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Targeted Deletion of CCR2 Impairs Deep Vein Thombosis Resolution in a Mouse Model[1,2]

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CCR2 is required for monocyte recruitment in many inflammatory processes, as well as conferring Th1 lymphokine responses. Deep vein thrombosis (DVT) resolution represents a specific inflammatory response whereby the thrombus must be dissolved for restoration of blood flow. Using a stasis model of DVT in the mouse, we investigated the role of CCR2 on DVT resolution. Genetic deletion of CCR2 ($CCR2^{-/-}$) was associated with larger thrombi at early and later time points, increased thrombus collagen, fewer thrombus monocytes (F4/80), and significantly impaired neovascularization. IL-2 and IFN-γ were significantly reduced in early $CCR2^{-/-}$ thrombi, whereas MCP-1 was significantly increased, and Th2 lymphokines were unaffected. Supplementation of $CCR2^{-/-}$ mice with IFN-γ normalized early thrombus resolution without increasing monocyte influx. Neither Ab depletion of IFN-γ nor genetic deletion of IFN-γ impaired early DVT resolution. Early fibrinolysis was not impaired in $CCR2^{-/-}$ mice, but a significant reduction in both matrix metalloproteinase (MMP)-2 and MMP-9 activity was observed. However, only MMP-9 activity was restored with administration of IFN-γ. We conclude that an early CCR2-dependent Th1 lymphokine response predominates in normal DVT resolution, mediates this in part by MMP-9 activation, but is not solely dependent on IFN-γ. *The Journal of Immunology,* **2006, 177: 3388–3397.**

Deep vein thrombosis (DVT)[4] is a major clinical problem, the incidence of which has not decreased over the last 20 years (1). Indeed, the U.S. Surgeon General has named DVT a national health problem in 2005. Anticoagulant therapy is efficacious when used prophylactically, but it carries a significant risk of major bleeding when used for long term therapy (2). Direct thrombolysis has not been established as a standard of care, given unremarkable clinical benefits (3). The mechanism of thrombus resolution is complex and involves plasmin-mediated fibrinolysis, as well as proteinase-mediated collagen breakdown (4–6).

Prior studies have suggested an important role for chemokines in DVT resolution. Chemokines are ubiquitous peptide inflammatory mediators that act as leukocyte chemotactic agents as well as cell activators. Although four main chemokine families exist, the ones most relevant to DVT resolution are the cysteine-cysteine (CC) and cysteine-X-cysteine (CXC) chemokine types. For example, both CXC and CC chemokines are present within a resolving thrombus (4), and exogenous administration of IL-8 (CXC chemokine) and MCP-1 (a CC chemokine) have been shown to accelerate thrombus resolution in experimental models (7, 8). Many pathophysiological processes involve CCR2 signaling, including peritoneal and pulmonary inflammation, and atherogenesis (9–11). It is likely that chemokine-directed leukocytes primarily mediate the DVT resolution. Monocytes are particularly important, and MCP-1 drives monocyte chemotaxis via CCR2 signaling (12–14). Similarly, monocytes expressing CXCR2 were also shown to be essential for early thrombus resolution, possibly by promoting fibrinolysis (5).

The CCR2 receptor also directs lymphokine responses via the Th1-Th2 axis. A typical Th1 lymphokine is IFN-γ, whereas Th2 lymphokines are exemplified by IL-4 and IL-13 (15, 16). These mediators are produced by activated monocytes as well as certain lymphocytes. Numerous prior studies have confirmed that CCR2 gene deletion ($CCR2^{-/-}$) confers a loss of Th1 lymphokine response and thus can act as a functional model for such (10, 17).

Restoration of blood flow in a thrombosed vein also involves neovascularization with functional flow channels, thrombus retraction, and fibrinolysis (7, 18, 19). Histological studies have confirmed the appearance of intrathrombus clefts and neovascular channels that evolve mostly within the first 4 wk (7, 18, 20). Pleotropic angiogenic growth factors released by polymorphonuclear neutrophils (PMNs) and monocytes are known to be present in the resolving thrombus and include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (21–23). The pathobiology of angiogenesis also involves CCR2 signaling (13, 24, 25). Fibrinolysis of venous thrombi is thought to be primarily mediated by urokinase plasminogen activator (uPA) from monocytic cells (26), as evidenced from impaired venous thrombus resolution in mice lacking uPA but not tissue-type plasminogen activator (27). Linked to the plasminogen activators are the matrix metalloproteinases (MMP), particularly MMP-2 and MMP-9, and which modulate collagen and matrix turnover in vessel wall remodeling and early wound healing (6, 28, 29).

The aim of this study was to characterize $CCR2^{+}$ leukocyte kinetics over time within the thrombus, delineate the role of the

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[4] Abbreviations used in this paper: DVT, deep vein thrombosis; PMN, polymorphonuclear neutrophil; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; uPA, urokinase plasminogen activator; MMP, matrix metalloproteinase; WT, wild type; IVC, inferior vena cava; vWF, von Willebrand factor; hpf, high power field; PAI-1, plasminogen activator inhibitor-1.

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early lymphokine response, and correlate this with thrombus resolution. We document that early DVT resolution is associated with a Th1 lymphokine response and that deficiency of CCR2 signaling is associated with impaired DVT resolution, in part because of reduced MMP-9 activity, and not fibrinolysis, suggesting a reciprocal relation between the plasminogen system and the MMPs. Furthermore, although exogenous administration of the Th1 lymphokine IFN-γ reverses the early CCR2$^{-/-}$-impaired DVT resolution, it is not necessary for this process.

Materials and Methods

Mouse model

The rodent model of stasis-induced DVT has been well described (4, 5, 7, 30) and used B6/129 (wild-type (WT) controls, F2 hybrids; The Jackson Laboraory) and B6/129 CCR2$^{-/-}$ mice (10) weighing 20–30 g, between 4 and 6 wk old. The mice underwent general inhalational anesthesia and, via a laparotomy, the inferior vena cava (IVC) was ligated with a 6-0 polypropylene suture. This consistently produces a stasis thrombus for tissue analysis. Mice were sacrificed at 2, 4, 8, 12, and 21 days. At harvest, the thrombosed IVC was weighed and measured, snap frozen, and stored at −70°C or placed in formalin, followed by ethanol, for histological analysis. Baseline comparisons of serum clotting factors with measurement of activated partial thromboplastin time and total clotting time of WT and CCR2$^{-/-}$ mice was done, as was peripheral differential leukocyte counts, using an automated system as described (5).

In a separate set of experiments, a group of WT mice underwent ligation and treated with i.p. saline, control nonspecific IgG, or 1 ml of 10 μg/ml anti-murine IFN-γ antisera (31). Another group, CCR2$^{-/-}$ mice, were given exogenous IFN-γ (R&D Systems; 50,000 IU i.p.) (32). All agents were administered at 24 h pre- and postligation. These groups were all harvested at 4 days. Finally, WT C57/BL6 and the corresponding IFN-$\gamma^{-/-}$ mice (all from The Jackson Laboratory) underwent the same procedure of IVC ligation and harvest at 4 days. Genetic deletion of IFN-γ was confirmed by RT-PCR genotyping using tail snip sample analysis per protocol (data not shown; www.med.umich.edu/tamc/pcr.html). The University of Michigan Committee on Use and Care of Animals approved this research protocol.

Laser doppler of IVC

The Lisca laser doppler (Lisca) was used to assess in vivo microvascular IVC blood flow, based on techniques described (33). After laparotomy, the pre-IVC ligation, immediate postligation, and harvest blood flow through the exposed IVC region of interest was assessed; a 5-s scan with 30-s interval, over four scanning cycles, was performed. Depth was constant and was adjusted for each mouse to ensure the best estimation of the midcoronal IVC section. These scans were saved, and accompanying image software was used to estimate the mean color flow by using a standardized area of analysis. The intensities were reported as percent of baseline blood flows, specific to each animal to ensure consistency.

