Original article

Effects of macrophage-dependent peroxisome proliferator-activated receptor γ signalling on adhesion formation after abdominal surgery in an experimental model

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Background: The pathophysiology of adhesion formation after abdominal and pelvic surgery is still largely unknown. The aim of the study was to investigate the role of macrophage polarization and the effect of peroxisome proliferator-activated receptor (PPAR) γ stimulation on adhesion formation in an animal model.

Methods: Peritoneal adhesion formation was induced by the creation of ischaemic buttons within the peritoneal wall and the formation of a colonic anastomosis in wild-type, interleukin (IL) 10-deficient (IL-10$^{-/-}$), IL-4-deficient (IL-4$^{-/-}$) and CD11b-Cre/PPARγ$^{fl/fl}$ mice. Adhesions were assessed at regular intervals, and cell preparations were isolated from ischaemic buttons and normal peritoneum. These samples were analysed for macrophage differentiation and its markers, and expression of cytokines by quantitative PCR, fluorescence microscopy, arginase activity and pathological examination. Some animals underwent pioglitazone (PPAR-γ agonist) or vehicle treatment to inhibit adhesion formation. Anastomotic healing was evaluated by bursting pressure measurement and collagen gene expression.

Results: Macrophage M2 marker expression and arginase activity were raised in buttons without adhesions compared with buttons with adhesions. IL-4$^{-/-}$ and IL-10$^{-/-}$ mice were not affected, whereas CD11b-Cre/PPARγ$^{fl/fl}$ mice showed decreased arginase activity and increased adhesion formation. Perioperative pioglitazone treatment increased arginase activity and decreased adhesion formation in wild-type but not CD11b-Cre/PPARγ$^{fl/fl}$ mice. Pioglitazone had no effect on anastomotic healing.

Conclusion: Endogenous macrophage-specific PPAR-γ signalling affected arginase activity and macrophage polarization, and counter-regulated peritoneal adhesion manifestation. Pharmacological PPAR-γ agonism induced a shift towards macrophage M2 polarization and ameliorated adhesion formation in a macrophage-dependent manner.

Surgical relevance

Postoperative adhesion formation is frequently seen after abdominal surgery and occurs in response to peritoneal trauma. The pathogenesis is still unknown but includes an imbalance in fibrinolysis, collagen production and inflammatory mechanisms. Little is known about the role of macrophages during adhesion formation.

In an experimental model, macrophage M2 marker expression was associated with reduced peritoneal adhesion formation and involved PPAR-γ-mediated arginase activity. Macrophage-specific PPAR-γ deficiency resulted in reduced arginase activity and aggravated adhesion formation. Pioglitazone, a PPAR-γ agonist, induced M2 polarization and reduced postoperative adhesion formation without compromising anastomotic healing in mice.

Pioglitazone ameliorated postoperative adhesion formation without compromising intestinal wound healing. Therefore, perioperative PPAR-γ agonism might be a promising strategy for prevention of adhesion formation after abdominal surgery.

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Introduction

Intra-abdominal adhesions may cause bowel obstruction, chronic abdominal pain, infertility and dyspareunia. More than one-third of patients are readmitted to hospital because of adhesions within 10 years after open abdominal or pelvic surgery[1]. Adhesions may also be responsible for the higher complication rates observed in reoperations[2] that may lead to poor quality of life.

The pathophysiology of intra-abdominal adhesion formation is, however, still poorly understood[3]. It is now widely accepted that injury to the peritoneal surface and the subsequent wound healing processes contribute to adhesion formation. Peritoneal adhesions do not originate exclusively from dysregulated collagen production and fibrinolysis, but also involve inflammatory responses[3]. Recent studies[4–7] have demonstrated the presence of leucocyte populations, including mast cells, T lymphocytes and macrophages, within adhesions. Macrophages probably play a key role, as systematic depletion of macrophages aggravated adhesion formation in a mouse model[8].

Macrophage function depends mainly on differentiation status. Cytokine and mediator expression profiles can be used to evaluate whether macrophage differentiation is towards a proinflammatory M1 phenotype, important during host defence, or an M2 phenotype, primarily involved in downregulation of inflammation, initiating wound repair and phagocytosis of apoptotic neutrophils and cell debris[9]. Levels of proinflammatory cytokines, such as tumour necrosis factor (TNF) α, interleukin (IL) 6 and interferon γ, have been shown to be raised in the M1 phenotype and in adhesive tissue[10], but are also expressed to some extent within the M2 phenotype. The cytokine and marker panel indicating changes to the M2 phenotype includes mannose receptor (MR) 1 (CD206), chitinase 3-like protein 3 (YM1) and arginase (Arg) 1[9].

Drivers of the M2 phenotype include Th2-derived IL-4 or IL-10[9] but also peroxisome proliferator-activated receptor (PPAR) γ[11], a ligand-activated nuclear receptor with potent anti-inflammatory properties that can be activated by endogenous ligands, such as lipid mediators or synthetic ligands from the class of thiazolidinediones including pioglitazone.

Although macrophage differentiation affects wound healing in several organs, such as skin, liver and lung[12], the role of macrophage differentiation is still controversial. Some reports have shown a profibrotic impact by Arg-1[13] and MR-1[14] expressing macrophages in fibrotic tissue[9], whereas others have described antifibrotic functions in animal models of cardiac fibrosis, Crohn's disease, renal fibrosis and arteriosclerosis[15]. Additionally, deletion of PPAR-γ in macrophages has been shown to suppress M2-like differentiation and to aggravate liver fibrosis in mice[16]. Here, the role of macrophage differentiation during intra-abdominal adhesion formation was investigated.

Methods

Experiments were performed with 6–8-week-old male wild-type (WT) C57BL/6J (Janvier, Saint Berthevin, France), and IL-$10^{-/-}$ and IL-$4^{-/-}$ (The Jackson Laboratory, Bar Harbor, Maine, USA) mice. CD11b lineage (predominantly affecting macrophage/monocyte) depletion of PPAR-γ was analysed in CD11b-Cre/$PPAR\gamma^{fl/fl}$ mice. $PPAR\gamma^{fl/fl}$ mice, without any cell-specific Cre expression and normal PPAR-γ activity, were used as controls. All mice had a mean bodyweight of 20–25 g at the time of use. The study was approved by the committee for animal experiments of North-Rhine Westphalia and performed in accordance with federal law regarding the protection of animals. Animals were maintained in specific pathogen-free housing on a 12-h light–dark cycle, and had free access to commercially available rodent chow and tap water. They were grouped up to five per cage. Animals were observed for signs of pain (weight loss, quality of fur). No adverse advent was observed during the experiments. The manuscript was written according to the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines.

All chemicals were purchased from Sigma (Taufkirchen, Germany) unless stated otherwise.

