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Title page

A novel peptide agonist of FPRL1 (ALX) displays anti-inflammatory and cardioprotective effects

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Running title page

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Abbreviations:

GPCR: G-protein-coupled receptor; FPR: Formyl-peptide receptor; FPRL1: Formyl-peptide receptor-like1; PBMCs: peripheral blood mononuclear cells; I/R: ischemia-reperfusion; LXA4: lipoxin A_4; PMNs: polymorphonuclear neutrophils; CI: Cell Index; LCA: left coronary artery; AAR: Area at Risk; IS: Infarct Size; LV: left ventricular; AN: area of necrosis; HPF: high power field

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Abstract

Activation of the Formyl-peptide receptor-like1 (FPRL1) pathway has recently gained high recognition for its significance in therapy of inflammatory diseases. Agonism at FPRL1 affords a beneficial effect in animal models of acute inflammatory conditions, as well as in chronic inflammatory diseases. CGEN-855A is a novel 21 amino acid peptide agonist for FPRL1 and also activates FPRL2. CGEN-855A was discovered using a computational platform designed to predict novel GPCR peptide agonists cleaved from secreted proteins by convertase proteolysis. *In vivo*, CGEN-855A displays anti-inflammatory activity manifested as 50% inhibition of PMN recruitment to inflamed air pouch, and provides protection against ischemia-reperfusion mediated injury to the myocardium in both murine and rat models (36 and 25% reduction in infarct size, respectively). Both these activities are accompanied by inhibition of PMN recruitment to the injured organ. The secretion of inflammatory cytokines, including IL-6, IL-1β, and TNFα, was not affected upon incubation of human peripheral blood mononuclear cells (PBMCs) with CGEN-855A, while IL-8 secretion was elevated up to 2 fold upon treatment with highest CGEN-855A dose only. Collectively, these new data support a potential role for CGEN-855A in the treatment of reperfusion-mediated injury and in other acute and chronic inflammatory conditions.

Introduction

Uncontrolled inflammation is a major component in the etiology of many diseases and pathological conditions. Abundant evidence substantiates a critical role for neutrophils in the myocardial ischemia-reperfusion (I/R)- mediated injury (Vinten-Johansen, 2004). Neutrophils are recruited to the myocardial area at risk by pro-inflammatory signals during the very early phase of reperfusion. These activated neutrophils contribute to tissue damage by releasing proteolytic enzymes, cytokines and reactive oxygen species. In accordance with these findings, several experimental therapies targeting neutrophil activation and/or recruitment reduced myocardial I/R injury in animal models. Among these, agonists of Formyl-peptide receptor-like 1 (FPRL1) display cardioprotective effects in models of I/R, in part by negative regulation of PMN activity (Leonard et al., 2002; Gavins et al., 2003; Bannenberg et al., 2004; Gavins et al., 2005).

FPRL1, also known as ALXR (lipoxin A_4 receptor) or CCR12, belongs to the FPR (formyl-peptide receptor) related family of G-protein-coupled receptors (GPCRs) that also includes FPR and FPRL2. It is expressed primarily on neutrophils and monocytes, and is activated by a wide variety of endogenous and exogenous ligands, most of which are non-specific (Le et al., 2002; Chiang et al., 2006). The prominent endogenous FPRL1 ligands are derivates of lipoxin i.e., lipoxin A_4 (LXA4) and the aspirin-triggered lipoxins (ATLs) (Bannenberg et al., 2004), as well as the glucocorticoid-regulated protein annexin 1 and its N-terminal derived peptide, Ac2-26 (Perretti et al., 1993). These ligands display anti-inflammatory properties via the FPRL1 pathway in various experimental animal models of acute and chronic inflammation,

hence substantiating the therapeutic potential of FPRL1 agonists. Lipoxin- and annexin 1-related molecules reduced inflammation induced by zymosan A in the air pouch (Perretti et al., 2002) and peritonitis (Bannenberg et al., 2004) models, and afforded protection against I/R-related damage in various organs including heart, lung, kidney, bowel, cerebrum and mesentery (Cuzzocrea et al., 1999; La et al., 2001; Leonard et al., 2002; Gavins et al., 2003; Bannenberg et al., 2004; Gavins et al., 2005). In addition, these ligands are efficacious in models of asthma and pleurisy (Bandeira-Melo et al., 2000; Bandeira-Melo et al., 2005) while lipoxin derivates also ameliorated colitis induced by various agents including DSS, TNBS or aspirin (Fiorucci et al., 2002; Gewirtz et al., 2002; Fiorucci et al., 2004). The mechanism underlying the anti-inflammatory activity afforded upon FPRL1 activation by these ligands involves resolution of inflammation through differential-regulation of leukocyte activity and life span. Activation of FPRL1 leads to inhibition of PMN migration, hence preventing neutrophil-mediated tissue injury while promoting non–phlogistic monocytes emigration which is not accompanied by degranulation; thus allowing clearance of apoptotic cells by macrophage phagocytosis (Chiang et al., 2006).

The growing evidence supporting the anti-inflammatory and tissue-protective effects of FPRL1 ligands prompted us to search for novel ligands for this receptor. This was achieved using a computational biology discovery platform utilizing machine learning algorithms designed to predict novel GPCR peptide ligands cleaved from secreted proteins (extracted from the Swiss-Prot protein database) by convertase proteolysis. Therefore, the ligands identified might also exist endogenously due to naturally occurring proteolysis. The predicted ligands were synthesized and screened for

activation of 152 GPCRs by calcium flux and cAMP assays. After intense screening efforts, a novel peptide agonist of FPRL1 and FPRL2 was discovered and designated CGEN-855A. CGEN-855A has no significant homology to known GPCR ligands, and is highly specific to FPRL1 and FPRL2, out of the 152 GPCRs screened, that also included the other member of the family, FPR (Shemesh et al., 2008). Herein, we investigated the FPRL1-CGEN-855A interaction focusing on anti-inflammatory and cardioprotective activities.

