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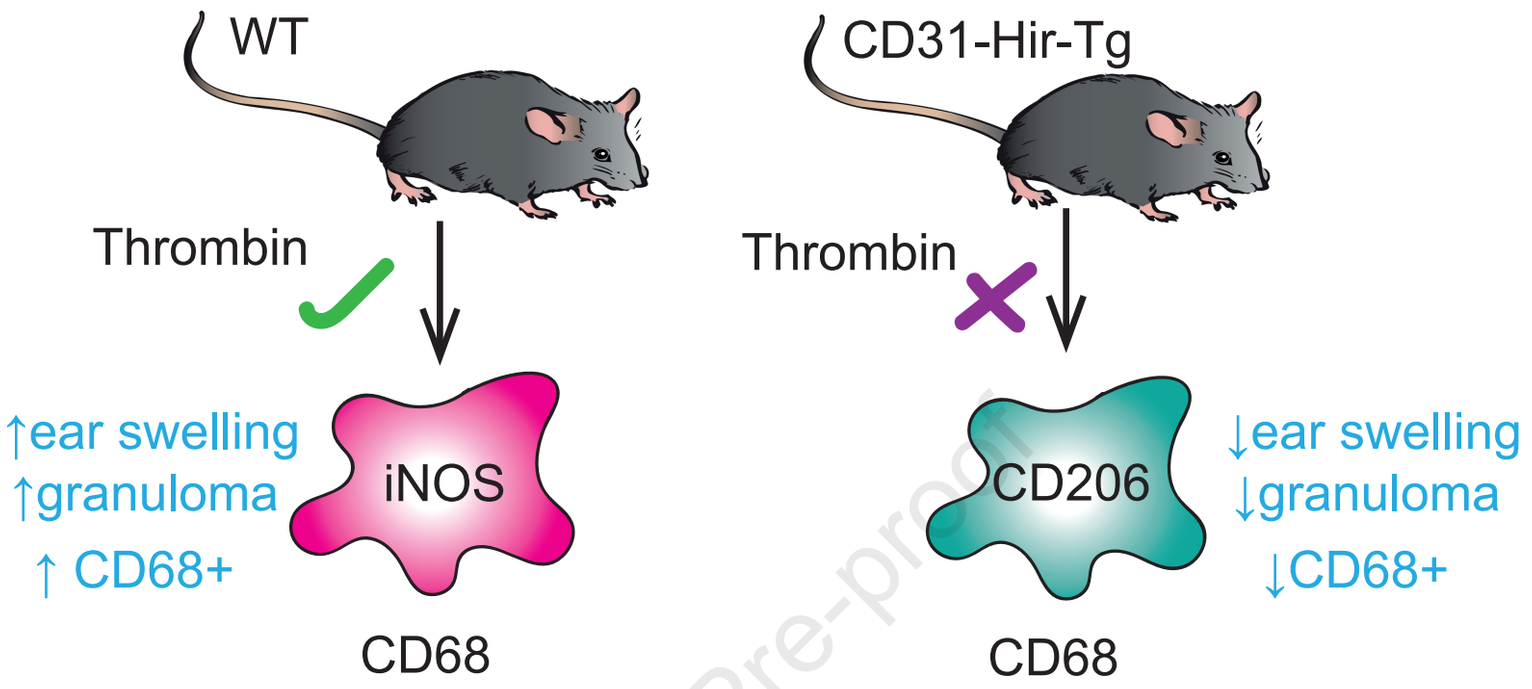
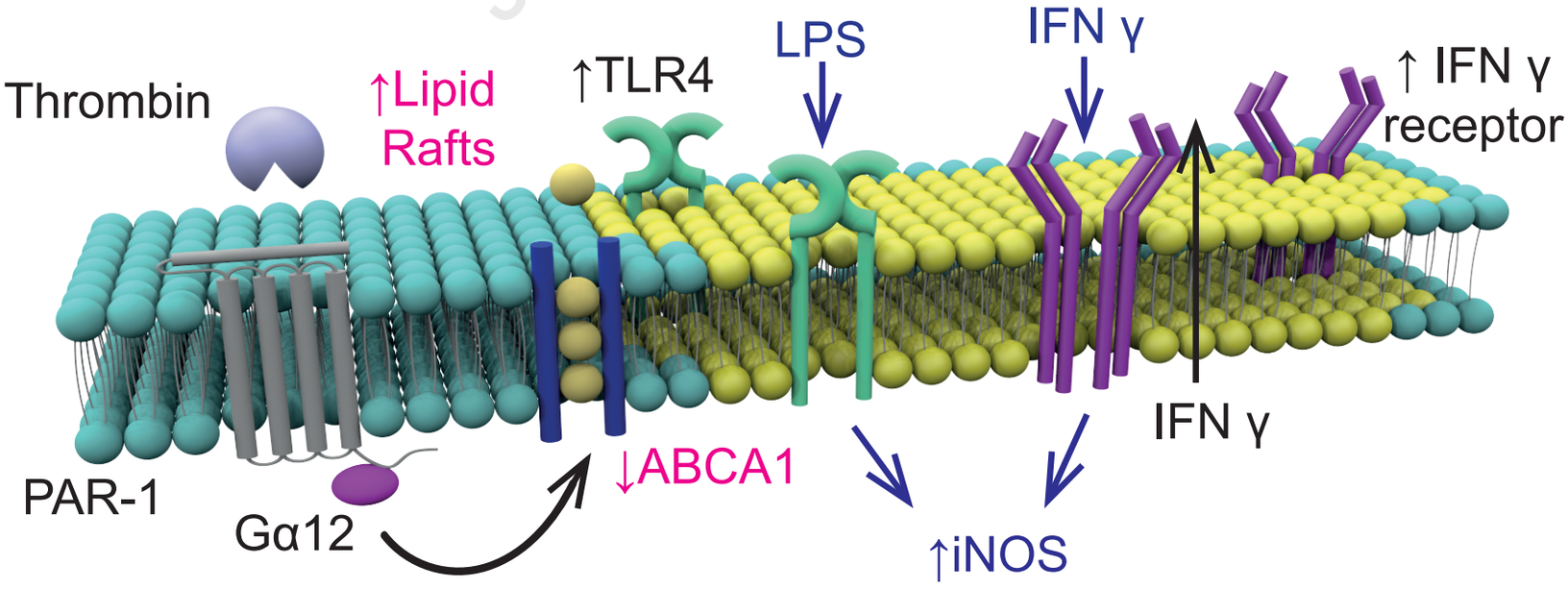
Oxazolone induced DTH



Thrombin primed macrophages



PAR-1 signalling on macrophages is required for effective in vivo delayed type hypersensitivity responses.

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Short Title: Thrombin primes exquisite sensitivity of macrophage to IFNγ

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Summary

Delayed type hypersensitivity (DTH) responses underpin chronic inflammation. Using a model of oxazolone-induced dermatitis and a combination of transgenic mice, adoptive cell transfer, and selective agonists/antagonists against protease activated receptors (PAR), we show that that PAR-1 signalling on macrophages by thrombin is required for effective granuloma formation. Using BM-derived macrophages (BMM) in vitro, we show that thrombin signalling induced; a) downregulation of cell membrane reverse cholesterol transporter ABCA1; b) increased expression of IFNγ receptor and enhanced co-localisation within increased areas of cholesterol-rich membrane microdomains. These two key phenotypic changes combined to make thrombin-primed BMM sensitive to M1 polarisation by 1000-fold less IFNγ, compared to resting BMM. We confirm that changes in ABCA1 expression were directly responsible for the exquisite sensitivity to IFNv in vitro and for the impact on granuloma formation in vivo. These data indicate that PAR-1 signalling plays a hitherto unrecognised and critical role in DTH responses.

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Abbreviations

ABCA1 ATP-binding cassette transporter
APC Antigen presenting cells
AMR Antibody mediated rejection
ApoE Apolipoprotein E
BM Bone marrow
BMM BM-derived macrophages
CTB Cholera Toxin B
DC Dendritic cells
DTH Delayed type hypersensitivity
EC Endothelial cell
ePCR Endothelial protein C receptor
ES Ear swelling
IFNγ Interferon γ
LPS Lipopolysaccharides
MALT Mucosa-associated lymphoid tissue
MCSF Macrophage colony-stimulating factor
MMP Matrix metalloproteinase
NK Natural killer
PAR Protease-activated receptor
TF Tissue factor
Tg Transgenic
TLR Toll-like receptor
TNF-β Tumour necrosis factor beta
WT Wild type

Introduction

Macrophages are heterogeneous and versatile cells found in virtually all tissues of adult mammals. Activation of macrophages has emerged as a key area of immunology, tissue homeostasis, disease pathogenesis, and resolving and non-resolving inflammation. Early literature described them dichotomously as M1 or M2 macrophages (Mills, 2015), with M1 macrophages being the classical inflammatory macrophages induced by T cell dependent (Interferon γ (IFNγ)) and T cell independent (lipopolysaccharides (LPS)) pathways. These promote upregulation of Th1 pro inflammatory chemokines and cytokines such as IL-6, IL-12 and IL-23. They upregulate HLA-DR thus having a role in antigen presentation and induce nitric oxide production. In contrast to M1 macrophages, M2 macrophages are anti- inflammatory having roles in tissue homeostasis and repair and have roles in the Th2 response. M2 macrophages are classically induced by IL-4 or IL-13. As time has progressed these two are recognised as extreme phenotypes, with subtypes described in vivo appearing more plastic and often expressing characteristics of both. Subsets with a predominant M2 phenotype (M2a-d) have been defined, having anti-inflammatory roles in the Th2 response (M2a), suppression of tumour growth (M2b), immune regulation and tissue remodelling (M2c) and angiogenesis (M2d). These subsets have different polarising stimuli eg. IL4/13 – M2a, immune complexes and toll-like receptor (TLR) ligands – M2b, IL-10 & TGF- beta- M2c and IL-6 for M2d macrophages. Further subsets have been defined in the field of atherosclerosis research (Adamson and Leitinger, 2011) including further anti-inflammatory atheroprotective subtypes M(Hb) M(heam) and Mox (Moore et al., 2013). What Is becoming clear is the classical/alternative model of macrophage activation does

not take into account the subtle changes occurring in the cells microenvironment which can have tangible changes to the cells phenotype without fully polarising the cells.