Pioglitazone treatment

Animals were gavaged daily with 1·25 mg pioglitazone (Import Gerke Pharma, Grevenbroich, Germany) dissolved in 0·5 per cent methylcellulose for 6 days, beginning 3 days before surgery. Control groups received 0·5 per cent methylcellulose as vehicle. Pioglitazone-treated animals and control groups were treated simultaneously, but housed in separate cages.

Surgery

Ischaemic button experiments

Surgery was performed under aseptic conditions. Anaesthesia was induced using isoflurane (Abbott, Wiesbaden, Germany). For analgesia, animals received carprofen 5 mg/kg bodyweight subcutaneously. Peritoneal adhesion formation was induced by the construction of four buttons on the peritoneal wall (*Fig. S1*, supporting information)[17]. Via a median laparotomy, the peritoneum was lifted with a clamp, and a ligature was applied by first stitching through

the base of the button and then ligating the peritoneum. Two buttons were placed on both sides of the peritoneum using a Vicryl® 6/0 suture (Ethicon, Somerville, New Jersey, USA). The abdomen was closed with a double-layered suture of the peritoneum (Vicryl® 5/0) and skin (silk 5/0; Braun, Sempach, Switzerland). Surgery was performed in all groups on day 0. Mice were killed on postoperative day (POD) 1, 3 or 7. Tissue samples were divided into buttons with and without adhesions. Before harvesting, adhesive tissue was stripped off buttons with adhesions. Unmanipulated peritoneal wall served as control. Eight mice were used per group for ischaemic button surgery, and five in the control group.

Colonic anastomosis

A standardized anastomosis model in mice was used, as described previously[18]. In brief, the abdomen was opened via a 3-cm midline incision. The ascending colon (1 cm from the caecum) was transected, strictly avoiding damage to the vessels. An end-to-end anastomosis was constructed with 12 interrupted full-thickness sutures (Vicryl® 8/0) before closure of the belly with a double-layered suture of the peritoneum (Vicryl® 5/0) and skin (silk 5/0). Eight animals were included per group for these experiments.

Functional analysis

Adhesion formation

Adhesion formation after ischaemic button surgery was quantified by counting adhesions on POD 7. In addition, an adhesion score was used[19]: score 0, no adhesions; score 1, thin, pellucid adhesions; score 2, tensile adhesion; score 3, inseparable and vascularized adhesion; score 4, entire abdomen linked by adhesions.

Adhesion formation in the colonic anastomosis experiments was quantified as the percentage of adhesions at the site of anastomosis; 0 per cent indicated absence of adhesions and 100 per cent indicated complete coverage of the circumference of the anastomosis. Adhesions around the anastomosis were also scored according to the method of Zuhlke and colleagues[20], whereby grade 0 represents no adhesions and grade 4 indicates manifest adhesions, dissectible only with sharp instruments.

Anastomotic bursting pressure

Anastomotic bursting pressure was measured directly after death by sampling a 3-cm colonic segment including the anastomotic site[18]. The anastomosis specimen was ligated at the distal end and connected via a catheter at the proximal end to a pressure transducer. Krebs–Henseleit buffer was infused at a constant rate via an infusion pump, and intraluminal pressure was recorded by means of a pressure transducer (Biopac Systems, Goleta, California, USA). A sudden loss of pressure indicated that the anastomosis had burst, and anastomotic bursting pressure was defined as the maximum intraluminal pressure before leakage.

Immunofluorescence

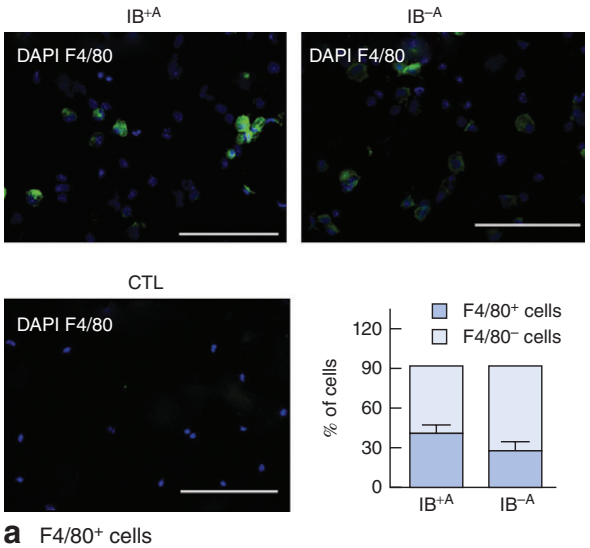
Cells from the control group, and buttons with and without adhesions were isolated after enzymatic digestion in a solution containing collagenase II (Worthington, Lakewood, New Jersey, USA), Dispase® II (La Roche, Mannheim, Germany), DNase (La Roche), bovine serum albumin and trypsin inhibitor. Cells were centrifuged on to glass slides (cytospin method) and stained with rat antimouse F4/80 (1 : 200) antibody (BM8; Life Technologies, Darmstadt, Germany) and Arg-1 (1 : 200) antibody (N20; Santa Cruz Biotechnology, Dallas, Texas, USA), followed by secondary donkey antirat Alexa 488 (Life Technologies) and donkey antigoat Cy3 (Dianova, Hamburg, Germany) antibodies. The nucleus was stained using 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies). $F4/80^+$ and $Arg\text{-}1^+$ cells were counted in five randomly chosen areas in each specimen at a magnification of ×200.

Quantitative PCR

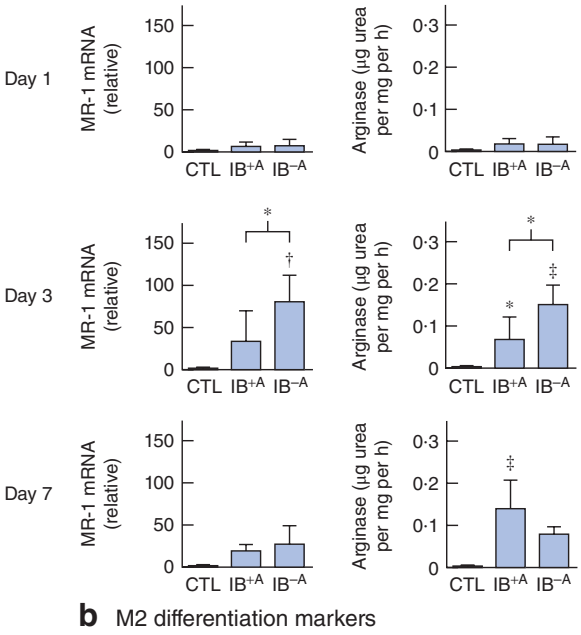
Gene expression of M1 and M2 markers was analysed by PCR. Reagents were from Life Technologies unless specified otherwise. Total RNA was extracted with Trizol® reagent using a tissue homogenizer (Precellys® 24; Peqlab, Erlangen, Germany) followed by DNase I treatment. cDNA was synthesized using a High Capacity cDNA rt kit. Expression of mRNA was quantified in triplicate by reverse transcription–PCR with specific probes/primers (*Table S1*, supporting information). The PCR was performed in Power SYBR® Green or Universal PCR Master Mix by amplification of 10 ng cDNA for 40 cycles (95°C for 15 s, 60°C for 1 min) on an AbiPrism® 7900HT (Life Technologies). Data quantification was performed by the $\Delta\Delta C_T$ method and value normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. IL-4 data were displayed as the ratio of IL-4 to GAPDH.