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Methods

Peptide synthesis: Peptide CGEN-855A (TIPMFVPESTSKLQKFTSWFM-amide) was synthesized and purified in acetate salt by Sigma Aldrich, Israel.

Radioligand competition binding assay: The assay was performed by MDS Pharma Services (Cat # 226200). Briefly, purified membranes of CHO cells transiently transfected with FPRL1 were incubated at room temperature for 90 min, with 0.025 nM [^{125}I] WKYMVm (W peptide) in the absence or presence of increasing concentrations of either CGEN-855A or CKβ8-1 (a.a 46-137). Unbound tracer was washed, and bound label was counted using a TopCounts Microplate Scintillation and Luminescence Counter (PerkinElmer Life Sciences).

Stable transfection of FPRL1 in CHO cells: Human FPRL1 cDNA was amplified from a commercial cDNA clone in pcDNA3 (Forward primer: 5'CTAGCTAGCCACCATGGAAACCAACTTCTCC; Reverse primer: 5' CGACCGGTTCACATTGCCTGTAACTCAGTC), inserting a NheI cloning site and an AgeI site at the 5' and 3' end of the cDNA, respectively. The construct was verified by sequencing. CHO-K1 cells (300,000/well) were transfected using 6 μl of FuGENE (Roche Mannheim, Germany) and 2 μg of either FPRL1-encoding construct or mock vector. Two days later, the medium was changed to selection medium (F12, 10% FBS, 10μg/ml puromycin) for 2 weeks. Pools of stably transfected cells were selected by puromycin-resistance. Integration into the genome was verified by PCR using external

primers resulting from the vector. Expression was validated by FACS analysis using anti-FPRL1 antibodies (R&D Systems, Minneapolis, MN).

Cell Impedance Measurements: Real time electronic cell sensing was carried out using RCD96 E-plate device (ACEA Biosciences Inc., CA). E-plates (ACEA Biosciences Inc., San Diego, CA) were coated with 120µl of 1mg/mL gelatin (40min, 37ºC), washed, and 0.1ml F12-HAM nutrient mixture (Biological Industries, Beit Haemek, Israel) was added. After recording background levels, CHO-K1 cells stably transfected with the FPRL1 were seeded in 5% FCS- complete F-12 medium, at 2-3x10^4/well and incubated for 22-26 hr at 37ºC, 5% CO_2 in a humidified atmosphere. Cell Index (CI; arbitrary units defined as the cell-electrode impedance of cells containing well subtracted of the background impedance of the medium) was continuously recorded. At CI values ≥1, the medium was replaced with 120µl serum-free F12-HAM nutrient mixture and CI readings were allowed to stabilize for 5 min. The peptide (prepared in double distilled water + 0.1% BSA) was added at 5µl/well in triplicates and CI was measured in 71 s intervals. CI was normalized to T_0 (last recorded point prior to peptide addition) by integrated software. Presented are ΔCI values, reflecting impedance changes (Solly et al., 2004).

Calcium mobilization assay: CHO-K1 cells were transiently co-transfected with pcDNA3.1 constructs encoding $G_{\alpha 16}$ and either FPR1or FPRL1, using a lipid technique. Five hours later, the cells were re-plated into 96-well plates (60,000 cells\well), grown overnight, and loaded with Fluo4-NW (Invitrogen, Eugene, OR) according to the manufacturer's recommendations. Fluorescence was monitored by FlexStation™ plate

reader (Molecular Devices). Seventeen seconds following initiation of reading, cells were stimulated with the indicated agonist (prepared in PBS + 0.1% BSA) in triplicate.

Aequeorin assay: The assay was carried out by Euroscreen. CHO-K1 cells stably expressing FPRL2, Gα16 and mitochondrial apoaequorin were plated at 10^6cells/ml in assay medium (DMEM-F12 medium +0.1% BSA), and incubated with 5mM Coelenterazine H (Molecular Probes, Burlington, Canada) overnight at room temperature. Cells were then washed in assay medium, resuspended, and plated onto 96-well plate at 10^5cells/ml. The ligand was prepared in assay medium and added to the cells. Emission was recorded over 60 s using a FDSS™ reader (Hamamatsu, Japan).

Neutrophil infiltration into murine air pouch: Male out-bred Swiss albino mice (T.O. strain; Harlan UK, Oxon, England), weighing ~25g were used. Dorsal air pouches were raised by subcutaneous injection of 2.5 ml of sterile air 6 and 3 days before treatment. CGEN-855A and Ac2-26 (Perretti et al., 1993), were dissolved in sterile pyrogen free PBS (Gibco, Grand Island, NY) and administered intravenously at 200µl (n=8), followed immediately by an intra pouch challenge with 1 mg zymosan A (Sigma - Aldrich, Steinheim, Germany). Alternatively, CGEN-855A or vehicle were administered into the pouch (in situ) in the absence of zymosan A challenge. Four hours later, lavage fluids were washed with 2 ml of ice cold PBS containing 3 mM EDTA and kept on ice. An aliquot of the lavage fluid was stained for neutrophils with PE-conjugated anti-Gr-1 monoclonal antibody (BD Biosciences Pharmingen, San Jose, Calif) or isotype control (rat IgG2b) and analyzed using FACScan analyser (Becton Dickinson, Cowley, UK).

