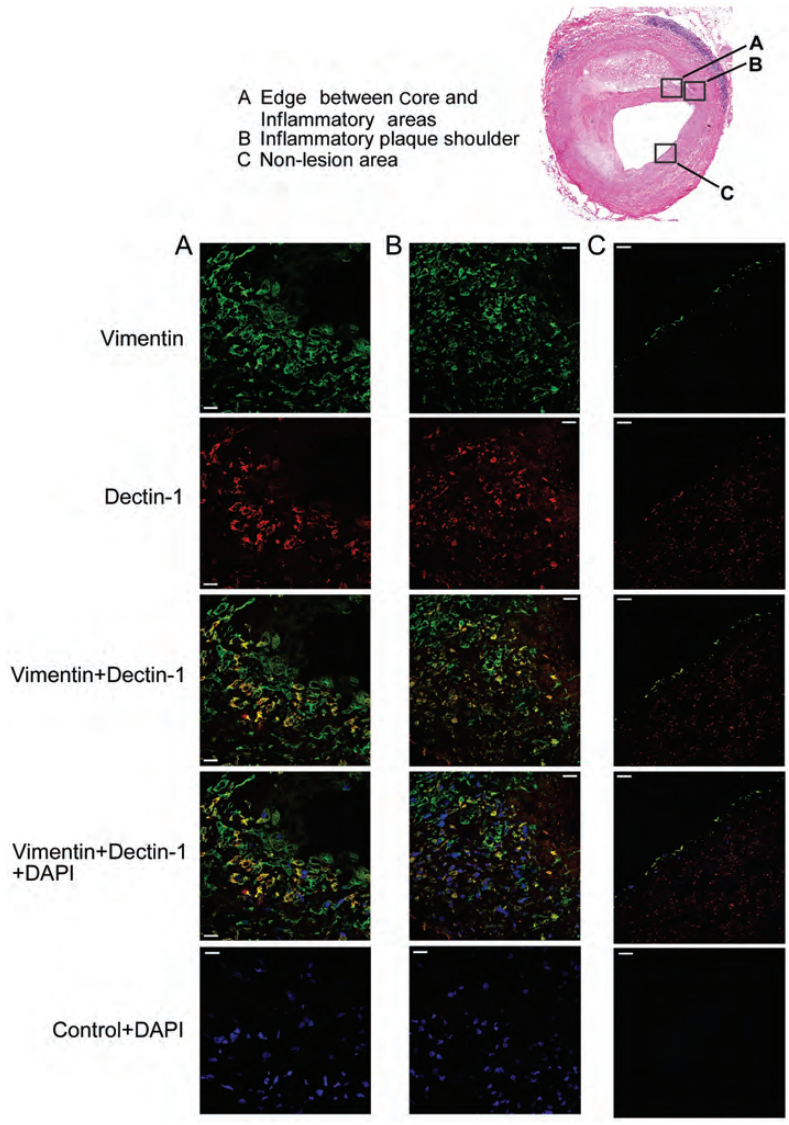

Figure 5 Co-localization of Dectin-1 and vimentin in inflammatory areas of human coronary atherosclerotic plaque tissue sections. Atherosclerotic tissue was stained with both Dectin-1 and vimentin. The three panels (*A*, *B*, *C*) show the corresponding different areas of the vessel wall stained for Dectin-1 and vimentin. Co-localized staining is predominantly seen near the core and the plaque shoulder, whereas the non-lesion side of the intima shows less staining. Controls with no primary antibodies are shown at the bottom of each panel. Scale bar—all panels (*A*), (*B*), (*C*) = 20 μm. Figures are representative of staining of five different specimens.

Since we recently identified Dectin-1 as an important receptor for stimulating monocyte superoxide anion production and CLRs have been shown to bind non-carbohydrate ligands especially from dying cells, thereby sensing immunogenic cell death.[31] We hypothesized that Dectin-1 might also have endogenous ligands that would promote oxidative stress in inflammatory sites. Identification of vimentin as an endogenous ligand for Dectin-1 (*Figures 1* and *2*) was at first surprising as it is an intermediate filament protein found in many cell types including macrophages, lymphocytes, neutrophils, endothelial cells, and fibroblasts.[32–34] Upon further investigation of the literature, we found that vimentin has been reported to also reside extracellularly being detected on the cell surface of viable, as well apoptotic cells, and released or secreted upon cell activation.[27,35–37] Among these studies, Mor-Vaknin *et al.*[27] showed that activated macrophages secrete vimentin in response to proinflammatory stimulus. We confirmed this observation by showing that MCP-1 activation of primary monocytes promotes vimentin release (*Figure 1G*). Thus in proinflammatory sites, soluble vimentin could be available to interact with Dectin-1.