Arginase assay

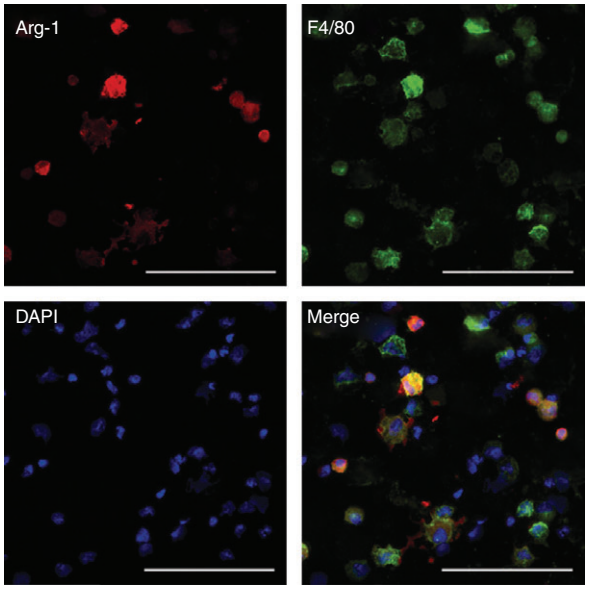
Arginase activity was determined as described previously[21]. In brief, proteins were isolated with 0·1 per cent Triton X-100, activated with 10 mmol/l manganese



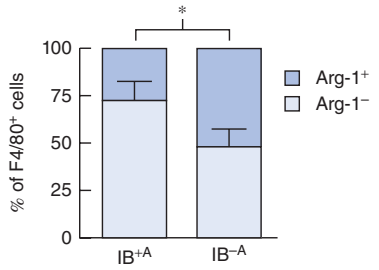
a F4/80⁺ cells



b M2 differentiation markers



c Arg-1 localization on F4/80⁺ cells



d Arg-1 expression on F4/80⁺ cells

Fig. 1 Macrophage infiltration and markers of M2 differentiation in ischaemic buttons. Ischaemic button (IB) tissue was collected from wild-type mice on days 1, 3 and 7 after surgery, and separated into buttons with (IB^{+A}) and without (IB^{-A}) adhesions. Unmanipulated peritoneal wall served as control tissue (CTL). **a** Representative immunofluorescence staining of $F4/80^+$ macrophages and nuclei (stained with 4′,6-diamidino-2-phenylindole, DAPI), and quantification of $F4/80^+$ and $F4/80^-$ cell populations relative to total cell counts in IB^{+A} and IB^{-A} groups on day 3 after surgery (original magnification ×400; scale bar 100 μm). Images are representative of five independent experiments. **b** Gene expression of mannose receptor (MR) 1 mRNA, relative to levels in CTL, and arginase activity. **c** Representative immunofluorescence staining for macrophage surface marker F4/80, M2 macrophage marker arginase (Arg-1) and nuclei (DAPI) in single-cell cytospin preparations of ischaemic button specimens (original magnification ×400; scale bar 100 μm). Images are representative of five independent experiments. **d** Quantification of $F4/80^+Arg\text{-}1^+$ and $F4/80^+Arg\text{-}1^-$ cells in IB^{+A} and IB^{-A} groups on day 3. Values are mean(s.d.) ($n = 5–8$ for all groups). $^*P < 0{\cdot}050$, $\dagger P < 0{\cdot}010$, $\ddagger P < 0{\cdot}001$ *versus* CTL unless indicated otherwise (one-way ANOVA with Bonferroni's *post hoc* test)

chloride and 50 mmol/l Tris–hydrochloric acid, and 0·5 mol/l L-arginine was added as substrate. The reaction was stopped by addition of 10 per cent sulphuric acid and 30 per cent phosphoric acid in water. Absorbance of the assay product urea was measured photometrically at 450 nm.

Statistical analysis

Continuous data are presented as mean(s.d.). Statistical analysis was performed using one- or two-way ANOVA with Bonferroni's *post hoc* test, or Students *t* test, with Prism® 5.02 software (GraphPad, La Jolla, California, USA). $P < 0{\cdot}050$ was considered statistically significant.

Results

Infiltrated M2 macrophages are associated with diminished adhesion formation

Adhesion formation started 24 h after ischaemic button placement, and the number and strength of adhesions increased up to POD 7. Immunofluorescence analyses showed equal numbers of infiltrated F4/80$^+$ macrophages within cytospin specimens of buttons with and without adhesions, whereas no macrophages were present in the unmanipulated peritoneal wall (*Fig. 1a*). Furthermore, transcription of the inflammatory mediators monocyte chemoattractant protein 1 and IL-6 was analysed, demonstrating the presence of inflammation up to POD 7 (*Fig. S2*, supporting information).

Ischaemic button specimens were examined for M2 differentiation markers including MR-1 and arginase. Raised gene expression of MR-1 was present in buttons without adhesions compared with controls ($P = 0{\cdot}002$) and buttons with adhesions ($P = 0{\cdot}039$) on POD 3 (*Fig. 1b*). Arginase activity also increased in the early postoperative phase in buttons without adhesions compared with those with adhesions (0·15(0·05) *versus* 0·07(0·05) μg urea per mg per h; $P = 0{\cdot}012$) on POD 3 (*Fig. 1b*). On POD 7 increased arginase activity was observed in buttons with adhesions compared with controls ($P < 0{\cdot}001$). Immunofluorescence analyses identified F4/80$^+$ macrophages as the exclusive cellular target of arginase expression (*Fig. 1c*). Corresponding to the arginase activity levels, larger numbers of arginase-expressing macrophages were found in buttons without *versus* with adhesions in WT mice (52·5(9·7) *versus* 28·0(10·7) per cent; $P = 0{\cdot}013$) (*Fig. 1d*).