Myocardial I/R model in mice: Male Albino mice (Harlan UK, Oxon, England) weighing ~30g were anesthetized and left coronary artery (LCA) ligation was performed using a 7/0 silk suture (Ethicon, W593 7/0 BVl, Edinburgh, UK). After 25 min of myocardial ischemia, the LCA was re-opened to allow reperfusion. Mice (n=6, each group) were treated with CGEN-855A or with vehicle (PBS) at 200 µl per mouse. i.v. immediately after reperfusion. To assess the Area at Risk (AAR), the LCA was re-occluded two hours after reperfusion, and Evans blue dye (1 ml of 2% wv^{-1}) was injected i.v. The heart was cut into four to five horizontal slices. After removing the right ventricular wall, the AAR (unstained) and non-ischaemic (blue) myocardium were separated and weighed. The AAR is expressed as percent of the total left ventricular (LV) weight. The Infarct Size (IS) was assessed by cutting the AAR into small pieces, and incubating them with *p*-nitro-blue tetrazolium (NBT, 0.5 $mgml^{-1}$, 20 min at 37°C), and calculated as a percentage of necrotic tissue relative to the AAR mass.

Plasma Troponin I concentration: Plasma was collected at the end of the reperfusion by centrifugation of whole blood at 4°C 3000 rpm for 10 minutes. Plasma troponin I was quantified in duplicate by ELISA (Bio-Quant inc., San Diego, CA), according to manufacturer's instructions.

Myocardial I/R model in rats: Male Sprague-Dawley rats weighing 370-380 g were used. The LCA was occluded with a 6-0 proline (Ethicon, NJ) ligature for 30 min and reperfused for 3 h. The rats (n=9 or 5 for different experiments, as indicated) were

treated intravenously with CGEN-855A or vehicle (saline) at 1 ml/kg, administered 5 minutes before reperfusion or postconditioning. Postconditioning was applied using an algorithm of 10 seconds reperfusion interrupted by 10 seconds of reocclusion repeated for three cycles prior to full reperfusion (Kin et al., 2005). The LCA was reoccluded and the AAR was delineated by injecting 1.5 ml of 20% Unisperse blue dye via the external jugular vein. The heart was excised and placed into 0.9% saline. The LV was separated from the remaining cardiac tissue and thinly (2 mm) cross-sectioned before separating the AAR (unstained) from the blue stained non-ischemic zone. The AAR was incubated for 10 min in a 1% solution of phosphate-buffered 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C, enabling assessment of the area of necrosis (AN). The infarct size (IS) was calculated as a percentage of the AAR (AN/AAR).

Detection of PMN by immunohistochemistry: After determination of AAR, the left ventricular tissue samples from non-ischemic and ischemic zones were divided in half transmurally, fixed in 4% paraformaldehyde 1 hour and transferred to 15% sucrose overnight. The samples were embedded in optimal cutting temperature compound (O.C.T., Sakura Finetek), and frozen in liquid nitrogen. Tissue samples (7 μm thick) were cut using a Hacker-Bright cryostat and mounted onto coated Vectabond (Vector Laboratories, Burlingame, CA) slides, refrozen and stored at -70°C. The cryostat sections were incubated with monoclonal anti-rat CD18 antibody (BD Pharmingen, San Jose, Calif), washed in PBS and incubated with a biotinylated horse anti-mouse IgG (Vector Laboratories), stained using ABC-peroxidase (Vector Laboratories) and substrated with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO). A non-immune IgG

was used as a control. PMN accumulation is expressed as the number of $CD18^+$ cells/mm^2.

PBMCs preparation: Citrated blood was obtained from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over equal volume of Histopaque 1077 (Sigma, St. Louis, MO) at 800g, for 15 min, at 24ºC. PBMCs were collected from the interface and washed with modified HBSS (10min, 250g, 24ºC) before resuspension in RPMI 1640 supplemented with 10% FCS.

Cytokine assays: Freshly prepared PBMCs were suspended at 10^6 cells/ml in RPMI1640 supplemented with 10% FCS, treated in duplicates with the indicated concentration of CGEN-855A or IL-1β for 24 hr and incubated at 37ºC, 5% CO_2, in a humidified atmosphere. After 24 hours, samples were centrifuged, and supernatants were collected and kept at -80ºC until analyzed. The content of IL-6, IL-8, IL-1β and TNF-α in the supernatants was analyzed, in duplicate, using ELISA (Biosource, Camarillo, CA.).

Statistics: All data are expressed as means ± standard error of the mean (SEM). All data were analyzed using SigmaStat 3.5 for Windows statistical software package (SPSS, Chicago, IL). A one-way analysis of variance (ANOVA) (infarct size, area at risk) was used, with post-hoc analysis between groups using the Student–Newman–Keuls test correcting for multiple comparisons. Infarct size was analyzed for all groups together. A *p*-value of less than 0.05 is considered significant.

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Results

CGEN-855A competes with W peptide for binding to FPRL1

CGEN-855A was tested for its ability to compete with [^{125}I] WKYMVm (W peptide), a high-affinity ligand of FPRL1 (Christophe et al., 2001), on its binding to membrane preparations from FPRL1 transiently transfected cells. CKβ8-1 (aa 46-137), was used as a positive control (Elagoz et al., 2004). The value of 0% inhibition (i.e. 100% binding of radioligand) was determined in the absence of either inhibitory peptide. CGEN-855A displaced the radiolabeled-W peptide in a saturable manner with an IC_{50} of 189nM and a K_i of 54.1nM (Figure 1).

