

Protease-activated receptor 1 activation is necessary for monocyte chemoattractant protein 1-dependent leukocyte recruitment *in vivo*

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Thrombin, acting through a family of protease-activated receptors (PARs), is known to amplify inflammatory responses, but the *in vivo* importance of PARs in inflammation is not fully appreciated. In a mouse heart-to-rat transplant model, where it is possible to distinguish graft (mouse) from systemic (rat) chemokines, we show that donor PAR-1 is required to generate the local monocyte chemoattractant protein (MCP)-1 needed to recruit rat natural killer cells and macrophages into the hearts. We have confirmed the importance of this mechanism in a second model of thioglycollate-induced peritonitis and also show that PAR-1 is important for the production of MCP-3 and MCP-5. Despite the presence of multiple other mediators capable of stimulating chemokine production in these models, these data provide the first evidence that thrombin and PAR activation are required *in vivo* to initiate inflammatory cell recruitment.

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Thrombin and other coagulation proteases mediate a variety of effects independently of thrombosis through specific protease-activated receptors (PARs), stimulation of which can amplify inflammation initiated by several diverse stimuli (1). Although the proinflammatory consequences of PAR stimulation have been implicated in several diseases (2–4), it has yet to be established whether thrombin or PAR activation provides a unique stimulus responsible for a nonthrombotic manifestation of inflammation.

Working in a model of acute humoral xenograft rejection (mouse heart into rat), we previously reported that inhibiting thrombin generation or inhibiting thrombin itself inside the graft (using organs from transgenic [Tg] mice expressing endothelial cell [EC]-tethered anti-coagulants) completely inhibited humoral rejection so that hearts were rejected by infiltrating T lymphocytes (5). These findings were surprising because the rejected hearts had significant Ig and C deposition on graft ECs. Inhibiting thrombosis by depleting fibrinogen from the recipients

(using a snake venom protein, ANCROD) failed to achieve the same degree of survival, and under these conditions, infiltrating NK cells and macrophages (MΦs), rather than T cells, were observed in rejected grafts (6). From these studies we hypothesized that thrombin was providing a stimulus in the humoral rejection process that was necessary for the infiltration of NK cells and MΦs. We have tested this hypothesis and confirmed our conclusions in a second model of thioglycollate-induced MΦ recruitment into the peritoneum.

RESULTS AND DISCUSSION

Donor monocyte chemoattractant protein (MCP)-1 is required for NK cell and MΦ recruitment

We hypothesized that infiltration of NK cells and MΦs into rejecting mouse hearts was due to the establishment of a chemokine gradient, most likely MCP-1, a CC chemokine known to be essential for NK cell and MΦ recruitment.

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We confirmed that rejection of WT hearts was associated with elevated plasma levels of both recipient and donor MCP-1 (Fig. 1 a). Hearts from MCP-1 KO mice were transplanted into ANCROD-treated rats. As previously reported, pre-transplant fibrinogen levels were depleted to 5% of the levels seen in control rats, and hearts rejected by ANCROD-treated rats contained no evidence of fibrin deposition (5). Fibrinogen levels were maintained at 5–8% control values to the time of rejection (0.23 ± 0.03 in ANCROD-treated rats vs. 2.78 ± 0.4 g/liter in controls; $n = 3$; $P < 0.0001$). MCP-1 KO hearts had a mean survival time (MST) of 5.5 ± 0.22 d (vs. 3.83 ± 0.31 d for WT plus ANCROD; $n = 6$; $P = 0.004$) and at rejection had fewer infiltrating NK cells and MΦs (Fig. 1, b and c) (6), despite high rat MCP-1 levels (Fig. 1 a). Thus, donor but not recipient MCP-1 was associated with infiltration by rat inflammatory cells and graft rejection. In vitro experi-

ments confirmed CCR2 expression by rat NK cells (as other species; reference 7) and showed that mouse MCP-1 acted as a chemoattractant for rat cells (not depicted).

Inhibition of PAR-1 activation inhibits donor MCP-1 production

Rejected hearts from Tg mice (CD31-Hir-Tg) expressing a tethered hirudin fusion protein (a direct specific inhibitor of thrombin) on activated ECs (5) showed minimal NK cell or MΦ infiltration, except when rats were given an inhibitory anti-hirudin mAb (Fig. 1, b and c). Rejection was associated with low plasma levels of both mouse and rat MCP-1 (Fig. 1 a), suggesting that both were thrombin dependent. When measured on day 3 and normalized for zero using values from recipients of MCP-1 KOs, circulating mouse MCP-1 levels in ANCROD-treated recipients of WT hearts were >10 times