PPAR-γ modulates macrophage differentiation and affects adhesion formation

Gene expression analysis of three possible inducers of M2 differentiation (IL-4, IL-10 and PPAR-γ) showed total

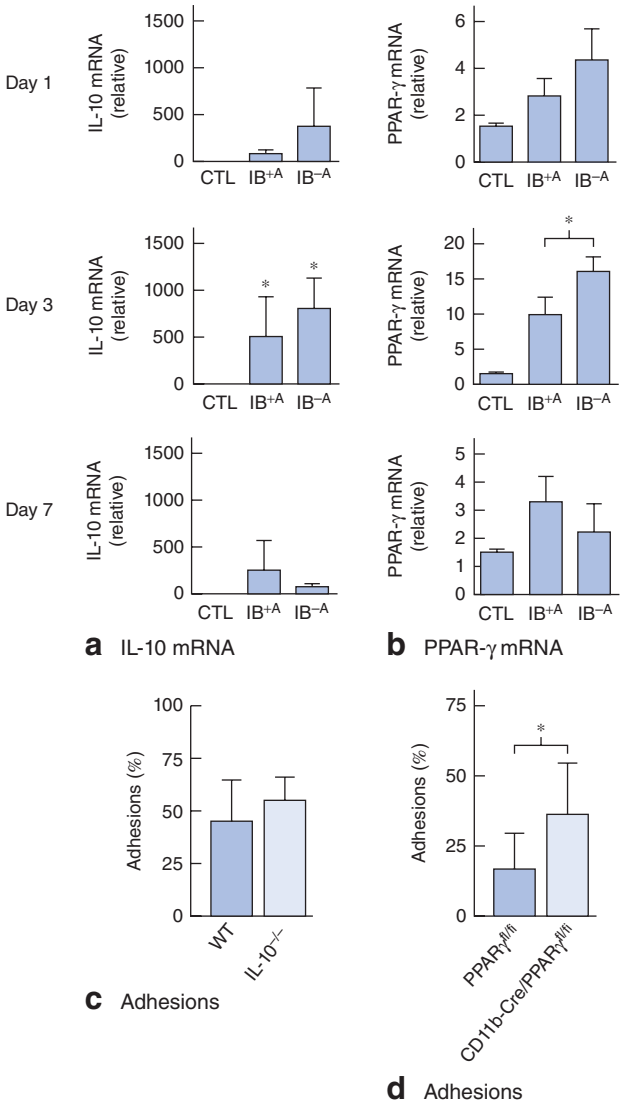


Fig. 2 Contribution of interleukin (IL) 10 and macrophage-specific peroxisome proliferator-activated receptor (PPAR) γ activity to postoperative adhesion formation. Ischaemic button (IB) tissue was collected from wild-type (WT), IL-10 deficient (IL-10$^{-/-}$) and CD11b-Cre/PPARγ$^{fl/fl}$ mice on days 1, 3 and 7 after surgery, and separated into buttons with (IB^{+A}) and without (IB^{-A}) adhesions. Unmanipulated peritoneal wall served as control tissue (CTL). **a**,**b** Gene expression of **a** IL-10 and **b** PPAR-γ mRNA levels relative to CTL. **c**,**d** Adhesion formation in **c** IL-10$^{-/-}$ *versus* WT and **b** CD11b-Cre/PPARγ$^{fl/fl}$ *versus* PPARγ$^{fl/fl}$ mice on day 7. Values are mean(s.d.) ($n = 5$–8 for all groups). *$P < 0{\cdot}050$ *versus* CTL unless indicated otherwise (**a**,**b** one-way ANOVA with Bonferroni's *post hoc* test, **d** Student's *t* test)

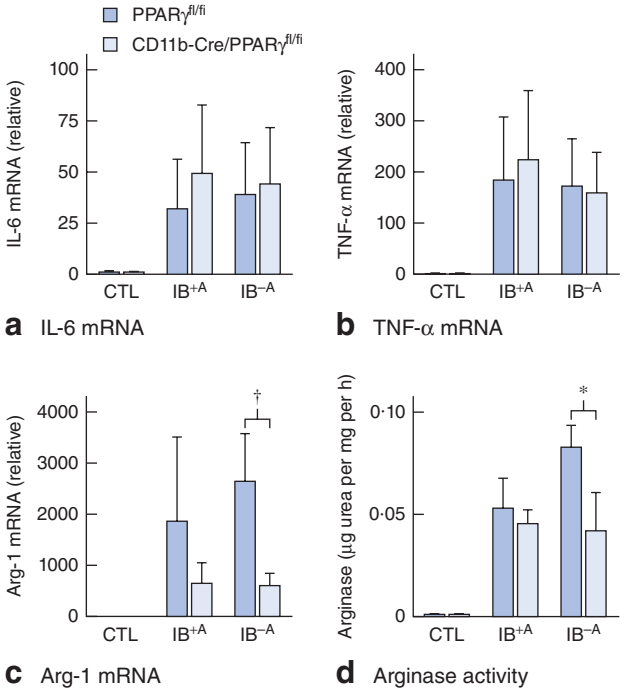


Fig. 3 Impact of macrophage-specific peroxisome proliferator-activated receptor (PPAR) γ deficiency on mRNA expression of M1 (interleukin (IL) 6 and tumour necrosis factor (TNF) α) and M2 (arginase (Arg) 1) markers, and arginase activity. Ischaemic button (IB) tissue was collected from CD11b-Cre/PPARγ$^{fl/fl}$ mice and corresponding PPARγ$^{fl/fl}$ control mice on day 3 after surgery, and separated into buttons with (IB^{+A}) and without (IB^{-A}) adhesions. Unmanipulated peritoneal wall served as control tissue (CTL). **a–c** Gene expression of **a** IL-6, **b** TNF-α and **c** Arg-1 mRNA levels relative to CTL. **d** Arginase activity measured indirectly by L-arginine to urea conversion. Values are mean(s.d.) ($n = 5–8$ for all groups). *$P < 0·050$, †$P < 0·010$ (two-way ANOVA with Bonferroni's *post hoc* test)

