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Identification of IgG1 isotype phosphorylcholine antibodies for the treatment of inflammatory cardiovascular diseases

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Short title: Identifying a new PC antibody

Abstract

Phosphorylcholine (PC) is an important pro-inflammatory damage associated molecular pattern. Previous data has shown that natural IgM anti-PC protect against cardiovascular disease. We aimed to develop a monoclonal PC IgG antibody with anti-inflammatory and anti-atherosclerotic properties.

A chimeric anti-PC (PC-mAb(T15), consisting of a human IgG1 Fc and a mouse T15/E06 Fab) was produced, this was shown to bind specifically to epitopes in human atherosclerotic tissues. Inflammation-driven accelerated atherosclerosis was induced by femoral-artery-cuff-placement in ApoE3*Leiden mice. This results in rapid induction of inflammatory genes and altered expression of genes associated with ER stress and choline metabolism in the lesions. Treatment with PC-mAb(T15) reduced accelerated atherosclerosis via reduced expression of endoplasmic reticulum-stress markers and CCL2 production.

Recombinant anti-PC Fab fragments were identified by phage display and cloned into fully human IgG1 backbones creating a human monoclonal IgG1 anti-PC (PC-mAbs) that specifically bind PC, apoptotic cells and oxLDL. Based on preventing macrophage oxLDL-uptake and CCL2 production, 4 monoclonal PC-mAbs were selected which to various extent reduced vascular inflammation and lesion development. Additional optimization and validation of 2 PC-mAb antibodies resulted in selection of PC-mAb X19-A05, which inhibited accelerated atherosclerosis. Clinical grade production of this antibody (ATH3G10) significantly attenuated vascular inflammation and accelerated atherosclerosis and was tolerated in safety studies in rats and cynomolgus monkeys.

In conclusion, chimeric anti-PCs can prevent accelerated atherosclerosis by inhibiting vascular inflammation directly and through reduced macrophage oxLDL-uptake resulting in decreased lesions. PC-mAb represents a novel strategy for cardiovascular disease prevention.

Keywords: Vascular disease, therapeutics, inflammation, atherosclerosis, restenosis

Introduction

In atherosclerotic diseases, cellular stress and inflammation generate damage associated molecular pattern molecules (DAMPs). One of these DAMPs is phosphorylcholine (PC), the polar headgroup of the dominating membrane phospholipid (PL) phosphatidylcholine[1]. Enzymatic hydrolysis and oxidative modification of phosphatidylcholine in cell and LDL membranes, especially fatty acids in the sn-2 position, lead to formation of bioactive PC containing lipids (often referred to as oxPL)[2]. In human plasma the main carrier of oxPLs are lipoprotein a (Lp(a)[2], and these Lp(a) associated OxPLs are able to induce arterial inflammation[3].

Many of these PC containing lipids are recognized by the innate immune system and stimulate powerful biological processes such as endothelial dysfunction, apoptosis and endoplasmic reticulum (ER)-stress[4, 5]. They are considered important mediators of vascular inflammation rendering oxPL as a promising therapeutic target[1]. There are several innate immune system receptors recognizing PC including proteins, scavenger receptors of phagocytic cells and natural antibodies (IgM anti-PC)[6], in general these receptors are part of the system for clearance of damaged particles and cells (efferocytosis)[7]. The murine T15/E06 IgM antibody is well known to inhibit oxLDL uptake into macrophages[8] and enhance efferocytosis[9, 10]. A pathophysiological role of PC containing oxPLs in atherosclerosis and ischaemia-reperfusion injury was shown by the protective effect of anti-PC lacking effector function[11, 12].

Immunization leading to high anti-PC levels can prevent native and accelerated atherosclerosis in mice by inhibition of oxLDL-uptake and inflammatory foam cell formation[13, 14]. Epidemiological data suggests that IgM anti-PC protects against cardiovascular disease development and that low levels of IgM anti-PC are associated with increased risks for cardiovascular events[15-19]. Acute coronary syndrome (ACS) patients with low levels of IgM anti-PC have a worse prognosis than patients with higher levels[20], although this was not confirmed in another study[21]. Experimental data shows that T15/E06 natural IgM PC antibodies have profound anti-inflammatory properties (unrelated to foam cell formation) and induce enhanced clearance of apoptotic cells[7, 22, 23]. This suggests that anti-PC could be therapeutically effective.

Human serum contains anti-PCs of several subclasses[24] and not all have associations to cardiovascular disease[25]. PC is also a pathogen associated molecular pattern (PAMP), present e.g. in the polysaccharide capsule of Streptococcus pneumoniae. While T15/E06 was also effective against *Streptococcus* infections in mice, there was major variability in IgG anti-PCs effects[26-28]. In man, the relation between carotid atherosclerosis and anti-PC may depend on the Ig isotype[25].

These results indicate that although PC is a chemically defined entity, presentation of the PC epitope depends on the local environment such as the exact modification of the fatty acid component leading to generation of PC epitopes, and that different IgG anti-PCs may be more specific for the variation in presentation of PC than the natural IgM anti-PC.

IgM antibodies are rapidly eliminated from plasma, and there are currently no therapeutic IgMs available. The aim was to generate a fully human monoclonal IgG1anti-PC (PC-mAb) that has the potential to block vascular inflammation, can inhibit uptake of oxLDL by macrophages and prevent vascular disease. As a reproducible model of such disease we used cuff induced femoral artery lesions in mice[29], an established model that was further validated by transcriptomic analysis at several time points after cuff placement, to confirm that lesion-development is inflammation driven and results in changes in choline metabolism. Drug effects were evaluated at two time points, 3 and 14 days after cuff placement. For validation purposes, we first generated a chimeric mouse/human IgG1 (PC-mAb(T15)) with a human IgG1 Fc and the murine T15/E06 Fab fragment. This antibody prevented vascular inflammation both in vitro and vivo, providing support that IgG PC-mAbs can be effective against vascular disease. We then identified multiple fully human monoclonal PC-mAbs using phage-display library screening and cloned the Fab fragments into a human IgG1 backbone. These human monoclonal PC-mAb IgG1 constructs were tested for their in vitro and in vivo anti-inflammatory and anti-atherosclerotic effects. The clinical grade antibody (ATH3G10) showed significant inhibition of vascular inflammation and prevented accelerated atherosclerosis in the murine cuff model and was well tolerated in rats and cynomolgus monkeys. ATH3G10 is now being tested in clinical studies (EudraCT Number:2018-003676-12; ClinicalTrials.gov Identifier: NCT03991143).