Type IV or Delayed type hypersensitivity (DTH) is the archetypal antigen-specific cell mediated immune response involving CD4+ T cells and monocytes/macrophages. In the sensitisation phase antigen presenting cells (APC) present the hapten (eg oxazolone) to naïve T cells. The T cells then expand to a group of hapten specific T-helper1 (TH1) cells. In the effector phase re-challenge with the same hapten leads to rapid expansion of the of the sensitised TH1 cells which then interact with resting macrophages leading to macrophage activation via IFNγ and tumour necrosis factor beta (TNF-β) (Chen et al., 2019). These interactions underpins the chronic inflammatory lesions characteristic of inflammatory bowel disease, chronic infection, sarcoidosis and rejection of transplanted kidneys (Black, 1999).

Thrombin is a serine protease generated at the site of tissue injury and is the main effector enzyme in the coagulation cascade (Coughlin, 2005, Shrivastava et al., 2013). Thrombin generation is initiated by tissue factor (TF) a transmembrane protein found on the adventitia of vessels as well as on tissue macrophages, dendritic cells and at low levels on circulating monocytes. In addition to the well described role of thrombin in coagulation, it has a direct effect on a wide array of cell types such as smooth muscle cells, platelets and endothelial cells (EC) (Cunningham et al., 2000). These cellular responses of thrombin are mediated through a family of G protein coupled protease activated receptors (PARs), designated PAR-1-4 (Coughlin, 2005). PARs are characterised by an activation mechanism whereby proteolytic cleavage at specific sites within the extracellular amino-terminus leads to the exposure of an amino-terminal “tethered ligand“ domain. This new amino terminus is then able to effect

transmembrane signalling (Vu et al., 1991) . Thrombin is able to cleave PAR-1,-3, and -4 but not PAR-2 (Cunningham et al., 2000). Tissue factor, Factor Xa (FXa), Factor VIIa (FVIIa), Trypsin and mast cell tryptase, amongst others, are able to signal through PAR-2 (Camerer et al., 2000). Whilst there is a wealth of data exploring the role of thrombin as an inflammatory mediator there is yet to be a robust description of how thrombin acts on innate immune cells. This prompted us to investigate how thrombin signalling in monocyte/macrophages impacts the DTH response. We show that thrombin signalling through PAR-1 signalling plays a hitherto unrecognised and critical role in DTH responses, inducing downregulation of cell membrane reverse cholesterol transporter ATP-binding cassette transporter 1 (ABCA1) and increased expression of IFNγ receptor. These two key phenotypic changes combined to make thrombin-primed bone marrow derived macrophages extremely sensitive to M1 polarisation.

Results

Inhibition of thrombin on CD31+ myeloid cells inhibits DTH responses to oxazolone

In order to investigate the role of thrombin in DTH responses, we induced a DTH response in the ear skin in response to oxazolone in either C57BL/6 wild-type (WT) or CD31-Hir-Tg mice. CD31-Hir-Tg mice express a fusion protein containing the direct thrombin inhibitor hirudin on all CD31+ cells including all circulating monocytes (Figure 1A) (Chen et al., 2004a). CD31-Hir-Tg mice had significantly reduced ear swelling (ES) compared to WT at 24 ($p=0.0019$) and 48 ($p=0.0024$) hours after re-challenge with oxazolone (Figure 1B). Immunofluorescence analysis of the ear sections revealed a reduction in the total number of macrophages as assessed by reduced CD68+ expression within the ear lesion from 4.9% in WT to 0.5% in CD31-Hr-Tg ($p <0.001$) (Figure 1C), a reduction in the number of granulomas per section (Figure 1D) and a shift in the phenotype of recruited cells to a more anti-inflammatory profile with significantly reduced ratio of iNOS:CD206 expression on CD68+ cells coupled with an increase in IL-10 expression. (Figure 1E-G).

As the transgenic fusion protein in CD31-Hir-Tg mice is expressed on all CD31+ cells, we generated bone marrow (BM) chimeras with wild-type (CD45.1) mice to isolate expression on either BM-derived elements (platelets and monocytes) (Chen et al., 2004a) or EC alone. Cells expressing CD45.1 allele (WT) can be distinguished from cells expressing the CD45.2 allele (CD31-Hir-Tg) allowing the easy tracking of donor and host leukocytes. Engraftment at day 30 was >95%. CD45.1 mouse recipients of CD31-Hir-Tg BM had a similar ear swelling phenotype to parental CD31-Hir-Tg mice (Figure 1H), whereas CD31-Hir-Tg recipients of CD45.1 BM had a WT phenotype. Similarly, CD68 expression within the ear was reduced in the CD45.1 recipients of

transgenic (CD-31-Hir-Tg) bone marrow in comparison to CD31-Hir-Tg recipients of CD45.1 BM (Figure 1I).

There was a reduced T-cell (CD3+) infiltration into the ears of the CD31-Hir-Tg mice but no difference in IFNγ expression within the lesion (Figure 2A). To assess whether the expression of the transgenic fusion protein influenced T cell priming, CD4+ T cells were isolated from the spleens of CD31-Hir-Tg or WT mice 5 days after initial exposure to oxazolone. These sensitised CD4+ T cells were then injected via tail vein into oxazolone naïve WT mice who then underwent the usual re-challenge step with oxazolone. Recipients of CD31-Hir-Tg CD4 T cells had similar degrees of ES as recipients of WT controls (Figure 2B), indicating that CD4+ T cell priming in CD31-Hir-Tg mice was 'normal' and suggesting that the protective effect of the transgenic fusion protein was due to its expression on monocytes. There was no difference in circulating coagulation parameters: D-dimers, fibrinogen, thrombin antithrombin complex, TF or thrombin activity between the WT and CD31-Hir-Tg mice (Figure S1), suggesting there was no systemic activation of coagulation proteases nor consumption of fibrinogen. However, the inflammation in control ears was accompanied by widespread local fibrin deposits, which were significantly diminished and appeared to be located predominantly only subepithelial in the oxazolone treated ears of CD31-Hir-Tg mice, suggesting that the DTH response did involve local activation of coagulation proteases (Figure S2).

PTL060 is a cytotopic thrombin inhibitor based on Hirulog. On IV injection a mirostyl tail anchors it into the lipid bilayer of circulating monocytes (and other cells) (Chen et al., 2020). When C57BL/6 mice undergoing DTH were treated with 10µg/g IV PTL060 on day 3 and day 5 (3 hours before re-challenge) (Figure 2C) there was a reduction in ES compared to saline control

(p=0.0121) (Figure 2D). Examination of the ears by immunohistochemistry revealed, in comparison to saline controls, PTL060 lead to a reduction in CD68 infiltration from 4.9% to 1.3% (p <0.0001) (Figure 2E) and the adoption of a more anti-inflammatory profile with an increase in CD68+ cells expressing CD206 33% to 40% (p= 0.0332) and completely inhibited iNOS expression on CD68+ cells (11% to 0% <0.0001) (Figure 2F).

We postulated that the transgenic fusion protein was most likely influencing phenotype by blocking thrombin activation of PAR-1. Therefore, prior to re-challenge, mice were treated with intraperitoneal PAR-1 agonists or antagonists. WT mice treated with a PAR-1 agonist (TFLLR-NH2) had an increase in ES (p=0.0279), CD68 expression (p=<0.0001) with increased iNOS expression (p=0.0212) when compared to saline controls (Figure 2G,H&I), whereas those treated with a PAR-1 antagonist (RWJ 56110) had reduced ES (p=0.0322), CD68 expression (0.0036), iNOS expression (p=0.0104) compared to saline controls (Figure 2G,H&I). Treatment with PAR-4 agonist (GYPGQV trifluoroacetate salt) or antagonist (tcY-NH2) had no impact on the outcome of DTH. Although a PAR-3 agonist (H-Ser-Phe-Asn-Gly-Gly-Pro-NH2) increased ES in WT mice (Figure 2G), it did not significantly increase ear swelling in CD31-Hir-Tg mice, whereas those treated with a PAR-1 agonist developed significantly increased ES (p=0.0219) (Figure 2G), [1] suggesting that only the provision of a PAR-1 signal on CD31-Hir-Tg cells was sufficient to overcome the effect of thrombin inhibition.

All these data suggest local generation of thrombin at the site of antigen re-challenge leads to activation of PAR-1 that critically contributes to the development of the recall response;

[1] Experiments using different PAR-1 agonists and antagonists yielded entirely consistent results (data not shown).

182 inhibition of thrombin on monocytes/macrophages, either through transgenic expression of
183 hirudin or local tethering of hirulog significantly inhibits the DTH.

