Estrogen Receptor-Alpha Promotes Alternative Macrophage Activation during Cutaneous Repair

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Efficient local monocyte/macrophage recruitment is critical for tissue repair. Recruited macrophages are polarized toward classical (proinflammatory) or alternative (prohealing) activation in response to cytokines, with tight temporal regulation crucial for efficient wound repair. Estrogen acts as a potent anti-inflammatory regulator of cutaneous healing. However, an understanding of estrogen/estrogen receptor (ER) contribution to macrophage polarization and subsequent local effects on wound healing is lacking. Here we identify, to our knowledge previously unreported, a role whereby estrogen receptor α (ERα) signaling preferentially polarizes macrophages from a range of sources to an alternative phenotype. Cell-specific ER ablation studies confirm an *in vivo* role for inflammatory cell *ERα*, but not *ERβ*, in poor healing associated with an altered cytokine profile and fewer alternatively activated macrophages. Furthermore, we reveal intrinsic changes in ERα-deficient macrophages, which are unable to respond to alternative activation signals *in vitro*. Collectively, our data reveal that inflammatory cell-expressed *ERα* promotes alternative macrophage polarization, which is beneficial for timely healing. Given the diverse physiological roles of ERs, these findings will likely be of relevance to many pathologies involving excessive inflammation.

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INTRODUCTION

Cutaneous wound repair consists of numerous overlapping events, initiated by injury to the skin, broadly grouped into the inflammatory response, the proliferative response, and the remodeling phase. The inflammatory response is a crucial component of cutaneous healing, as evidenced by severely delayed repair following *in vivo* macrophage ablation (Goren *et al.*, 2009). Moreover, a dysregulated inflammatory response, often seen with age or co-pathology (e.g., diabetes), prevents the repair process from progressing efficiently (Loots *et al.*, 1998).

We have previously demonstrated the importance of the sex steroid estrogen in delayed healing in the elderly (Hardman and Ashcroft, 2008), where estrogen replacement accelerates healing in aged humans and hormone-deprived animal models (Ashcroft *et al.*, 1997; Hardman *et al.*, 2008) and protects against developing a chronic wound (Margolis *et al.*, 2002). Our previous studies have revealed estrogen to be a global regulator of healing, influencing numerous cell types, including reducing inflammatory cell influx (Emmerson *et al.*, 2009). Estrogen is also potently anti-inflammatory in other tissues: estradiol reduces the inflammatory response to cholera toxin injection (Josefsson *et al.*, 1992) and is anti-inflammatory in the brain, protecting against neurodegeneration (Vegeto *et al.*, 2003); and estradiol dampens the expression of numerous proinflammatory cytokines, including TNF-α, MCP-1, Interleukin (IL)-1β and -6, and macrophage migration inhibitory factor (Pfeilschifter *et al.*, 2002; Hardman *et al.*, 2005). Estrogen signals via two nuclear hormone receptors: estrogen receptor α (ERα) and estrogen receptor β (ERβ), and, crucially, both ERs are present on macrophages (Harkonen and Vaananen, 2006). In the context of skin repair, our data show that although the ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) alone promotes healing in an Ovariectomized (Ovx) mouse model, both DPN and the ERα agonist 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) (PPT) reduce local wound inflammation (Campbell *et al.*, 2010).

Broadly speaking, macrophages can be polarized toward two distinct pathways. Classically activated (CA) macrophages, dependent on the cytokines IFN-γ and TNF-α, upregulate the enzyme-inducible nitric oxide synthase (iNOS) (Mosser and Zhang, 2008) and produce a variety of proinflammatory cytokines that are important for host defense, including IL-1, IL-6, and IL-23. Conversely, alternatively activated (AA)

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Abbreviations: AA, alternatively activated; BMDMs, bone marrow-derived macrophages; CA, classically activated; ER, estrogen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; iNOS, inducible nitric oxide synthase; Ovx, Ovariectomized

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macrophages, dependent on IL-4 and IL-13 released from T_H2 lymphocytes in response to tissue injury, upregulate the enzymes Arginase 1, Fizz, and Ym1 (Mosser and Zhang, 2008). The balance between iNOS and arginase activity, which both compete for the common substrate L-arginine, is tightly regulated during repair (Albina *et al.*, 1990). Although CA and AA macrophages are locally present in wounds, little is known about their specific contributions to healing. Our previous data have shown that arginase 1 is 82-fold downregulated in human nonhealing wounds, indicating its potential importance for healing (Hardman and Ashcroft, 2008). Conversely, an absence of iNOS delays healing, whereas iNOS upregulation correlates with faster healing (Yamasaki *et al.*, 1998). Intriguingly, both iNOS and arginase are upregulated in chronic venous and diabetic ulcers (Jude *et al.*, 1999; Abd-El-Aleem *et al.*, 2000). These and other data have led to the proposal that local macrophage polarization state has a major influence on healing (Deonarine *et al.*, 2007).

The role of estrogen in macrophage polarization under physiological conditions remains unclear. It has, however, been proposed that estrogen inhibits the production of T_H1 cytokines, while stimulating the production of T_H2 cytokines (Salem, 2004). Moreover, reduced wound levels of the AA markers Fizz and Ym1 have been reported following Ovx, which was rescued with systemic estrogen or progesterone replacement (Routley and Ashcroft, 2009). In this study, we demonstrate that estrogen's anti-inflammatory effects on cutaneous wound healing are ERα-mediated, with ERα directly promoting alternative macrophage polarization *in vitro* in a variety of macrophage populations.

RESULTS

Estrogen and ER-selective agonists promote alternative macrophage activation

It is now widely accepted that macrophages become polarized during healing, playing distinct roles. However, estrogen's ability to influence macrophage polarization remains to be studied in detail, with the specific role of ERs being unclear. To test these roles, we first used bone marrow-derived macrophages (BMDMs), pretreated with either 17β-estradiol or ER-selective agonists, PPT or DPN, and subsequently stimulated with either IFN-γ/lipopolysaccharide (LPS) or IL-4 to induce CA and AA polarization, respectively. Changes in *Nos2* (classical) and *Arg1* (alternative) gene expression 6 or 24 hours post stimulation (Figure 1a–d) were analyzed via real-time PCR. As expected, *Nos2* expression was strongly induced by classical stimulation, whereas *Arg1* was induced following alternative stimulation. Intriguingly, pretreatment with either 17β-estradiol or the ERβ agonist DPN significantly dampened the 6-hour post stimulation increase in *Nos2* expression (Figure 1a). In the absence of ER ligand, no increase in *Arg1* was observed 6 hours post IL-4 stimulation (Figure 1b), in line with previous findings suggesting that Arg1 is a late marker of AA macrophages (Menzies *et al.*, 2009). Intriguingly, pretreatment with the ERα agonist, PPT, strongly induced *Arg1* expression after only 6 hours of stimulation (Figure 1b), suggesting a direct ERα-mediated transcriptional effect. By 24 hours, IL-4 stimulation strongly induced *Arg1* expression in all groups, indicating that the ERα predisposition was relatively short-lived (Figure 1d).