CGEN-855A activates FPRL1 and FPRL2 in a dose-dependent manner

A high and uniform expression of FPRL1 was detected in CHO cells that were stably transfected with FPRL1 but not in mock transfected cells (Figure 2a). Activation of these FPRL1-transfected cells with CGEN-855A resulted in an elevation of cell impedance index in a dose-dependent manner, with an EC_{50} of 381nM (Figure 2b). This activation was not observed after challenging mock transfected cells with CGEN-855A.

CGEN-855A elicited a cellular response in cells expressing either FPRL1 or FPRL2 but not in FPR expressing cells (Figure 2c and 2d). fMLP and W peptide were included as positive controls for FPRL1 and FPR, respectively. Furthermore, CGEN-855A did not induce calcium flux in any of the other 149 GPCRs that were tested in the original screen leading to its identification, although these receptors responded to relevant positive controls (Shemesh et al, 2008).

CGEN-855A exhibits anti-inflammatory activity in a model of acute inflammation

An intra-pouch challenge with zymosan A triggered a marked accumulation of neutrophils in the air pouch, as determined by FACS analysis of Gr-1$^+$ cells (Figure 3). Administration of CGEN-855A at 50 and 200μg/mouse (corresponding to 2 and 8mg/kg), reduced the accumulation of neutrophils triggered by zymosan A by 48.8% and 23.3%, respectively (Figure 3a). Statistical significance was achieved only for the group treated with 50μg/mouse but not with 200μg/mouse. Altogether, the extent of inhibition achieved after treating the mice with 50μg/mouse CGEN-855A is comparable to that obtained by administration of Ac2-26 at 200μg/mouse.

To validate that CGEN-855A does not elicit pro-inflammatory activity, we also tested its direct effect upon administration into the air pouch in the absence of zymosan A. As shown in Figure 3b, intra-pouch administration of 100μg CGEN-855A did not induce neutrophil recruitment into the air pouch when used alone.

CGEN-855A displays cardioprotection in animal models of I/R-induced myocardial infarction

The inhibitory activity on neutrophil migration demonstrated by CGEN-855A in the air pouch model, prompted us to study its effect on I/R-induced myocardial injury. When administered i.v. at 30 or 60 μg/mouse (corresponding to 1 or 2mg/kg, respectively) immediately prior to reperfusion, CGEN-855A afforded significant and dose-dependent cardioprotection, as illustrated by the reduction in infarct size (36% reduction at the highest dose, Figure 4a). As expected, the AAR was similar in all groups, with AAR/LV values ranging between 50-52% (data not shown). In addition, plasma

levels of troponin I, an established marker of myocardial damage, were also reduced in a dose-dependent manner (50% reduction at the highest dose, Figure 4b), with a pattern mirroring that observed for reduction of infarct size.

In addition, a rat model of I/R was used in order to compare the cardioprotective effect of CGEN-855A to that of postconditioning- a mechanical maneuver defined as a series of brief (i.e. seconds) interruptions of reperfusion following a specific prescribed algorithm, applied at the very onset of reperfusion, that was shown to trigger cardioprotective responses to reperfusion injury in animal models and in clinical studies (Vinten-Johansen et al., 2007). Administration of CGEN-855A at 2 mg/kg reduced infarct size to a similar extent as postconditioning (Figure 5a; 43.6±2.9 and 41.2±2.7%, respectively, compared with 57.0±2.3% in the control group). Interestingly, the combination of CGEN-855A with postconditioning did not further reduce infarct size (44.6% ± 1.3%).

Finally, PMN accumulation in the AAR was analyzed to confirm that the cardioprotective activity provided by CGEN-855A is due to inhibition of PMN recruitment. CGEN-855A significantly attenuated PMN accumulation to the AAR compared to vehicle (30.1 ± 0.6 *vs.* 43.2 ± 0.7 PMNs/HPF) (Figure 5 b, c). This attenuation was comparable to that achieved by postconditioning (34.8±1.5).

CGEN-855A does not affect cytokine secretion by human PBMCs

The human and murine families of FPRs are diverse, and might be differently affected by certain compounds. This is of special importance due to the apparent inconsistency in the effects mediated by FPRL1 agonists. Thus, we studied the effect of

CGEN-855A on the secretion of inflammatory cytokines by human cells. Incubation of PBMCs with CGEN-855A at 0.25, 2.5 or 25 μg/ml (corresponding to 0.1, 1 and 10 μM) did not affect secretion of IL-6, IL-1β or TNFα (Figure 6 a-c). A moderate elevation in IL-8 levels (up to 2 fold) was observed upon cells' treatment with the highest dose CGEN-855A (Figure 6d). IL-1β (100 ng/ml), which was used as positive control, induced high levels of cytokine secretion.