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188 Macrophage responses to thrombin

189 To assess how thrombin signalling influences the behaviour of WT macrophages, BM isolates
190 were incubated with 25ng/ml macrophage colony-stimulating factor (MCSF) for 5 days, which
191 was found to be the time at which PAR-1 expression was maximal (Figure 3A). Cells were then
192 stimulated for a further 24 hours with either thrombin or maintained in MCSF alone as a control.
193 There was no change in iNOS or CD206 expression compared to baseline in response to
194 thrombin (Figure 3B). ELISA confirmed a significant increase in IFNγ concentration in cell culture
195 supernatants from thrombin stimulated cells compared to controls (304.6 pg/ml vs 119.9pg/ml
196 respectively p=0.0185) as well as a significant reduction in IL-10 production (454pg/ml vs.
197 309pg/ml p=0.0286) (Figure 3C).
198 ABCA1 plays a critical role in lipid homeostasis and orchestrates the principal cellular pathway
199 leading to cholesterol efflux (Pradel et al., 2009). We found that ABCA1 expression was highest in
200 MCSF-matured BM cells that were treated with IL4 for 24 hours and lowest after culture for 24
201 hours with a combination of LPS and IFNγ (Figure 3D). This is in keeping with previously
202 published data (Singaraja et al., 2002). Next, we evaluated what role thrombin had on ABCA1
203 expression. Thrombin, but not active site inhibited thrombin, down-regulated surface ABCA1
204 expression by flow cytometric analysis from 51.27% to 16.28% after 24 hours culture (p=0.0024)
205 (Figure 3D). Thrombin mediated reduction in ABCA1 was also seen on western blot (p=0.0303)

(Figure 3E). This was shown to be reliant on the G protein subunit Gα 12 as inhibiting this with small interfering RNA (siRNA) prevented thrombin mediated ABCA1 down regulation (Figure 3F). Thrombin cleaved PAR-1 is known to transactivate PAR-2 (O'Brien et al., 2000). Blocking the PAR-2 signal with the PAR-2 antagonist FSLLRY-NH2 prior to thrombin stimulation did not affect the outcome of thrombin on ABCA1 expression (Figure 3D). This thrombin mediated ABCA1 down regulation was mimicked by culturing cells with the PAR-1 agonist peptide (TFLLR-NH2) (Figure 3G) and inhibited by antagonising signalling through PAR-1 (Figure S3). Only at very high dose did PAR-4 agonist peptide (GYPGQV trifluoroacetate salt) impact ABCA1 expression (Figure 3G), whereas a PAR-3 agonist (H-Ser-Phe-Asn-Gly-Gly-Pro-NH2) failed to influence ABCA1 expression (Figure S4). Delivery of a signal through matrix metalloproteinase (MMP) 13 did not affect ABCA1 expression (Figure 3H). Non canonical PAR-1 signalling can occur through the endothelial protein C receptor (ePCR) (Zhao et al., 2014). Pre culturing the cells with an ePCR neutralising antibody did not affect thrombin's ability to reduce ABCA1 expression (Figure 3I).

ABCA1 has been linked to the formation of lipid rich microdomains in the external leaflet of the plasma membrane (Zhu et al., 2010). These discrete lipid domains, representing organised accumulations of cholesterol and glycosphingolipids, play a key role in inflammatory signalling due to the high concentration of cell receptors residing within the 'lipid rafts' (Pike, 2003). To evaluate the role thrombin signalling had on lipid rafts, bone marrow macrophages (BMM) where incubated for 24 hours in complete media with MCSF or Thrombin. After 24 hours cells were stained using Vybrant™ Alexa Fluor™ 488 Lipid Raft Labelling Kit. The thrombin treated cells had increased expression of Cholera Toxin B (CTB) on the cell surface, correlating with increased lipid raft formation ($p < 0.0001$) (Figure 4A). Surface expression of TLR4 increased upon thrombin

stimulation (MFI increased from 47.01 to 79.02 (p=0.0427)) and there was also increased colocalization of the receptor within the lipid rafts (46.04% vs. 66.03% p= 0.0004) (Figure 4B). Thrombin stimulation increased surface expression of IFNγ receptor (MFI 435.6 vs 477.4) (p=0.0287) and these also showed increased expression within the lipid rafts from 2.39% expression to 8.73% p=0.0031 (Figure 4C).

Thrombin primes BM-derived macrophages to be hyperresponsive to M1 polarising signals

Given thrombin's apparent role in augmentation of lipid raft composition, specifically with the increase in both the LPS and IFNγ receptor – both moderators of the M1 phenotype, we considered that thrombin was priming the cells which could potentially translate to increased responsiveness to LPS or IFNγ. For these experiments BMM were incubated for 24 hours with thrombin or MCSF alone as a control followed by increasing concentrations of LPS and/or IFNγ. Thrombin stimulated cells were more sensitive to the combination of low dose LPS/IFNγ, evidenced by increased proportion of iNOS expression (29.1% vs 89.3% p= 0.0079) and increased MFI (1543 vs. 9096 p=0.0040) (Figure 5A). Thrombin stimulated cells were exquisitely sensitive to very low dose IFNγ (in the absence of LPS) with increasing concentrations resulting in enhanced iNOS expression in a dose dependent manner (Figure 5B). Similarly, but to a lesser extent, the cells were also sensitive to low dose LPS (without IFNγ) (Figure 5C). These enhanced responses to low dose LPS appeared to be due entirely to thrombin-mediated increases in IFNγ secretion, as they were abolished by increasing amounts of an IFNγ blocking antibody (Figure 5D).

LPS stimulation of the BMM increased the expression of iNOS (p=0.0286), TNFα (p=0.0030), RANTES (p=0.0079), IL6 (p=0.0286) and IL-1β (p=0.0287) by qPCR. Pre-treatment with thrombin increased further iNOS expression (p=0.0286) during LPS stimulation but this heightened sensitivity to LPS was not seen in TNFα, RANTES, IL6 or IL-1β expression. (Figure 5E). LPS and PAR-2 have been shown to synergistically enhance inflammatory signalling (Ostrowska et al., 2007). There was no difference in PAR-2 expression during thrombin stimulation so the enhanced responses to low dose LPS cannot be attributed to increased PAR-2 expression (Figure S5).

Thrombin-mediated down regulation of ABCA1 has been described to be via upregulation of the ubiquitin–proteasome system component cullin 3 (Raghavan et al., 2018). To assess the importance of ABCA1 to thrombin mediated heightened sensitivity to low dose M1 stimuli, cullin 3 siRNA was used to maintain ABCA1 expression (Figure 6A&B, S6) in the face of thrombin stimulation. This inhibition of thrombin mediated ABCA1 down regulation by cullin 3 siRNA was associated with a failure to increase cell membrane lipid rafts (Figure 6C) and a loss of the hypersensitivity to low dose LPS/IFNγ seen after exposure to thrombin (Figure 6D).

Taken together, all these data indicate that thrombin, through PAR-1 signalling, prime BMM to polarisation by IFNγ and TLR-4 agonists. This is via an increase in expression of IFNγ, IFNγ receptor and TLR-4 and co-localisation of both receptors in membrane lipid rich microdomains, due to the associated downregulation of ABCA1 by cullin 3.

ABCA1 is critical to the phenotype of ES in delayed type hypersensitivity

To confirm that these mechanistic steps were operational in the DTH responses in vivo, we confirmed that CD31-Hri-Tg mice showed increased ABCA1 expression compared to WT mice (Figure 7A) after second exposure to oxazolone. BM isolates from CD31-Hir-Tg were not sensitive to thrombin and thus maintained ABCA1 expression in the face of thrombin (Figure 7B). Finally, CD31-Hir-Tg mice were treated with IP probucol for 3 days prior to oxazolone re-challenge (Figure 7C). Probucol inhibits ABCA1 mediated cellular lipid efflux but does not affect ABCA1 surface expression (Favari et al., 2004). The probucol treated CD31-Hir-Tg had an increase in ES at 24 and 48 hours compared to saline-treated control CD31-Hir-Tg mice (Figure 7D), associated with increased infiltration by CD68+ cells (Figure 7E), expressing reduced levels of CD206 but increased levels of iNOS (without any change in ABCA1 expression) (Figure 7F).

Discussion

In this study we describe how the serine protease thrombin is able to prime macrophages to become exquisitely responsive to low doses of LPS and IFNγ. We confirm the reports of others (Chinetti-Gbaguidi et al., 2015) that ABCA1 is a marker of IL-4 stimulated anti-inflammatory macrophages. Moreover, we describe the link between thrombin stimulation, lipid raft composition alteration and increased sensitivity to M1 stimuli. Finally, to our knowledge, we provide the first report of ABCA1's key role in the development of normal DTH responses and the first report that thrombin-mediated PAR-1 signalling provides the stimulus in vivo for ABCA1 downregulation.

Thrombin is the main effector protein in the coagulation cascade (Manabe et al., 2009) but is able to directly affect a wide array of cells types such as smooth muscle cells, platelets and endothelial cells (Cunningham et al., 2000) via signalling through PARs. We have previously described the roles that thrombin plays in acute and chronic vascular inflammation using CD31-Hir-Tg mice (Chen et al., 2008b, Chen et al., 2006, Chen et al., 2008a). In a mouse-to-rat model of heart transplantation, hearts from CD31-Hir-Tg mice rejected significantly later compared to WT hearts (Chen et al., 2004b), due to inhibition of both intravascular thrombosis associated with antibody-mediated rejection, and inhibition of thrombin-dependent CCL2 chemokine gradients necessary for monocyte recruitment (Chen et al., 2008b, Chen et al., 2006) in this model. Aortas from these mice, when transplanted into Apolipoprotein E (ApoE)-/- mice fed a high fat diet, fail to express CCL2 and MIF and do not develop atherosclerosis, in contrast to the florid lesions seen in control WT aortas (Chen et al., 2020). Recently, we have reported pre transplant perfusion into rat or primate organs with PTL060 (or related compounds) prevents the

intravascular thrombosis associated with antibody mediated rejection (AMR) (Manook et al., 2017, Karegli et al., 2017). Most recently, we have showed that intravenous delivery of PTL060 into ApoE-/- mice fed a high fat diet leads to widespread coating of the endothelium, inhibits expression of both CCL2 and MIF and prevents atheroma formation (Chen et al., 2020). Importantly in this work, intravenous delivery of PTL060 also led to widespread uptake onto the membranes of circulating leukocytes and was associated with significant regression of atherosclerotic plaques when treatment was started 16 weeks after the beginning of the high fat diet (Chen et al., 2020). In this model, the direct effect of PTL060 on monocytes was the dominant mechanism driving atheroma regression, as the same phenotype was achieved by adoptive transfer of PTL060-coated monocytes.