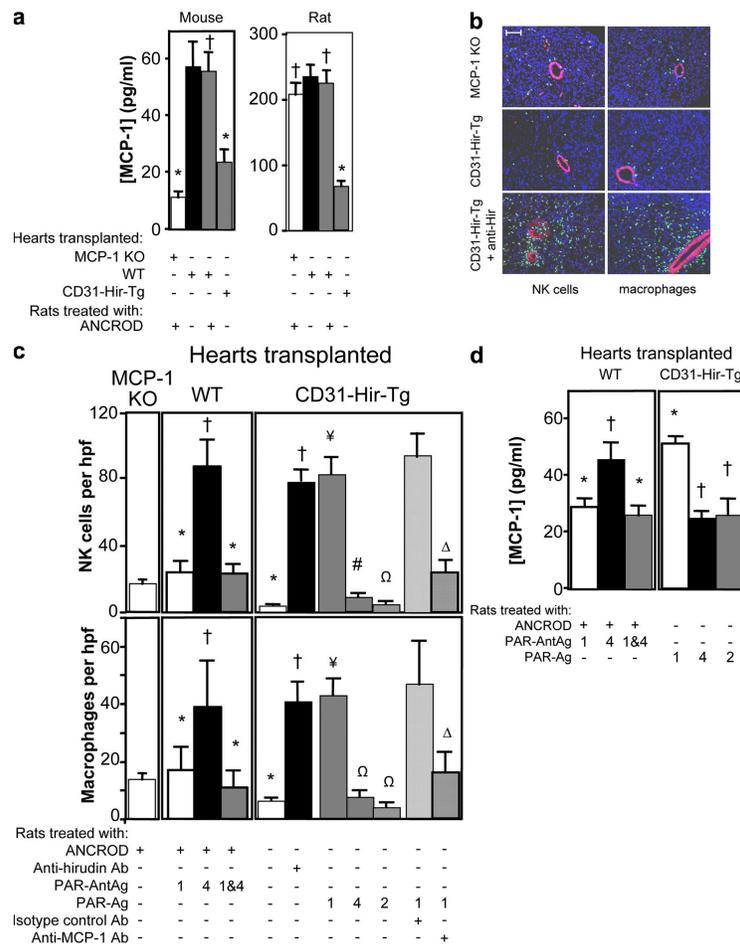


Figure 1. Role of donor versus recipient MCP-1 and PAR-1 versus PAR-4 in leukocyte recruitment in vivo. (a) Mouse and rat MCP-1 levels in rat plasma on the day of rejection (between days 2 [WT] and 7 [CD31-Hir-Tg]). $n = 6$. Compared with WT hearts in normal rats: *, $P \leq 0.002$; †p, NS. (b) Immunohistology of rejected hearts from MCP-1 KO or CD31-Hir-Tg hearts. Sections stained with DAPI (blue) and α -smooth muscle actin (red) and green, with CD161 (NK cells) or CD68 (MΦs). Bar, 100 μ m. (c) Cells infiltrating rejected hearts. Results are reported as cells per hpf \pm SEM. At least 100 cells from six fields on multiple sections were counted. Numbers in the grid represent the specific PAR antAg or Ag that rats received. WT compared with no antAgs: *, $P \leq 0.003$; †p, NS. CD31-Hir-Tg compared with MCP-1 KO: *, $P \leq 0.004$. Compared with CD31-Hir-Tg: †, $P \leq 0.001$; ¥, $P \leq 0.0003$; Ω, $P = NS$; #, $P = 0.01$. Compared with isotype control: Δ, $P \leq 0.007$. (d) Mouse MCP-1 levels on the day of rejection. WT, $n = 6$. Compared with WT hearts in defibrinogenated rats (a): *, $P = 0.001$; †, $P = NS$. CD31-Hir-Tg, $n = 6$. Compared with CD31-Hir-Tg hearts (a): *, $P = 0.001$; †, $P = NS$.

those seen in recipients of CD31-Hir-Tg hearts (37.9 ± 7.16 vs. 3.6 ± 1.9 pg/ml; $n = 6$; $P = 0.01$), indicating that >90% of mouse MCP-1 generation was inhibited by the hirudin. Therefore, we hypothesized that hirudin, by inhibiting thrombin, prevented the generation of mouse MCP-1.

To test this hypothesis, WT hearts were transplanted into rats given ANCROD with highly selective antagonists (antAg) to PAR-1, PAR-4, or both. Generation of mouse MCP-1 was inhibited only by the PAR-1 antAg to levels similar to those in animals transplanted with CD31-Hir-Tg hearts (compare Fig. 1, d and a). The PAR-1 antAg also inhibited NK and M Φ infiltration (Fig. 1 c) and prolonged survival (MST 5.83 ± 0.31 d; $n = 6$; $P = 0.003$ compared with ANCROD alone). Although the PAR-4 antAg prolonged survival (MST 5.17 ± 0.31 d; $n = 6$; $P = 0.006$ vs. WT plus ANCROD), rejection was accompanied by significant NK cell and M Φ infiltration (Fig. 1 c). Both antAgs together had no additional impact compared with PAR-1 antAg alone (MST 6.33 ± 0.33 d; $n = 6$; $P = NS$). The selectivity of these antAgs was confirmed in vitro (see Fig. 2). Donor MCP-1 production, recruitment of NK cells and M Φ s, and graft survival were not altered when rat recipients of CD31-Hir-Tg hearts were treated with PAR-1 or PAR-4 antAgs (not depicted).