We observed the presence of significant co-localized staining for extracellular vimentin and Dectin-1 expressed by CD68-positive macrophages in inflammatory regions of the atherosclerotic lesions. Although highly regarded as a selective marker for macrophages and dendritic cells, CD68 antibodies have been reported to stain lymphocytes, stromal cells, and endothelial cells in fixed tissue specimens. This may explain our observation of only 57% colocalization of these markers.[38]

The physiological significance of released or secreted vimentin is not completely understood but strongly suggests important surface or extracellular-associated functions. Many of the endogenous danger

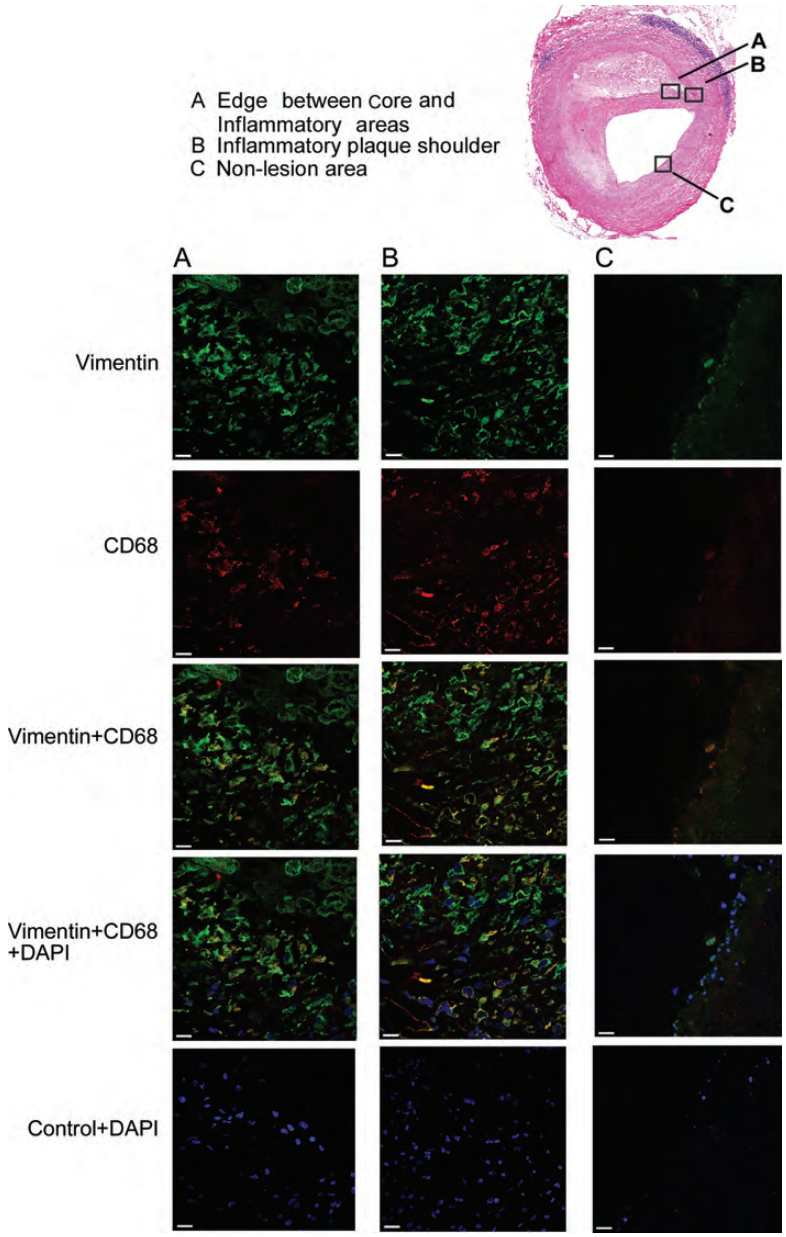

Figure 6 Co-localization of CD68 and vimentin in inflammatory areas of human coronary atherosclerotic plaque tissue sections. Double staining of atherosclerotic plaques from human carotid artery tissue sections shows that the immunoreactivity of vimentin and CD68 co-localize in the inflammatory areas within the coronary vessel wall. The three panels show different areas of the vessel wall stained for vimentin and CD68. Co-localized staining is predominantly observed near the core and in the plaque shoulder, whereas the non-lesion side of the intima shows less staining. Controls without primary antibodies are shown at the bottom of each panel. Scale bar—(*A*–*C*) = 20 μm. Figures are representative of staining of five different specimens.