The data in a contact dermatitis model, presented here, is entirely consistent with our data in atherosclerosis, but provide a much greater mechanistic insight into the role and importance of thrombin in monocyte/macrophage polarisation in vivo. Expression of a hirudin fusion protein on monocytes prevented ES after second exposure to oxazolone and shifted the phenotype of DTH lesions away from an M1 spectrum towards M2. This was PAR-1- and ABCA1-dependent. Furthermore, we have demonstrated that PTL060 also deliverers a protective phenotype in this additional model system.

Our in vitro experiments revealed the mechanistic basis of these findings. Thrombin, via PAR-1-mediated ABCA1 downregulation increased the expression of IFNγR and shifted the receptors into cholesterol rich microdomains, resulting in a massively increased sensitivity to IFNγ-mediated polarisation. At the same time, TLR4 expression was increased within the same lipid rafts, and thrombin induced secretion of picomolar concentrations of IFNγ, which in

combination, enhanced the sensitivity of cells to LPS-mediated polarisation. Thrombin's nuanced role in LPS stimulation was further highlighted when pre-treatment with thrombin changed the expression of some (iNOS) but not all Myd88 and TRIF dependent genes during TLR4 stimulation (Leifer and Medvedev, 2016). In vitro there appeared to be a correlation between high dose PAR-4 stimulation and ABCA1 expression. This is not entirely surprising as it is well documented that the PAR-4 receptor lacks the hirudin-like domain therefore higher concentrations of thrombin are required to initiate cellular signalling (Xu et al., 1998). We did not see any evidence of PAR-4 signalling affecting the outcome of the in vivo findings. In contrast, a PAR-3 agonist, even at high doses failed to impact on ABCA1 expression, and although the same agent caused increase ES in WT mice, it did not partially reverse the phenotype of the CD31-Hir-Tg mice in the same way as a PAR-1 agonist, suggesting a minor role, if any for PAR-3 in this model. That said, a definitive conclusion about the role of PAR-3 is difficult in the absence of reliable reagents to antagonise PAR-3 activation.

We believe that in this model, the thrombin is generated on the surface of myeloid cells, which are known to express TF (Rao and Pendurthi, 2012). We were able to demonstrate evidence of local fibrin generation in the WT mice but not the transgenic strain. Interestingly there was no evidence of systemic activation of coagulation.

Other groups have previously reported on the impact of PAR-1 signalling on monocyte/macrophage function. In RAW cells thrombin has been shown to induce iNOS (Kang et al., 2003). In human THP1 cells thrombin has been linked to IL-8 production (Kang et al., 2003). In a model of Citrobacter rodentium–induced colitis, PAR-1 signalling on monocytes was shown to be key to promoting Th17-type immune response via IL-23 (Saeed et al., 2017). PAR-1 signalling

has been shown to enhance the Poly I:C induction of the antiviral response via TLR3 in bone marrow macrophages (Antoniak et al., 2017). Recently López-Zambrano et al reported that thrombin signalling, in part through PAR-1, was sufficient to induce M1 polarisation in bone marrow macrophages (Lopez-Zambrano et al., 2020). The difference between our data and this work is likely to be due to the use of L929 conditioned medium to differentiate the BMM instead of purified MCSF. Taken together our data is consistent with the underlying implication that thrombin primes monocytes to make enhanced responses to microenvironmental polarisation cues. Priming of monocytes has been described by others. Askenase et al. have recently described how monocytes are primed for regulatory function prior to egress from the bone marrow using a model of gastrointestinal infection. In this model, natural killer (NK) cell derived IFNγ promoted regulatory programming in monocyte progenitors controlled by systemic IL-12 produced by Batf3- dependent dendritic cells (DCs) in the mucosa-associated lymphoid tissue (MALT) (Askenase et al., 2015). Our data suggest that the sensitivity of monocytes to distal priming by systemic cytokines may be regulated by cell-intrinsic mechanisms controlling the encryption and de-encryption of tissue factor on myeloid cells, which is known to regulate their ability to generate cell surface thrombin and other coagulation proteases (Chen and Hogg, 2013).

ABCA1 is a major regulator of cellular cholesterol and phospholipid homeostasis (Singaraja et al., 2002). It has a key role in atherosclerosis, mediating the efflux of cholesterol and phospholipids and thus reducing the atherosclerotic plaque burden (Pradel et al., 2009). Our data is consistent with other reports that ABCA1 is linked to an anti-inflammatory M2 phenotype (Pradel et al., 2009) and augments the report from Raghavan et al, which first revealed that thrombin down

regulates ABCA1 expression (Raghavan et al., 2018)via cullin 3 expression, which is a component of cullin-RING E3 ubiquitin ligase complex involved in protein ubiquitination (Dubiel et al., 2018). ABCA1 has been shown to disrupt cholesterol rich microdomains via redistribution of cholesterol from rafts to non-rafts through its ATPase-related functions (Zhu et al., 2010). In our study we were able to show a direct link between thrombin stimulation, ABCA1 down regulation, increase in lipid-rich microdomains at the cell membrane and increased sensitivity to IFNγ, which, along with the secretion of picomolar concentrations of IFNγ, was the basis for the increased sensitivity to LPS. This is consistent with previous reports that human monocytes when cultured with IFNγ have heightened responses to bacterial LPS (Hayes and Zoon, 1993).

In summary, we have provided the first evidence that thrombin-mediated PAR-1 signalling on the surface of monocytes, leading to ABCA1 downregulation and an associated sensitivity to IFNγ and TLR stimulation is critically involved in the development of normal DTH responses in vivo. Targeting this pathway could potentially offer a way to modulate innate immune responsiveness and to control inflammatory responses in multiple diseases.

Limitations of the Study

A potential limitation of this work is that we have not confirmed results in mice deficient in PAR, particularly PAR-1. Our rationale is that both the priming/sensitisation and re-challenge phases would be influenced by the lack of PAR-1 signalling. Our approach instead relied upon using highly specific agonists and antagonists to allow us to isolate only the re-challenge phase for study.

We have also not addressed the role of PAR-2 signalling in this model, as our ongoing experiments dissecting the impact of PAR-2 stimulation suggest complex interactions between PAR-2 and PAR-1 stimulation which require further interrogation and will be the subject of a subsequent report. Others have reported that PAR-2 signalling in contact dermatitis contributes to the inflammatory response (Seeliger et al., 2003).

Resource Availability

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Hannah Wilkinson (hannah.wilkinson@kcl.ac.uk).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This study did not generate datasets or codes.

Methods

All methods can be found in the accompanying Transparent Methods supplemental file.

Acknowledgments

Author contributions: H. Wilkinson designed and performed all the experiments and wrote the manuscript. A. Dorling designed the experiments, supervised the overall project and assisted in manuscript preparation. H. Leonard assisted with in vitro experimentation. P. Goossens provided

guidance in lipid raft staining techniques. M. Robson, T. Lawrence and D. Chen assisted in experimental design and manuscript review. J. McVey assisted in data review and manuscript writing. Support for this work was received through an MRC research training fellow grant MR/P018513/1

Declaration of Interests

The authors declare no competing interests

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Figure 1. Results of oxazolone induced delated type hypersensitivity experiments in transgenic mice

(A) Surface CD31 expression on monocytes (as defined as CD45+ CD11b+ Ly6G- Ly6c+) on the peripheral blood of CD31-Hir-Tg mice. (B) Difference in ES at 24 hours. Derived by subtracting thickness of right ear (oxazolone) from that of left (control) ear. WT group n= 6, transgenic group n=6. (C) Immunofluorescence (IF) analysis of CD68 expression with the ear. Expression calculated by % lesion area occupied by CD68+ cells. (D) Granuloma assessed as average number per section at 100x magnification. A granuloma was defined as a collection of CD68+/CD3+ cells outpouching from the epidermis. (E-G) IF analysis of proportion of CD68+ cells expressing iNOS (E), CD206 (F) or IL-10 (G). Graphs show percentage of CD68 cells that co-stain with iNOS or CD206, or in the case of IL-10, the % lesional area occupied by IL-10+ cells. Representative images show (CD68 -red; CD206 or iNOS-green; DAPI-blue) E= epidermis, AC= auricular cartilage. The scale bar shows 200µm in distance. (H) Bone marrow chimeric mice underwent oxazolone induced DTH: graph shows change in ES at 24 hours compared to vehicle control ear. WT recipients of WT bone marrow and CD31-Hir-Tg recipients of CD31-Hir-Tg bone marrow represent experimental controls. Group numbers WT (CD45.1)-> WT (CD45.1) n=3, CD31-Hir-Tg -> WT (CD45.1) n=6, CD31-Hir-Tg -> CD31-Hir-Tg n=6, WT (CD45.1) -> CD31-Hir-Tg n=6. (I) IF results of macrophage infiltration (CD68 -red; DAPI-blue) within the ear of the bone marrow chimeric mice. Associated graph shows expression calculated by % lesion area taken up by CD68+ cells when corrected for background florescence. E= epidermis, AC= auricular cartilage. The scale bar shows 200 µm in distance. Data represented as mean ± SEM. *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.