PAR-1 activation promotes donor MCP-1 production

In contrast, when rats transplanted with hearts from CD31-Hir-Tg mice were given highly selective agonist (Ag) peptides for PAR-1, PAR-4, or PAR-2, the PAR-1 Ag promoted significant mouse MCP-1 generation (Fig. 1 d), infiltration by NK cells and M Φ s (Fig. 1 c), and accelerated rejection (MST 4 ± 0.26 d vs. 6.67 ± 0.21 d for PAR-4 Ag, $P = 0.0006$; 6.33 ± 0.21 d for PAR-2 Ag, $P = 0.0006$; or 6.67 ± 0.33 d for rats receiving no Ag, $P = 0.0006$; $n = 6$ each group). These data show that signaling through PAR-1 alone was sufficient to promote donor MCP-1 production and infiltration by NK cells and M Φ s. To show that MCP-1 was responsible for recruitment, recipients of CD31-Hir-Tg hearts were given an inhibitory anti-MCP-1 mAb with the PAR-1 Ag. Compared with controls, these hearts had few NK cells or M Φ s (Fig. 1 c) and survived longer (MST 5.33 ± 0.21 d vs. 3.83 ± 0.17 d; $n = 6$; $P = 0.001$).

Production of donor MCP-1 is dependent on activation of donor PAR-1

In vitro experiments with purified mouse heart microvascular ECs showed that MCP-1 production was mediated predominantly through PAR-1 (Fig. 2, a–d). In vivo, hearts from PAR-1 KO mice were transplanted into ANCROD-treated rats. Compared with defibrinogenated rats receiving WT hearts with PAR-1 antAg, levels of mouse MCP-1 were as low (compare Figs. 2 e and 1 d), hearts were as poorly infiltrated by either NK cells or M Φ s (compare Figs. 2 f and 1 c), and survival was comparable (MST 5.17 ± 0.31 d; $n = 6$; $P = NS$), and this was significantly longer than that in ANCROD-treated animals given WT hearts without PAR-1 antAg ($P = 0.02$). Therefore, generation of the donor MCP-1 was dependent

on direct PAR-1 activation on donor tissue, rather than being stimulated indirectly by the actions of rat cytokines generated in a thrombin-dependent way.

Donor PAR-1 activation enhances production of donor MCP-3 and MCP-5, but not recipient chemokines or cytokines

There were fewer NK cells and M Φ s in PAR-1 KO or CD31-Hir-Tg hearts than in MCP-1 KO hearts, despite the fact that both made more MCP-1, suggesting that additional donor