Histological analysis/immunohistochemical staining/neovascular channel quantification/Trichrome staining

Immunohistochemical staining was performed on the paraffin-embedded tissue sections (10 μm) as described (7, 18, 19, 34). Anti-PMN (1/1000; Accurate Chemical and Scientific), anti-F4/80 (1/100; Serotec), anti-CCR2 (1/100; Santa Cruz Biotechnology), anti-uPA (1/250; Santa Cruz Biotechnology), and anti-von Willebrand factor (vWF; 1/100; Serotec) were used. Secondary Ab and color development was done using a Vecta ABC kit manufacturer's instructions. In a blinded manner, positively stained cells or neovascular channels in five high power fields (hpf; ×1000) radially within the thrombus were counted and totaled. Neovessels were counted only if a nucleus and cellular morphology were present (5). Other sections were processed per standard protocol for Trichrome staining (5, 7, 19).

Chemokine/cytokine/vascular growth factor ELISA

After thrombus-vein wall separation, the thrombus was placed in complete lysis buffer at 0°C (Boehringer Mannheim), homogenized, sonicated for 10 s, and centrifuged at 10,000 × *g* for 5 min, after which the supernatant was collected. Quantification of peptide mediators was normalized to total protein in the sample, by a modified Bradford assay per manufacturer's instructions (Pierce) with serial dilutions of BSA (Sigma-Aldrich) as standards. Tissue homogenate ELISAs for mouse MIP-1α, RANTES, MCP-1, TGFβ, IFN-γ, IL-2, IL-4, IL-5, and IL-13 were performed with species-specific primary Abs quantified using a double ligand technique, as has been described for similar chemokines (4, 5, 35). Mouse bFGF and VEGF were analyzed by using a commercial ELISA according to the manufacturer's instructions (R&D Systems).

Collagen assay

Thrombus collagen content was estimated by a commercially available kit according to the manufacturer's instructions (Bicolor), as described (19). Collagen amount was then corrected to thrombus weight for each sample (micrograms per milliliter per milligram of thrombus).

Fibrin staining protocol

Fibrin staining with anti-human fibrinogen Ab (1/100; Dako A/S), which cross-reacts with rat and mouse fibrin, was used for quantitative fibrin content analysis (5, 36). Computer imaging analysis was done to determine relative staining intensities as described.

Real time PCR

RNA was isolated from the thrombus homogenate using the Trizol reagent as described (6, 37). Expression of uPA, CCR2, MCP-1, and β-actin mRNA was determined from primer sequences derived using premier software (Premier Biosoft International) based on primary cDNA sequences from GenBank (http://www.ncbi.nlm.nih.gov-/GenBank/): β-actin: sense, GAGGGAAATCGTGCGTGACAT; antisense, AGAAGGAAGGC-TGG AAAAGAG; CCR2: sense, GGAGTGGGAAGAAGTATGT; antisense, TCAACCTTGGCAAGATAA; MCP-1: sense, TCCCTGTCATGCTTC TGG; antisense, CTGCTGGTGATCCTCTTGTA; uPA: sense, TATGCAG CCCCAC-TACTATGGCTC; antisense, GAAGTGTGAGACCCTCGT GTAGAC. For quantification of mRNA levels, expression of the target gene in ratio to β-actin expression was calculated by the formula: target gene expression/β-actin expression = $2^{-(\Delta Ct)}$ (38).

Gelatin SDS-PAGE substrate zymography

As described (39), precast 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin were used to determine MMP-2 and MMP-9 activity (unless otherwise stated, all zymography supplies were from Novex) of the thrombus and vein wall homogenate. Separate densitometry analysis was performed using a FOTO/Analyst CCD CAMERA (Fotodyne) and GEL-Pro Analyzer software version 3.1 (Media Cybernetics). Total activity (pro- and active) MMP activity was summed and normalized to thrombus weights (milligrams).

Western immunoblots

Protein was extracted from vein wall thrombus samples by overnight incubation in 1% SDS (6, 37). Western blotting for uPA and plasminogen activator inhibitor-1 (PAI-1) was performed as previously described (6). Primary Abs (uPA, Santa Cruz Biotechnology; PAI-1, BD Pharmingen) were diluted in Tris-buffered saline with Tween 20 to a dilution of 1/5000, and after secondary Ab application, the labeled proteins were detected via the application of ECL chemiluminescence agents (Detection reagents and film; Amersham Biosciences). Images were detected and densitometry performed as described for zymography.

Statistical analysis

Data were reported as mean ± SEM. All data were subjected to statistical analysis by *t* test to compare experimental groups with appropriate controls. Analysis was performed with Sigma Stat version 2.0 (SPSS), and graphic representations were created using Sigma Plot. $p < 0.05$ was assigned as significant.

Results

DVT resolution is impaired in CCR2$^{-/-}$ mice

Venous thrombi were significantly larger at 4, 12, and 21 days in the CCR2$^{-/-}$ mice, as compared with control (n = 13–15; Fig. 1*A*). At these time points, thrombus size was ~25–40% larger in CCR2$^{-/-}$ than in controls. Although WT DVT sizes decreased linearly over 21 days, this was delayed in the CCR2$^{-/-}$ mice. Serum clotting parameters of activated partial thromboplastin time and total clotting time were similar between the WT and CCR2$^{-/-}$ mice (data not shown), suggesting no intrinsic alteration in clotting factor production or activation.