FACs analysis and measurement of nitric oxide revealed induction of $iNOS^+$ cells and activity, with little influence of hormone pretreatement (Figure 1e and g). By contrast, 17β-estradiol and PPT strongly increased the number of $Relm\alpha^+$ cells (AA macrophage marker) (Figure 1f) and, crucially, significantly increased arginase activity measured through the production of urea (Figure 1h). We note that IL-4 stimulation in combination with 17β-estradiol strongly induced *ERα* expression, demonstrating reciprocal signaling effects (Supplementary Figure S1a online). Moreover, *ERβ* expression was significantly reduced following stimulation toward either classical or alternative activation (Supplementary Figure S1b online).

We next confirmed the observed effects of PPT and DPN pretreatment in combination with IFN-γ/LPS or IL-4 stimulation in additional cell types. In isolated peritoneal macrophages, polarization mirrored that observed in BMDMs, with clear effects of pretreatment at a 6 hours post stimulation time point (Figure 2a and b), with little effect being seen after 24 hours of stimulation (Figure 2c and d). Finally, to confirm physiological relevance, we directly isolated macrophages from murine wounds and subjected them to PPT and DPN pretreatment in combination with IFN-γ/LPS or IL-4 stimulation. In these wound-derived cells, the magnitude of induction of M1 (*Nos2*) or M2 (*Arg1*) gene expression following exposure to respective stimuli was far less than in BMDMs or peritoneal macrophages (Figure 2e and f). This observation, which was confirmed using the additional markers *TNFa* (M1) and *Ym1* (M2), suggests that these cells are already partially polarized or activated (Figure 2g and h). Crucially, PPT stimulation of wound-derived macrophages replicated the effects observed in BMDMs strongly inducing both M1 and M2 polarization markers, suggesting that signaling through ERα directly influences macrophage polarization. The fact that ERα influences the polarization of macrophages from a range of sources *in vitro* strongly suggests that it will be functionally important during inflammation *in vivo*.

Inflammatory-specific *ERα*-null (LysM-ERα) mice display delayed healing upon exogenous estrogen treatment

Estrogen is known to dampen local wound recruitment of innate immune cells; however, the cell-specific contribution of each ER has yet to be determined. To investigate this, wild-type (WT) and inflammatory cell-specific *ER*-null (*LysM-ERα* and *LysM-ERβ)* mice underwent incisional wounding in the absence of estrogen (Ovx) or following exogenous estrogen replacement (Figure 3a). In the absence of estrogen, healing was delayed irrespective of genotype, as has been previously reported for Ovx WT mice (Emmerson *et al.*, 2009) (Figure 3b and c—white bars). However, estrogen treatment revealed genotype-specific differences in healing response. Inflammatory cell deletion of *ERα* (*LysM-ERα*) resulted in a marked healing delay at 3 days post wounding (i.e., equivalent wound area to Ovx; Figure 3a and b). By contrast, estrogen replacement in *LysM-ERβ* mice was able to reduce wound area and improve healing, similarly to WT mice. By 7 days post