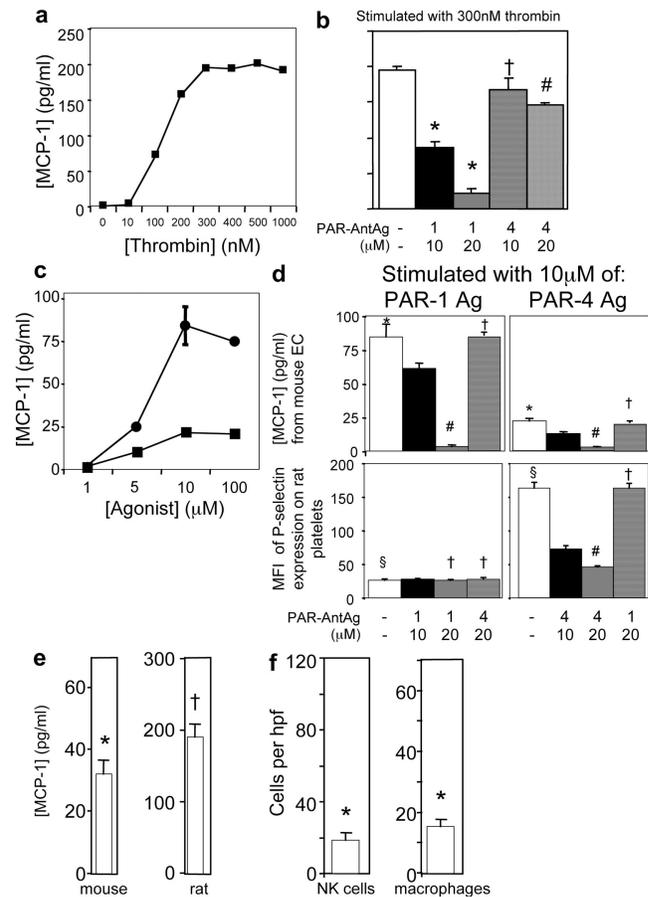


Figure 2. Role of donor PAR-1. (a) MCP-1 made by ECs stimulated by thrombin for 5 h. SEM bars are included but are too small to see. $n = 3$. Repeated twice. (b) MCP-1 (\pm SEM) made by ECs stimulated by 300 nM thrombin in the presence of PAR antAgs as indicated. $n = 3$. Compared with medium: *, $P \leq 0.004$; #, $P = 0.01$; †, $P = NS$. (c) MCP-1 (\pm SEM) made by ECs stimulated by PAR-1 Ag (●) or PAR-4 Ag (■). $n = 3$. (d) Shows the selectivity of the PAR Ags and antAgs. MCP-1 made by ECs and P-selectin on rat platelets after stimulation with 10 μ M PAR-1 Ag or PAR-4 Ag in the presence of PAR antAgs as indicated. $n = 3$. Comparing PAR-1 Ag to PAR-4 Ag: for mouse EC, *, $P = 0.03$; for rat platelets, †, $P = 0.0001$. Within each panel, compared with medium control: #, $P \leq 0.05$; †, $P = NS$. (b–d) Representative of two experiments. (e) Mouse and rat MCP-1 levels on day of rejection in defibrinogenated rats given hearts from PAR-1 KO mice. $n = 5$. Compared with WT hearts in defibrinogenated rats (Fig. 1 a): *, $P = 0.02$; †, $P = NS$. (f) NK cells and M Φ s infiltrating rejected PAR-1 KO hearts in defibrinogenated rats. Compared with WT hearts in defibrinogenated rats: *, $P \leq 0.001$.

chemokines may play a role in recruiting mononuclear cells. Consistent with this, mouse MCP-3 and MCP-5 were found circulating at lower levels in recipients of PAR-1 KO and CD31-Tg hearts than in recipients of WT hearts (Fig. 3, a and b). In the latter, levels were significantly inhibited by the PAR-1 antAg, whereas conversely, after CD31-Hir-Tg hearts were transplanted, levels were increased by the PAR-1 Ag (Fig. 3, a and b). Two other observations appear significant. First, consistent with a previous report (8), mouse MCP-3 was found at negligible levels after transplantation of MCP-1 KO hearts and after rats were given the anti-MCP-1 antibody (Fig. 3 a), suggesting that MCP-3 production is MCP-1 dependent. Second, donor MCP-5 levels were significantly inhibited and enhanced by the PAR-4 antAg or Ag, respectively (Fig. 3 b).

In contrast, rat MCP-1 was found at comparable levels in defibrinogenated recipients of WT and PAR-1 KO hearts (compare Figs. 2 e and 1 a), as were rat IFN- γ , TNF- α , and IL-6 (Fig. 3, c-e), indicating that donor PAR-1 was not involved. The concentrations of all four were also high in rats receiving MCP-1 KO or WT hearts in the context of an anti-MCP-1 antibody (Figs. 1 a and 3, c-e, and not depicted for rat MCP-1), indicating that levels did not correlate with the degree of mononuclear cell infiltration.

Nevertheless, rat MCP-1 (not depicted), IFN- γ , IL-6, and to a lesser extent TNF- α were significantly suppressed by the PAR-1 antAg when administered to ANCROD-treated rats and enhanced by the PAR-1 but not PAR-4 Ag when administered to rats receiving CD31-Hir-Tg hearts (Fig. 3, c-e). Both rat NK cells and M Φ s expressed PAR-1 and PAR-4 by flow cytometry (not depicted), so these data are most consistent with the hypothesis that thrombin, generated in the graft, amplifies chemokine and cytokine production by rat mononuclear cells via PAR-1 but is insufficient, by itself, to promote infiltration in the absence of mouse MCP-1 generation.

PAR-1 is important for MCP-1-dependent recruitment in a nontransplantation model

M Φ recruitment into the peritoneum after instillation of thioglycollate is known to be MCP-1 dependent (9), and we confirmed this using MCP-1 KO mice (Fig. 4, a and b). Using PAR-1 KO mice we demonstrated that levels of MCP-1, MCP-3, and MCP-5 (Fig. 4, c-e) and M Φ recruitment (Fig. 4, b and c) were dependent on PAR-1. WT mice injected with PAR-1 antAg and CD31-Hir-Tg mice had significantly reduced levels of circulating MCP-1, MCP-3, and MCP-5 and accordingly showed significantly reduced M Φ recruitment (Fig. 4, a-e). Finally, injection of PAR-1 Ag into the Tg mice significantly enhanced levels of circulating chemokines and M Φ recruitment (Fig. 4, b-e). No mice received ANCROD in these experiments. Therefore, PAR-1 is required to generate MCP-1, MCP-3, and MCP-5 for inflammatory cell recruitment.