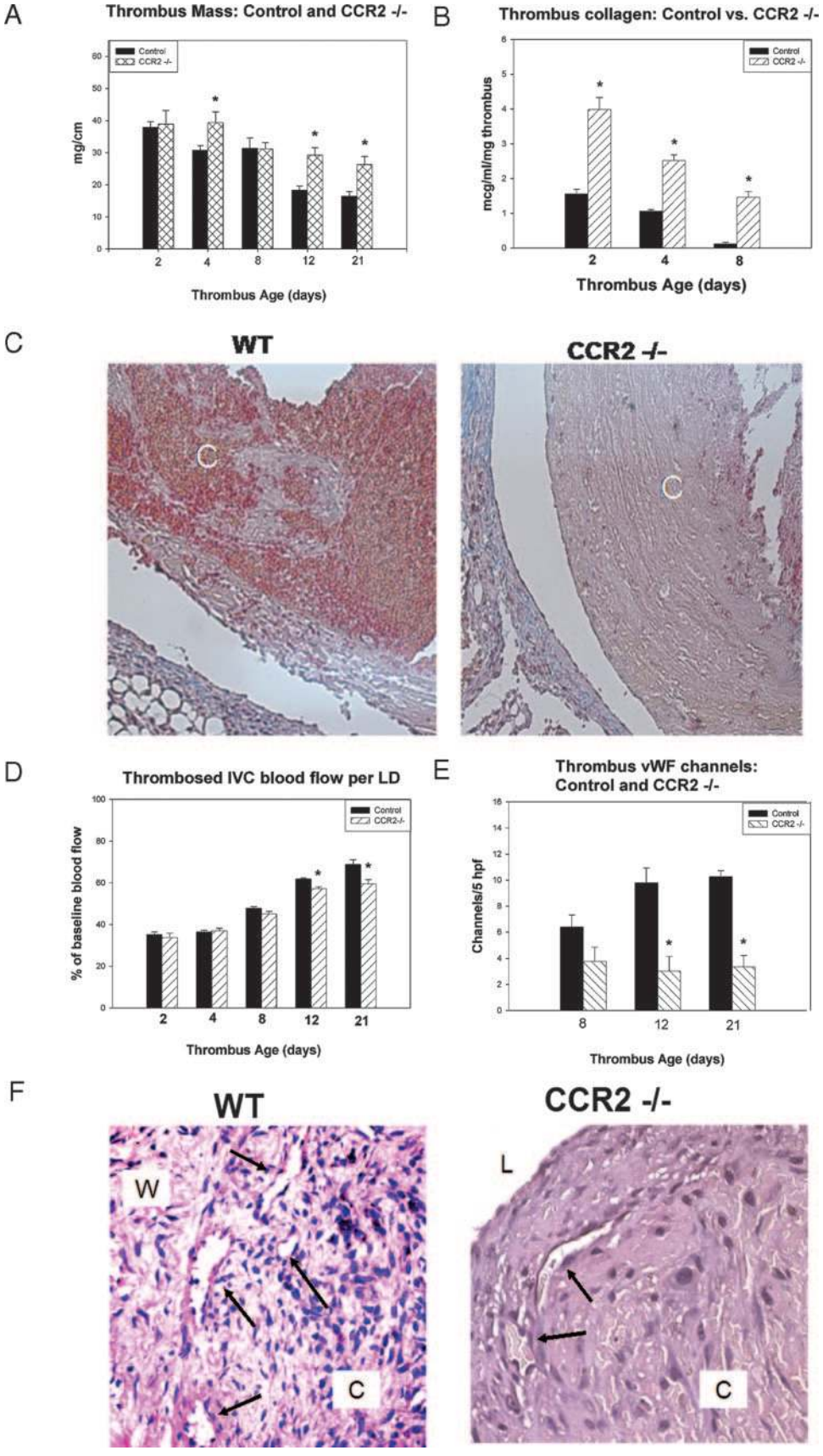

FIGURE 1. *A,* Thrombus resolution is reflected by the thrombosed IVC weight divided by length (mg/cm). The thrombus size was significantly greater in CCR2$^{-/-}$ mice as compared with controls at 4, 12 and 21 days. *, $p < 0.05$; $n = 13–15$. *B,* Total thrombus collagen was assessed by a colorimetric assay. At days 2–8, collagen was significantly greater in the CCR2$^{-/-}$ thrombi than in controls. *, $p < 0.05$, $n = 5$. *C,* Representative histological section of the thrombosed IVC (×400). Note the central portion of the thrombus is blue-white consistent with the presence of collagen. Less of this staining appearance is noted in the WT controls. C, clot. *D,* Laser doppler (LD) imaging of the pre- and harvest IVC comparisons of blood flow shows less IVC blood flow at later time points, 12 and 21 days, in the CCR2$^{-/-}$ mice as compared with controls. *, $p < 0.05$; $n = 5–13$. *E,* Thrombus neovascular channels were quantified by positive vWF staining. No significant staining is present before 8 days. Note significantly fewer channels in 12 and 21 days of CCR2$^{-/-}$ thrombi as compared with controls. *, $p < 0.05$; $n = 4–5$. *F,* Immunohistological representative example of a 12-day thrombosed IVC section (×400) stained for vWF. Note significantly more positive channels in the control thrombi than in CCR2$^{-/-}$ thrombi. Arrows, Channels; W, wall.

When evaluating the thrombus proper (for which consistent extraction is possible before 8 days), total collagen levels were significantly elevated by 30–60% in CCR2$^{-/-}$ thrombi at all time points, as compared with WT controls (Fig. 1*B*, $n = 5$, $p < 0.001$). Trichrome staining of thrombosed IVC sections qualitatively confirmed that increased thrombus collagen was present at these early

time points in CCR2$^{-/-}$ mice (Fig. 1*C*). Immunostaining of separate thrombi sections showed the collagen subtype to be primarily type III, with essentially no type I collagen staining (data not shown).

Later venous thrombus resolution is associated with neovascularization (5, 20, 23). Laser doppler imaging detects blood flow within and around the thrombosed IVC (33). In WT controls, blood flow returns to nearly 70% of normal nonthrombosed IVC flow by 21 days. However, 20–30% less blood flow was observed at later time points (days 12 and 21) in CCR2$^{-/-}$ thrombosed IVC as compared with WTs (Fig. 1*D*) ($n = 5$–9, $p < 0.05$). Consistent with these observations were ~3-fold fewer vWF-positive channels at days 12 and 21 in CCR2$^{-/-}$ mice thrombi than in WT controls (Fig. 1, *E* and *F*; $n = 4$–6, $p < 0.05$). This was particularly striking at day 21 between the groups. Because thrombus neovascularization was less at days 12 and 21 in the CCR2$^{-/-}$ thrombi than in WT controls, the proangiogenic factor VEGF was assessed by ELISA. VEGF levels were low (days 2–8; range, 15–75 pg/mg thrombus) within the thrombus and were not significantly different between the CCR2$^{-/-}$ and WT mice at any time point.

CCR2$^{-/-}$ mice have fewer thrombus monocytes and uPA-positive cells

CCR2 signaling directs monocyte chemotaxis and activity to areas of inflammation (9, 11, 13). In WT thrombus, F4/80$^+$ cell influx peaked at 8 days. As compared with WT, F4/80$^+$-staining monocytes were significantly reduced at all time points in CCR2$^{-/-}$ thrombi except at day 21 (Fig. 2, *A* and *B*; $n = 3$–4, $p < 0.05$). In WT only, CCR2$^+$-staining cells increased linearly from days 2 to 12, but absolute cell numbers at all times were less than the F4/80 monocytes (Fig. 2, *C* and *D*). Phenotypically, CCR2$^+$ cells were mononuclear type cells. As a corollary measure, thrombus CCR2 gene expression rose from day 2 and peaked at day 12 (2 days, $7.3 \pm .3 \times 10^{-5}$; 12 days, $5.0 \pm 3 \times 10^{-4}$ gene/β-actin, $n = 4$). No CCR2 gene expression was detectable in CCR2$^{-/-}$ mice.

No differences were observed in PMN number within the thrombus between the WT and CCR2$^{-/-}$ mice, with highest numbers at 2 days (26–34 cells/5 hpf) and lowest at 8 days (4–5 cells/5 hpf). Peripheral leukocyte counts were not significantly different comparing WT controls with CCR2$^{-/-}$ (8.2 ± 1 vs $6.5 \pm 0.6 \times 10^3$ cells/ml; $n = 11$–17, $p = 0.20$). The number of circulating monocytes were less in the CCR2$^{-/-}$ mice (0.31 ± 0.04 vs $0.51 \pm 0.08 \times 10^3$ cells/ml; $n = 11$–17, $p = 0.02$).

Prior experiments have shown a correlation between uPA$^+$-staining cells and thrombus resolution (5, 27). No difference in uPA$^+$-staining cells was present before day 8 in WT and CCR2$^{-/-}$ thrombi. However, ~40% fewer uPA$^+$ cells at 8 and 12 days in the CCR2$^{-/-}$ thrombi than in WT controls was found (Fig. 2, *E* and *F*; $n = 5$–6, $p < 0.05$). However, analysis of the thrombus homogenate did not show significant differences in uPA gene expression at these time points (8 days, 4.3 ± 2 vs $0.96 \pm 0.1 \times 10^{-7}$; $n = 5$, $p = 0.3$; 12 days, 5.2 ± 3 vs $5.8 \pm 0.4 \times 10^{-8}$, $n = 5$, $p = 0.6$).