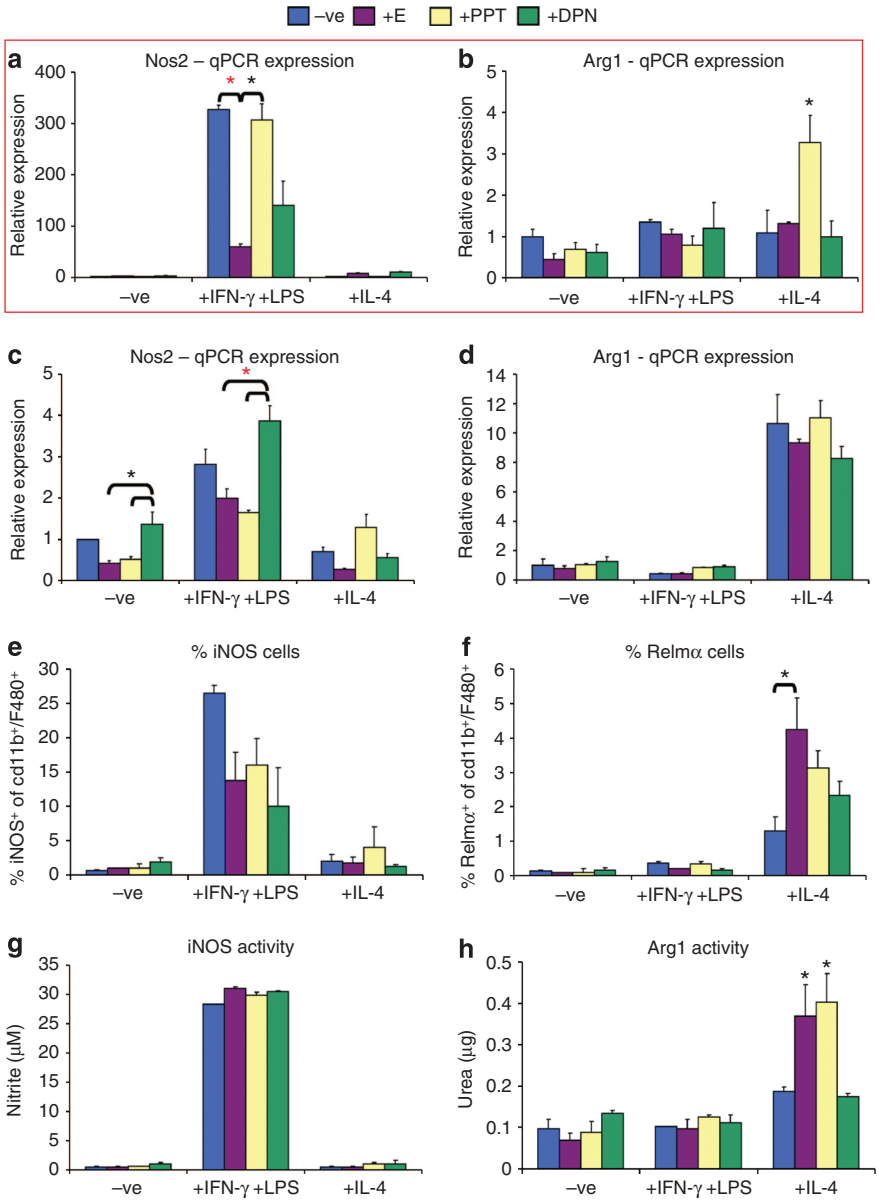

Figure 1. Estrogen receptor (ER)-selective agonists have differential effects on *in vitro* macrophage polarization. Bone marrow-derived macrophages (BMDMs) were pretreated with either 17β-estradiol (E; 10^{-7} M), an ERα agonist (PPT; 10^{-6} M), ERβ agonist (DPN; 10^{-6} M), or left untreated for 16 hours, after which cells were polarized to classical (+IFN-γ/+LPS) (CA) or alternative (+IL-4) activation (AA) or left untreated. (**a**–**d**) Real-time PCR for *Nos2* (CA marker) (**a**, **c**) or *Arg1* (AA marker) (**b**, **d**), 6 hours (red box) or 24 hours post stimulation. (**e**, **f**) After 24 hours of stimulation, cells were isolated, and flow cytometry analysis was performed using markers inducible nitric oxide synthase (iNOS) (**e**) and Relmα (AA marker) (**f**). Data are presented as a percentage of $cd11b^{+}/F480^{+}$-positive cells. (**g**, **h**) iNOS activity (**g**), nitric oxide production, and arginase activity (**h**) were measured in macrophages at 24 hours post stimulation. Estrogen dampens classical activation, whereas both estrogen and estrogen receptor α (ERα) stimulation promote alternative macrophage polarization. Mean + SEM, $n = 3$ replicates per group and are representative of three separate experiments. (Black*) $P < 0.05$, (red*) $P < 0.01$. DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; PPT, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl).

wounding, the *LysM-ERα* healing delay was even more pronounced (Figure 3c). Despite clear promotion of re-epithelialization in WT mice at 3 (Figure 3d) and 7 (Figure 3e) days post wounding, estrogen failed to promote re-epithelialization in either *LysM-ERα* or *LysM-ERβ* mice. Thus, inflammatory cell ERα is clearly essential for estrogen's beneficial effects on skin repair, with an absence of ERα delaying healing.

Estrogen's effects on wound inflammatory cell composition are predominantly ERα mediated

The delayed healing phenotype observed with estrogen replacement in *LysM-ERα* mice was accompanied by elevated local neutrophils at both 3 (Figure 4a and c) and 7 (Figure 4b and d) days post wounding (i.e., estrogen treatment did not reduce neutrophil recruitment in comparison with Ovx). These findings were repeated for local macrophage numbers, where

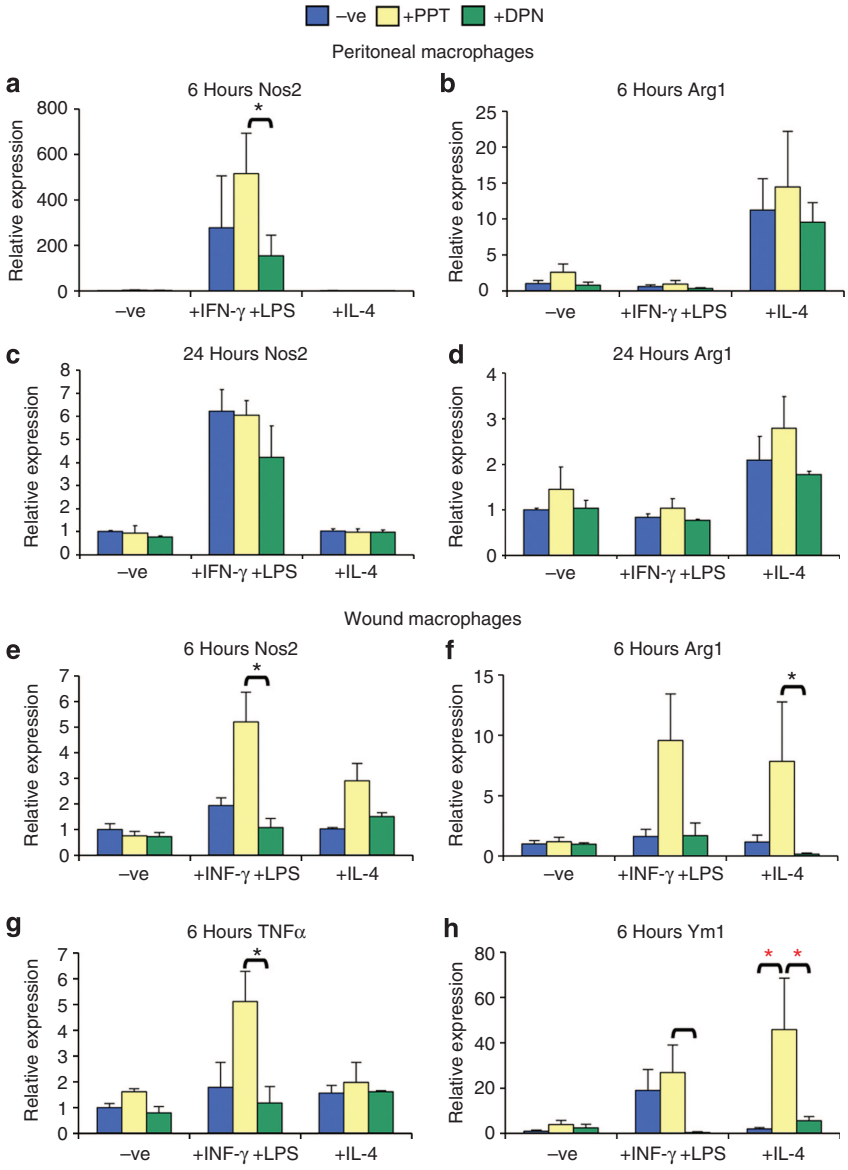

Figure 2. Estrogen receptor (ER)-selective agonists differentially influence *in vitro* polarization of peritoneal and wound-derived macrophages. Macrophages obtained from the peritoneum or isolated from wound tissue were treated with either an estrogen receptor α (ERα) agonist (PPT; 10^{-6} M), ERβ agonist (DPN; 10^{-6} M), or left untreated with subsequent polarization to classical (+IFN-γ/+LPS) (CA) or alternative (+IL-4) activation (AA). (**a**–**d**) Real-time PCR for *Nos2* (CA marker) (**a**, **c**) or *Arg1* (AA marker) (**b**, **d**) in peritoneal macrophages 6 or 24 hours post stimulation. (**e**–**h**) Real-time PCR for *Nos2, Tnfα* (CA markers) (**e**, **g**) or *Arg1, Ym1* (AA markers) (**f**, **h**) in wound-derived macrophages 6 hours post stimulation. ERα treatment augments both classical and alternative macrophage polarization. Mean + SEM, $n = 3$ replicates per group and are representative of three separate experiments. (Black*) $P < 0.05$, (red*) $P < 0.01$. DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; PPT, 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl).

estrogen-treated *LysM-ERα* wounds exhibited excessive macrophage influx at both 3 (Figure 4e and g) and 7 (Figure 4f and h) days post wounding. By contrast, estrogen treatment of both WT and *LysM-ERβ* mice led to reduced wound neutrophil and macrophage influx. Delayed healing in estrogen-treated *LysM-ERα* wounds was accompanied by potentially causative increased expression of key neutrophil chemokines and receptors *Cxcl1* and *Cxcr2* (Supplementary Figure S2a online), and macrophage-associated chemokines *Ccl2* and *Ccr2* (Supplementary Figure S2b online), as compared with WT. Collectively, leukocyte *ERα* expression appears to be essential for the anti-inflammatory effects of estrogen on innate immune cells, and macrophage signaling through ERβ alone appears to confer little anti-inflammatory effects *in vivo*.