Discussion

FPRL1 is a promiscuous receptor, activated in vitro by a variety of ligands which greatly vary in their biological features including origin, nature, size and specificity (Le et al., 2002). The biological activities induced by interaction of an individual ligand with FPRL1 are inconsistent- while some induce pro-inflammatory responses, others, namely annexin 1 and Ac2-26 as well as LXA4, promote resolution of inflammation; the latter findings have underpinned current clinical programs aiming at discovering novel FPRL1 agonists for treatment of acute and chronic inflammatory conditions. We demonstrated here that CGEN-855A activates FPRL1 and display important anti-inflammatory properties by reducing PMNs recruitment to inflamed sites. CGEN-855A produced a similar efficacy to that of Ac2-26 in the air pouch model but a lower dose was required to achieve similar degrees of inhibition (50 vs. 200 μg/mouse corresponding to 20 and 80 nmol, respectively). The smaller reduction in PMN accumulation obtained with administration of higher doses of CGEN-855A might result from receptor desensitization, and might indicate that even lower doses would be sufficient to exert important checkpoint functions on the experimental inflammatory response. Nevertheless, these results implied on the effective therapeutic range of CGEN-855A, and when administered at lower doses in the disease-related model, the I/R-induced myocardial infarction (Fig. 4; 10, 30 and 60 μg/mouse), a clear dose-dependent effect was observed both in infarct size and troponin levels in the plasma.

Due to the perplexing effect mediated via FPRL1, the possibility that CGEN-885A might elicit pro-inflammatory responses was eliminated as PMNs were not recruited to the air pouch upon intra-pouch administration of CGEN-855A alone.

Furthermore, although a moderate elevation in IL-8 secretion was observed upon treating human cells with the highest tested concentration of CGEN-855A, it did not induce any prominent elevation in secretion of the other pro-inflammatory cytokines tested, supporting a lack of pro-inflammatory, or activating downstream effects, upon human FPRL1 agonism by this compound. In addition, the biological implications of FPRL2 activation by CGEN-855A are difficult to foresee since the biological role of FPRL2 is unknown and an endogenous agonist for FPRL2 was only recently reported (Gao et al., 2007).

Although controversial, previous findings substantiate a role for PMNs and specifically neutrophils in the early stage of reperfusion-injury (reviewed by Vinten-Johansen, 2004) and several studies describe experimental interventions aimed at inhibiting PMN recruitment at the time of reperfusion or shortly before. PMN inhibition was achieved by leukodepletion using neutrophil anti-serum (Kin et al., 2006), or by antibodies targeting adhesion molecules such as P- and E- selectins (Lefer et al., 1994), CD11/CD18 (Ma et al., 1991), ICAM-1 (Ma et al., 1992; Ioculano et al., 1994; Zhao et al., 1997; Zhao et al., 2003) or PECAM-1 (Gumina et al., 1996). These strategies provided up to ~50% reduction in infarct size. However, none of these anti-PMN approaches have been shown to consistently be effective in the clinic (Vinten-Johansen, 2004; Frangogiannis, 2006; Yellon and Hausenloy, 2007). On the other hand, postconditioning has shown significant protection when applied to patients (Tissier et al., 2007; Thibault et al., 2008).

When tested in mouse and rat models of I/R, CGEN-855A afforded a significant cardioprotective effect manifested by reduced infarct size (by 36% and 25% in the mouse

and rat model, respectively), that was further confirmed by reduced levels of troponin I in plasma (shown in mice). In accordance with the inhibitory effect of CGEN-855A on PMN recruitment to the inflamed site attained by the air pouch model, the cardioprotective activity of CGEN-855A in rats was accompanied by reduced recruitment of PMN to the AAR. The extent of cardioprotection obtained by CGEN-855A treatment is comparable to that observed by other groups using FPRL1 agonists in similar models (Gavins et al., 2005) and comparable to that observed with postconditioning. Of note, no additive protection was elicited upon combination of the two treatments. It is possible that both FPRL1 and postconditioning exert their effect through similar pathways, i.e. PMN-mediated injury. Indeed, studies report that postconditioning is associated with a reduction in PMN accumulation, adherence to coronary vascular endothelium, endothelial dysfunction, and cytokines relevant to the PMN recruitment process (Zhao et al., 2003; Halkos et al., 2004). Overall, these data might suggest that the apparently partial cardioprotective effect observed in these models of I/R is the maximal effect that can be achieved via inhibition of PMNs recruitment and that processes mediated by other cells, such as T cells (Varda-Bloom et al., 2000; Spagnoli et al., 2002; Lim et al., 2003) and endothelial cells (Melo et al., 2004), also play a role in the myocardial damage resulting from I/R.

Collectively, these data provide strong evidence that activation of the FPRL1 pathway could be beneficial for the treatment of acute and chronic inflammation. The results presented herein support further development of CGEN-855A as a potential candidate for therapeutic control of inflammatory diseases, in general, and for the treatment of reperfusion-related cardiovascular damage, in particular.

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Footnotes

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Legends for Figures

Figure 1. CGEN-855A binds to FPRL1. Membranes from PFRL1 transfected CHO cells were incubated with [^{125}I] WKYMVm in the absence or presence of increasing concentrations of either CGEN-855A (●) or CKβ8-1 (■). Results are presented as mean ± SD of duplicates.

Figure 2. CGEN-855A specifically activates FPRL1 in a dose dependent manner. A, CHO stably transfected with either FPRL1 (thick line) or mock vector (thin line) were stained with PE- conjugated anti-human FPRL1 Ab or with IgG2b isotype control Ab (dashed line) and surface expression of FPRL1 was analyzed by FACScan (Becton Dickinson). **B,** Stable pools of FPRL1 were seeded on E-plates and stimulated with CGEN-855A at 25, 10, 3.3, 1.1, 0.37, 0.12 μM. Mock transfected cells were stimulated with 25 and 10 μM CGEN-855A. Cell impedance was recorded continuously in intervals of 71 s and presented as normalized CI. Insert presents normalized CI of FPRL1 (black bars) and mock (white bars) trasfected cells as mean ± SD of triplicates at one time point (12.5 min). **C,** CHO-K1 cells transiently transfected with either FPRL1 or FPR1 and Gα16 were loaded with Fluo4-NW. Calcium flux response was measured using FlexStation^{TM} (Molecular Devices), upon cells stimulation with CGEN-855A at 1μM. W peptide and fMLP (1μM each) were included as positive controls for FPR and FPRL1, respectively. Assay was conducted in triplicates, mean ± SD is presented. **D**, CHO cells stably expressing FPRL2, Gα16 and mitochondrial apoaequorin were incubated with Coelenterazine H and activated with CGEN-855A at 0.3 1 3 10 30 100

300 1000 3000 10000 nM. Results are expressed as percentage of activation compared to the reference agonist.