Figure 2. Assessing how transgenic expression of hirudin influences T cell priming in type IV hypersensitivity and evaluating role of PAR signaling

(A) CD3 and IFNγ expression in the WT or CD31-Hir-Tg mice after DTH. CD3 or IFNγ expression calculated by % lesion area taken up by CD3+ or IFNγ cells when corrected for background WT group n= 6, transgenic group n=6. (B) Adoptive transfer of oxazolone primed WT or CD31-Hir-Tg CD4 cells into WT mice before oxazolone applied to ear. Change in ear thickness, compared to vehicle challenge alone, was measured at 24 hours. WT control mice received IV saline instead of cells but abdominal oxazolone challenge on day 0 then ear re-challenge on day 5. 'Re-challenge alone' mice were oxazolone naive mice that received only 1% oxazolone in acetone and olive oil to the right ear. WT group n=6, re-challenge alone n=2, WT CD4 -> WT n=4, Tg CD4 -> WT n=6. (C) PTL060 experimental protocol. (D) ES Results of WT (C57BL/6) mice treated with IV 10mcg/g PTL060 (n=6) or equivalent volume saline (n=4) on day 3 & 5 after sensitization in oxazolone induced DTH model. (E) IF analysis of CD68 expression within the ear of PTL060 treated group vs saline. (F) IF analysis of iNOS and CD206 expression on CD68+ cells in the PTL060 treated group vs saline. (G) The effect of PAR signaling on DTH responses. Before a re-challenge on day 5, WT or CD31-Hir-tg mice received 10microM/g Intraperitoneal (IP) PAR-1 agonist (TFLLR-NH2) (n= 5) or antagonist (RWJ 56110) (n=5) or PAR-4 agonist (GYPGQV trifluoroacetate salt) (n=5) or antagonist (tcY-NH2) (n=4) or PAR-3 agonist (H-Ser-Phe-Asn-Gly-Gly-Pro-NH2) (n=5). The ears were then painted with oxazolone or vehicle alone. Data represents change in ES at 24 hours compared to control ear. (H) IF analysis of CD68 expression within WT ears. Expression calculated by % lesion area taken up by CD68+ cells when corrected for background. (I) IF

582 analysis of iNOS expression on CD68+ cells in WT ears. Data represented as mean ± SEM. *P ≤
583 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.

584 **Figure 3. Thrombin induces a pro inflammatory state without fully polarising the cultured**
585 **macrophages and down regulates ABCA1 expression through PAR1**

586 (A) Surface expression of PAR-1,-2 &-4 on bone marrow macrophages cultured for 5 days in
587 complete bone marrow medium analysed by flow cytometry. (B) Intracellular flow cytometric
588 analysis of CD206 or iNOS expression on bone marrow macrophages cultured for 5 days in
589 complete bone marrow medium and stimulated for 24 hours with 25ng/ml MCSF or 50units/ml
590 of thrombin. Representative flow cytometry profiles are shown to the right. (C) Cell culture
591 supernatants taken from cells treated for 24 hours with 25ng/ml MCSF or 50units/ml thrombin
592 were analysed by ELISA. IFNγ ELISA n=5, IL-10 ELISA n=4. * P ≤ 0.05. (D) ABCA1 expression,
593 analysed by flow cytometry, on F4/80 CD11b positive cells after 5 days in bone marrow culture
594 followed by 24 hours stimulation with 25ng/ml MCSF, 100ng/ml LPS and 50ng/ml IFNγ for M1
595 cells and 25ng/ml IL4 for M2 cells or 50units/ml thrombin or equimolar active site inhibited
596 thrombin (FIIai), or the PAR-2 antagonist FSLLRY-NH2 for 2 hours prior to thrombin stimulation.
597 Data taken from at least 4 experiments (E) Western Blot of MCSF or Thrombin treated cells.
598 Representative gel shown to the right. ABCA1 band confirmed at approx. 250 kDA. (F) Surface
599 ABCA1 expression of cells transfected with siRNA to Gα12 or negative control siRNA for 24 hours
600 then thrombin or MCSF for 24 hours. Data analysed by flow cytometry. Data taken from 3
601 experiments. (G) ABCA1 expression, analysed by flow cytometry, on F4/80 CD11b positive cells
602 after 5 days in bone marrow culture followed by 24 hours stimulation with 25ng/ml MCSF,
603 thrombin, or increasing amounts of TFLLR-NH2 (PAR-1 agonist peptide) or GYPGQV

trifluoroacetate salt (PAR-4 agonist peptide). Data represents percentage change in expression from control (MCSF) stimulated cells. (H&I) Change in surface ABCA1 expression analysed by flow cytometry of bone marrow macrophages cultured for 24 hours with MMP-13 (H) or pre cultured for 2 hours with a neutralizing ePCR antibody (eBio1560 (1560) Thermofischer scientific) prior to thrombin stimulation (I). Data shown as percentage change from baseline expression of MCSF-media maintained cells in 3 different experiments. Data represented as mean ± SEM. *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.

Figure 4. Thrombin increases the lipid raft content of cells

(A) CTB (green) or DAPI (blue) staining of MCSF and thrombin treated cells. The scale bar shows 10µm in distance. Associated graph shows graphical representation of CTB intensity (B) TLR4 surface expression as measured by IF on MCSF or thrombin treated cells. Cells were prepared for lipid raft CTB staining as above and co-stained with fluorochrome conjugated anti TLR4 ab. The graphs represent, from left to right, the % of cells in positive gate, MFI of TLR4 on cells in the positive gate, and proportion of cells showing co-localisation of CTB with TLR4. Co staining calculated using ICY cell imaging software using Pearson correlation coefficient of both CTB staining and TLR4 receptor staining. (C) IFNγ surface expression as measured by IF on MCSF or thrombin treated cells. Cells were prepared for lipid raft CTB staining as above and co-stained with fluorochrome conjugated anti IFNγ ab. The graphs represent, from left to right, the % of cells in positive gate, MFI of IFNγ on cells in the positive gate, and proportion of cells showing co-localisation of CTB with IFNγ. Co staining calculated using ICY cell imaging software using Pearson correlation coefficient of both CTB staining and IFNγ receptor staining. Data shown from 4

separate experiments. Data represented as mean ± SEM. *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.

Figure 5. Priming with thrombin increases sensitivity to low dose LPS and IFNγ

(A) Intracellular flow cytometric analysis of % of iNOS+ cells in positive gate and MFI of iNOS expression by cells in the positive gate. Cells were murine bone marrow macrophages primed for 24 hours with thrombin or control (MCSF) n=6 prior to 24-hour stimulation with low dose M1 stimuli 0.01 ng/ml LPS & 50 ng/ml IFNγ n=6. (B+C) BMM were primed for 24 hours with MCSF or 50units/ml thrombin as indicated then stimulated for a further 24 hours with escalating amounts of either IFNγ alone (B) or LPS alone (C). Cells were then analysed by intracellular flow cytometry for iNOS expression. Data represents % positive cells. (D) The effect of IFNγ blockade on heightened sensitivity to LPS alone. BMM were cultured for 5 days with 25ng/ml MCSF then stimulated for 24 hours with thrombin. Media was replaced with fresh media containing escalating doses IFNγ blocker (IFNγαag) (Abcam) for 1 hour. All wells were then treated with low dose LPS (10ng/ml) +/- thrombin for 24 hours. iNOS expression was then analysed by flow cytometry. Data shown change in iNOS expression between control and thrombin treated cells. Each data point represents a single experiment, bars represent mean of data. (E) qPCR data for the expression of TNFα, IL-1β, IL-6, RANTES and iNOS. BMM were stimulated for 24 hours with thrombin or maintained in complete media. After 24 hours the media was removed and replaced with fresh media containing 10ng/ml LPS +/- thrombin. Cells were removed for qPCR analysis 4 hours later. Data shown relative to MCSF control cells. Data shown from 4 separate experiments. Error bars are means of data. Data represented as mean ± SEM. *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.

Figure 6. ABCA1 is essential for heightened sensitivity of thrombin primed cells to LPS and IFNγ

(A) Cullin 3 expression, analysed by qPCR, after 24 hours transfection of BMM cultured for 24 hours with cullin 3 siRNA, negative control siRNA or control cells maintained in complete media containing 25ng/ml MCSF. (B) The above cells were then stimulated for 24 hours with thrombin and surface ABCA1 expression was assessed by flow cytometry. (C) CTB staining of lipid rafts of the three experimental cell groups after 24 hours siRNA (or control) transfection and then 24 hours of thrombin. Cells were counterstained with DAPI then analyzed using inverted confocal microscope @ 60x magnification (oil immersion) and analyzed using NIS-Elements software. The scale bar shows 10μm in distance. (D) The cells were then treated for a further 4 hours with 0.01ng/ml LPS and 50ng/ml IFNγ with or without thrombin and then analysed by flow cytometry for intracellular iNOS expression. Data shown from 3 different experiments. Data represented as mean ± SEM. *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.

Figure. 7 The importance of ABCA1 in vivo

(A) ABCA1 expression in the ears of WT or CD31-Hir-Tg mice after oxazolone induced DTH measured by IHC. Represented as % of CD68+ cells co-expressing ABCA1 WT group n= 6, transgenic group n=6. (B) Flow cytometric analysis of surface ABCA1 expression of cultured WT or CD31-Hir-Tg BMM treated with 24 hours 50U/ml thrombin or MCSF control. (C) IP probucol experiments. CD31-Hir-Tg mice were challenged with 5% oxazolone on day 0. Then from day 2-5 they received 1mg/kg IP probucol (n=4) or control (n=4) before re-challenge with oxazolone on day 5. (D) Data represents difference in ES at 24 hours. (E) IP probucol experiments. IF analysis of CD68 expression with the ear. Expression calculated by % lesion area taken up by CD68+ cells when corrected for background. Representative image CD68-red, DAPI-blue. E= epidermis, AC=

669 auricular cartilage. The scale bar shows 200µm in distance (F) IP probucol experiments. IF
670 analysis of CD206, iNOS & ABCA1 expression on CD68+ cells. Data represented as mean ± SEM.
671 *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.