signals, which appear outside of cells, do not have secretory signal peptides in their sequences and are secreted through alternative secretory pathways.[30] Similarly, vimentin also has no secretory signal sequence.[39] The carboxy terminus of vimentin contains a di-acidic motif(Asp-X-Glu) used for selective protein export.[40] Active secretion of vimentin has been reported to be up- and down-regulated during pro- and anti-inflammatory conditions, respectively.[27] Secreted vimentin has also been shown to induce the oxidative burst and interact with the complement cascade favouring bacterial killing.[27] Cell surface expression on monocytes was up-regulated in tuberculosis infection and involved in triggering the lysis of infected cells.[41] A recent study by Ise *et al.*[42] showed the rod II domain of vimentin that is present on the cell surface has lectin-like activity toward *N*-acetylglucosamine. They propose that extracellular physiological ligands include O-GlcNAc proteins.

Studies suggest that there might be conformational structural changes of soluble vimentin upon extracellular release[35,37] and further studies are required to determine the structure and role of the secreted vimentin in atherogenesis. Regardless, we observed direct strong binding of vimentin to Dectin-1 using BIACORE. Of greater importance, we found that vimentin induces NADPH oxidase-derived O_2^- production in human monocytes in a Dectin-1-dependent manner. The levels of

O_2^- production were almost as robust as those induced by zymosan, one of the most potent stimulators of the NADPH oxidase and O_2^- production. Corroborating our binding studies and O_2^- assays are the previous studies about the location of the O_2^- components and production of O_2^- in atherosclerotic lesions.[29]

Although vimentin was also shown to bind to Dectin-2, albeit with much lower affinity, we do not believe that Dectin-2 participates in either the O_2^- production or in the staining of atherosclerotic lesions. The induction of O_2^- through Dectin-1 is dependent on signalling through the ITAM-like cytoplasmic domain of Dectin-1 via Syk, Src, and PKCδ.[2] Dectin-2 has no cytoplasmic signalling domain. Furthermore, the antibody to Dectin-1 used for immunohistochemistry has no reactivity with Dectin-2.

The expression of TLRs (2 and 4) by macrophages was found to be significantly up-regulated in atherosclerotic lesions when compared with normal arteries. But the distribution of Dectin-1 has not been previously explored.[3,4] Our studies showed the co-localization of Dectin-1 and vimentin in areas of active inflammation and O_2^- production in human atherosclerotic plaque tissue sections. Further study is required to see if Dectin-1 is also a crucial active innate immune receptor similar to the TLRs in the pathogenesis of human atherosclerosis. Understanding and modulating the activities of endogenously generated danger signals and their receptors in atherosclerosis may enable us to bring the inflammatory process under control. Therapeutic strategies are being developed to modulate the expression of these endogenous danger signals to prevent and treat atherosclerosis and other chronic inflammatory diseases. We suggest that Dectin-1 may serve as a novel target arresting proinflammatory processes.

We conclude that vimentin is a non-microbial endogenous ligand for Dectin-1 that is capable of activating monocyte macrophages. Vimentin was identified in human atherosclerotic tissue lesions and was extracellular in areas of inflammation and in the necrotic core. Our results were obtained with advanced atherosclerotic lesions. In future studies, it will be interesting to determine how early this interaction may occur during lesion development. The extracellular localization of vimentin in macrophage-rich active inflammatory areas, the shoulders and the necrotic core of atherosclerotic plaques, strongly suggests its active role in atherogenesis. Recently, cell surface vimentin has been proposed as a marker for detection of early atherosclerosis.[32] The interesting finding of co-localization of vimentin with Dectin-1 receptor in areas previously shown to be actively involved in O_2^- production by NADPH oxidase in human atherosclerotic lesions suggests its crucial role in inducing the oxidative stress. We propose that the presence of extracellular vimentin due to necrosis and active release in lesions may contribute to the chronicity of the disease and that blocking the binding between vimentin and Dectin-1 may control disease progression by relieving oxidative stress.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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