LysM-ERα wounds have reduced AA macrophages

Our initial *in vitro* data (Figures 1 and 2) suggest that estrogen is able to promote alternative macrophage polarization specifically through ERα. To corroborate this *in vivo,* immunohistochemistry for the iNOS (CA marker) and Arg1 (AA marker) was performed. Estrogen treatment reduced the number of

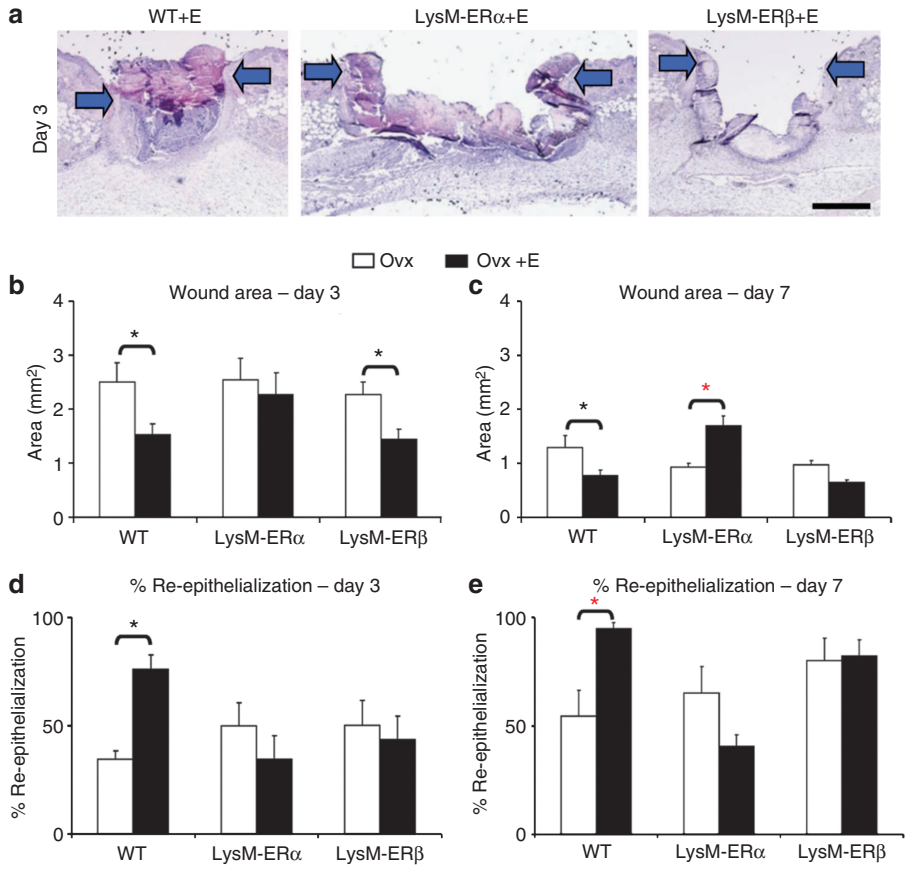

Figure 3. Exogenous estrogen treatment delays cutaneous healing in inflammatory cell-specific *ERα*-null (*LysM-ERα*) mice. (**a**) Representative micrographs of hematoxylin and eosin (H&E) staining from estrogen (+E)-treated wild-type (WT) and inflammatory cell-specific *ER*-null (*LysM-ERα* and *LysM-ERβ*) day 3 incisional wounds. (**b**, **c**) Wound area quantification (granulation tissue) of ovariectomized (Ovx) and estrogen-treated (Ovx + E) WT, *LysM-ERα*, and *LysM-ERβ* wounds at 3 (**b**) and 7 (**c**) days post wounding reveals a failure of estrogen to promote healing in *LysM-ERα*-null mice. (**d**, **e**) Estrogen promotes re-epithelialization in WT wounds at 3 (**d**) and 7 (**e**) days post wounding, with no effect in *LysM-ERα* and *LysM-ERβ* wounds. Scale bar = 400 μm. Mean + SEM, $n = 6$ mice per group. (Black*) $P < 0.05$, (red*) $P < 0.01$.

iNOS$^+$ cells, irrespective of genotype, at 3 days post wounding (Figure 5a). However, by 7 days post wounding, although this trend continued in WT and *LysM-ERβ* wounds, estrogen treatment increased iNOS$^+$ cells in *LysM-ERα* wounds (Figure 5b). Thus, the exaggerated healing delay in *LysM-ERα* mice at 7 days post wounding is associated with local macrophages remaining in a CA state. Further profiling reveals that in WT wounds estrogen drives the switch to increased AA macrophages, measured by increased Arg1$^+$ (Figure 5c and d) cells in the granulation tissue, which complements our *in vitro* data (Figure 1). Crucially, estrogen-treated *LysM-ERα* wounds were specifically associated with reduced numbers of AA macrophages, quantified through reduced Arg1$^+$ cells (Figure 5c and d) at both 3 and 7 days post wounding. The macrophage specificity of these findings was confirmed by colocalization studies using iNOS, Arg1, and Mac3 (pan-macrophage marker), revealing reduced AA macrophages in *LysM-ERα* mouse wounds (Figure 5e). The finding that estrogen treatment in *LysM-ERα* wounds fails to promote alternative macrophage polarization was further confirmed by immunohistochemical staining of Ym1$^+$ (AA marker) cells (Supplementary Figure S3b online) and quantitative reverse transcriptase in real-time (qPCR) expression of additional AA markers from isolated wound tissue (Supplementary Figure S3c online). These data reveal that estrogen promotes alternative macrophage activation *in vivo* via inflammatory cell ERα.