Materials and Methods

An extended version of the material and methods can be found in the online material section.

Mice

All mouse experiments were performed in compliance with Dutch government guidelines and conform to the guidelines from Directive 2010/63/EU of the European Parliament on protection of animals used for scientific purposes and were approved by the Institutional Committee for Animal Welfare of the Leiden University Medical Center (LUMC approval numbers 09224, 10207, 11208, 13137). Transgenic male C57BL/6J ApoE*3-Leiden mice were used due to their specific response to the hypercholesterolemic diet[30] and their stronger response to vascular interventions[31]. The mice (bred in our own laboratory) were aged 10-12 weeks at the start of the dietary run-in period and were fed a high cholesterol high fat diet (AB Diets) to induce hypercholesterolemia three weeks prior to surgery and continued throughout the entire experiment. Mice were randomized based on plasma cholesterol levels (Roche Diagnostics, 1489437) and body weight. All animals received food and water ad libitum.

For the 3 days antibody testing experiments mice received one intraperitoneal injection at the time of surgery. For the 14 days experiments mice received intraperitoneal injections at the time of surgery and twice weekly (4 injections in total). As negative controls sterile 0.9% NaCl or human IgG1 streptavidin antibodies (control anti-streptavidin IgG1, Dyax Corp; a Takeda company) were used.

cuff model

Mice were anesthetized with an intraperitoneal injection of midazolam (5 mg/kg, Roche Diagnostics), dexmedetomidine (0.5 mg/kg, Orion,) and fentanyl (0.05 mg/kg, Janssen Pharmaceutical). Toe pinching was used to check depth of anaesthesia. Femoral arteries were isolated and sheathed with a rigid non-constrictive polyethylene cuff (Portex). After the surgery, the anaesthesia was antagonized with a subcutaneous injection of atipamezole (2.5 mg/kg, Orion) and flumazenil (0.5 mg/kg, Fresenius Kabi). Buprenorphine (0.1 mg/kg, MSD Animal Health) was provided (subcutaneous injection) directly after surgery to relieve pain. Mice in the antibody studies were sacrificed at 3 days (acute inflammation) or 14 days (accelerated atherosclerosis) after cuff placement. At sacrifice mice were anesthetized with the aforementioned midazolam/dexmedetomidine/fentanyl mix and were euthanized via exsanguination followed by PBS perfusion (transcriptome analysis) and perfusion fixation (histology studies). Cuffed femoral arteries

were harvested and snap frozen (transcriptome analysis) or fixed overnight in 3.7% formaldehyde and processed to paraffin (histology studies).

Human atherosclerotic material

Endarterectomy tissues were obtained in accordance with guidelines set out by the 'Code for Proper Secondary Use of Human Tissue' of the Dutch Federation of Biomedical Scientific Societies (Federa) and conform to the principles outlined in the Declaration of Helsinki.

Toxicity studies

Toxicity studies of 4 and 26 weeks duration were performed (table 1). These studies were conducted at Charles River Laboratories, Edinburgh, all animal housing and procedures were fully compliant with EU Directive 2010/63, UK legislation and AAALAC (Charles River Labs, Edinburgh). Weekly intravenous doses of 4, 10 and 40mg/kg were used in all studies. The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight changes, food consumption, ophthalmology, electrocardiology, blood pressure, respiratory rate, clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), immunogenicity, toxicokinetic parameters, gross necropsy findings, organ weights, and histopathologic examinations.

Statistical analysis

All data are presented as mean ± standard deviation, unless otherwise indicated. Overall comparisons between data from groups were performed with one-way Anova, for non-parametric testing the Kruskal-Wallis test was used. If significant differences were found, groups were compared using a Mann-Whitney sum test. All statistical analyses were performed with GraphPad Prism 8. P-values less than 0.05 were regarded as statistically significant. For RNA seq data, to determine differential expression compared to baseline empirical Bayes moderated t-statistics were computed using the limma package (3.28.14). Benjamini-Hochberg adjusted P-values less than 0.05 were regarded as statistically significant.

Results

Cuff placement results in an early induction of inflammatory and ER stress genes and sustained changes in choline metabolism gene expression

To select a proper model to test the effect of PC antibodies in vivo we focused on the femoral artery cuff model and profiled the transcriptome of arterial segments in response to injury. Dimension reduction through multi-dimensional scaling revealed distinct and tight clustering of early timepoints (baseline, 6 hours, 1,3 day) and more common clustering of later timepoints (7,14 days) (**fig 1A**). KEGG pathway over-representation analysis of up-regulated genes across timepoints showed an early inflammatory signature at timepoints 6 hours, 1 days and 3 days with pathways like TNF signalling, IL-17 signalling, NF-kB signalling and B-cell receptor signalling all significantly over-represented (**fig 1B**). Pathways over-represented in down-regulated genes tended to be consistent across all timepoints and included multiple extracellular signalling and second messenger pathways including ECM-receptor interaction, focal adhesion, cGMP PKG signalling and calcium signalling pathways (**fig 1C**). Along with surgical stress, key mediators of endoplasmic reticulum (ER) stress including Atf4, Atf6 and spliced Xbp1 rapidly and transiently increased, peaking at 6 hours post-surgery and returning to baseline levels by 3 days (fig 1D). Consistent with this increase, numerous transcriptional targets of these key mediators were also significantly increased at early timepoints including Hspa5, Atf4 and Gadd34 (fig 1E). Interestingly, genes related to choline metabolism (KEGG mmu05231) showed distinct patterns of expression across the timepoints and this gene set was significantly differentially expressed at both day 3 (**fig 1F**) and day 14 (**fig 1G**) compared to baseline. Genes specifically related to interconversion of choline and phosphorylcholine were also significantly regulated over time. At 6 hours Chka, which converts choline to phosphorylcholine, was significantly up-regulated and tended to be upregulated across timepoints (fig 1H,I). Across all timepoints Chpt1 which generates phosphatidylcholine was significantly down-regulated (fig 1H,I). Other genes which shunt choline away from phosphorylcholine (Chdh, Ache) tended to either be significantly down-regulated or unchanged across timepoints (fig 1H,I). Other genes which generate choline from phosphatidylcholine (Gpcpd1, Lypla1, Pla2) tended to either be significantly up-regulated or unchanged (fig 1H,I). Based on the different gene regulation patterns further studies were conducted at day 3 after cuff placement to investigate inflammation-driven processes and at day 14 to investigate vascular remodelling and accelerated atherosclerosis.