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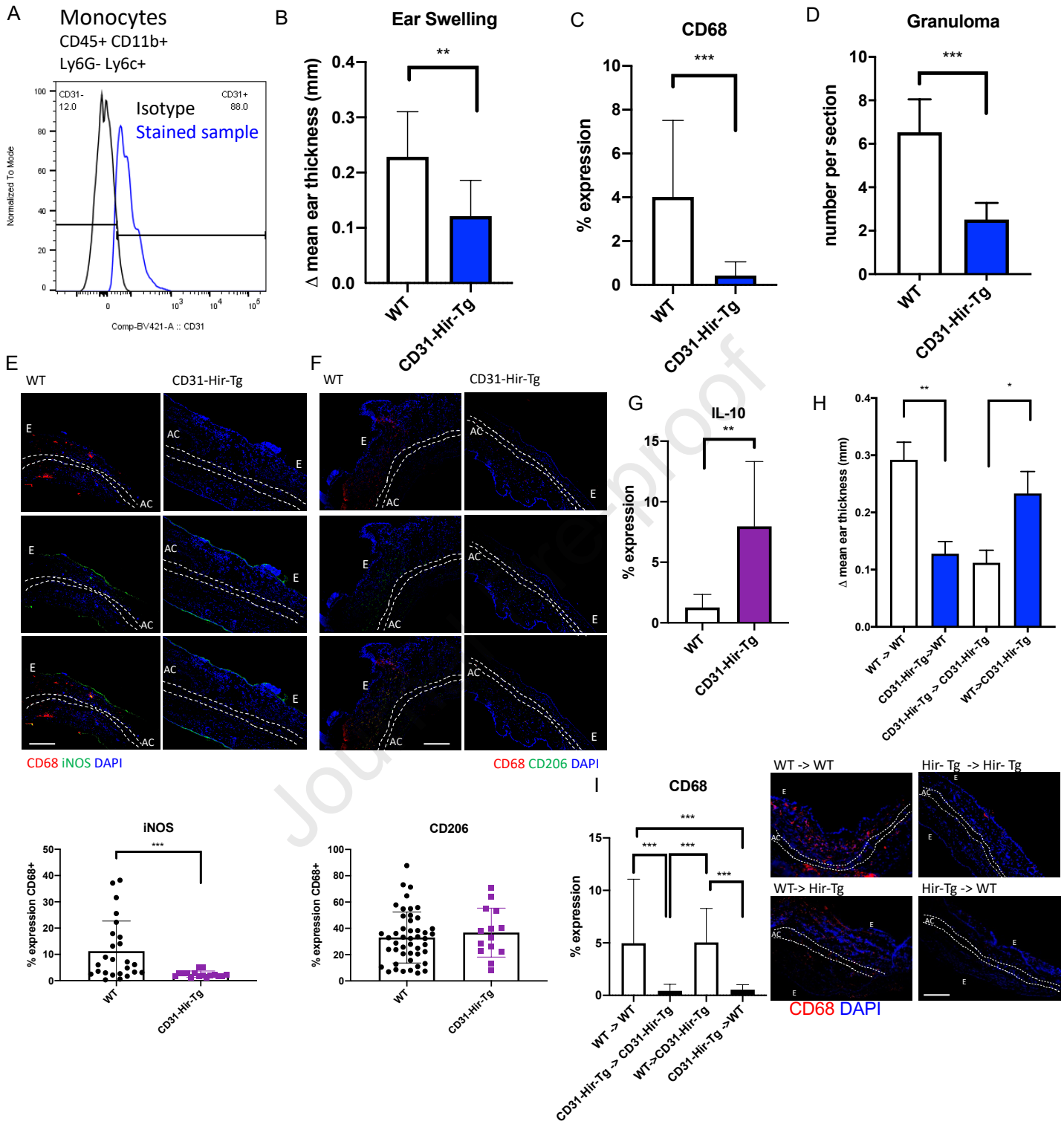
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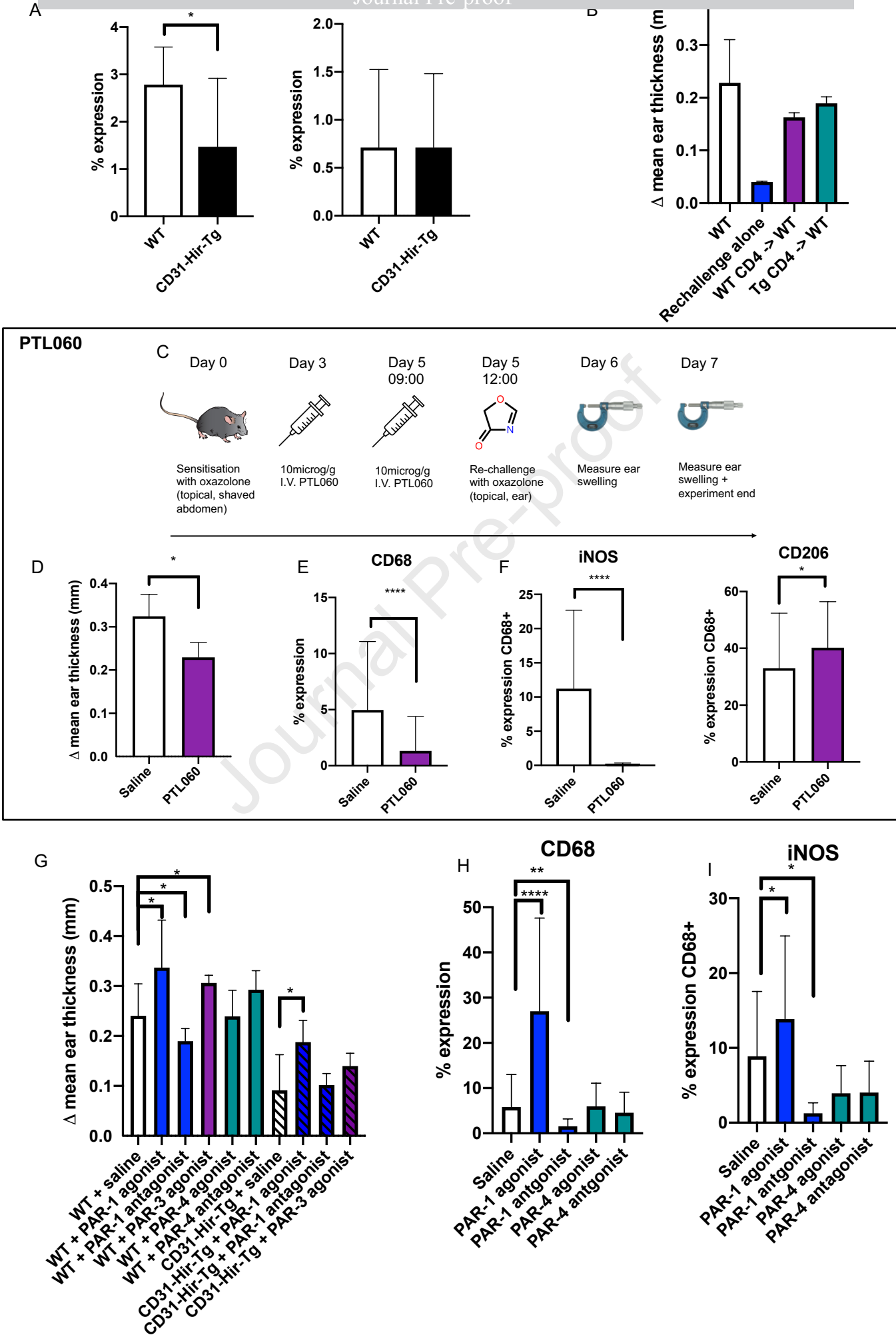