DVT in CCR2$^{-/-}$ mice have an altered chemokine milieu and an impaired Th1 lymphokine response

Thrombus mediators are produced both in the thrombus and by the surrounding vein wall cells, given the intimate physical proximity of the thrombus-vein wall interface (5, 18, 33). To evaluate the thrombus milieu, only the thrombus (and not wall) was evaluated at 2, 4, and 8 days after DVT (for which complete thrombus extraction is possible) for analysis (Fig. 3*A*). MCP-1 protein levels by

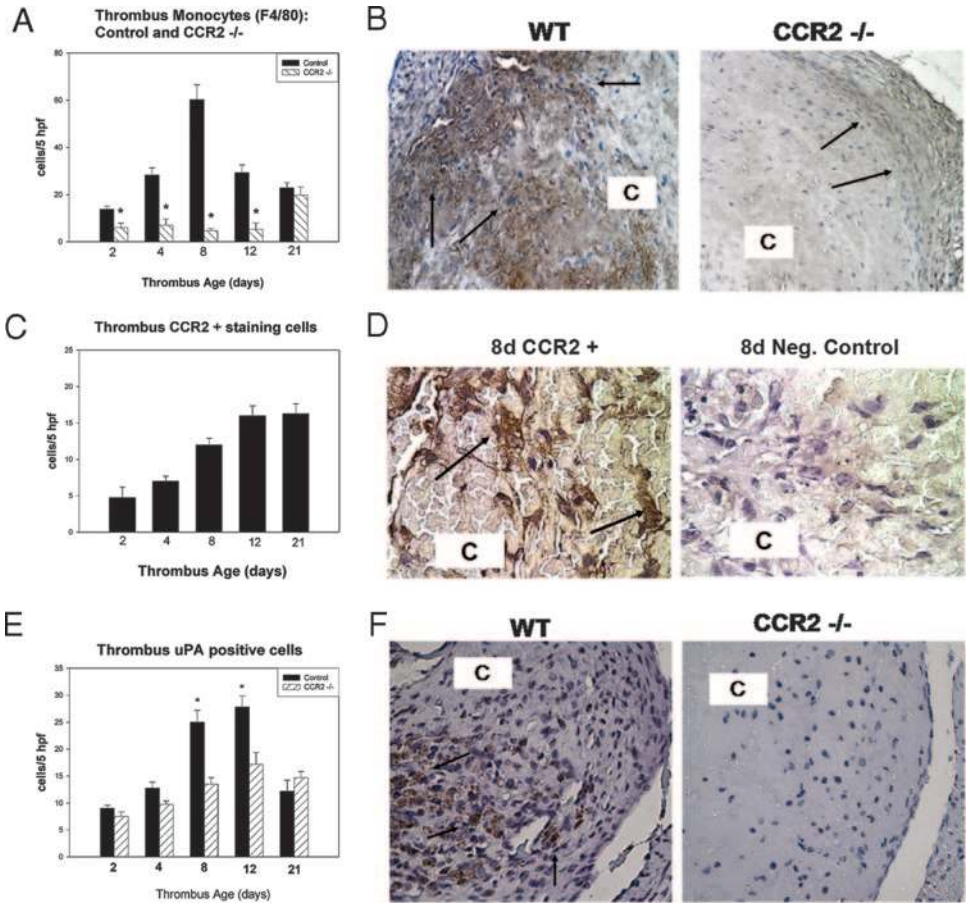

FIGURE 2. *A,* Quantification of F4/80$^+$ cells within the thrombus. Significantly fewer positive cells are present in CCR2$^{-/-}$ thrombi sections than in WT controls through 12 days. *, $p < 0.05$; $n = 3$–4 per time point. *B,* Representative IVC histology section of 8-day WT and CCR2$^{-/-}$ F4/80$^+$ cells (×400; arrows). Note the perithrombus distribution of cells, and significantly less positive staining is noted in the CCR2$^{-/-}$ mice, as well as overall less cellularity. *C,* Quantification of thrombus CCR2$^+$ cell staining is shown. Note the linear increase over time to level out after 12 days. $n = 3$–4 per time point. *D,* Representative 8-day (8d) thrombus IVC section stained for CCR2$^+$ cells (×1000; arrows). The cellular morphology is of mononuclear cell type. Neg., Negative. *E,* Quantification of uPA$^+$ cellular staining. Fewer uPA + cells are present at 8 and 12 days in CCR2$^{-/-}$ thrombi as compared with WT controls. *, $p < 0.05$, $n = 4$–5. *F,* Representative IVC section of uPA Ag immunostaining at 12 day time point (×400), arrows. Note significantly fewer + staining cells in the CCR2$^{-/-}$ thrombi and overall diminished cellularity. C, Clot.

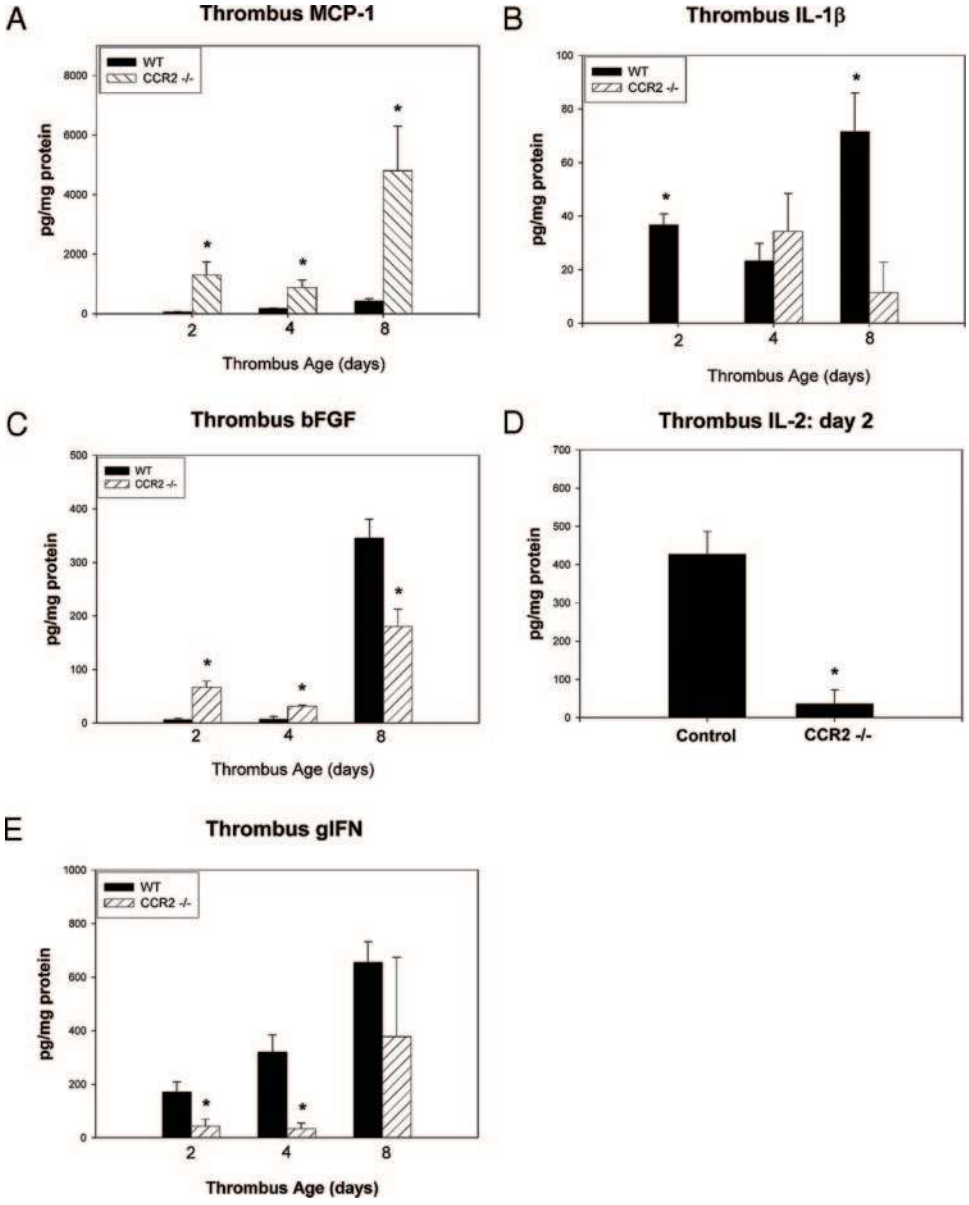

FIGURE 3. Thrombus homogenate ELISA for peptide mediators, corrected for total protein in the sample. *A,* MCP-1; *B,* IL-1β; *C,* bFGF; *D,* IL-2; *E,* IFN-γ (gIFN). All are corrected as picograms per milligram of protein. $*$, $p < 0.05$, $n = 5$ for each time point.