Figure 3. CGEN-855A inhibits PMNs migration into mouse air-pouch inflamed with Zymosan A. **A,** Zymosan A (1mg) was injected intra-pouch immediately following i.v. treatment with either CGEN-855A, Ac2-26 or vehicle as indicated. Lavage fluid was collected after 4 hr, stained with anti-Gr-1 antibody and analyzed by FACScan (Becton Dickinson). Irrelevant rat IgG2b antibody was used as isotype control. Shown is the number of Gr-1^+ cells recovered in the lavage fluids (mean ± SEM of n=8). *P<0.05 *vs*. vehicle group. **B,** CGEN-855A (0.1mg), Zymosan A (1mg) or vehicle were injected intra-pouch. Lavage fluids were collected and analyzed as described in panel A.

Figure 4. CGEN-855A reduces I/R- mediated myocardial injury in mice. Mice were subjected to 25 min ischaemia followed by 120 min reperfusion, by LCA occlusion. Vehicle (PBS) or CGEN-855A were administered at indicated doses immediately after reperfusion. **A,** Myocardial infarct was determined as described under materials and methods and expressed as percentage of AAR. Data presented as mean ± SEM of $n = 6$. *$P < 0.05$ and ** *$P<0.01$ vs.* vehicle group. **B,** Plasma samples were tested for troponin I using ELISA. Values were extrapolated from a calibration curve and presented as mean ± SD of duplicates. **P<0.05* and ** *P<0.01 vs.* vehicle group.

Figure 5. CGEN-855A reduces I/R- mediated myocardial injury and PMN's recruitment in rats. Rats were subjected to 30 min ischaemia followed by 180 min

reperfusion, by LADCA occlusion. Vehicle (Saline) or CGEN-855A were administered 5 minutes before reperfusion while postconditioning was applied immediately prior to terminal reperfusion. **A,** Myocardial infarct was determined as described under materials and methods and expressed as percentage of AAR. Data presented as mean ± SEM of n = 9. *$P < 0.05$ *vs.* vehicle group. **B,** PMN'S accumulation in the AAR tissue presented as mean ± SEM of n=5 **P<0.05 vs.* vehicle group. **C,** Representative sections of AAR stained for PMN'S accumulation by immunohistochemistry using anti-CD11 and anti-CD18 antibodies is presented for each study group as indicated. Magnification is x200.

Figure 6. CGEN-855A does not affect cytokine secretion by PBMCs. PBMCs were incubated for 24hr with CGEN-855A at 0.25, 2.5 and 25μg/ml (corresponding to 0.1, 1 and 10μM). The levels of IL -6 (A), IL-1β (B), TNF-α (C), and IL-8 (D) in the supernatants were evaluated by ELISA. Presented are means ± SEM of duplicate ELISA from duplicate assay samples of two donors. **P<0.05* and ** *P<0.01 vs.* untreated cells.