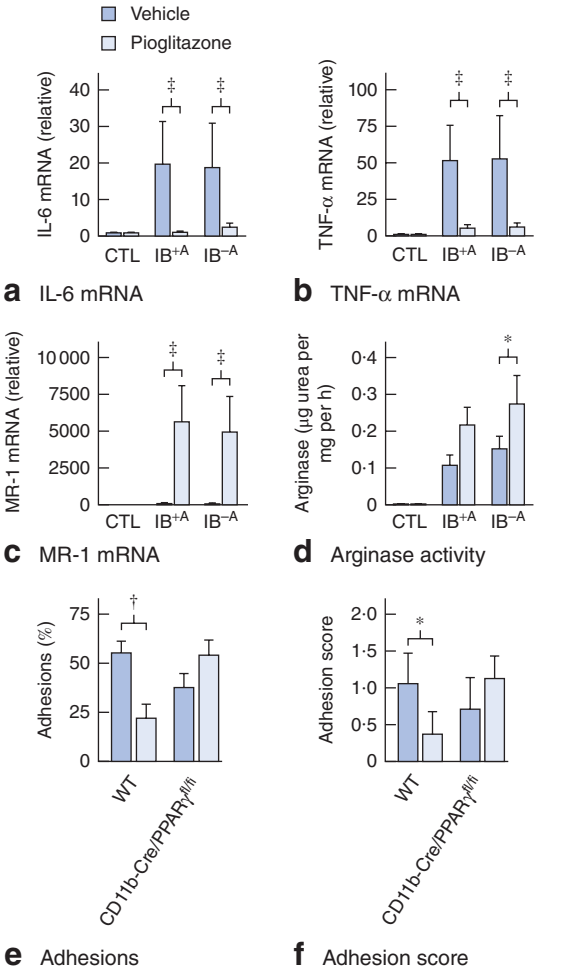


Fig. 4 Effect of pioglitazone on mRNA expression of M1 (interleukin (IL) 6 and tumour necrosis factor (TNF) α) and M2 (mannose receptor (MR) 1 and arginase activity) markers, and adhesion formation in wild-type (WT) mice and CD11b-Cre/PPARγ$^{fl/fl}$ mice. Mice received a daily gavage of pioglitazone for 7 consecutive days, starting 3 days before surgery. Controls were treated with vehicle. Ischaemic button (IB) tissue was collected on days 3 and 7 after surgery, and separated into buttons with (IB^{+A}) and without (IB^{-A}) adhesions. Unmanipulated peritoneal wall served as control tissue (CTL). **a–c** Gene expression of **a** IL-6, **b** TNF-α and **c** MR-1 mRNA on day 3 relative to CTL. **d** Arginase activity was measured indirectly by L-arginine to urea conversion on day 3. **e** Adhesion formation was determined on day 7 and **f** verified by an adhesion score. Values are mean(s.d.) ($n = 5–7$ for all groups). *$P < 0·050$, †$P < 0·010$, ‡$P < 0·001$ (**a–d** two-way ANOVA with Bonferroni's *post hoc* test, **e**,**f** Student's *t* test)

absence of IL-4 transcription in ischaemic buttons, and no differences in adhesion formation in IL-4$^{-/-}$ compared with WT mice (*Fig. S3*, supporting information). However, IL-10 and PPAR-γ mRNAs were significantly upregulated compared with levels in control tissue (*Fig. 2a*,*b*). Levels of IL-10 mRNA did not differ between buttons with and without adhesions, but PPAR-γ mRNA levels were increased in those without adhesions on POD 3 ($P = 0·012$) (*Fig. 2b*). The role of IL-10 and PPAR-γ in adhesion formation was analysed by use of IL-10$^{-/-}$ and CD11b-Cre/PPARγ$^{fl/fl}$ mice. The latter lack PPAR-γ exclusively in CD11b^{+} cells such as macrophages and

monocytes. Numbers of adhesions and adhesion score were not altered in IL-10$^{-/-}$ compared with WT mice (*Fig. 2c*), but were raised in CD11b-Cre/PPARγ$^{fl/fl}$ mice compared with PPARγ$^{fl/fl}$ mice on POD 7 (36·1(18·2) *versus* 16·7(12·9) per cent; $P = 0·042$) (*Fig. 2d*; *Fig. S4*, supporting information).

It was questioned whether PPAR-γ affects macrophage differentiation during adhesion formation. Expression of IL-6 and TNF-α mRNA was not altered in buttons without adhesions (*Fig. 3a*,*b*). However, Arg-1 mRNA expression and arginase activity were reduced in CD11b-Cre/PPARγ$^{fl/fl}$ mice compared with PPARγ$^{fl/fl}$ control mice ($P = 0·005$ and $P = 0·013$ respectively) (*Fig. 3c*,*d*). This suggests that Arg-1 is expressed in a PPAR-γ-dependent manner and might have a functional impact on adhesion formation.

Pioglitazone drives arginine 1 expression in macrophages and prevents adhesion formation

Gene expression analysis in pioglitazone-treated mice revealed a significant reduction in IL-6 mRNA and TNF-α mRNA in buttons with and without adhesions on POD 3 (all $P < 0·001$) (*Fig. 4a*,*b*), whereas MR-1 mRNA was upregulated compared with values in the vehicle-treated group ($P < 0·001$) (*Fig. 4c*). Arginase activity was also upregulated in buttons without adhesions after pioglitazone treatment compared with the vehicle group ($P = 0·020$) (*Fig. 4d*). By POD 7, pioglitazone treatment resulted in a reduced number of adhesions in WT mice (21·9(20·9) per cent *versus* 55·0(19·7) per cent in vehicle-treated group; $P = 0·003$) (*Fig. 4e*). Pioglitazone also decreased the severity of adhesions, as indicated by a lower adhesion score than in the vehicle-treated group (0·37(0·29) *versus* 1·05(0·42); $P = 0·015$) (*Fig. 4f*). Pioglitazone did not reduce numbers of adhesions (54·2(18·2) *versus* 37·5(14·1) per cent for pioglitazone *versus* vehicle-treated groups), nor did it affect adhesion scoring (1·08(0·28) *versus* 0·74(0·35)) in CD11b-Cre/PPARγ$^{fl/fl}$ mice (*Fig. 4e*,*f*).

Pioglitazone did not compromise anastomotic healing but reduced anastomotic adhesion formation

There was no difference in anastomotic bursting pressure between pioglitazone- and vehicle-treated mice on POD 7 (197·5(106·4) *versus* 190·7(94·2) mmHg) (*Fig. 5a*). Additionally, anastomotic gene expression of collagen type I and III transcripts was not altered by pioglitazone (*Fig. 5b*,*c*). The adhesion-covered anastomotic circumference and strength of adhesions was significantly lower after pioglitazone treatment than in the vehicle-treated group ($P < 0·001$ and $P = 0·016$ respectively) (*Fig. 5d*,*e*).