ELISA were significantly elevated at all time points within the thrombus ($n = 5$, $p < 0.001$) in CCR2$^{-/-}$ mice, peaking at 8 days. Thrombus MCP-1 gene expression was higher at 4 days in CCR2$^{-/-}$ mice by 7-fold (3.1 ± 0.5 vs $0.43 \pm 0.03 \times 10^{-2}$ gene-β-actin ratio; $n = 4$, $p = 0.002$). Levels of the CC chemokines MIP-1α and RANTES, which interact via CCR5 and not CCR2 (17), were not significantly different in the thrombus between the two groups through 8 days (data not shown). The proinflammatory mediator IL-1β was significantly reduced at days 2 and 8 in the CCR2$^{-/-}$ thrombi (Fig. 3*B*). The profibrotic factor bFGF was elevated at days 2 and 4, but a reciprocal relation was found at 8 days in CCR2$^{-/-}$ thrombi as compared with WT controls (Fig. 3*C*), whereas TGFβ levels were low and not significantly different between groups within the thrombus (range, 2–8 ng/mg protein).

The Th1 lymphokine levels were reduced in CCR2$^{-/-}$ thrombi as compared with WT controls. Thrombus IL-2 was significantly reduced at 2 days in CCR2$^{-/-}$ as compared with WT controls and was not detectable at other time points in either group (Fig. 3*D*). Thrombus IFN-γ at days 2 and 4 was decreased by nearly 80% (Fig. 3*E*; $n = 5$–6, $p < 0.01$). In contrast, no increase in the Th2 lymphokines, IL-4, IL-5, and IL-13 were found in the CCR2$^{-/-}$ thrombi as compared with WT controls, and no significant differences were found between the groups (data not shown).

The Th1 lymphokine IFN-γ is sufficient but not necessary for early DVT resolution

Given the strong association between early impaired DVT resolution, decreased IFN-γ levels and decreased monocyte influx, the 4-day time point was examined in detail. To better delineate the role of IFN-γ in modulating early DVT resolution, an addition and subtraction strategy was used. As shown previously, DVT sizes were significantly greater in CCR2$^{-/-}$ mice than were WT controls (Fig. 4*A*). The saline and IgG controls were combined as one group for purposes of analysis, given that there were no observed differences in any of the DVT resolution parameters. The impaired DVT resolution in CCR2$^{-/-}$ mice was totally reversed by exogenous IFN-γ, with an actual nonsignificant reduction in DVT size compared with controls (Fig. 4*A*; $n = 7$). In contrast, Ab depletion of IFN-γ of WT controls was not associated with an increase in DVT size.

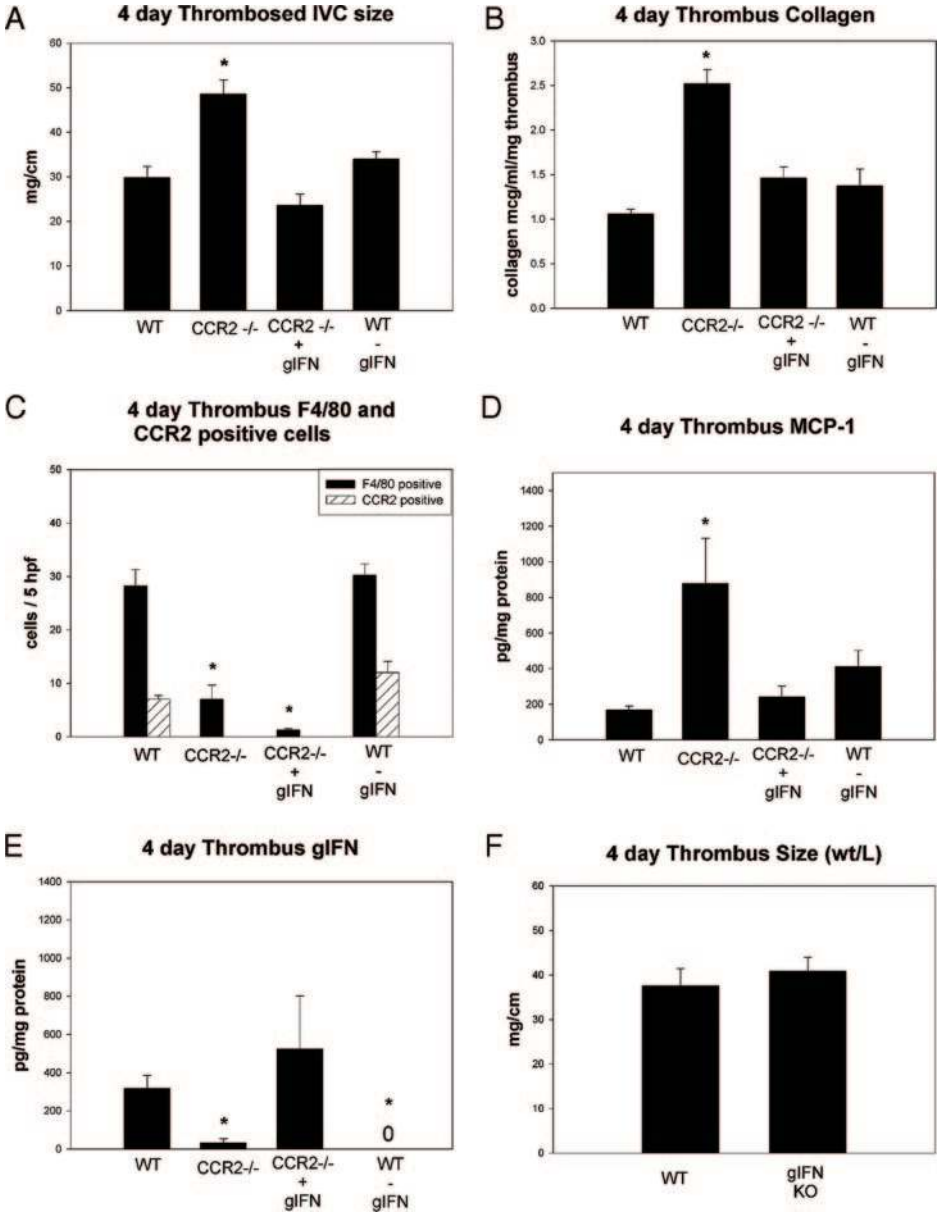

FIGURE 4. *A,* DVT sizes were significantly greater only in CCR2$^{-/-}$ mice, as compared with the other groups. Administration of IFN-γ restored DVT resolution, but inhibition of IFN-γ by Ab depletion did not impair DVT resolution in WT mice. $n = 7$ each group. *, $p < 0.05$. *B,* Total collagen in 4-day thrombus was greater only in CCR2$^{-/-}$ mice and was significantly reduced with IFN-γ (gIFN) administration. *, $p < 0.05$, $n = 5$–7. *C,* Comparison of cellular thrombus content in various conditions. Note that the CCR2$^{-/-}$ mice had significantly fewer F4/80^{+} cells and were not altered with the addition of IFN-γ. Similarly, anti-IFN-γ inhibition was not associated with reduced F4/80^{+} cells or reduced CCR2^{+} cell influx. *, $p < 0.05$, $n = 3$–5. *D,* Thrombus MCP-1 levels as measured by ELISA were elevated in CCR2$^{-/-}$ thrombi, reduced in CCR2$^{-/-}$ when exogenous IFN-γ was administered, and not affected by IFN-γ inhibition. *, $p < 0.05$, $n = 5$ each time point. *E,* Confirmation of prior experiments showed reduced levels of IFN-γ in CCR2$^{-/-}$ thrombi. This was restored by exogenous IFN-γ administration and was significantly reduced in WT by anti-IFN-γ Ab. $n = 4$–5 each time point. *, $p < 0.05$. *F,* The same IVC ligation model was used in C57BL/6 and C57BL/6 IFN-γ$^{-/-}$ mice to produce DVT. No significant difference in DVT sizes was observed. Note relative size of the WT C57BL/6 control thrombus weight (wt)/length was similar to the WT B6/129 control thrombus weight/length shown in the other experiments.