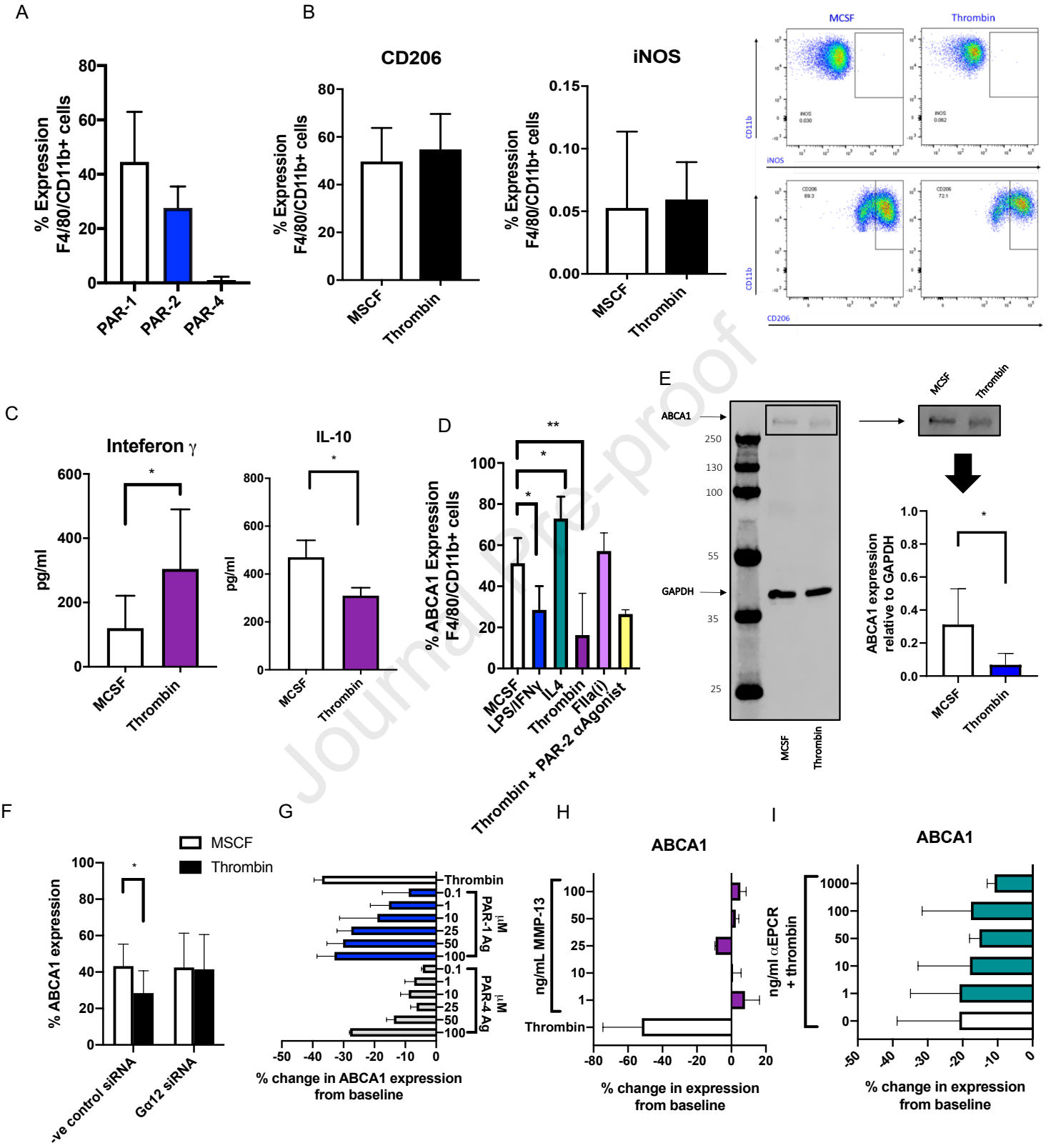

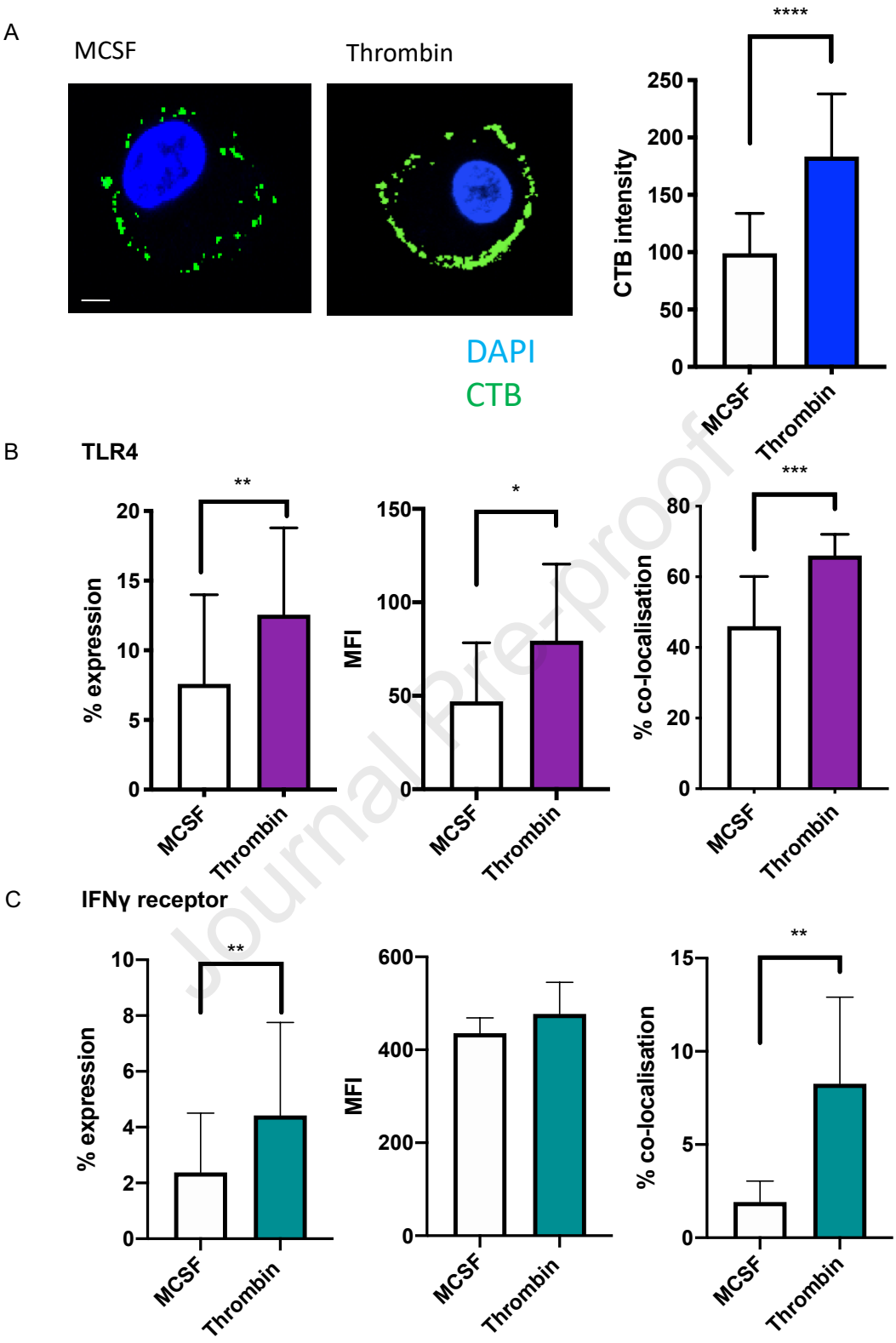

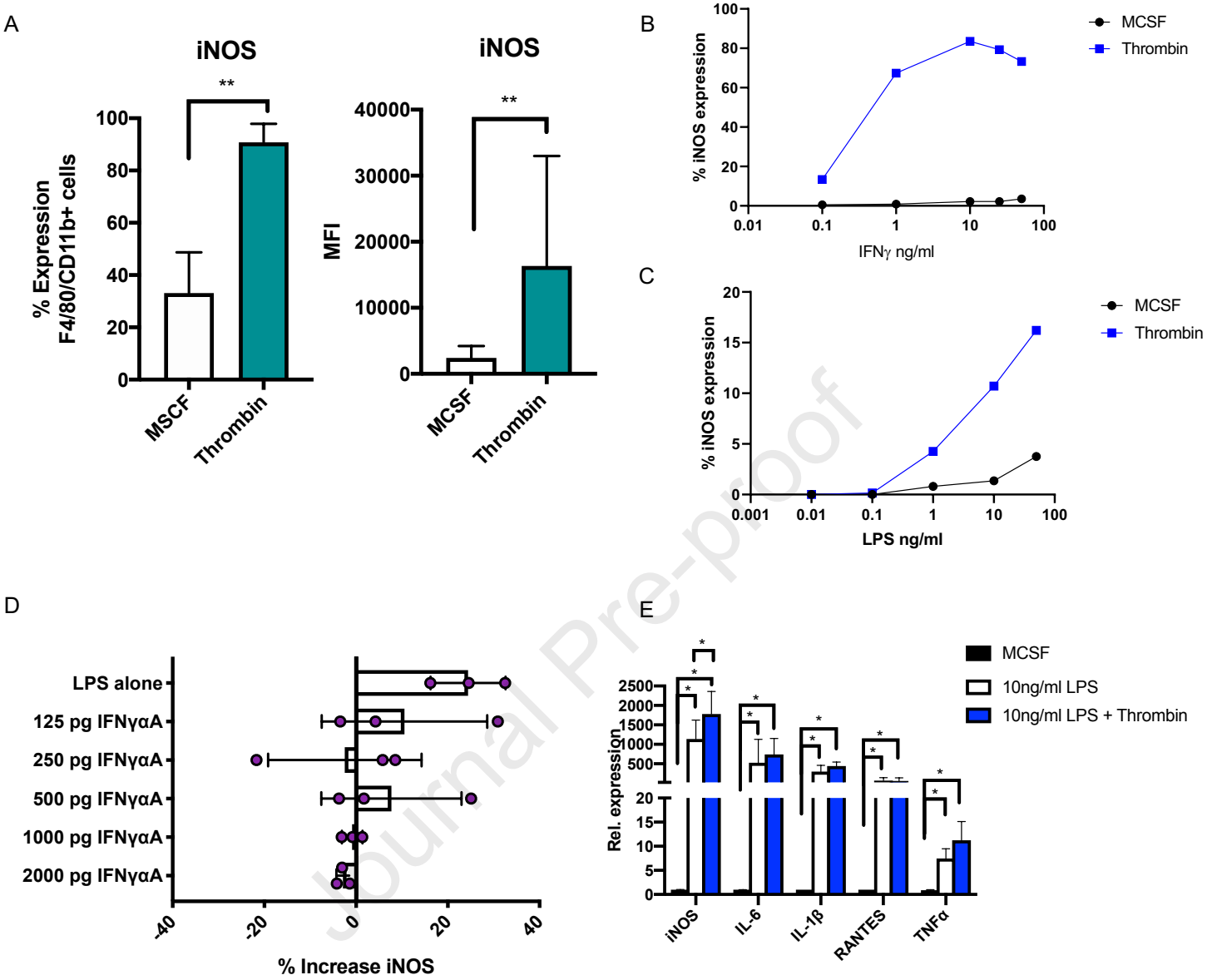

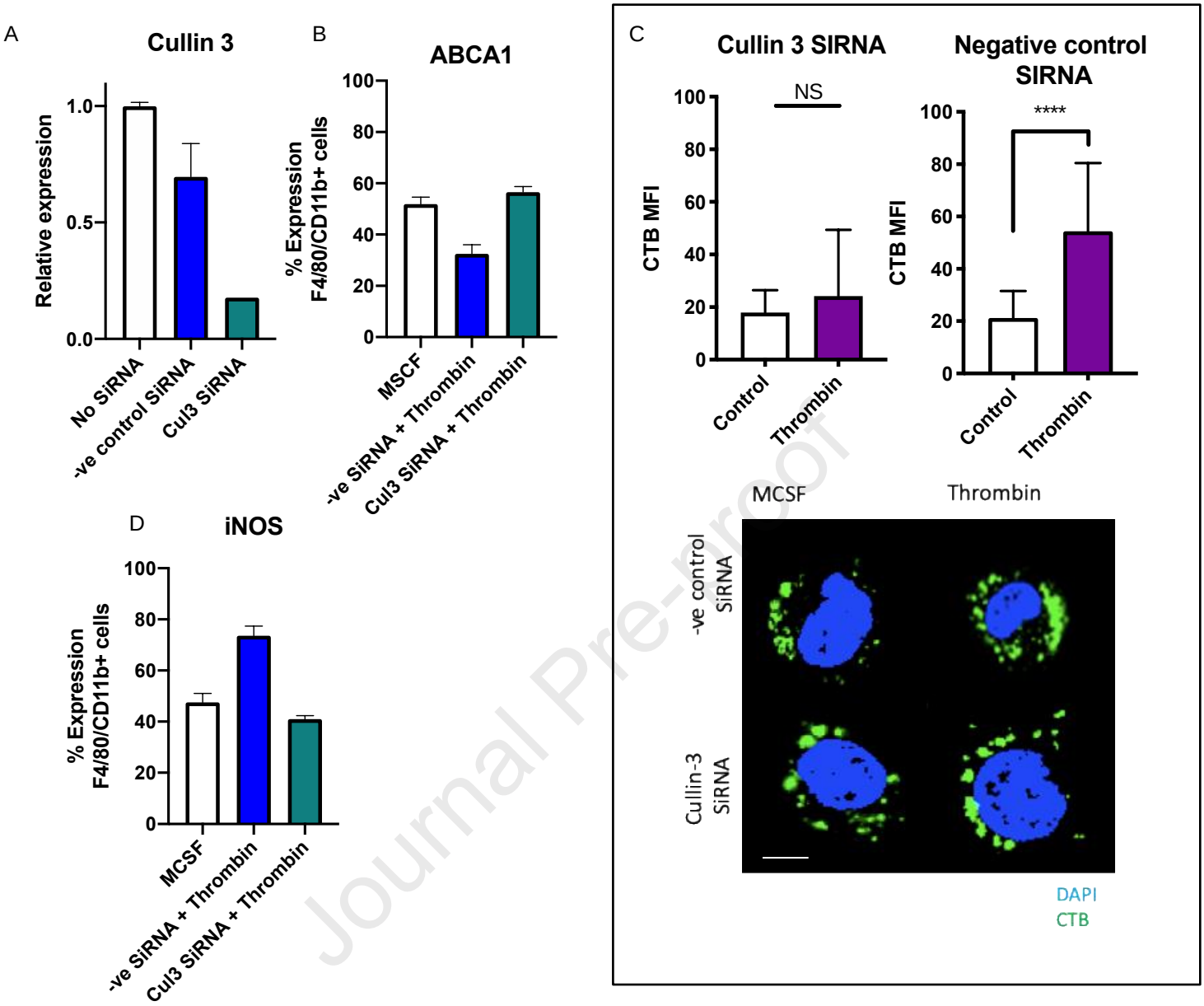

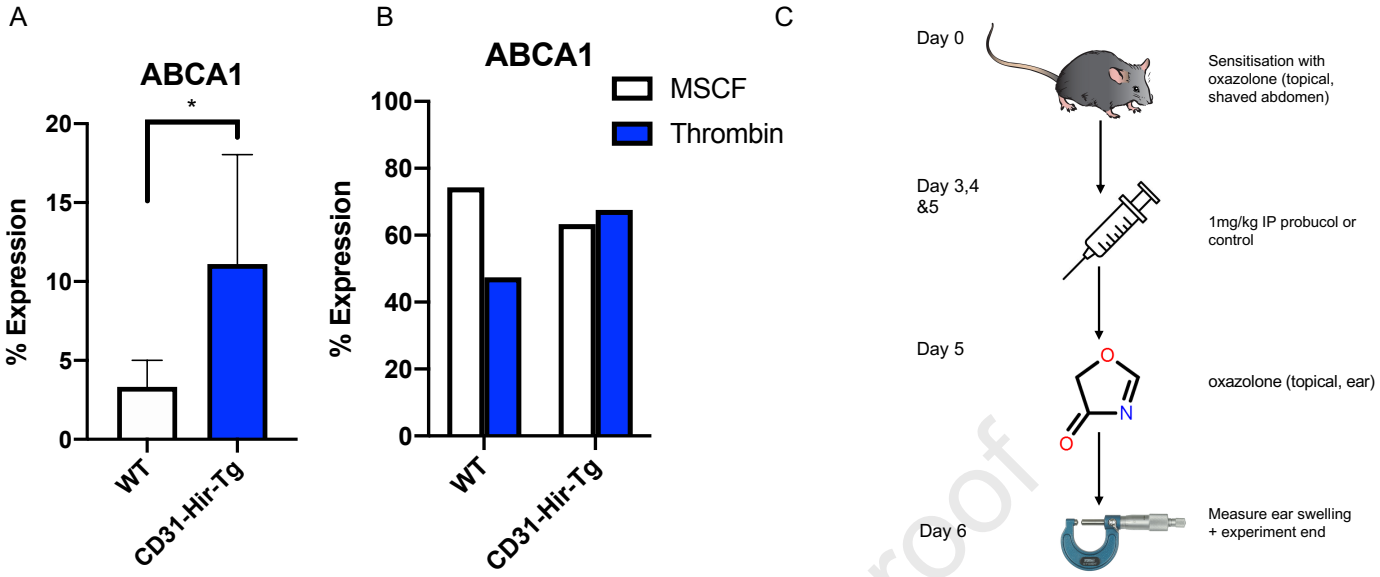

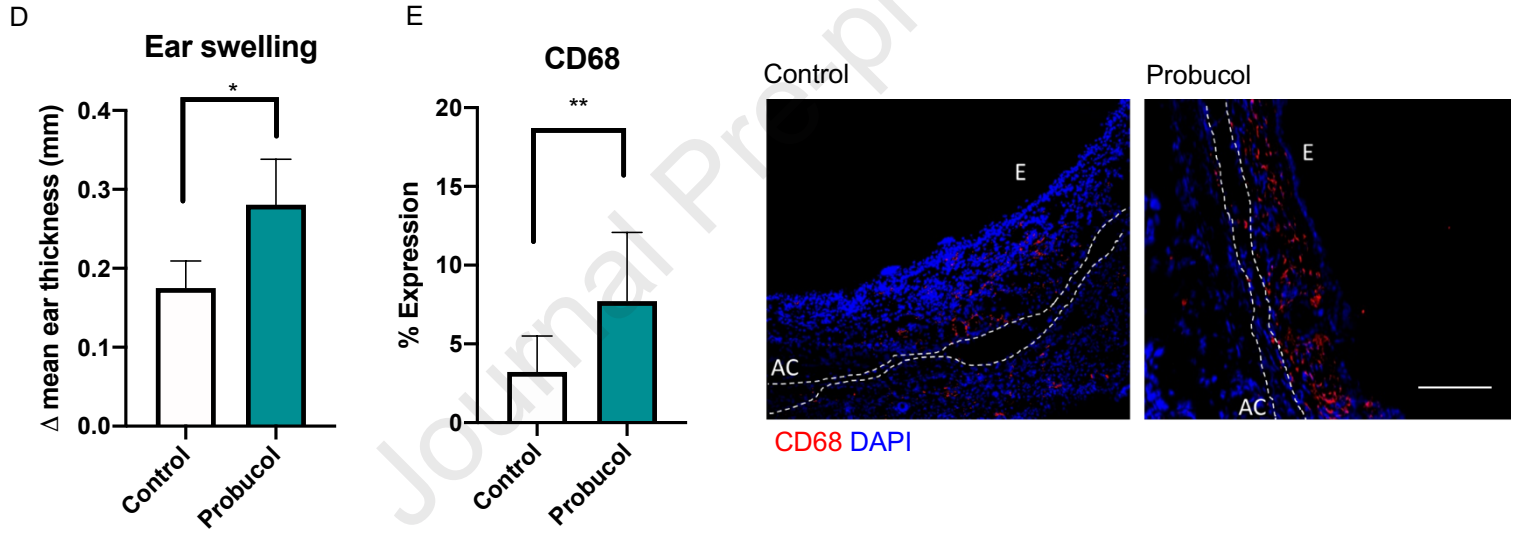

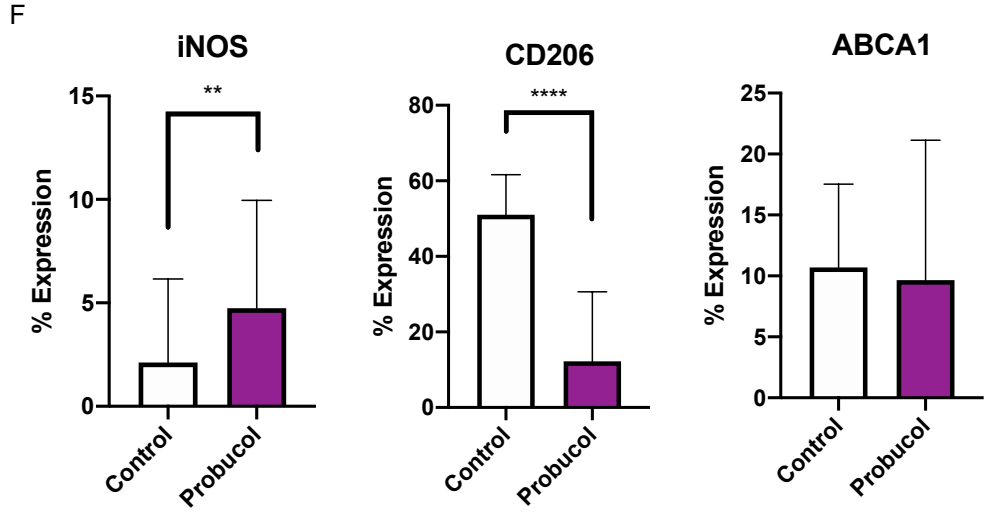

Inhibiting thrombin signalling on monocytes is protective in type IV hypersensitivity

PTL060, a thrombin inhibitor, reduces inflammation in type IV hypersensitivity

Thrombin primes macrophages to be highly sensitive to IFNγ and LPS

Thrombin increases lipid rafts on macrophages in an ABCA1 dependent manner