Estrogen fails to promote alternative macrophage activation in the absence of ERα *in vitro*

To confirm our *in vivo* findings suggesting that inflammatory cell ERα is required for estrogen to promote alternative macrophage activation, BMDMs were isolated from conditional null mice pretreated with estrogen and stimulated to polarize into CA and AA macrophages. Our results reveal that, in the absence of macrophage ERα, CA macrophages display substantially increased *Nos2* gene expression (Figure 6a), mirrored at the protein level by increased numbers of iNOS$^+$ cells within stimulated ERα-deficient macrophages (Figure 6c). However, we note that nitric oxide production after IFN-γ/LPS

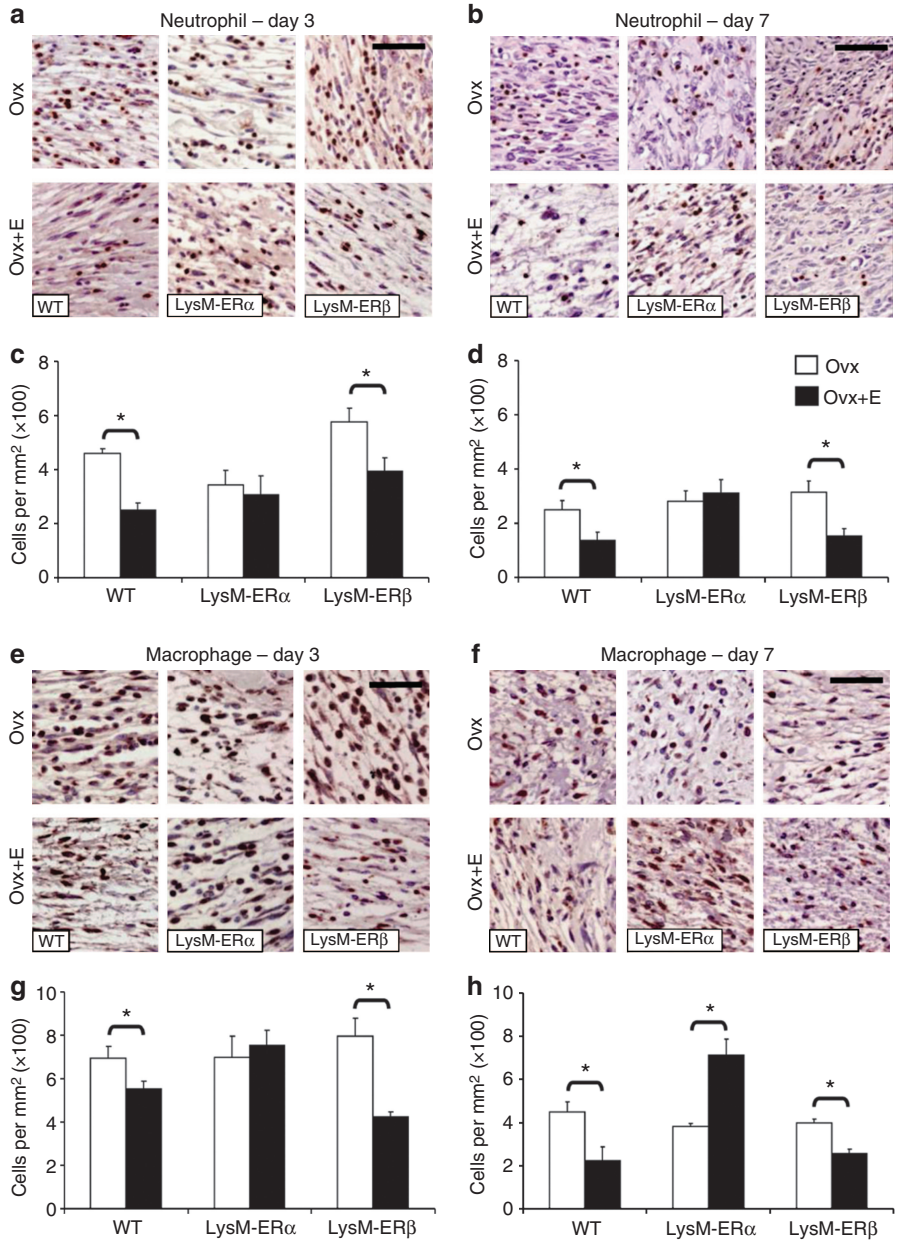

Figure 4. Estrogen treatment of inflammatory cell-specific *ERα*-null (*LysM-ERα*) mice results in an excessively prolonged local inflammatory response. (**a**, **b**) Representative neutrophil immunohistochemistry from day 3 (**a**) and day 7 (**b**) wild type (WT), *LysM-ERα*, and *LysM-ERβ* ovariectomized (Ovx) and estrogen-treated (Ovx + E) wounds. (**c**, **d**) Quantification reveals that estrogen fails to reduce neutrophil numbers in estrogen-treated *LysM-ERα* wounds. (**e**, **f**) Representative macrophage immunohistochemistry from day 3 (**e**) and day 7 (**f**) WT, *LysM-ERα*, and *LysM-ERβ* wounds. (**g**, **h**) Quantification reveals unchanged or increased numbers in estrogen-treated *LysM-ERα* wounds at 3 (**g**) and 7 (**h**) days post wounding, respectively. Scale bar = 50 μm. Mean + SEM, $n = 6$ mice per group. (*) $P < 0.05$.

stimulation was equivalent across all genotypes at this time point (Figure 6e). Importantly, with respect to alternative activation, ERα-deficient macrophages exhibited significantly reduced expression of *Arg1* compared with WT and ERβ-deficient macrophages (Figure 6b), reduced numbers of Relmα$^+$ cells (Figure 6d), and, of functional importance, a profound reduction in arginase activity (Figure 6f). Thus, in line with *in vivo* data (Figure 5), ERα-deficient macrophages appear to be intrinsically unable to adopt an AA phenotype.

DISCUSSION

Over the past decade, studies from our group and others have highlighted the importance of estrogen deficiency in delayed cutaneous wound healing in the elderly (Ashcroft *et al.*, 1997; Margolis *et al.*, 2002). Replacement of estrogen substantially