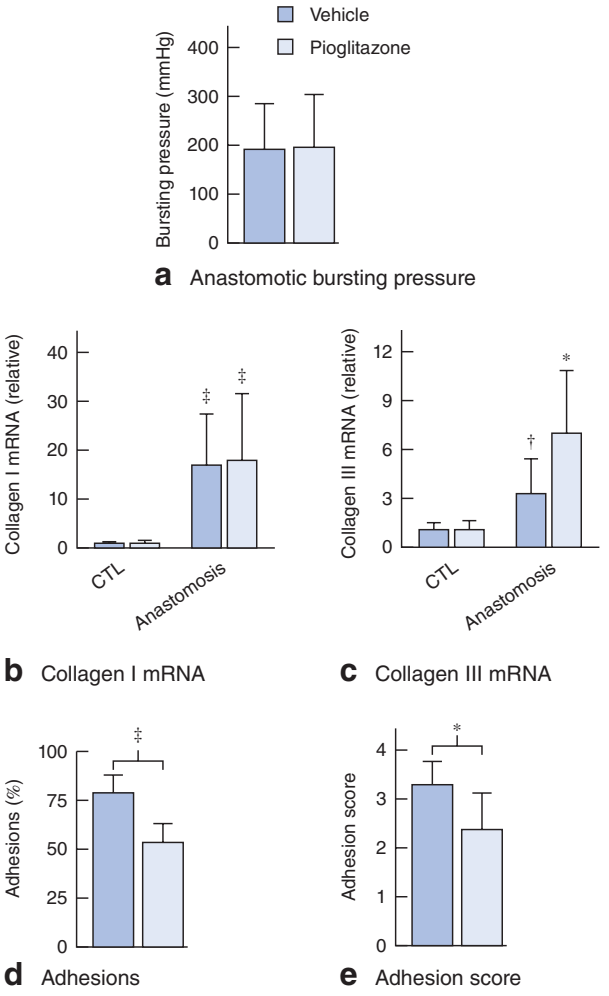


Fig. 5 Effect of pioglitazone on healing and adhesion formation at the colonic anastomosis. Wild-type mice received a daily gavage of pioglitazone or vehicle for 7 consecutive days and underwent colonic anastomosis surgery on day 4. Results for the anastomotic tissue were compared with those for an unmanipulated colonic specimen (CTL) from the same animal. **a** Anastomotic bursting pressure measured 7 days after surgery. **b**,**c** Gene expression of **b** collagen I and **c** collagen III mRNA measured within the anastomosis relative to CTL. Collagen expression was compared between anastomosis and CTL after pioglitazone or vehicle treatment. **d** Adhesion formation at the circumference of the anastomosis 7 days after surgery and **e** scored according to ease of dissection. Values are mean(s.d.) ($n = 8$ for all groups). *$P < 0·050$, †$P < 0·010$, ‡$P < 0·001$ *versus* CTL unless indicated otherwise (**a**,**d**,**e** Student's *t* test, **b**,**c** two-way ANOVA with Bonferroni's *post hoc* test)

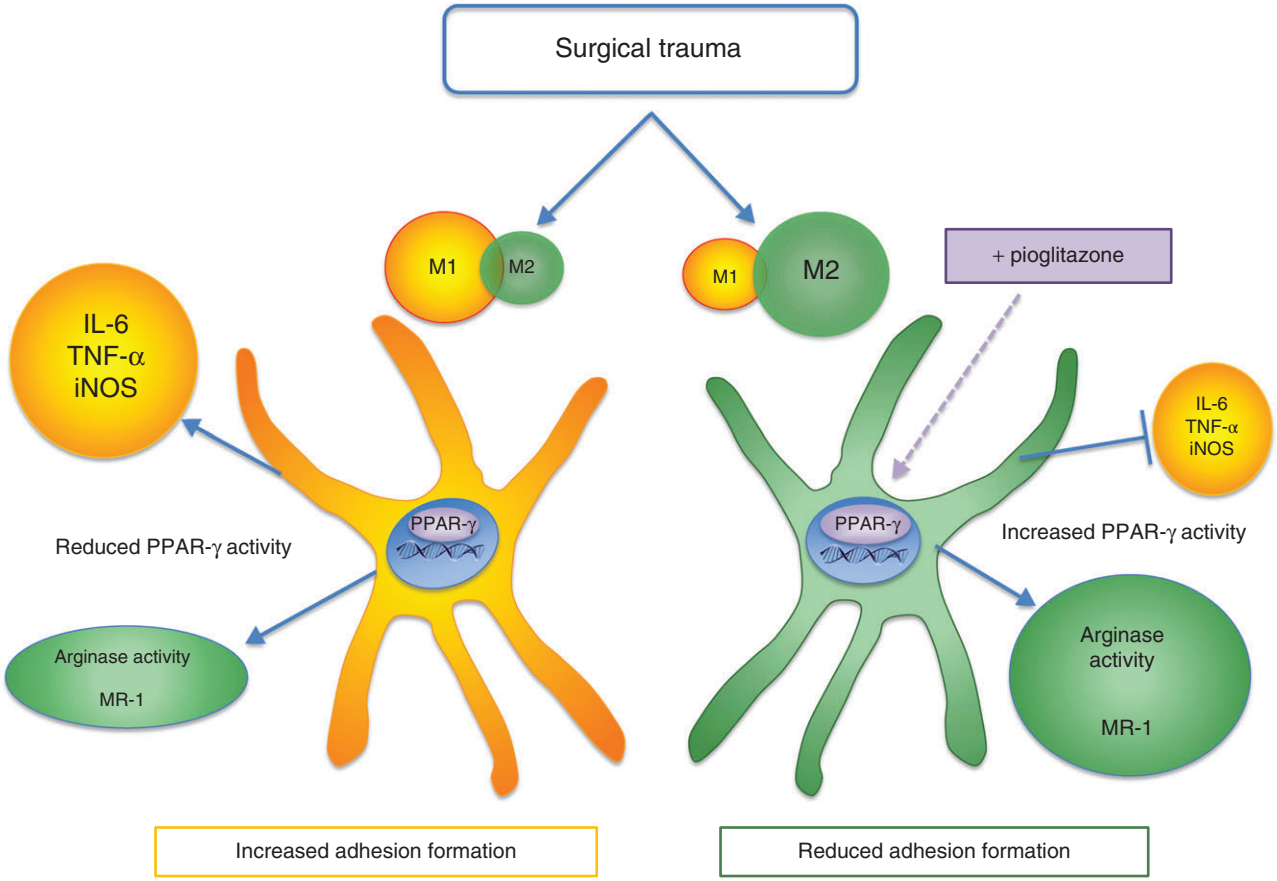


Fig. 6 Role of the peroxisome proliferator-activated receptor (PPAR) γ pathway and its intervention in M2 macrophage polarization during abdominal adhesion formation. Macrophage activation and infiltration to the site of a peritoneal lesion occurs during abdominal surgery. Excessive expression of interleukin (IL) 6, tumour necrosis factor (TNF) α and inducible ntiric oxide synthese (iNOS) indicates M1 macrophage polarization (left pathway) and increases adhesion formation, presumably via local induction of dysbalanced collagen formation/degradation. Counter-regulatory mechanisms include PPAR-γ-dependent M2 macrophage polarization (right pathway), indicated by increased mannose receptor (MR) 1 expression and arginase activity. Dysbalanced macrophage polarization with an M1 greater than M2 phenotype may originate from disturbed or inappropriate PPAR-γ activity and coincides with augmented adhesion formation. However, the mechanism leading to the insufficient local PPAR-γ activation remains to be determined. Nevertheless, pioglitazone treatment reduces postoperative adhesion formation, indicating that PPAR-γ agonism corrects the disturbances in macrophage polarization by supporting M2 polarization, as shown by increased MR-1 expression and arginase activity

Discussion

In this study, perioperative pharmacological and endogenous PPAR-γ agonism reduced adhesion formation in mice. This correlated with alteration of macrophage differentiation shown by increased arginase activity and MR-1 expression. Furthermore, PPAR-γ agonism did not compromise anastomotic healing.