Chimeric PC-mAb(T15) binds to human atherosclerotic lesions, inhibits in vivo vascular inflammation and prevents accelerated atherosclerotic lesion formation

Immunohistochemical staining with chimeric PC-mAb(T15) showed strong and specific binding to cells including macrophages within human atherosclerotic lesions (**fig 2A**). Specific binding of PC-mAb(T15) to PC was confirmed by ELISA (data not shown). Therapeutic effectiveness of PC-mAb(T15) was tested in the mouse cuff model for post-interventional accelerated atherosclerosis. Plasma cholesterol levels or body weight were not affected by PC-mAb(T15) (**suppl table 1**). PC-mAb(T15) reduced endothelial-adherence and vessel infiltration of monocytes/macrophages to the injured arterial segments 3d after injury (**fig 2B**). Together this led to a 60% significant reduction in macrophages in the vessel wall compared to both control groups (**fig 2C & suppl fig 1A, B**). Comparable results were found for CD45+ leukocytes (**suppl fig 1C, D**). ER stress related proteins are signs of pro-inflammatory responses as shown in human atherosclerotic lesions in which HSPA5 (**suppl fig 1E**) and DDIT3 (**suppl fig 1F**) are highly expressed. Both HSPA5 and DDIT3 are also found in murine lesions (**suppl fig 1G, H)**. PC-mAb(T15) treatment showed trends towards reduction of $HSPA5^+$ cells (**suppl fig 1I-J**) whereas $DDIT3^+$ cells attached to the endothelium and in the media were significantly reduced with resp. 80 and 67% (**suppl fig 1K-L**). These results indicate that PC-mAb(T15) prevents early inflammatory responses, well before generation of local foam cells in this model (26).

Effects of PC-mAb(T15) on accelerated atherosclerosis and remodelling were evaluated 14d after cuff placement (**fig 2D-I, suppl fig 2**). PC-mAb(T15) in comparison to both control and IgG control group (**fig 2D**) significantly reduced neointima formation (82 and 76%, **fig 2E**), intima/media ratio (79 and 72%, **fig 1F**), as well as luminal stenosis (68 and 60%, **fig 1G**). PC-mAb(T15) treatment resulted in a significant increase in SMCs in the media (87% compared to control and 37% compared to control IgG) (**suppl fig 2D, E**) whereas no differences could be detected in the neointima (**fig 2H**). PC-mAb(T15) significantly reduced macrophage infiltration in the neointima (resp. 74 and 69%, **fig 2I, suppl fig 2F, G**). Furthermore, PC-mAb(T15) reduced the number of CD45+ leukocytes in both media and neointima (**suppl fig 2H-J)** as well as cells displaying ER-stress marker HSPA5 (**suppl fig 2K-M**) and the percentage of cells displaying DDIT3 (**suppl fig 2N-P**). Thus passive immunisation with PC-mAb(T15) prevents vascular inflammation and lesion formation in part by reducing ER stress.

PC-mAb(T15) and human polyclonal IgG anti-PC do not block oxLDL-uptake by macrophages, unlike polyclonal anti-PC IgM

The murine IgM T15/E06 natural antibody and polyclonal human IgM anti-PC[32] is known to block scavenger receptor-mediated uptake of oxLDL[13]. As expected, polyclonal anti-PC IgM showed an inhibition of DiI-Labelled Cu-oxLDL uptake by human monocyte-derived macrophages (**fig 3A**), which was dose-dependent (**suppl fig3A**). In contrast to anti-PC IgM, serum derived polyclonal anti-PC IgGs did not show an inhibition of Cu-oxLDL uptake (**fig 3B, suppl fig3B**). PC-mAb(T15) also did not prevent Cu-oxLDL uptake in human macrophages. Unlike IgM, the Fc-region of IgG bears a highly conserved N-glycosylation site that is essential for Fc receptor-mediated activity by macrophages and could lead to increased oxLDL uptake of oxLDL-immune-complexes by macrophages via Fc receptors. As such a mechanism could mask inhibition of scavenger receptor-mediated uptake, we excluded this possibility by using both IgG1 and IgG4 isotype chimeric PC-mAb(T15) (**fig 3C**). As IgG4 antibodies are less prone to bind Fc receptors the experiment was also performed including Fc-receptor blockade by anti-CD32 and anti-CD64 antibodies. However, this had no effect on Cu-ox-LDL uptake (**fig 3D**). Even though PC-mAb(T15) has anti-inflammatory effects in vivo, transfer of the T15/E06 variable region from IgM to the IgG format abolished the scavenger receptor blocking effects.

Development of monoclonal human PC-mAbs

Because the ability to block oxLDL uptake is important, we decided to optimize the PC-mAb using phage display and binding to PC-BSA as an initial screen, with secondary screens including inhibition of oxLDL uptake and binding to apoptotic cells in vitro. Screening of 10660 different phage yielded 1511 positive hits, defined as a >3fold stronger signal by the individual phage for binding to BSA- or ferritin-conjugated PC compared to IgG controls. 54 antibodies were recovered after recombinant reformatting to full length IgG. Binding specificity was assessed using a Biacore SPR assay and ELISA for binding to either control, linker, PC-BSA and PC-KLH, allowing selection of antibodies with potential therapeutic effectiveness. 27 antibodies with the best binding affinities to PC-BSA and PC-ferritin, were investigated for their effects on macrophage oxLDL-uptake (data not shown). Nine recombinant anti-PC IgG antibodies inhibited oxLDL-uptake similarly or better than polyclonal IgM anti-PC on a weight basis (**Suppl fig 3C, D**).

These 9 monoclonal antibodies displayed an approximately 1000-fold higher affinity for PC than polyclonal IgG anti-PC (**fig 3E**). M99-B05 and X9-C01, were analysed for binding to Cu-oxidized LDL

and both displayed profoundly increased binding affinity compared to chimeric PC-mAb(T15) (**fig 3F**). The high avidity of murine IgM T15/E06 to PC compared to the IgG isotype explains the effectiveness of the IgM T15/E06[22] on oxLDL uptake.

Antibodies with high affinity for PC and oxLDL were tested for binding to apoptotic Jurkat cells using FACS analysis. Only marginally increased binding to cells considered as early apoptotic (annexin $A5^+$ PI^-) was found. Some antibodies, including M99-B05 and X9-C01, were found to bind strongly to late apoptotic (annexin $A5^+$ PI^+) Jurkat cells (**fig 3G, suppl fig 3E-G)**. Five selected antibodies were finally tested for their ability to block oxLDL-induced release of CCL2 from monocytes of which M99-B05 and X9-C01 effectively blocked this release in a dose-dependent fashion with an IC50 in the 1-3 nM range (data not shown) whilst other tested antibodies were less effective (**fig 3H**).