The importance of MCP-1 for mononuclear cell recruitment has been shown in various animal models of disease (9-13). Our results confirm that peritoneal M Φ recruitment after thioglycollate is significantly reduced in MCP-1 KO mice, and we demonstrate for the first time the importance

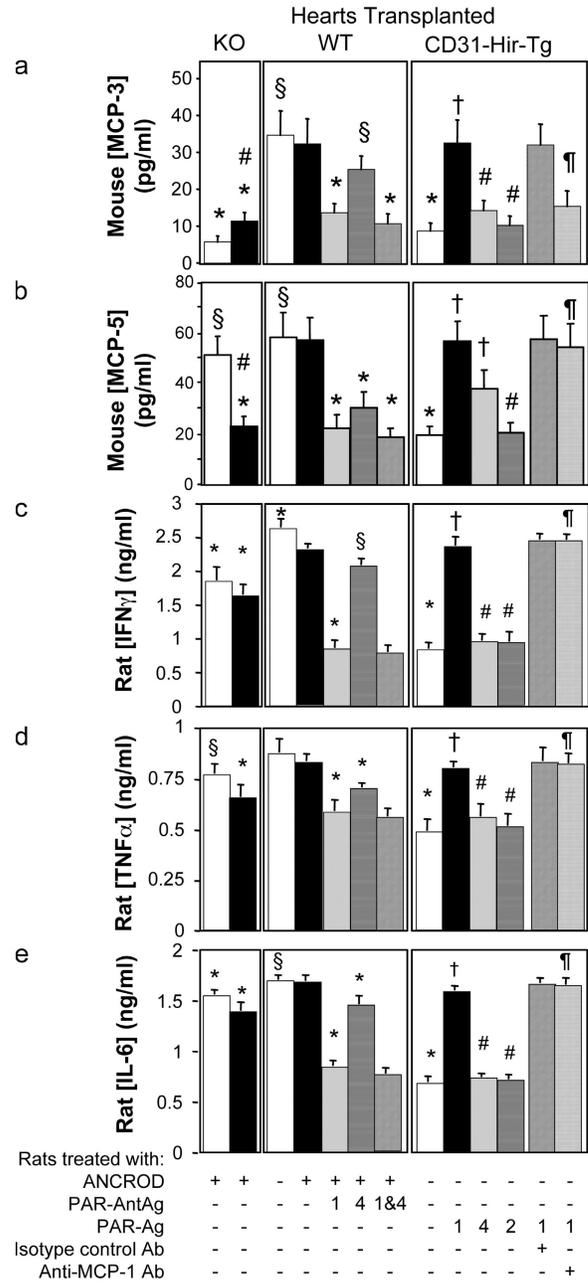


Figure 3. Mouse MCP-3, MCP-5, and rat cytokines. All graphs are organized in a similar pattern. (a) Mouse MCP-3, (b) mouse MCP-5, (c) rat IFN- γ , (d) rat TNF- α , and (e) rat IL-6. In KO column: white, MCP-1 KO; black, PAR-1 KO. Statistics: (a) MCP-3: compared with WT into defibrinogenated rats: *, $P \leq 0.01$; §, $P = NS$; CD31-Hir-Tg into normal rats: †, $P < 0.05$; #, $P = NS$; isotype control: ¶, $P < 0.05$; (b) MCP-5: compared with WT into defibrinogenated rats: *, $P < 0.02$; §, $P = NS$; CD31-Hir-Tg into normal rats: †, $P < 0.05$; #, $P = NS$; isotype control: ¶, $P = NS$; (c) IFN- γ : compared with WT into defibrinogenated rats: *, $P \leq 0.04$; §, $P = NS$; CD31-Hir-Tg into normal rats: †, $P < 0.02$; #, $P = NS$; isotype control: ¶, $P = NS$; (d) TNF- α : compared with WT into defibrinogenated rats: *, $P \leq 0.04$; §, $P = NS$; CD31-Hir-Tg into unmanipulated rats: †, $P = 0.002$; #, $P = NS$; isotype control: ¶, $P = NS$; (e) IL-6: compared with WT into defibrinogenated rats: *, $P \leq 0.04$; §, $P = NS$; CD31-Hir-Tg into unmanipulated rats: †, $P = 0.002$; #, $P = NS$; isotype control: ¶, $P = NS$.