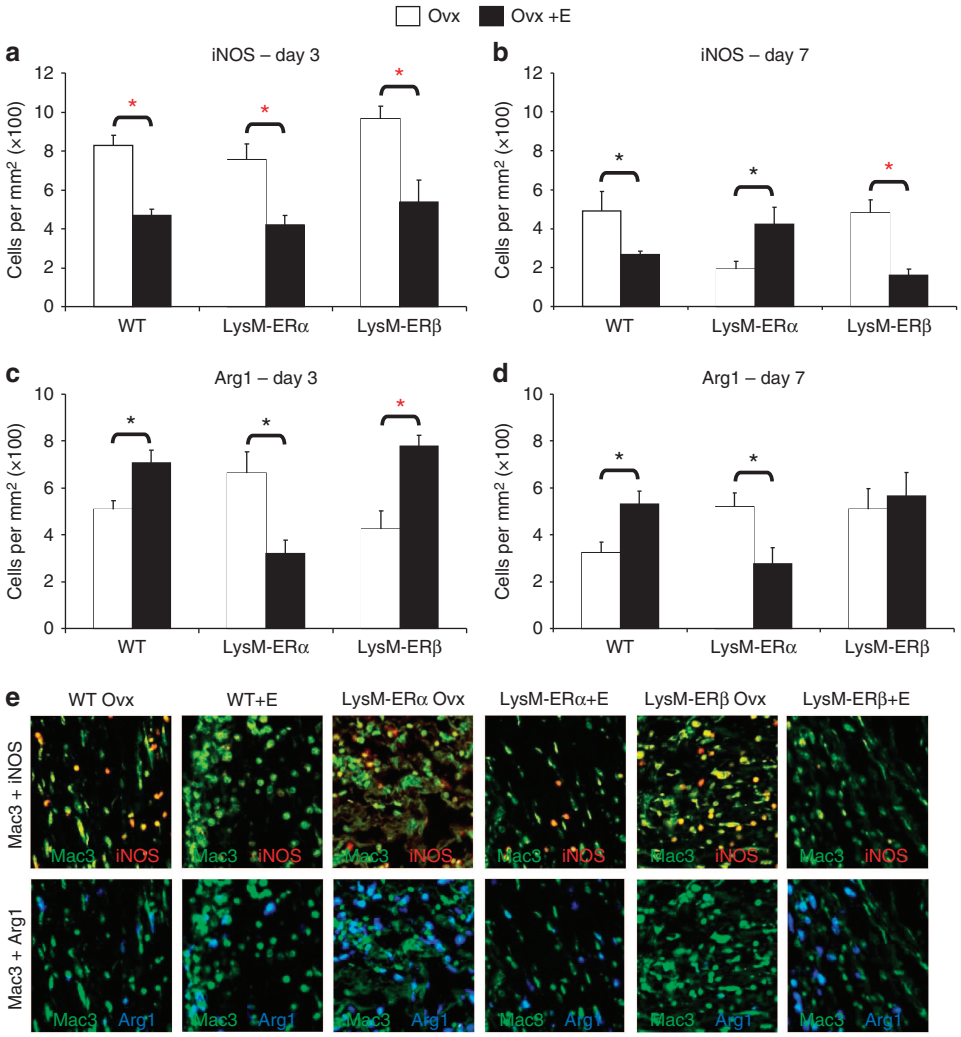

Figure 5. Altered macrophage polarization in estrogen-treated *LysM-ERα* mice. (**a**, **b**) Immunohistochemical quantification of inducible nitric oxide synthase (iNOS)$^+$ cells (classically activated (CA) macrophage marker) in ovariectomized (Ovx) and estrogen-treated (+E) wild type (WT), *LysM-ERα*, and *LysM-ERβ* wounds at 3 (**a**) and 7 (**b**) days post wounding. (**c**, **d**) Immunohistochemical quantification of Arg1$^+$ cells (alternatively activated (AA) macrophage marker) in ovariectomized (Ovx) and estrogen-treated (+E) WT, *LysM-ERα*, and *LysM-ERβ* wounds at 3 (**c**) and 7 (**d**) days post wounding. Estrogen-treated *LysM-ERα* wounds have reduced numbers of AA macrophages at both 3 and 7 days post wounding. (**e**) Colocalization staining of iNOS$^+$ macrophages (Mac3 + iNOS) and Arg1 + macrophages (Mac3 + Arg1) at day 3 post wounding revealed similar results. Scale bar = 50 μm. Mean + SEM, $n = 6$ mice per group. (Black*) $P < 0.05$, (red*) $P < 0.01$.

accelerates healing in both aged humans and estrogen-depleted animal models (Ashcroft *et al.*, 1997) and is dependent on cell-specific ER isoform expression (Campbell *et al.*, 2010). Importantly, estrogen is able to dampen inflammation, a key causative factor in chronic wound ontogenesis. Macrophage polarization has emerged as a key factor in the progression of a diverse range of disease pathologies (Loke *et al.*, 2007; Byers and Holtzman, 2011); however, the contribution of estrogen and ER signaling to macrophage polarization during wound healing has yet to be determined. Here, using a combination of *in vitro* and *in vivo* experiments (inflammatory cell-specific (LysM-cre) *ER null* mice), we reveal the importance of inflammatory cell ERα for effective healing. Further, our data reveal that ERα-mediated promotion of alternative macrophage activation is a key factor in the effective progression of tissue repair.

Although both ERs are inflammatory cell expressed, our data specifically implicate inflammatory cell ERα in estrogen's healing-promoting effects. This observation appears at odds with our recent data revealing that in the epidermis ERβ mediates estrogen's beneficial effects on wound healing (Campbell *et al.*, 2010). In fact, it would appear that ER function is cell-specific during healing, with both epidermal ERβ and inflammatory cell ERα required for effective promotion of healing. This 'yin-yang' relationship has been demonstrated in other tissues (Lindberg *et al.*, 2003) and is extensively described in hormone-related cancers where ERα often promotes cell proliferation, whereas ERβ inhibits ERα's proliferative effects (Poola and Speirs, 2001; Attia and Ederveen, 2012). Indeed, at the level of gene expression, ERs often lead to diametrically opposed regulation (Williams *et al.*, 2008).