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Fig. 1

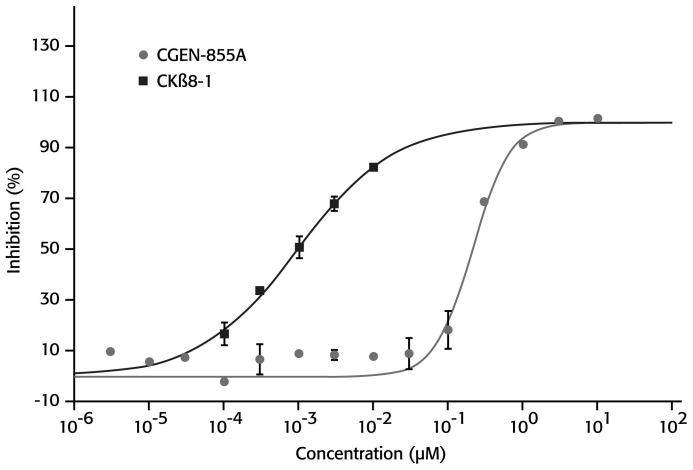

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Fig. 2

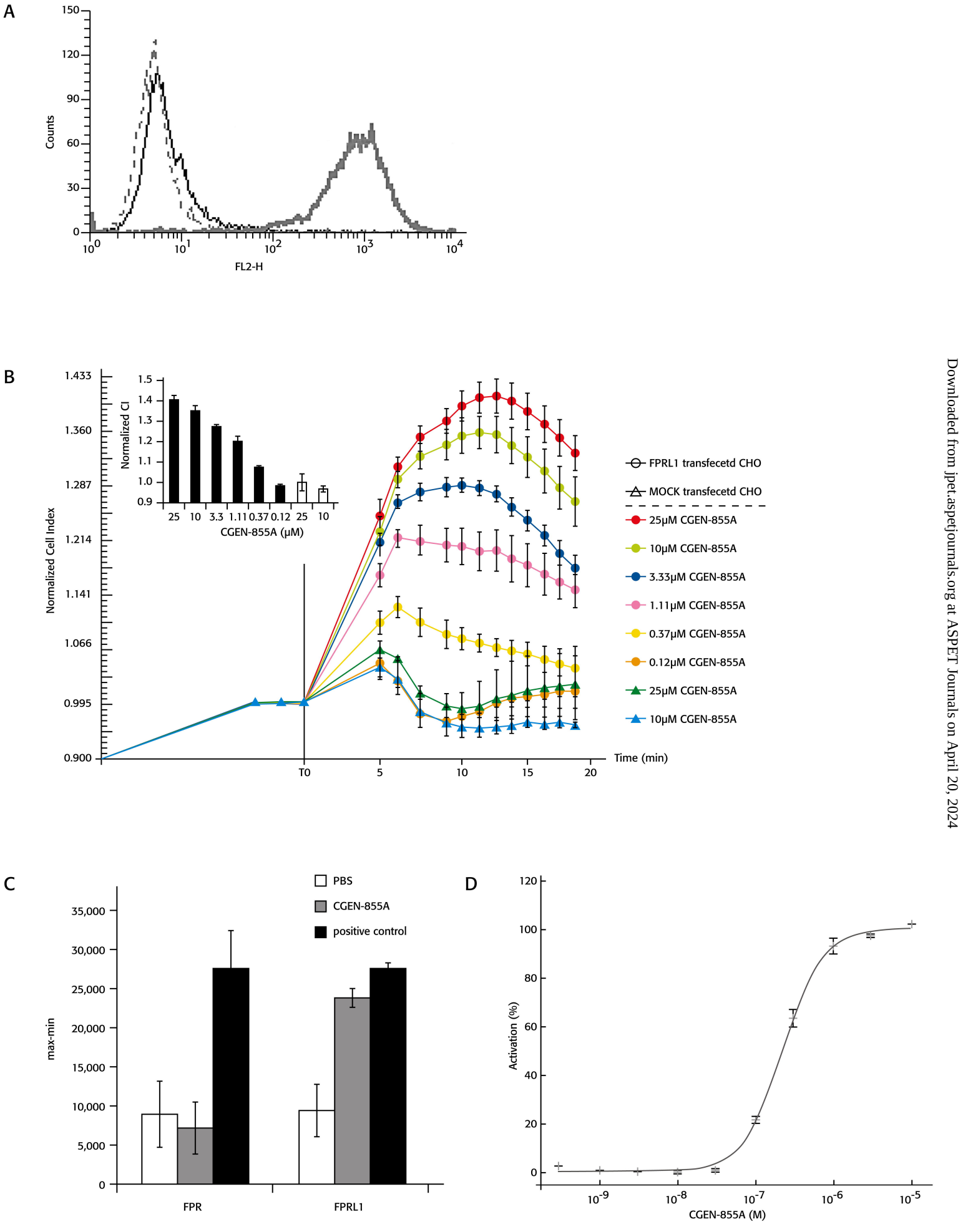

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Fig. 3