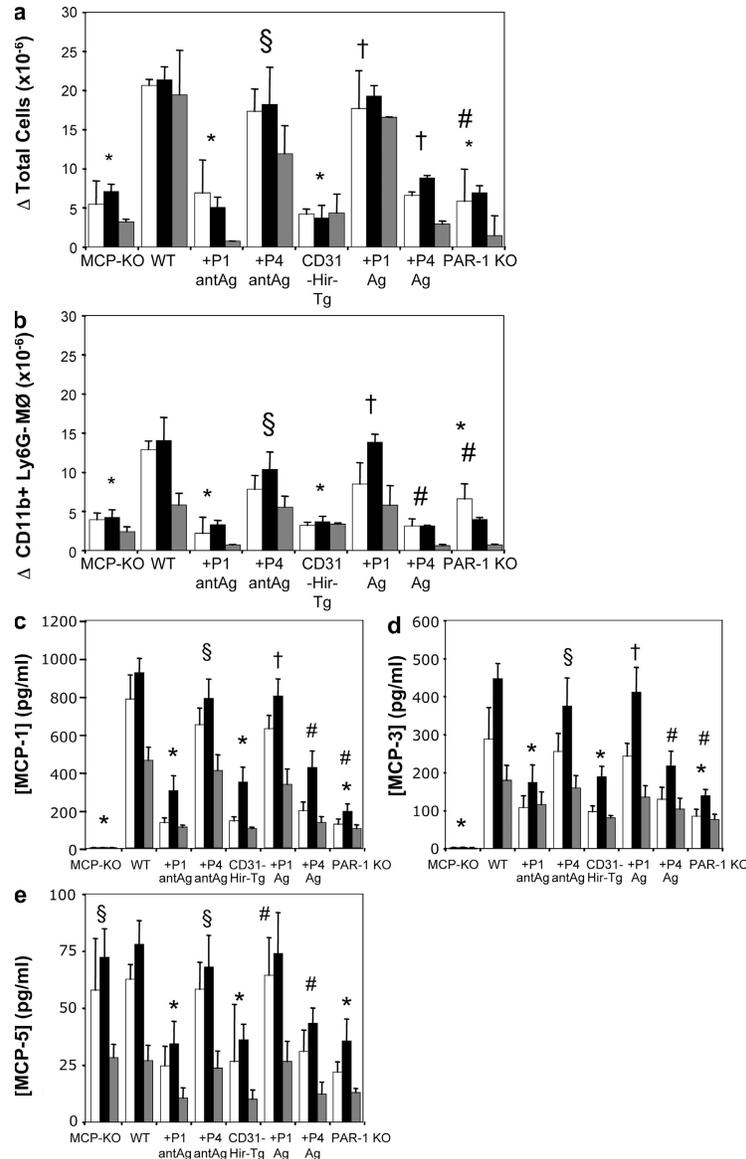


Figure 4. Thioglycollate peritonitis model. (a and b) Δ Total peritoneal cells (a) or Δ CD11b+Ly6G-M Φ s (b) isolated 48 (white), 72 (black), and 96 (gray) h after thioglycollate. $n = 3$ animals per group. Total cells counted manually. Percentage of M Φ s derived by flow cytometric analysis using anti-CD11b and Ly6G. Δ , numbers derived by subtracting the average cells in mice given control saline from experimental values after thioglycollate. Comparing peak cells to: WT: *, $P \leq 0.05$; §, $P = NS$; CD31-Hir-Tg: †, $P \leq 0.05$; #, $P = NS$. (c-e) Δ , MCP-1 (c), MCP-3 (d), and MCP-5 (e) concentrations in the same groups of mice as in a and b. Δ , concentrations derived by subtracting the average chemokine concentration in mice administered saline from the experimental values obtained after thioglycollate. Comparing peak [MCP-1] to: WT: *, $P < 0.007$; §, $P = NS$; CD31-Hir-Tg: †, $P < 0.02$; #, $P = NS$. Comparing peak [MCP-3] to: WT: *, $P \leq 0.01$; §, $P = NS$; CD31-Hir-Tg: †, $P < 0.05$; #, $P = NS$. Comparing peak [MCP-5] to: WT: *, $P < 0.02$; §, $P = NS$; CD31-Hir-Tg: †, $P < 0.05$; #, $P = NS$.

of this chemokine for NK cell and M Φ recruitment during acute humoral rejection. By performing the transplant study in a xenogeneic model, it was possible to differentiate between MCP-1 made by donor tissue from that made by recipient. Only the former was associated with infiltration into rejecting hearts.

Results from complimentary but distinct experimental approaches, using either WT or PAR-1 KO hearts transplanted into defibrogenated rats or CD31-Hir-Tg hearts transplanted

into unmanipulated rats, showed that PAR-1 activation on donor cells was necessary to generate sufficient quantities of donor MCP-1 to promote leukocyte infiltration. Similarly, MCP-1 generation and M Φ recruitment into the inflamed peritoneum after thioglycollate were PAR-1 dependent. Although this contrasts with findings from a previous study in PAR-1 KO mice, which nevertheless reported thrombin-dependent M Φ recruitment after thioglycollate (14), the fact that our distinct experimental approaches gave the same results

strengthens our conclusions over those made by Szaba and Smiley (14).