Macrophages represent a dynamic and functionally heterogeneous cell population; their function strongly depends on the differentiation status and their plasticity accounts for significant variation during healing processes[9,15,22]. Previous studies suggested an involvement of macrophages in adhesion formation[8], but their exact function and differentiation status is still unknown. In the present study, adhesion formation correlated inversely with M2 marker expression, as buttons without adhesions contained higher transcript levels of Arg-1 and MR-1. Furthermore, increased arginase activity and higher levels of Arg-1-expressing macrophages were observed in buttons without than in those with adhesions. Arg-1 is a key molecule in macrophage polarization[23]. The finding of diminished arginase activity on POD 3 and prolonged increased activity on POD 7 in buttons with adhesions, compared with those without adhesions, suggests putative resolution or at least a modulatory effect by altered

macrophage differentiation during adhesion formation. This is supported by a recent study[24] showing a reduction in hepatic fibrosis and collagen deposition linked to Arg-1-expressing M2 macrophage induction. Suppression of fibrosis by a shift to M2 differentiation is a promising therapeutic target for fibrotic diseases[15]. This could be due to the competitive effect of arginase activity and inducible nitric oxide synthase. As release of nitric oxide is known to increase collagen production in wound fibroblasts[25], increased arginase activity could suppress nitric oxide release and therefore reduce collagen production during adhesion formation. Pesce and colleagues[26] also hypothesized that arginase activity within macrophages competes with fibroblasts for L-arginine, a substrate for collagen production, resulting in reduced collagen production. However, contradictory results have also been published, describing a profibrotic role of M2 macrophages, by increasing collagen production and deposition during fibrosis[9].

Given the inverse correlation between M2 marker expression and adhesion formation in the present model, an analysis was undertake to determine which molecules drive macrophage differentiation towards an M2 phenotype during peritoneal healing. Neither IL-4- nor IL-10-mediated pathways, which are both well known to induce different M2-like macrophage subtypes[9], were involved in adhesion formation, although IL-10 was at least expressed and may have contributed to overall inflammation during the healing process. This is in agreement with the findings of Daley and co-workers[27], who demonstrated IL-4/IL-13-independent polarization of monocytes into M2 macrophages during murine wound healing after surgery[27]. In contrast, in the present study, expression of PPAR-γ, another molecule that induces M2 differentiation[28], was greater in buttons without adhesions than in buttons with adhesions. PPAR-γ modulates immune and metabolic functions in macrophages by inhibiting proinflammatory responses and upregulating anti-inflammatory cytokine expression[11]. Reduced arginase activity and expression was observed in CD11b-Cre/PPARγ$^{fl/fl}$ mice, which lack PPAR-γ specifically in monocytes and macrophages, indicating reduced M2 macrophage function. Furthermore, adhesion formation was increased in CD11b-Cre/PPARγ$^{fl/fl}$ mice, providing an axis between M2 differentiation, arginase activity and adhesion formation. Importantly, Arg-1 expression and arginase activity have been found to depend on PPAR-γ activation[29].

Pharmacological PPAR-γ agonism with pioglitazone prevented adhesion formation, and increased arginase activity and M2 marker gene expression. This was also shown in the vicinity of the colonic anastomosis. These data indicate that exogenous PPAR-γ agonism is powerful enough to overcome the detrimental impact of an additional surgical trauma. The protective effect of PPAR-γ agonism probably originates from the macrophage compartment, as pioglitazone failed to prevent adhesion formation in CD11b-Cre/PPARγ$^{fl/fl}$ mice. The role of the PPAR-γ pathway in adhesion formation is summarized in *Fig. 6*.

Of note, PPARγ$^{fl/fl}$ mice showed reduced adhesion formation compared with the C57BL6/J WT counterparts that were used as controls in all other experiments. This difference can be explained by their genetic background. C57BL6/J WT, but not the genetically modified mice with a C57BL/6NCrJ background, carry a deletion in the *Nnt* gene driving the metabolism leading to glucose intolerance[30]. As glucose intolerance is known to impair wound healing[31], this may be responsible for differences in postoperative adhesion formation between the two mouse strains.

This study suggests that PPAR-γ-mediated adhesion prevention depends on macrophage arginase function. This was confirmed by a recent study[32] showing a requirement for local macrophage-dependent arginase activity in cutaneous wound healing. Controversially, other authors[33] have demonstrated improved excisional wound healing by topical arginase inhibition. Further analyses of the role of arginase in peritoneal wound healing are required.

Perioperative pioglitazone treatment could be a strategy for the prevention of abdominal adhesion formation. The present data show that PPAR-γ agonism does not compromise anastomotic healing in mice, which generally involves arginase activity[34]. However, the anastomotic leakage model used in this study cannot be extrapolated to the clinical setting. Nevertheless, use of systemic pioglitazone to prevent adhesion formation may be complementary to the currently available antiadhesive agents such as biodegradable barriers. These barriers are limited to local application, and some are suspected to impair anastomotic healing[35].

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References

1 Ellis H, Moran BJ, Thompson JN, Parker MC, Wilson MS, Menzies D *et al*. Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. *Lancet* 1999; **353**: 1476–1480.