PC-mAbs are effective against vascular inflammation and remodelling in vivo

The anti-inflammatory properties of four antibodies (M4B2 was not continued due to low yields during production) were tested in vivo in the cuff model, with PC-mAb(T15) as positive control. Treatment with M99-B05 and PC-mAb(T15) reduced endothelial-adherence and extravasation of $CD45^+$ leukocytes (**fig 4A**) and macrophages (**fig 4B**) significantly, while the other PC-mAbs did not (images; **suppl. fig 4A**). Interestingly, the number of $CCL2^+$ cells in the vessel wall was significantly reduced by all three antibodies: PC-mAb(T15) 82%, M99-B05 82% and X9-C01 (74%) (**fig 4C, D**). Next, effects of M99-B05 and X9-C01 on accelerated atherosclerosis in the 14 days femoral artery cuff model were analysed. Both antibodies significantly prevented neointima formation (42 and 44% resp.) and reduced intima/media ratio (both 43%) and lumen stenosis (56 and 64% resp.) in comparison to control IgG (**fig 4E-G, suppl B-D)**. Based on all these previous data M99-B05 was selected for further optimization.

Optimization of monoclonal PC-mAb(M99-B05) into PC-mAb(X19-A05)

To optimize the properties of PC-mAb(M99-B05), we employed germlining, codon optimization and amino acid replacements for potential de-amidation sites which resulted in a series of mutants. These were tested for binding to PC and it was observed that some modifications of M99-B05 negatively affected binding affinity to PC (**fig 4H**). The mutant X19-A05 was selected as the optimal antibody, as it combines several beneficial modifications of M99-B05 while retaining good binding affinity to PC.

Two weeks treatment with X19-A05 twice weekly with 0.5, 2 and 10mg/kg dosages significantly prevented neointima formation, reduced I/M ratio and lumen stenosis, comparable to M99-B05 (**fig 4I-L, suppl fig 4E-G**).

X19-A05 is potent, as a low dosage of 0.5 mg/kg twice weekly is already sufficient to produce a 35% inhibition of neointima formation (**fig 4J**).

ATH3G10 is the selected drug product for clinical development

PC-mAb(X19-A05) was selected for further clinical development resulting in PC-mAb (ATH3G10). ATH3G10 is the IgG1za/kappa antibody clinical grade product, produced using a stable cell line with the same Fab fragments as X19-A05. Biological activity of ATH3G10 was investigated in the 14 days femoral artery cuff model using twice weekly i.p. injections in the doses 0.1 and 1 mg/kg (**fig 5A, suppl fig 5A-D**). Treatment resulted in significantly reduced neointima formation by both concentrations of resp. 22% and 27% compared to the control group (**fig 5B**). A 26% reduction in I/M ratio was observed for both groups (**fig 5C**). Furthermore, ATH3G10 reduced the macrophage content in both media and neointima (**fig 5D, E, suppl 5E, F**). ATH3G10 thus has anti-inflammatory and vascular protective properties.

ATH3G10 toxicology studies in rats and Cynomolgus monkeys

To proceed toward human studies we performed the required toxicity testing in rats and a non-rodent species the Cynomolgus monkey, which is the most common used species for antibody testing. For these type of human antibody studies the expectation is that mammals develop an immune response in the form of anti-drug antibodies (ADA) [33, 34]. The repeated toxicity studies in rats and monkeys treated up to 4 weeks did not show any drug related toxicity in any organ and ATH3G10 was considered well tolerated. In the 4 weeks toxicity studies, the highest tested dose 40mg/kg was considered as the No Observable Adverse Effect Level (NOAEL) in both species. In the 26-week toxicity study in monkeys, most female but no male monkeys developed ADA after several months of drug exposure. 2 female monkeys in the highest dose group had severe reactions due to an immune reaction to the human protein[35] leading to precipitation of immune complexes between ATH3G10 and complement in several tissues along with inflammation and bleeding in some organs. In the lower dose groups, no immune complexes were seen in the animals even if ADAs were detected. In the 4-week studies, ADA was observed in one female rat at termination, and in no Cynomolgus monkeys. Thus in rats and Cynomolgus monkeys ATH3G10 limited immunogenicity to the human

protein was observed. These toxicity studies allowed ATH3G10 to enter clinical studies that are currently ongoing (EudraCT Number: 2018-003676-12; ClinicalTrials.gov Identifier: NCT03991143).

Discussion

Human PC binding antibodies selected by phage-library display and converted to fully human monoclonal IgG1 targeting PC were used to demonstrate for the first time the therapeutic efficacy of IgG anti-PC. It has been shown in several studies that there is an inverse correlation of atherosclerosis and IgM as well as polyclonal IgG1 (but not IgG2) PC antibodies [13, 14, 25]. PC antibodies are known to prevent cardiovascular diseases via targeting of various processes; they are anti-apoptotic, anti-thrombotic, target oxLDL, are anti-inflammatory by targeting the NFκB pathway and prevent smooth muscle cell proliferation and migration[14-19, 36-38]. One or several mechanisms of anti-PC might contribute to a potential benefit, and the mechanism may actually differ between different disease situations. The current project aimed to develop a fully human monoclonal PC antibody (PC-mAb) for treatment of inflammation associated vascular disease. Therefore the strategy was to select a drug for clinical trials that has beneficial effects on arterial inflammation and neointima/lesion formation in vivo, rather than a prespecified molecular mechanism. Passive in vivo immunization with chimeric PC-mAb(T15) and selected fully human PC-mAbs prevented cuff-induced arterial inflammation and accelerated atherosclerosis. The selected human PC-mAb binds to apoptotic cells, has high affinity for oxLDL, and inhibited oxLDL-uptake by macrophages in vitro, a beneficial effect of polyclonal IgM anti-PC, but surprisingly not of human polyclonal IgG anti-PC or PC-mAb(T15).

PC plays a key role as DAMP in the inflammatory reactions in both native atherosclerosis and following vascular intervention strategies due to acute events[11, 39]. In the latter setting, where rapid onset of action is desired, passive rather than active vaccination is preferred. Up until now the experimental work validating anti-PC as a therapeutic strategy has largely been performed using the mouse T15/E06 IgM, whilst for clinical use, IgG-based antibody formulations are preferred[40]. To test if this format is effective, we used the chimeric PC-mAb(T15). Specific binding was observed in human atherosclerotic lesions, proving antibody binding to the target tissue. To minimize the risk for an immunologic reaction to mouse/human chimeric and human proteins in mice, we used an accelerated in vivo model of atherosclerosis[29].