Collagen levels in CCR2$^{-/-}$ thrombi were also significantly reduced by exogenous IFN-γ administration to nearly WT levels, whereas Ab depletion of IFN-γ was not associated with an elevated thrombus collagen (Fig. 4*B*; $n = 5$). Intrathrombus cellular analysis showed that monocytes (F4/80) were not restored to WT levels in those CCR2$^{-/-}$ that received IFN-γ. Consistent with the fact that DVT sizes were similar, no reduction in F4/80^{+} monocytes or CCR2^{+} cells were found in those that received anti-IFN-γ Ab (Fig. 4*C*).

From the prior experiments, thrombus MCP-1 levels showed an inverse relationship with monocyte influx and impaired DVT resolution. Whereas MCP-1 levels were significantly elevated in CCR2$^{-/-}$ thrombi as compared with WT, exogenous IFN-γ administration to CCR2$^{-/-}$ mice was associated with reduced MCP-1 levels similar to WT controls (Fig. 4*D*). Similarly, mice that received anti-IFN-γ Ab had no elevation of thrombus MCP-1. To confirm the addition and subtraction strategies altered the IFN-γ thrombus milieu, IFN-γ levels were measured by ELISA. Importantly, IFN-γ was elevated slightly above baseline WT levels in those CCR2$^{-/-}$ mice that received IFN-γ and was reduced to undetectable levels in WT mice receiving anti-IFN-γ Ab (Fig. 4*E*).

To further confirm that IFN-γ was not necessary for DVT resolution, mice with targeted deletion of IFN-γ (C57BL/6-IFN-γ$^{-/-}$) and WT controls (C57BL/6) underwent IVC ligation to produce DVT and were harvested at 4 days. Consistent with the Ab depletion experiments, no significant differences in thrombus size (Fig. 4*F*) or thrombus collagen (WT, 0.42 ± 0.18 vs IFN-γ$^{-/-}$ = 0.82 ± 0.2 μg/mg thrombus, $n = 3$–4, $p = 0.2$) were noted in the IFN-γ$^{-/-}$ mice as compared with WT controls. By ELISA, no detectable IFN-γ was present, and MCP-1 levels were similar to WT levels (data not shown). Importantly, despite murine genetic background differences from the prior experiments, thrombus size at 4 days in C57BL/6 controls were not significantly different than in B6/129 WT controls, suggesting no species-specific intrinsic differences in DVT resolution at this time point.

CCR2$^{-/-}$ mice have reduced local vein wall MMP-2 and MMP-9 activity, and MMP-9 activity is restored with IFN-γ administration

The Th1 lymphokine IFN-γ has multiple inflammatory activities, including MMP activation (28, 32). First, to exclude that differential fibrinolysis might account for the observed difference in

DVT size, comparison of fibrin content by quantitative image analysis was done (5). No difference in thrombus fibrin content was found between CCR2$^{-/-}$ and WT controls (Fig. 5*A*). This finding is also supported by the observation that the number of uPA^{+} cells were similar in both CCR2$^{-/-}$ and WT controls at the day 4 time point (Fig. 2*E*). To further assess possible differential plasminogen system activation, Ag levels of uPA and PAI-1 were determined by Western immunoblotting, and a ratio calculated to yield the fibrinolytic to anti-fibrinolytic balance (6). Unexpectedly, CCR2$^{-/-}$ thrombi had the greatest relative amount of uPA, with a ratio 5–8 fold greater than WT, CCR2$^{-/-}$ + IFN-γ, and WT-IFN-γ murine thrombi ($n = 4$–5, $p < 0.01$; Fig. 5*B*).

Given that differences in fibrinolysis between WT and CCR2$^{-/-}$ thrombi were unlikely the mechanism of impaired DVT resolution, investigation of the proteinases MMP-2 and MMP-9 was done. Densitometric analysis of MMP-2 and MMP-9 total gelatinolytic activity showed significantly reduced levels in the vein wall homogenate and a trend toward less activity in the thrombus proper in CCR2$^{-/-}$ as compared with WT controls. IFN-γ administration partially restored MMP-2 activity but was still significantly less than WT controls. Anti-IFN-γ was associated with a significant reduction in MMP-2 activity (Fig. 5*C*). Conversely, the CCR2$^{-/-}$ mice that received IFN-γ had a significant increase in MMP-9 (to 60% of WT levels, $p = 0.3$), but not MMP-2 (Fig. 5*D*). Anti-IFN-γ Ab administered to WT mice significantly reduced MMP-2 but not MMP-9 activity. Similarly, no difference in MMP-2 and MMP-9 activity was found in the IFN-$\gamma^{-/-}$ as compared with WT controls (data not shown). These data also suggest that the primary collagenolytic activity is from the vein wall itself, and not derived from cells within the thrombus.