Our data also show the importance of thrombin and PAR-1 for the generation of MCP-3 and MCP-5, two other ligands of CCR2. We have not addressed the precise role of these chemokines relative to MCP-1 in our models, nor the interplay between them, particularly between MCP-1 and MCP-3. However, our observations are consistent with those of Inouye et al. (8), who, on finding significantly reduced MCP-3 levels in MCP-1 KO mice, suggested that MCP-3 might be regulated by MCP-1.

Previous work has shown that thrombin can promote the release of preformed MCP-1 from intracellular vesicles (15) and induce MCP-1 synthesis from various cells (16, 17), either directly through PAR-mediated signaling or indirectly via release of inflammatory cytokines from bystander leukocytes (18, 19). Our work complements and extends all these previous data by showing the critical role that thrombin plays in promoting MCP-1 generation *in vivo*.

The fact that PAR-4 signaling played no role in our models may be partially explained by well-defined differences in the avidity of thrombin for PAR-1 compared with PAR-4 (20). However, our results also show that maximal activation of PAR-4 on microvascular ECs was incapable of stimulating significant MCP-1 production (Fig. 2 c). In contrast, *in vivo* production of MCP-5 appeared equally reliant on PAR-1 and PAR-4.

This work is the first to demonstrate that thrombin is necessary *in vivo* to promote NK cell and M Φ recruitment through locally generated tissue chemokines. Although from the data presented here we cannot rule out an indirect role for thrombin through, for instance a thrombin-dependent PAR-1 activator such as activated protein C, it is likely that thrombin is acting directly on PAR-1, as the importance of PAR-1 signaling by activated protein C at physiological concentrations is under debate. That thrombin should be so important in the heart transplant model is surprising, as the humoral immune response against both WT and Tg hearts is intact (5, 6), with significant deposits of IgM, IgG (of all isotypes), C3, and C9 on the ECs of rejecting grafts. Many of these factors, but especially C components, have been shown in other model systems to induce MCP-1 generation (21) or have direct chemotactic activity for M Φ s (22), as have IFN- γ , TNF- α , and IL-6 (16, 23–25). However, our data indicates that each of these was unable to promote significant donor MCP-1 production in the absence of PAR-1.

Interestingly, systemic administration of PAR Ags did not lead to massively elevated levels of these rat cytokines, despite the theoretical potential for PAR to be activated on all circulating cells. The most likely explanation is that a second signal, generated by contact with the xenogeneic heart, for instance, is required for cellular activation. Consistent with this, *in vitro* cultures using rat NK cells revealed that PAR-1 Ags were incapable of stimulating IFN- γ secretion unless mouse ECs were present, in which case PAR-1 activation significantly enhanced IFN- γ production (not depicted).

Thrombin generation during systemic inflammation is highly dependent on the procoagulant changes induced on endothelium (26). Why mammals have evolved or maintained, as an integral part of EC activation, the switch from an anticoagulant to a procoagulant phenotype is interesting because if it results in intravascular thrombosis it can pose a direct threat to the survival of the individual, as often occurs during acute severe sepsis. The conventional explanation is that generating intravascular fibrin clots allows an organism to isolate certain types of infection (27) thereby limiting spread, a concept supported by a recent analysis showing the importance of plasminogen activators as pathogenicity factors for some types of bacterial infection (28). Our data support an additional and complimentary explanation that there may be other advantages to the organism, based on thrombin-mediated signaling through PAR on vascular cells, that mediate effective production of chemokines such as MCP-1, resulting in the efficient recruitment of leukocytes to clear or contain a localized source of inflammation.

MATERIALS AND METHODS

Animals. Male inbred Lewis rats (200 g) were from Harlan Olac. Donor hearts were from 25–30-g WT, CD31-Hir-Tg (26), MCP-1-KO (reared by V. Perry, University of Southampton, Southampton, England, UK) (9), or PAR-1 KO mice (29). Tg organs were from heterozygous and KO were from homozygous mice, backcrossed onto WT for >10 generations. All animal procedures were approved by UK Home Office.

Reagents. ANCROD (EC 3.4.21.74), BSA, goat and rabbit serum, and SLIGRL (PAR-2 Ag) were from Sigma-Aldrich. TFLLR (PAR-1 Ag), GYP-GKF (PAR-4 Ag), meroxaptopropionyl-Phe-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH₂ (PAR-1 antAg), and Trans-cinnamoyl YPGKF-NH₂ (PAR-4 antAg) were from Peptides International Inc. Thrombin was from Enzyme Research Laboratories Ltd.

Cell culture. Mouse microvascular ECs were purified and passaged as described previously (26) and used at passages 1–3.