Transcriptomic analysis demonstrated a rapid induction of inflammatory signalling pathways, as well as a rapid induction of key transcriptional mediators of the ER stress response. Expression of choline metabolic genes was robustly altered in response to cuff placement. PC-mAb(T15) effectively reduced monocyte/macrophage adherence and extravasation with notably less CCL2 expression. Defective inflammatory resolution including ER stress is a known lead to the progression of

atherosclerotic lesions[41]. Here demonstrated by expression of ER stress markers HSPA5 and DDIT3 in human and murine atherosclerotic lesions. PC-mAb(T15) treatment led to retarded accelerated atherosclerosis and a less inflammatory phenotype, involving reduction of ER stress markers. In line with this, recent findings indicate that a fully human IgG1 clone against PC promotes clearance of dead cells and that IgG1 but not IgG2 anti-PC is associated with protection in relation to atherosclerosis[42].

Polyclonal human IgM anti-PC inhibited oxLDL-uptake by macrophages in vitro, whereas polyclonal IgG anti-PC and PC-mAb(T15) did not, probably due to low affinity to oxLDL (fig 4b). Post-interventional accelerated atherosclerosis is mainly triggered by injury-induced local inflammatory factors including cellular (ER) stress and DAMP expression and is not primarily the result of oxLDL-uptake[43, 44]. Although this concept supports why PC-mAb(T15) was effective in the murine cuff model, scavenger receptor mediated uptake of oxLDL does contribute to progression of atherosclerosis[3], and blocking oxLDL uptake should be beneficial. Therefore selected IgGs combining high affinity binding to the PC epitope on both oxLDL and apoptotic cells, and with inhibition of macrophage oxLDL uptake. Out of a total of 10660 phage clones, these selection criteria yielded four promising antibody clones. Further in vivo selection resulted in M99-B05 and X9-C1 which both reduced CCL2 expression in the vessel wall and reduced accelerated atherosclerosis, proving its long-term efficacy.

Codon optimized M99-B05, designated X19-A05, effectively inhibited neointima formation. A stable CHO cell line capable of producing ATH3G10, identical to X19-A05 in its Fab regions was developed for production of clinical grade material. ATH3G10 had preserved PC-affinity and therapeutic efficacy shown by significantly reduced neointima formation and I/M ratios in low dosages (0.1-1mg/kg). The observed effect of ATH3G10 seemed not as effective as the X19-A05 however, the cholesterol levels in the ATH3G10 study were higher (Suppl table 1) making it a more robust study. The present findings show that anti-PC has prominent effects on accelerated lesion development and also support the concept of PC as an important target for new therapeutics in vascular disease as also shown in recently published studies. PC-mAb preserves coronary flow reserve and attenuates atherosclerotic inflammation as determined by the uptake of 18F-fluorodeoxyglucose in atherosclerotic $LDLR^{-/-}ApoB^{100/100}$ mice[45]. Furthermore, PC-mAb showed preserved cardiac function and reduced infarct size in hypercholesterolemic mice [46] ATH3G10 did not cause adverse reactions in toxicity studies opening up the step towards clinical studies. These results combined with the strong epidemiologic support for the concept, suggest that ATH3G10 is a promising

therapeutic candidate for prevention of cardiovascular events in situations of increased inflammatory stress, and it is currently in clinical development.

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Author contribution

Conception; MdV,KP,PQ. Acquisition and analysis of data; MdV,ME,RdJ,MM,JK,EP, MN,SK,DS,ID,AB. Interpretation of data; MdV,ME,MM,AB,JM,J,JK,EN,JJ,KP,PQ. Manuscript drafting MdV,ME,KP,PQ, Manuscript revision; SK,AB,JM,JK,EN,JJ. Final approval; all.

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None

Conflict of Interest

KP, AB and ID are consultants to Athera Biotechnologies. JF, KP and AB are minor shareholder of Athera Biotechnologies, Stockholm, Sweden. JF and KP are named inventors on anti-PC-related patents. DS is a former employee of Dyax Pharmaceuticals, Cambridge, MA, USA. None of the other authors has conflicts of interest.

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Figure Legends

Figure 1. **Femoral arterial cuff placement causes rapid and sustained alterations to global transcriptome including ER stress and choline metabolism.** (A) Multi-dimensional scaling plot of transcriptome of cuffed arteries at baseline (0h) or multiple timepoints after cuffing (n=4-8 mice/timepoint. (B) KEGG pathway over-representation test of genes significantly up-regulated or downregulated (C) at multiple timepoints after cuffing. (D) Log2 fold changes of key upstream mediators of the ER stress response and downstream targets (E). (F) Gene set enrichment analysis of genes in the choline metabolism pathway at 3 and 14 (G) days after cuffing. (H) Diagram of major choline metabolic reactions, bold indicates metabolites and italic indicates genes carrying out the reactions. Red arrows indicate enzymes significantly up-regulated over the time course, blue significantly down-regulated and grey unchanged. (I) Log2 fold changes of genes shown in G. Empirical Bayes moderated t-statistics were computed to determine differential expression compared to baseline *p<0.1, **p< 0.05, ***p<0.001.

Figure 2. **Chimeric PC-mAb(T15) prevents accelerated atherosclerotic lesion formation**. (A) Representative human endarterectomy specimen (n=12) stained for phosphorylcholine (PC, yellow star) and macrophages (CD68, blue hash). (B) Representative cross-sections of macrophages (MAC-3) in cuffed arteries of ApoE3*Leiden mice receiving vehicle (control), human anti-streptavidin (control IgG) or anti-PC(T15) IgG after 3 days (n=5/group). (C) Quantification of macrophages (t3). (D) Representative cross-sections of cuffed arteries at day 14 (n=9-10/group). Quantification of neointima (E), intima/media ratio (F), % lumenstenosis (G), % smooth muscle cells (H) and % macrophages (I) mean±SD, Mann-Whitney *p<0.05, ***p<0.001, ****P<0.0001.