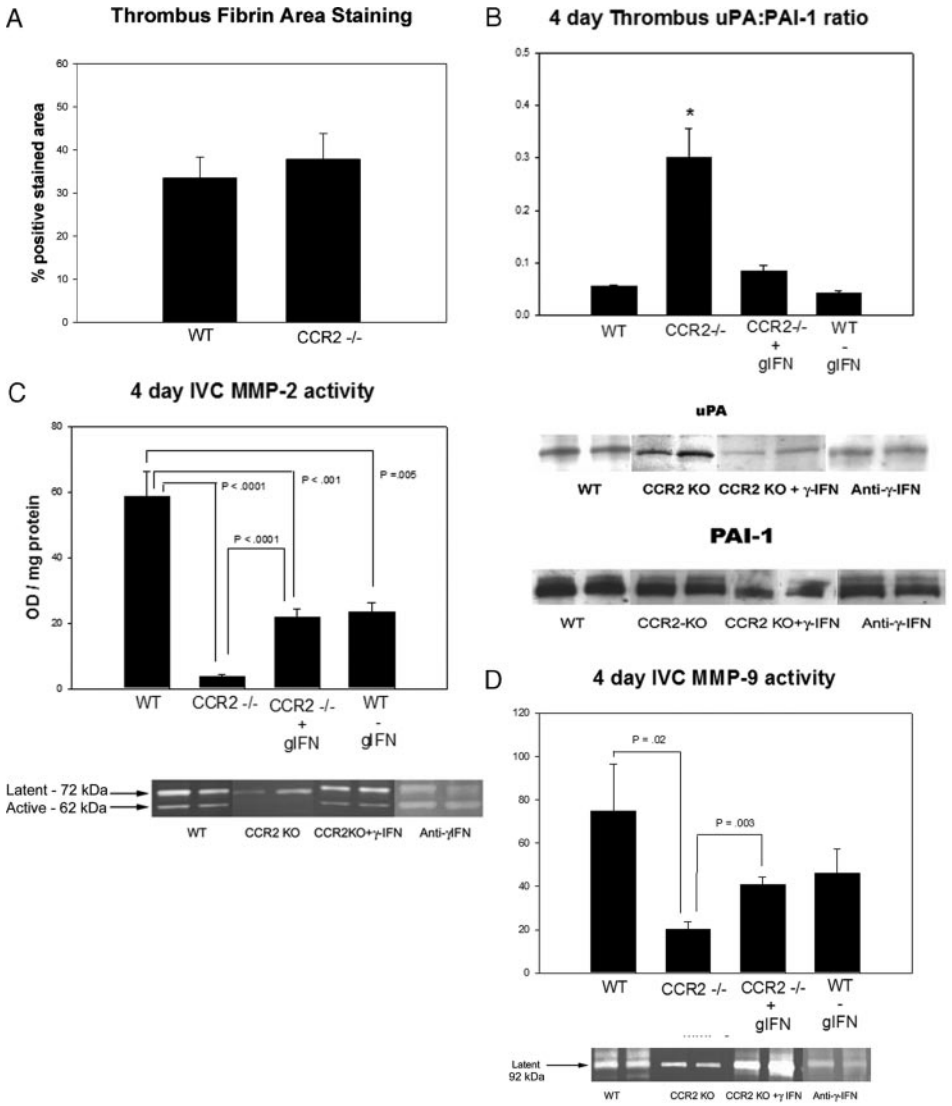

FIGURE 5. *A,* Comparison of 4-day fibrin content between WT and CCR2$^{-/-}$ showed no significant difference in positively stained area by image analysis, suggesting that fibrin content was not different between these groups. $n = 5$ for each group. *B,* Comparison of uPA and PAI-1 levels by Western immunoblotting were done to examine the relative fibrinolytic balance. Note that only CCR2$^{-/-}$ thrombi had a greater uPA content ratio whereas the CCR2$^{-/-}$ + IFN-γ (gIFN), and WT-IFN-γ were not different from WT alone. Representative Western immunoblot images are shown. KO, Knockout. *, $p < 0.05$, $n = 4$–5. *C,* IVC MMP-2 total activity was measured by zymography under conditions shown. As compared with WT controls, all groups had a significant reduction in MMP-2 activity. Note that CCR2$^{-/-}$ + IFN-γ had a partial restoration of MMP-2 that was significantly greater than CCR$^{-/-}$ + vehicle but did not reach control WT levels. Representative gel image is shown, and most zymographic activity is in the latent form. $n = 4$–5 mice per group. *D,* IVC MMP-9 activity as measured by zymography. Note that only CCR2$^{-/-}$ mice had a significant reduction in MMP-9 activity as compared with WT controls. Neither CCR2$^{-/-}$ + IFN-γ nor WT − IFN-γ MMP-9 activity was significantly less than WT controls. $n = 4$–5 mice per group. Representative gel image is shown, and most activity is in the latent form.

Discussion

Efficient thrombus clearance is essential for allowing prograde return of blood flow in the venous system after DVT and is clinically associated with less risk of postphlebitic syndrome (1, 40). Whereas other studies have suggested the early DVT environment is proinflammatory (4, 5, 8), the data herein support several mechanisms associated with CCR2 signaling activity that directly affects thrombus resolution (Fig. 6). Five interrelated conclusions can be drawn from the data: 1) a Th1 lymphokine response predominates in early DVT resolution; 2) loss of CCR2 signaling is associated with decreased thrombus neovascularization; 3) the MCP-1-CCR2 axis is important for monocyte influx into the resolving thrombus, independent of other CC chemokines; 4) IFN-γ is sufficient, but not necessary for DVT resolution; 5) vein wall MMP-9 may be more important than MMP-2 in early DVT resolution.

The data suggest that a CCR2-dependent Th1 lymphokine response occurs early in the resolving thrombus, and interference with this response is associated with impaired early and later DVT resolution. Specifically, a bimodal effect of the CCR2 signaling on leukocytes and surrounding vein wall cells after DVT seems to occur; namely, early events are probably due to early monocyte mediated events, such as thrombolysis and MMP activation, whereas later events may be related to other cellular healing processes, such as neovascularization and thrombus contraction. This notion is supported by the fact that $CCR2^{-/-}$ mice, functionally deficient in Th1 responses (10, 17), had larger DVT both early (day 4), and later, at days 12 and 21. The two primary Th1 lymphokines, IFN-γ and IL-2 (15, 41), were significantly decreased in the $CCR2^{-/-}$ thrombi, which is consistent with other disease models utilizing these mice (10, 14). How the loss of CCR2 signaling causes an impaired Th1 response has not been fully delineated, but it is probably due to decreased monocyte trafficking with loss of Th1-type lymphokine release (42). Overproduction of MCP-1, acting through a receptor independent of CCR2, may also inhibit the Th1 response, although this has been defined only in vitro (43). It is unlikely that lymphocytes play much of a role in this response as few are present in the thrombus beyond 2 days (4).

Later DVT resolution involves neovascularization (5, 20, 23). This was markedly impaired in $CCR2^{-/-}$ mice, with a significant reduction in thrombus neovascularization as measured by both in vivo laser doppler imaging and immunohistochemical analysis of neovascular channels as compared with WT mice. However, less difference in thrombus monocytes was found, including nearly identical numbers at day 21. This suggests that other processes that are not necessarily monocyte dependent may contribute to DVT resolution at the later time points, or that early monocyte presence confers long lasting effects on later DVT resolution. The proangiogenic activity of MCP-1 is well documented but is dependent on a functional CCR2 receptor, and despite significantly elevated thrombus MCP-1 in the $CCR2^{-/-}$ mice, impaired neovascularization was observed (13, 21). Others have found that $CCR2^{-/-}$ mice have impaired neovascular responses in various models of disease (12, 24). Although specific proangiogenic mediators were not evaluated at days 12 and 21 because of difficulty in clean separation of vein wall and thrombus, we speculate that reduced bFGF in $CCR2^{-/-}$ mice at 8 days (but not VEGF) may have contributed to this impaired later neovascular response. Basic FGF is a well-established proangiogenic and profibrotic mediator associated with DVT neovascularization (5). MMPs, in particular MMP-9, have proangiogenic activity by mediating basement membrane remodeling for endotheliazation of a tissue matrix (28, 29). Indeed, MMP-9 inhibition is associated with impaired neovascularization in an animal model of cardiac ischemia (44).

Monocytes are the most important leukocyte in modulation of DVT resolution. Monocytes release and direct smooth muscle cell proangiogenic mediator release, uPA, as well as MMP activity (5, 8, 26, 45). In support of this contention is that Ab inhibition and genetic deletion of IFN-γ did not impair DVT resolution, probably because these thrombi had a normal content of F4/80 and $CCR2^{+}$ cells. We speculate that these monocytes may mediate DVT resolution by promoting MMP release and activation from the adjacent vein wall cells. Other disease models involving $CCR2^{-/-}$ mice have also shown significant deficiencies in monocyte extravasation into inflammatory sites (9–11). Perhaps more important physiologically and consistent with our findings is that $CCR2^{+}$ monocytes are critical for influx of other monocyte subtypes, such as F4/80 cells (46). It was not surprising that DVT resolution was not impaired at 2 days, given that PMNs predominate in the thrombus and are critical for very early thrombus resolution (19). It was not as clear why 8-day $CCR2^{-/-}$ and WT mice had similar thrombus sizes, but other mechanisms may be more important at this time point and/or have attained a threshold of thrombus monocytes present to allow normal resolution. Alternatively, the activity of $CXCR2^{+}$ monocytic activity may predominate at this time point (5).