Cardiac transplantation. Heterotopic heart transplantation was performed as described previously (5). PAR Ags (8 picomoles/g twice daily), PAR antAgs (10 μ g/g once daily [o.d.]), and ANCROD (0.08 U/g o.d. beginning pretransplantation) (6) were administered *i.v.* Anti-MCP-1 antibody (400 μ g pretransplant and 200 μ g o.d. thereafter) or control Armenian hamster IgG (eBioscience) was administered *i.p.* Graft rejection was defined as loss of regular palpable contractions on daily palpation. Graft MSTs are expressed as days \pm SEM, and statistical significance was determined using a log-rank test.

Thioglycollate-induced peritonitis. This was performed as by Lu et al. (9). Mice received 1 ml sterile 4% thioglycollate broth (Sigma-Aldrich) or control saline *i.p.* Cells were harvested by peritoneal lavage with 3 ml of ice-cold HBSS and washed and resuspended in 1.2 ml PBS for flow cytometric analysis with a rat Cy5-labeled anti-CD11b and FITC-labeled anti-Ly6G (both from Abcam) or manual counting with a hemocytometer.

Immunohistology. Sections were prepared exactly as described previously (5). The following antibodies were used: sheep anti-hirudin (Enzyme Research), rat anti-mouse CD31, FITC-conjugated goat anti-rat CD3, mouse anti-rat IgG (BD Biosciences), mouse anti-rat IgM, mouse anti-human α -smooth muscle actin (Sigma-Aldrich), goat anti-rat C3, goat anti-rat IgG1, goat anti-mouse IgG3 (all from Autogen Bioclear), FITC-conjugated mouse anti-rat CD161 (3.2.3), purified mouse anti-rat CD68 (ED2; Serotec Ltd), or rabbit anti-rat C9 (provided by B.P. Morgan, University of Cardiff, Cardiff,

Wales, UK). Appropriate second layer staining was with a sheep anti-mouse IgG-FITC, donkey anti-goat IgG-FITC (Sigma-Aldrich), goat anti-rabbit IgG-Texas red (Dako), or horse anti-mouse IgG-Texas red (Vector Laboratories). Many sections were also stained with DAPI (Sigma-Aldrich). Sections were examined on an immunofluorescence microscope (Axiovert S100 TV; Carl Zeiss, Inc.). NK cells and MΦs were counted manually from six different high power fields (hpf) from each of three different rejected hearts to generate cell infiltration data. Results are presented as mean cells per hpf \pm SEM.

ELISA. Protocols for rat TNF- α , rat IL-6 (Metachem Diagnostics), rat IFN- γ (Thermo Fisher Scientific), rat (Assay Designs) or mouse MCP-1 (R&D Systems), mouse MCP-3 (AXXORA Ltd.), mouse MCP-5 (R&D Systems), and fibrinogen (Genway Biotech, Inc.) were performed according to the manufacturers' instructions, with absorbance measured at 450 nm on a plate reader (Titertek Multiskan Plus). Alternatively, fibrinogen was measured using the Clauss method as described previously (26). All samples were run in triplicate, and results were expressed as mean \pm SEM. Experiments using purified reagents revealed no cross reactivity of the anti-mouse ELISA reagents with rat MCP-1 (unpublished data).

Platelets. Platelet suspensions were prepared as described previously (26) and suspended in PBS solution (10^8 /ml) containing 2.5 mM Gly-pro-Arg-Pro peptide (Sigma-Aldrich) with 5 mg/ml BSA. P-selectin expression after thrombin or PAR Ag was analyzed using FITC-conjugated anti-CD62P (Santa Cruz Biotechnology, Inc.) on a Beckman Coulter EPICS XL flow cytometer.

NK cells. Rat peripheral blood mononuclear cells were isolated by Ficoll-hypaque centrifugation, washed, and resuspended in medium containing 3% FCS for magnetic selection using anti-rat NK cell mAb 3.2.3 (Endogen). Staining was with goat polyclonal anti-CCR2 antibody (Autogen Bioclear) or goat IgG isotype control (Abcam) followed by FITC-conjugated anti-goat IgG FITC (Sigma-Aldrich).

Statistical analyses. Results were analyzed using a log-rank or Student's non-paired *t* test, and values were regarded as statistically significant if $P < 0.05$.

We thank V. Perry for rearing the MCP-1 KO mice.

This work was supported by the Medical Research Council, UK.

D. Chen and A. Carpenter performed the experiments. J. Abrahams performed animal husbandry and breeding. R.C. Chambers reared the PAR-1 KO mice and critically appraised the manuscript. R.I. Lechler initiated the work and critically appraised the manuscript. J.H. McVey provided technical expertise and supervision and critically appraised the manuscript. A. Dorling supervised the work and wrote the paper.

The authors have no conflicting financial interests.

Submitted: 11 July 2007

Accepted: 28 May 2008

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