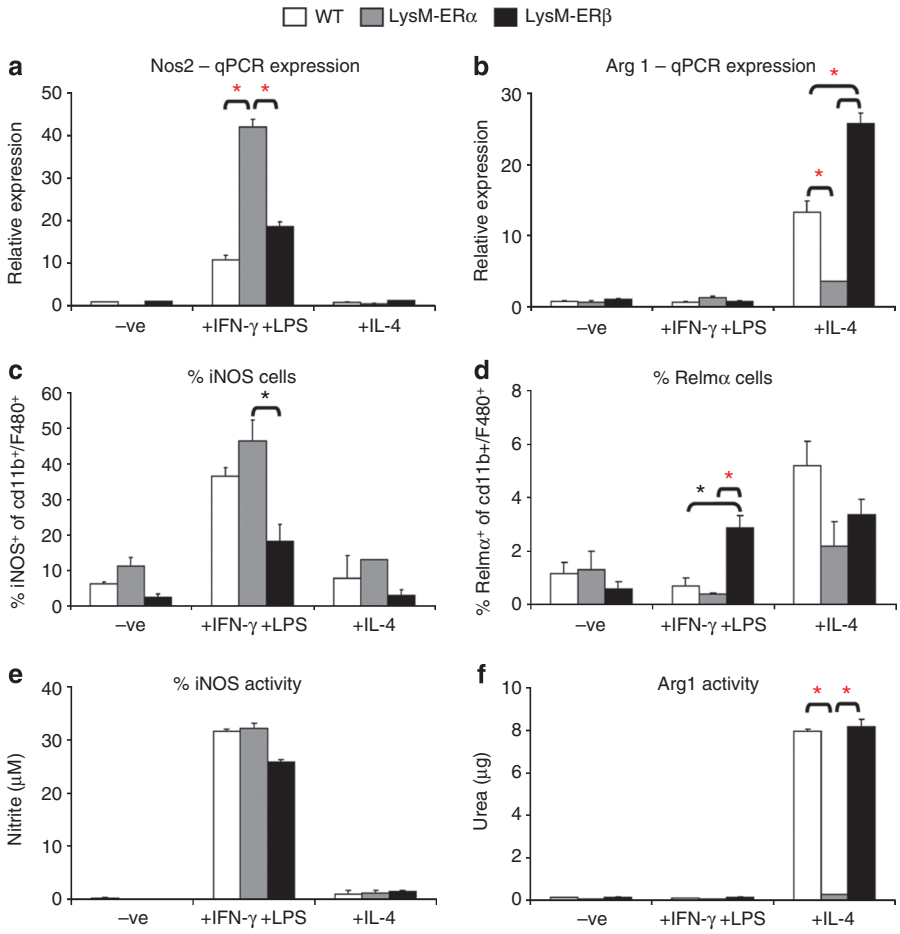

Figure 6. Estrogen receptor α (ERα) deletion impairs alternative macrophage activation *in vitro*. Bone marrow-derived macrophages (BMDMs) from wild type (WT), *LysM-ERα*, and *LysM-ERβ* mice were pretreated with 17β-estradiol (E; 10^{-7} M) for 16 hours, after which cells were polarized to either classically activated (CA) (+IFN-γ/+LPS) or alternatively activated (AA) macrophages (+IL-4) or left untreated. (**a**, **b**) Real-time PCR for *Nos2* (**a**) and *Arg1* (**b**) after 24 hours of polarization stimulation reveals a strong shift toward CA marker expression in *LysM-ERα* with significantly elevated expression of *Nos2*. (**c**, **d**) A similar profile is observed for flow cytometry analysis on cells isolated 24 hours post stimulation, using the markers inducible nitric oxide synthase (iNOS) (**c**) and Relmα (**d**) expressed as a percentage of $cd11b^+/F480^+$-positive cells. (**e**, **f**) Functional assays, nitric oxide production (**e**) and arginase activity (**f**), reveal a major reduction in arginase activity in *LysM-ERα* versus WT and *LysM-ERβ* cells following IL-4 stimulation. Mean + SEM, $n=3$ replicates per group and are representative of three separate experiments. (Black*) $P<0.05$, (red*) $P<0.01$.

Our data indicate that ERα is required to mediate estrogen's anti-inflammatory activity, and that inappropriate inflammatory cell influx in *LysM-ERα* mice is the probable cause of the observed delayed healing. Our findings fit with previous studies indicating that ERα is important for estrogen's anti-inflammatory activity: in T lymphocytes, ERα is required for estradiol-mediated protection against experimental autoimmune encephalomyelitis (Lelu *et al.*, 2011), and macrophage recruitment in acute and chronic brain injury is mediated through ERα (Polanczyk *et al.*, 2003). The corresponding increased expression of neutrophil and macrophage chemokines most likely drive the increased inflammatory cell influx. ERαKO mice display increased levels of proinflammatory chemokines (Ccl2, Ccl3, Ccl5, and Cxcl1) during neuroinflammation (Brown *et al.*, 2010), whereas the ERα-selective agonist PPT protects against influenza A virus pathogenesis via reduced Ccl2 expression (Robinson *et al.*, 2011). These findings suggest that key cytokines are involved in ERα-mediated inflammatory cell influx into tissue.

Although macrophage polarization is clearly linked to disease progression in a range of pathologies (Pesce *et al.*, 2009), the contribution to chronic wound healing is less clear. A previous study (Routley and Ashcroft, 2009) showed that estrogen administration promoted alternative macrophage polarization *in vivo*. We now show that alternative macrophage activation in the skin is mediated through ERα. Furthermore, our *in vitro* studies using macrophages from multiple sources indicate a role for ERα in not only promoting expression of AA markers but also functional activity. Importantly, we show that AA macrophage activation is associated with increased levels of *ERα* expression. In human monocytes/macrophages, estradiol is known to increase *ERα* expression, but has no effect on *ERβ* expression, and this becomes more pronounced during monocyte–macrophage

differentiation (Murphy *et al.*, 2009). Work exploring the role of ER in macrophage activation is currently limited, although Calippe *et al.* (2010) have recently shown that loss of ERα abolished the anti-inflammatory effects of estradiol on isolated peritoneal macrophages.

The underlying mechanisms that govern macrophage activation/polarization are not completely understood. AA macrophages are known to have impaired activation of NF-κB in response to LPS (Di Napoli *et al.*, 2005), and induction involves members of the JAK/signal transducer and activator of transcription (STAT) family (Zhu *et al.*, 2001). It has recently been shown that estrogen is able to inhibit NFκB-dependent inflammation by promoting synthesis of the negative regulator IκBα (Xing *et al.*, 2012). Conversely, a recent study shows that estrogen inhibits AA polarization in a tumor-associated macrophage cell line via the inhibition of the JAK1-STAT6 pathway (Yang *et al.*, 2012). The role of these pathways in cutaneous macrophage polarization remains unclear.

In summary, our data clearly indicate that the beneficial effects of estrogen on cutaneous healing are, in part, mediated through inflammatory cell ERα. Moreover, we suggest that ERα-mediated alternative macrophage activation is key for the promotion of tissue repair, a finding that could also be translated into other tissues/pathologies where ERα-mediated inflammation is important. Thus, differential tissue-specific ER-mediated effects on skin healing reveal important new targets for future therapies to promote effective healing.

MATERIALS AND METHODS

Animal experiments

All animal studies were approved by the UK Government Home Office (Project License 40/3203). C57BL/6 mice were purchased from Harlan Laboratory (Bicester, UK).

Isolation of BMDMs

BMDMs were isolated from C57Bl/6 mice or inflammatory cell-specific *ERα*- and *ERβ*-null mice and littermate controls, as described previously (Menzies *et al.*, 2009). Briefly, bone marrow cells were flushed with DMEM (without phenol red (Lonza, Slough, UK)) supplemented with 10% charcoal-stripped calf serum (Thermo Scientific, Loughborough, UK) and L-Glutamine (Invitrogen, Paisley, UK) + 1% prostrate specific antigen (PSA) (CellnTec, Buckingham, UK) using a 25G needle. The resultant cells were resuspended and plated at a density of 10^{-6} cells per ml. A concentration of 30 ng ml^{-1} macrophage colony stimulating factor (eBioscience, Hatfield, UK) was added to promote the differentiation of bone marrow cells into macrophages and cultured for 7–10 days at 37 °C and 5% CO_2. After differentiation, BMDMs were plated in 12-well plates (2 million cells per well) and pretreated with 17β-estradiol (10^{-7} M; Sigma Aldrich, Poole, UK), PPT 10^{-6} M (4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl); Tocris, Bristol, UK), or DPN 10^{-6} M (2,3-bis(4-Hydroxyphenyl)-propionitrile; Tocris) for 16 hours or left untreated. Subsequently, BMDMs were treated with either 1 μg/ml^{-1} LPS + 100 ng ml^{-1} IFN-γ or 20 ng ml^{-1} IL-4 for 24 hours to differentiate into CA or AA macrophages, respectively, or left untreated, and then used for expression analysis, flow cytometry, or enzymatic assays.