Figure 3. **Development and screening of monoclonal human PC-mAbs** Flow cytometry demonstrates that unlike murine IgM anti-PC(A), polyclonal anti-PC IgG does not block oxLDL-uptake by macrophages (B). Both IgG1 and IgG4 antibodies fail to display a inhibiting effect (C), excluding the potential enhanced Fc-receptor-regulated uptake of oxLDL.(D) including Fc-receptor blockade by anti-CD32 and anti-CD64 antibodies. Relative ranking of antibody affinity to PC (E) and oxLDL (F), measured through ELISA (expressed as OD). Flow cytometry of antibody affinity (G) for live cells (annexin V-PI-), early apoptotic cells (annexin V+PI-) and late apoptotic cells (annexin V+PI+), expressed as mean fluorescent intensity of each cell population. (H) CCL2 release assay of human monocytes stimulated with oxLDL alone or in combination with antibody clones, results of 2 individual experiments. One-way Anova.

Figure 4. **In vivo validation of PC-mAb antibodies** The anti-inflammatory properties of PC-mAbs were tested in vivo in the cuff model (3 days), with PC-mAb(T15) as positive control (n=7-10). Quantification of (A) %

leukocytes, (B) % macrophages. M99-B05 and X9-C01 were selected for further analysis. (C) Representative cross-sections of CCL2 staining, (D) quantification of $CCL2^+$ cells. M99-B05 and X9-C01 were tested in a 14 day cuff experiment (n=8-9) Quantification of neointima (E), intima/media ratio (F), % lumenstenosis (G). After codon optimization the PC-affinity of seven M99-B05 clones was tested (H). X19-A05 was selected for further testing in a 14 day cuff experiment. Representative cross-sections (I) of cuffed arteries of ApoE3*Leiden mice receiving human anti-streptavidin (control IgG), M99-B05 or X19-A05 (0.5, 2, 10mg/kg) (n=9-10). Quantification of neointima (J), intima/media ratio (K), % lumenstenosis (L). mean±SD, Mann-Whitney *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001.

Figure 5. **ATH3G10 prevents accelerated atherosclerosis** ATH3G10 was tested in a 14 day cuff experiment (n=10-12) in ApoE3*Leiden mice receiving vehicle (control), or ATH3G10 (0.1mg/kg or 1 mg/kg). **(**A) Representative cross-sections of elastin stained cuffed arteries after 14 days. (B) Quantification of neointima (C), intima/media ratio, (D) representative cross-sections of macrophage (MAC-3) staining. Quantification of (E) % macrophages in media and (F) % macrophages in neointima. mean±SD, Mann-Whitney *p<0.05, **p<0.01.

Figures/tables

Table 1. Overview of toxicity studies performed. For rats, additional groups of rats were treated equally, and used for toxicokinetics and immunogenicity studies.

species	duration	Doses (weekly, iv)	n/ group (m+f)	Recovery groups
SD rats	4 weeks	0; 3; 10 and 40 mg/kg bw	10 + 10	5 + 5
Cynomolgus	4 weeks	0; 3; 10 and 40 mg/kg bw	3 + 3	2 + 2
Cynomolgus	26 weeks	0; 3; 10 and 40 mg/kg bw	3 + 3	2 + 2