The observation that both MCP-1 gene expression and protein levels increases over time to peak at 8 days in WT controls and follows a similar bell-shaped curve to the influx of monocytes

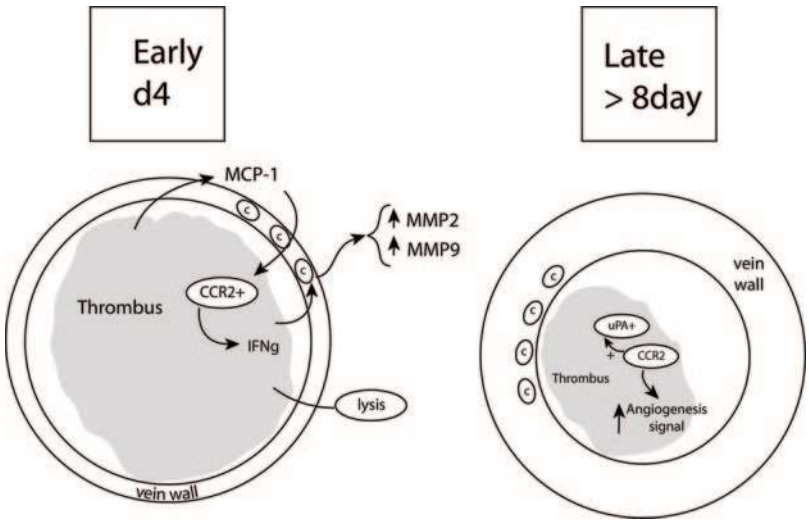

FIGURE 6. Proposed hypothesis of CCR2 signaling activity after stasis DVT in the mouse. Early events are primarily related to thrombolysis (collagenolysis) in part via MMP-2 and MMP-9. Later activities are related to thrombus neovascularization. D4, Day 4; C, vein wall cells.

suggests a central signaling role for the MCP-1-CCR2 axis in DVT resolution. Deletion of CCR2 was associated with greatly increased levels of MCP-1, suggesting loss of a normal feedback inhibition. Similar elevation of MCP-1 has been observed in other disease models with $CCR2^{-/-}$ mice (10, 35). Importantly, the other CC chemokines, such as RANTES and MIP-1α, presumably acting via competent CCR5 signaling, were not significantly upregulated in the $CCR2^{-/-}$ mice despite significantly fewer thrombus monocytes. This suggests that these other monocyte chemotactic signals do not compensate for the loss of CCR2 signaling and are probably not integral for DVT resolution. Lastly, although others have found that exogenous MCP-1 accelerated thrombus maturation (8), we have not found any significant effect at early time points of 4 and 8 days (our unpublished data) and suggest that other ligands of CCR2 such as MCP-2—MMP-5 may be important.

The presence of early collagen is consistent with a thrombus providing a healing matrix scaffold for scar formation (5, 19). Markedly increased thrombus collagen was observed in the $CCR2^{-/-}$ mice at early time points and at 4 days was associated with reduced vein wall levels of MMP-2 and MMP-9, suggesting impaired collagenolysis. This observation has also been described in a model of pulmonary and cardiac fibrosis in $CCR2^{-/-}$ mice, showing significantly reduced MMP-2 and MMP-9 activity (11, 47). Although MMP-9 has collagenolytic activity, it is primarily against type I collagen, for which little was present in the thrombus. More likely in this system is that MMP-9, and to a lesser extent, MMP-2 probably act against type IV collagen and laminin, both of which are present in the early thombus (Ref. 7 and our unpublished observations). Alternatively, a different collagenase, such as MMP-13, may be decreased with CCR2 gene deletion; thus, MMP-2 and MMP-9 may only be surrogate markers.

The association of CCR2 deletion with organ fibrosis is mixed in other disease models. For example, in the bleomycin pulmonary injury model, CCR2 deletion is associated with less late collagen accumulation (9, 11). In contrast, and more similar to the present findings, deletion of CCR2 was associated with significantly greater organ fibrosis in a pulmonary model of infection and a model of mechanical vascular injury (48, 49). Thus, the local environment and mechanism of injury may be the driving factor of how CCR2 signaling contributes to the fibrotic response. Although the Th2 responses are both anti-inflammatory and profibrogenic (15, 41), no elevation of Th2 lymphokines, such as IL-4 or IL-13, were found in the $CCR2^{-/-}$ thrombi to account for the increased thrombus collagen. However, it is possible that a relative imbalance existed in the $CCR2^{-/-}$ mice (i.e., significantly less Th1 lymphokines present) and that the Th2 lymphokines may have unimpeded activities.

Because deletion of CCR2 signaling is associated with impaired IFN-γ production (14, 28, 50) and IFN-γ is a known activator of a local MMP release (16), we asked whether exogenous replacement of this lymphokine could restore normal DVT resolution. Indeed, the DVT resolution was normalized with exogenous IFN-γ as measured by size, collagen content, and MCP-1 levels. Importantly, exogenous IFN-γ restored vein wall MMP-9, but not MMP-2, to nearly WT levels. This occurred despite significantly reduced thrombus F4/80 cells and no $CCR2^{+}$ cells. Conversely and consistent with these observations is that IFN-γ inhibition was associated with a significant reduction in MMP-2, but not MMP-9, and no impairment of DVT resolution. Although the presence of normal levels of MMP-9 may explain the lack of DVT inhibition, those WT mice that received anti-IFN-γ had a normal thrombus content of F4/80 and $CCR2^{+}$ cells. Taken together, these data support the notion that IFN-γ is sufficient but not necessary for DVT resolution. Given that IFN-γ Ab inhibition or genetic deletion did not impair DVT resolution, other signals including IL-1β and TNF-α may compensate for the loss of this signaling mediator (28). Consistently, thrombus IL-1β is present in the resolving WT thrombi and was significantly lower at 2 and 8 days after DVT in $CCR2^{-/-}$ mice. Finally, although IFN-γ can directly decrease collagen synthesis (51), we believe this is not a likely mechanism as anti-IFN-γ-treated mice had collagen levels similar to those of control WTs (Fig. 4*B*). Taken together, these findings suggest that a redundant system of MMP activation exists, and the most important common factor may be CCR2 signaling that mediates cross-talk between the vein wall and adjacent thrombus cells.

The plasmin system, by activation of uPA, is a primary mechanism of venous thrombolysis (27). It is now clear that arterial, pulmonary, and venous vascular beds may have differential thrombotic and fibrinolytic activities (52). Although we were not able to measure plasmin activity directly, the measures of uPA/PAI-1, quantification of thrombus uPA^{+} cells and gene expression at 4 days, and thrombus fibrin content all suggest that loss of CCR2 signaling did not inhibit this process. Although similar numbers of uPA^{+} cells were present, we speculate that each cell may have produced more uPA per cell to account for this difference. Indeed, the ratio of thrombus uPA to PAI-1 was elevated in the $CCR2^{-/-}$ mice but was normalized with the addition of IFN-γ (possibly because of restored MMP-9 activity). We speculate that later differences in DVT resolution associated with significantly fewer uPA^{+} cells in the $CCR2^{-/-}$ mice (at 8 and 12 days) were probably secondary to impaired monocyte influx, because monocytes are primary sources of uPA (26, 27). It is also unlikely that the $CCR2^{-/-}$ mice had any intrinsic procoagulant or fibrinolytic defects, given that as baseline clotting parameters were similar.

These findings have clinical applications. First, the addition of limited duration local pro-MMP-9 mediators may hasten thrombolysis in addition to the native or exogenously delivered plasminogen activators. More data are accruing that the longer a thrombus is in contact with the vein wall, the greater is the vein wall damage (40). Cellular specific modulation to hasten DVT resolution may be particularly fruitful for rapid reduction of thrombus burden without the complications of bleeding risk associated with anticoagulants.

Disclosures

The authors have no financial conflict of interest.

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