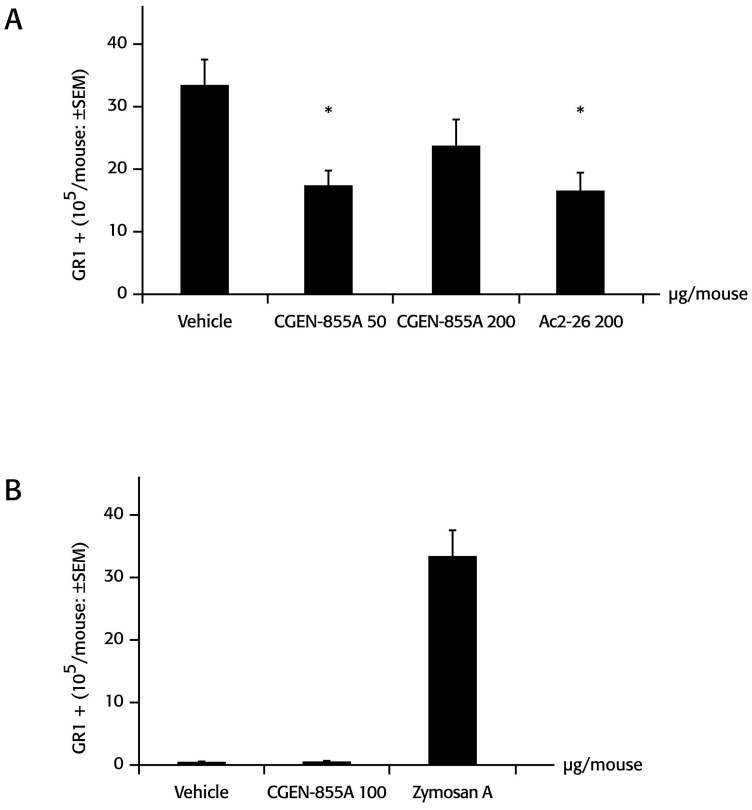

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Fig. 4

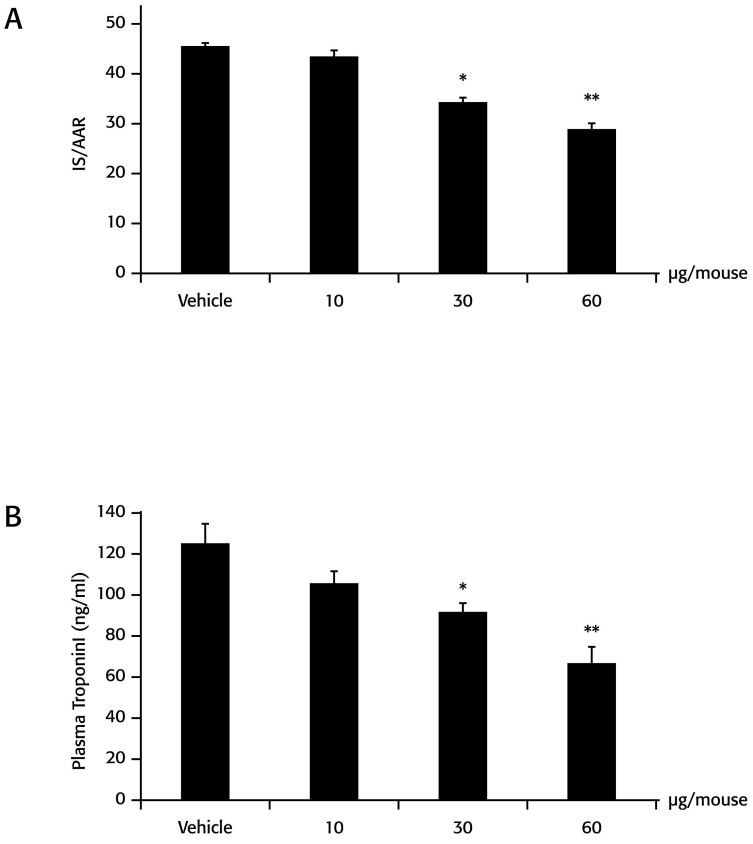

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Fig. 5

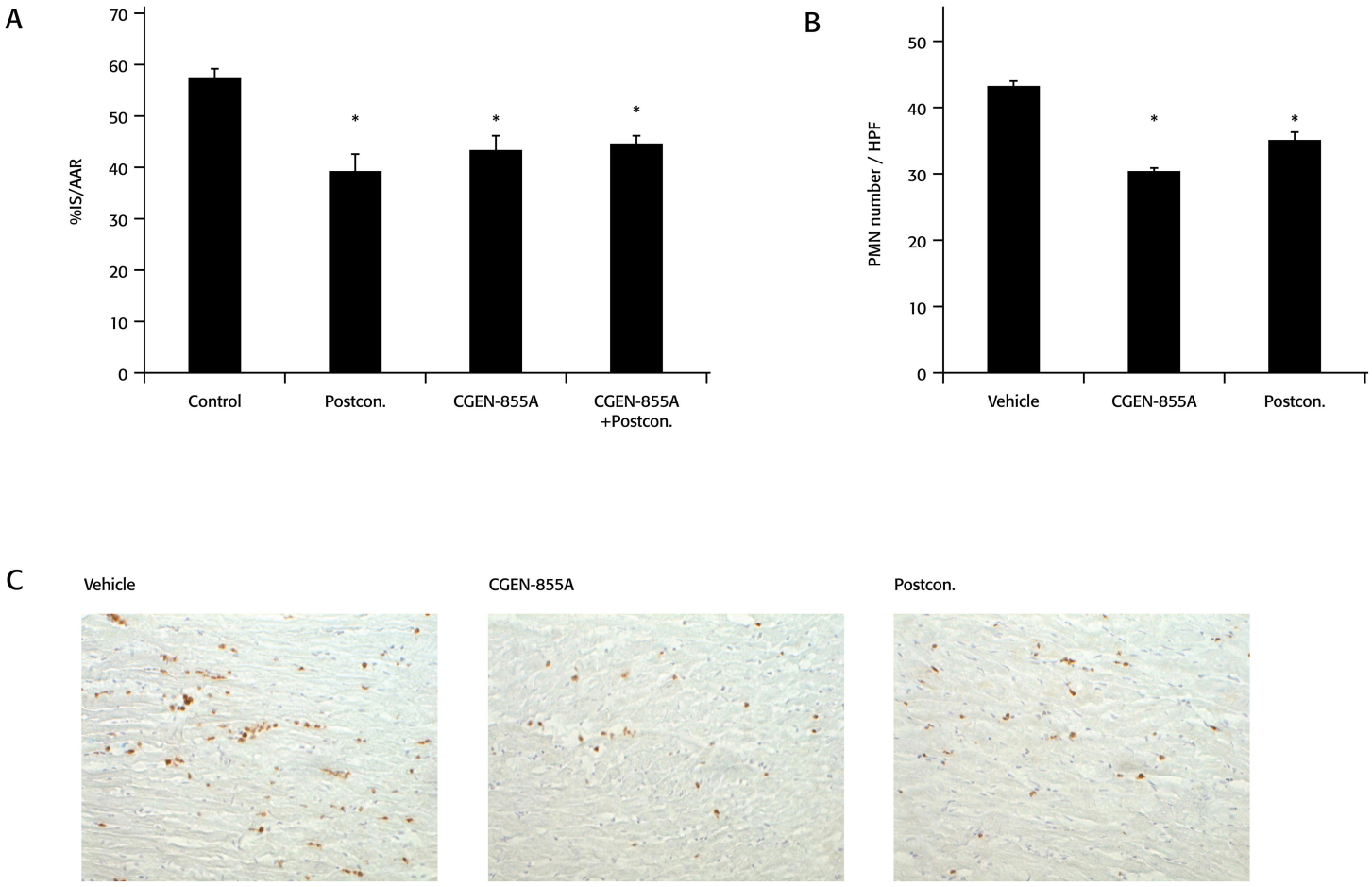

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Fig. 6