2 Ten Broek RP, Issa Y, van Santbrink EJ, Bouvy ND, Kruitwagen RF, Jeekel J *et al*. Burden of adhesions in abdominal and pelvic surgery: systematic review and meta-analysis. *BMJ* 2013; **347**: f5588.
3 Hellebrekers BW, Kooistra T. Pathogenesis of postoperative adhesion formation. *Br J Surg* 2011; **98**: 1503–1516.
4 Binnebosel M, Rosch R, Junge K, Lynen-Jansen P, Schumpelick V, Klinge U. Macrophage and T-lymphocyte infiltrates in human peritoneal adhesions indicate a chronic inflammatory disease. *World J Surg* 2008; **32**: 296–304.
5 Dizerega GS, Campeau JD. Peritoneal repair and post-surgical adhesion formation. *Hum Reprod Update* 2001; **7**: 547–555.
6 Xu X, Rivkind A, Pappo O, Pikarsky A, Levi-Schaffer F. Role of mast cells and myofibroblasts in human peritoneal adhesion formation. *Ann Surg* 2002; **236**: 593–601.
7 Ohashi K, Yoshimoto T, Kosaka H, Hirano T, Iimuro Y, Nakanishi K *et al*. Interferon gamma and plasminogen activator inhibitor 1 regulate adhesion formation after partial hepatectomy. *Br J Surg* 2014; **101**: 398–407.
8 Burnett SH, Beus BJ, Avdiushko R, Qualls J, Kaplan AM, Cohen DA. Development of peritoneal adhesions in macrophage depleted mice. *J Surg Res* 2006; **131**: 296–301.
9 Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity* 2010; **32**: 593–604.
10 ten Broek RP, Stommel MW, Strik C, van Laarhoven CJ, Keus F, van Goor H. Benefits and harms of adhesion barriers for abdominal surgery: a systematic review and meta-analysis. *Lancet* 2014; **383**: 48–59.
11 Nagy L, Szanto A, Szatmari I, Szeles L. Nuclear hormone receptors enable macrophages and dendritic cells to sense their lipid environment and shape their immune response. *Physiol Rev* 2012; **92**: 739–789.
12 Sindrilaru A, Scharffetter-Kochanek K. Disclosure of the culprits: macrophages – versatile regulators of wound healing. *Adv Wound Care (New Rochelle)* 2013; **2**: 357–368.
13 El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M *et al*. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol* 2008; **9**: 1399–1406.
14 Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 1992; **176**: 287–292.
15 Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* 2012; **18**: 1028–1040.
16 Morán-Salvador E, Titos E, Rius B, González-Périz A, García-Alonso V, López-Vicario C *et al*. Cell-specific PPARγ deficiency establishes anti-inflammatory and anti-fibrogenic properties for this nuclear receptor in non-parenchymal liver cells. *J Hepatol* 2013; **59**: 1045–1053.
17 Reed KL, Fruin AB, Bishop-Bartolomei KK, Gower AC, Nicolaou M, Stucchi AF *et al*. Neurokinin-1 receptor and substance P messenger RNA levels increase during intraabdominal adhesion formation. *J Surg Res* 2002; **108**: 165–172.
18 Pantelis D, Beissel A, Kahl P, Vilz TO, Stoffels B, Wehner S *et al*. Colonic anastomotic healing in the context of altered macrophage function and endotoxemia. *Int J Colorectal Dis* 2011; **26**: 737–746.
19 Sikkink CJ, de Man B, Bleichrodt RP, van Goor H. Auto-cross-linked hyaluronic acid gel does not reduce intra-abdominal adhesions or abscess formation in a rat model of peritonitis. *J Surg Res* 2006; **136**: 255–259.
20 Zühlke HV, Lorenz EM, Straub EM, Savvas V. [Pathophysiology and classification of adhesions.] *Langenbecks Arch Chir Suppl II Verh Dtsch Ges Chir* 1990; 1009–1016.
21 Corraliza IM, Campo ML, Soler G, Modolell M. Determination of arginase activity in macrophages: a micromethod. *J Immunol Methods* 1994; **174**: 231–235.
22 Schürmann C, Seitz O, Sader R, Pfeilschifter J, Goren I, Frank S. Role of wound macrophages in skin flap loss or survival in an experimental diabetes model. *Br J Surg* 2010; **97**: 1437–1451.
23 Rath M, Müller I, Kropf P, Closs EI, Munder M. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. *Front Immunol* 2014; **5**: 532.
24 Manuelpillai U, Lourensz D, Vaghjiani V, Tchongue J, Lacey D, Tee JY *et al*. Human amniotic epithelial cell transplantation induces markers of alternative macrophage activation and reduces established hepatic fibrosis. *PLoS One* 2012; **7**: e38631.
25 Schaffer MR, Efron PA, Thornton FJ, Klingel K, Gross SS, Barbul A. Nitric oxide, an autocrine regulator of wound fibroblast synthetic function. *J Immunol* 1997; **158**: 2375–2381.
26 Pesce JT, Ramalingam TR, Mentink-Kane MM, Wilson MS, El Kasmi KC, Smith AM *et al*. Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. *PLoS Pathog* 2009; **5**: e1000371.
27 Daley JM, Brancato SK, Thomay AA, Reichner JS, Albina JE. The phenotype of murine wound macrophages. *J Leukoc Biol* 2010; **87**: 59–67.
28 Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L *et al*. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 2007; **447**: 1116–1120.
29 Gallardo-Soler A, Gómez-Nieto C, Campo ML, Marathe C, Tontonoz P, Castrillo A *et al*. Arginase I induction by modified lipoproteins in macrophages: a peroxisome proliferator-activated receptor-gamma/delta-mediated effect that links lipid metabolism and immunity. *Mol Endocrinol* 2008; **22**: 1394–1402.

30 Freeman HC, Hugill A, Dear NT, Ashcroft FM, Cox RD. Deletion of nicotinamide nucleotide transhydrogenase: a new quantitive trait locus accounting for glucose intolerance in C57BL/6J mice. *Diabetes* 2006; **55**: 2153–2156.
31 Greer N, Foman NA, MacDonald R, Dorrian J, Fitzgerald P, Rutks I *et al*. Advanced wound care therapies for nonhealing diabetic, venous, and arterial ulcers: a systematic review. *Ann Intern Med* 2013; **159**: 532–542.
32 Campbell L, Saville CR, Murray PJ, Cruickshank SM, Hardman MJ. Local arginase 1 activity is required for cutaneous wound healing. *J Invest Dermatol* 2013; **133**: 2461–2470.
33 Kavalukas SL, Uzgare AR, Bivalacqua TJ, Barbul A. Arginase inhibition promotes wound healing in mice. *Surgery* 2012; **151**: 287–295.
34 Witte MB, Vogt N, Stuelten C, Gotoh T, Mori M, Becker HD. Arginase acts as an alternative pathway of L-arginine metabolism in experimental colon anastomosis. *J Gastrointest Surg* 2003; **7**: 378–385.
35 Beck DE, Cohen Z, Fleshman JW, Kaufman HS, van Goor H, Wolff BG. A prospective, randomized, multicenter, controlled study of the safety of Seprafilm adhesion barrier in abdominopelvic surgery of the intestine. *Dis Colon Rectum* 2003; **46**: 1310–1319.

Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Primer sets used for gene expression analyses by quantitative PCR (Word document)

Fig. S1 Representative intraoperative views of ischaemic buttons in wild-type mice with and without adhesions (Word document)

Fig. S2 Proinflammatory gene expression in ischaemic buttons from wild-type mice (Word document)

Fig. S3 Interleukin (IL) 4 expression and adhesion analysis in ischaemic buttons from wild-type and IL-4$^{-/-}$ mice (Word document)

Fig. S4 Adhesion scores on day 7 after ischaemic button surgery (Word document)