Figure 1

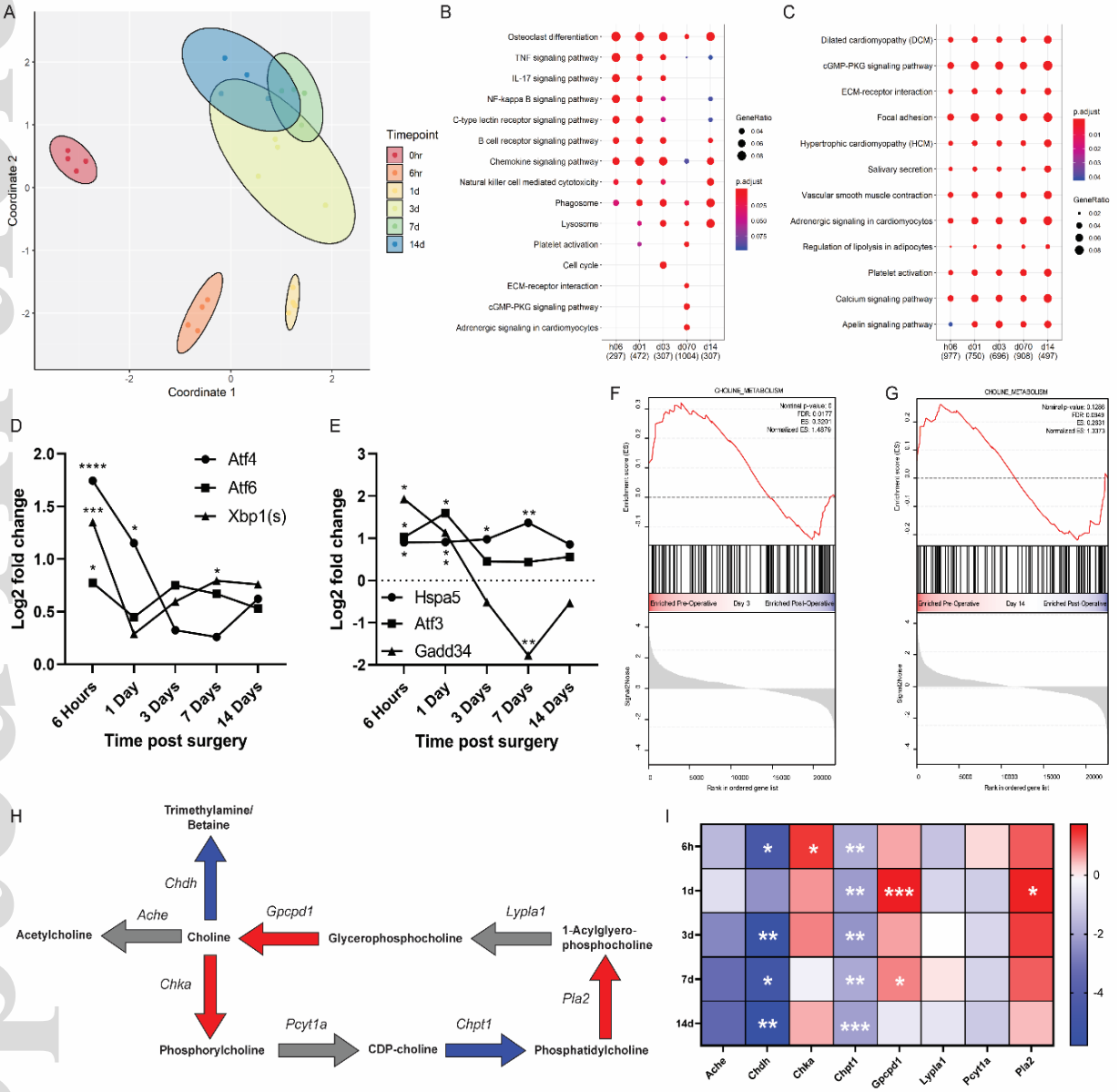
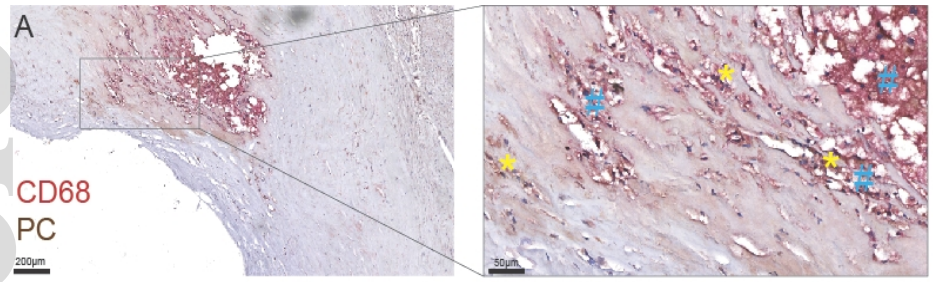
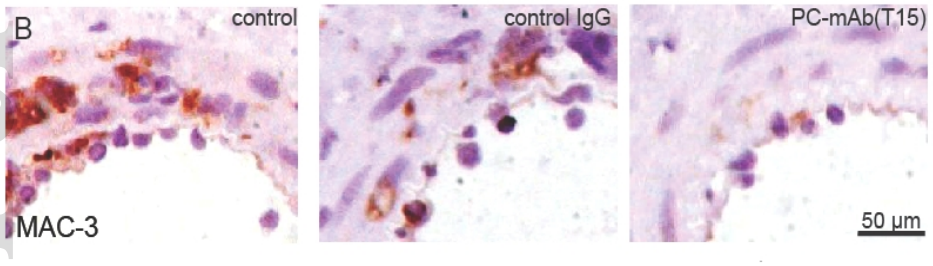
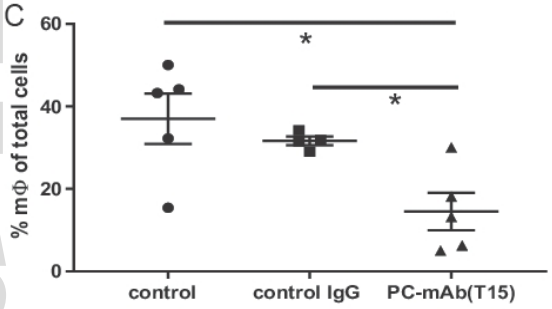
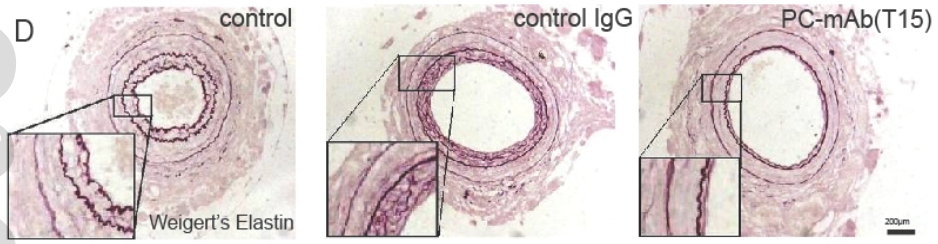


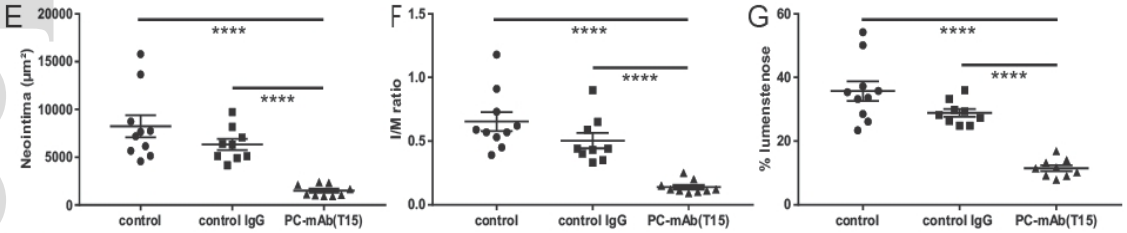
Figure 2











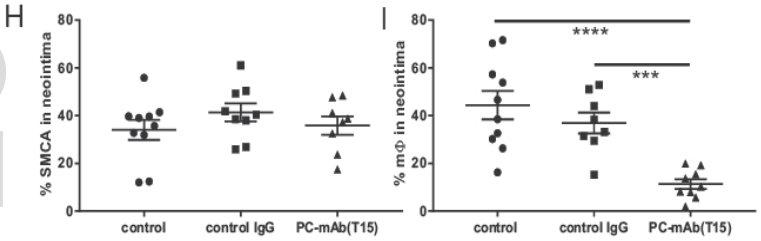


Figure 3

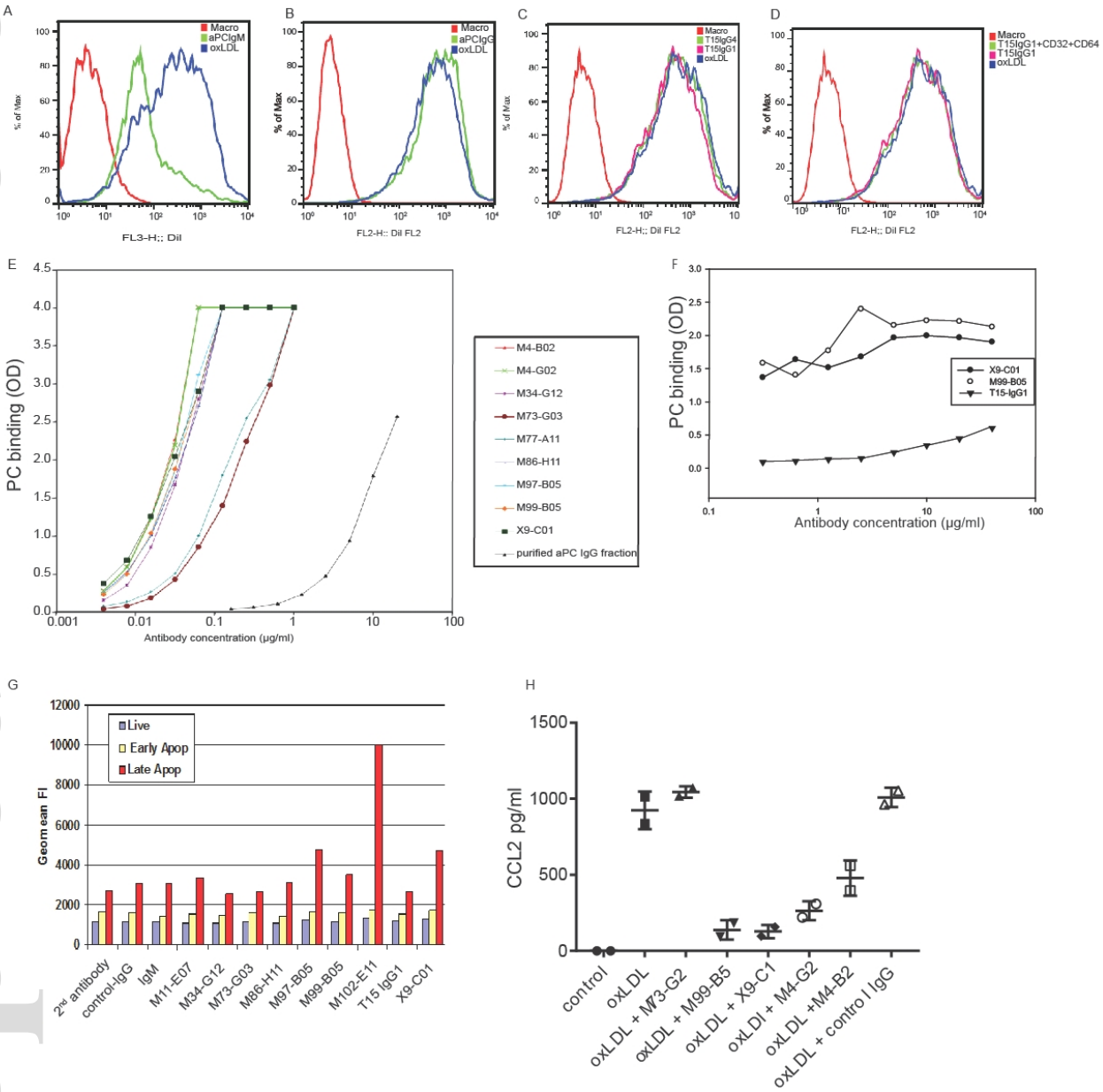


Figure 4

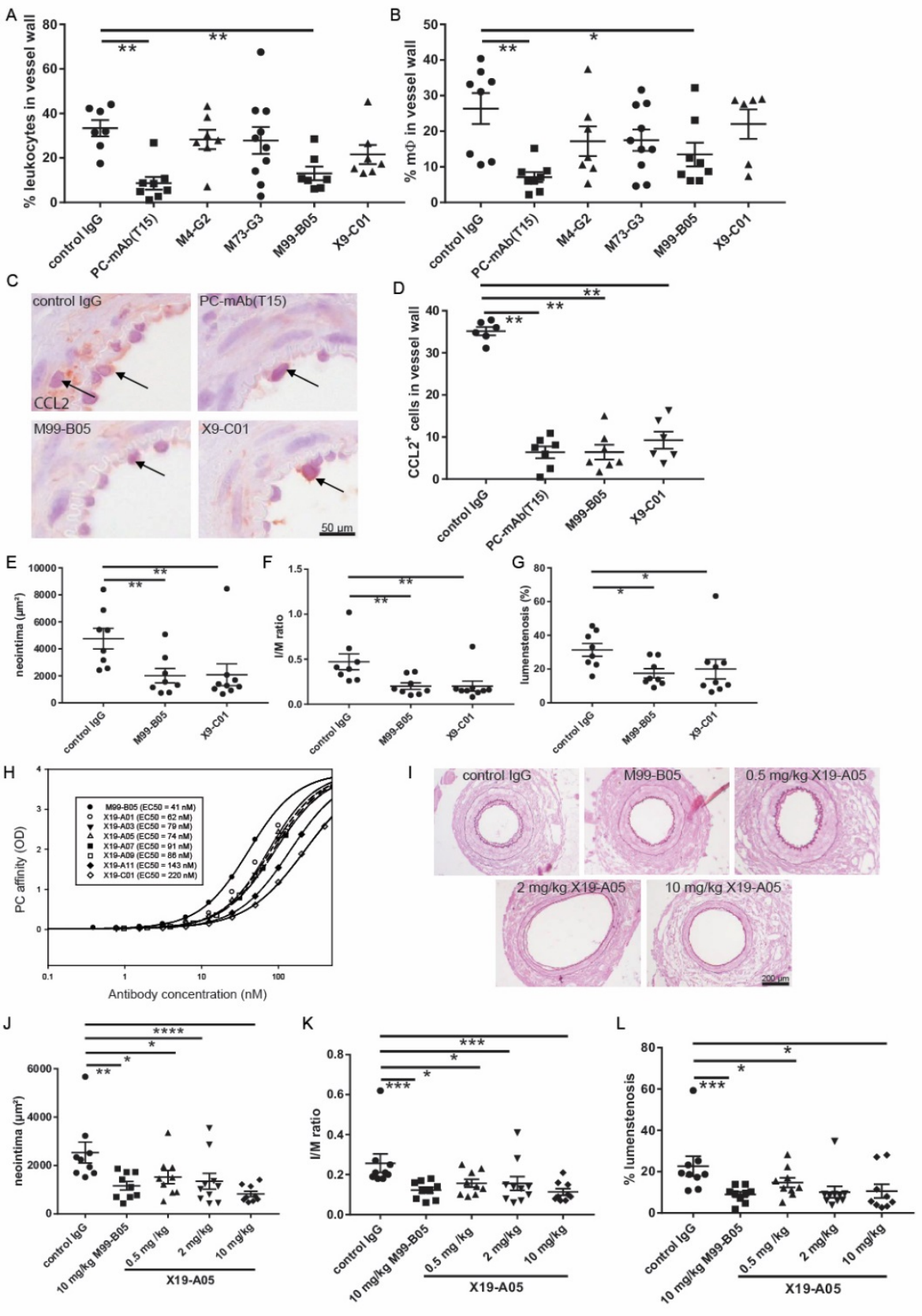


Figure 5