Isolation of peritoneal macrophages

Peritoneal macrophages were isolated from female mice by intraperitoneal lavage, as previously described (Emmerson *et al.*, 2010). A density of 10^6 cells per ml in suspension in RPMI medium (Invitrogen) supplemented with 10% charcoal-stripped calf serum (Thermo Scientific) were pretreated with PPT (10^{-6} M) or DPN (10^{-6} M) as for BMDCs. Subsequently, cells were treated with either 1 μg ml^{-1} LPS + 100 ng ml^{-1} IFN-γ or 20 ng ml^{-1} IL-4 or left untreated and collected for qPCR analysis at 6 or 24 hours.

Isolation of wound macrophages

Ten-week-old female mice were wounded according to our established protocol (Emmerson *et al.*, 2009). After killing, macrophages (Gr-1$^-$ CD11b$^+$) were isolated from excisional wounds (3 days post wounding). Tissue digestion was performed according to Mahdipour and Mace (2012). In brief, freshly harvested tissue was incubated overnight at 4 °C in Hanks buffered saline solution containing 1 mg ml^{-1} dispase I (Sigma), 10 mg ml^{-1} G418 (Sigma), and 3% fetal bovine serum, and then transferred to Hanks buffered saline solution containing 1 mg ml^{-1} collagenase D (Roche Diagnostics, Burgess Hill, Sussex, UK) and 75 U ml^{-1} DNase I (Qiagen, Crawley, UK) for 2 hours at 37 °C. Cell suspensions from both steps were pooled, filtered (70 μM cell strainer, BD Bioscience, Oxford, UK), centrifuged, and washed three times with phosphate-buffered saline + 3% fetal bovine serum and resuspended in phosphate-buffered saline + 3% fetal bovine serum for antibody incubation. Cells were blocked with Fc-block for 5 minutes at room temperature, and macrophages were isolated by first depleting Gr-1$^+$ cells by using DSB-X-biotin-labeled anti-Gr-1 and dynabeads (Life Technologies, Paisley, UK), followed by isolation of CD11b$^+$ cells using an APC-positive selection EasySep system (Stem Cell Technologies, Grenoble, France), according to the manufacturer's protocol. Isolated cells were plated at a density of 10^6 cells per ml and pretreated with PPT (10^{-6} M) or DPN (10^{-6} M) as for BMDCs. Subsequently, cells were treated with either 1 μg ml^{-1} LPS + 100 ng ml^{-1} IFN-γ or 20 ng ml^{-1} IL-4 or left untreated and collected for qPCR analysis at 6 hours.

RNA isolation and qPCR

RNA was isolated from cell lysate or whole-wound homogenate using the Purelink RNA kit (Life Technologies). cDNA was transcribed from 1 μg of RNA (Transcriptor reverse transcriptase; Roche). Quantitative real-time PCR was performed using MESSA GREEN qPCR SYBR Mastermix (Eurogentec, Southampton, UK) and an iQ qPCR thermal cycler (Bio-Rad Laboratories, Hemel Hempstead, UK). Each sample was serially diluted over three orders of magnitude, and all samples were run on the same 96-well plate. Expression ratios were determined relative to a standard sample and normalized using a value derived from the housekeeping gene *Gapdh*. Primer sequences are listed in Supplementary Table S1 online.

Flow cytometry

Cells were detached and transferred to FACs tubes and resuspended in staining buffer containing phosphate-buffered saline, 0.1% BSA, and 0.05% sodium azide. Fc receptors were blocked with anti-CD16/CD32 antibody (BD Biosciences) for 20 minutes on ice, followed by the addition of 1.5 μg ml^{-1} PerCP-conjugated 7AAD (eBioscience)

for a further 5 minutes. Cells were washed in phosphate-buffered saline containing 10% fetal calf serum and stained with 1.5 μg ml^{-1} Pe-Cy7-conjugated anti-mouse Cd11b (eBioscience) and Pacific Blue-conjugated anti-mouse F480 (AbD Serotec, Kidlington, UK) for 60 minutes on ice. For intracellular staining, cells were subsequently incubated in Cytofix/Cytoperm Fixation/Permeabilisation Solution according to the manufacturer's instructions (BD Biosciences) and stained with 1.5 μg ml^{-1} AF488-conjugated anti-mouse iNOS (BD Biosciences) and 3 μg ml^{-1} rabbit-polyclonal Relmα antibody (PeproTech EC, London, UK) for 45 minutes on ice, followed by 1.5 μg ml^{-1} phycoerythrin-conjugated secondary antibody for a further 30 minutes. Cells were washed before being resuspended in FACs buffer and analyzed on a BD LSRII flow cytometer.

Greiss assays for determination of NO production

Quantification of nitrite accumulation was used as a measure of NOS2 activity. A volume of 50 μl of Greiss reagent Part 1 (1% sulfanilamide in 5% phosphoric acid) was incubated with 50 μl of BMDM cell supernatant for 10 minutes. A volume of 50 μl of Greiss reagent Part 2 (0.1% *N*-(1-Naphthyl)ethylenediamine dihydrochloride in dH_2O) was added and incubated for 10 minutes in the dark and the absorbance was read at 570 nm. Nitrite production was determined by comparison with a standard curve generated from known $NaNO_2$ concentrations.

Arginase activity assay

Arginase activity was assessed by measuring the amount of urea production via metabolism of L-arginine by arginase (Corraliza *et al.*, 1994). Briefly, BMDMs were vortexed in 100 μl of 0.1% Triton X-100 (Sigma Aldrich), incubated for 30 minutes at room temperature, and 100 μl of assay buffer (10 mmol l^{-1} MnCl in 50 mmol l^{-1} Tris, pH 7.5) was added and heated at 55 °C for 10 minutes to activate the enzyme. Triplicate samples of 25 μl of cell lysate in buffer were incubated with 25 μl of 0.5 M L-arginine (Sigma Aldrich) for 60 minutes at 37 °C, after which the reaction was stopped by adding 400 μl of acid. A volume of 25 μl of 9% α-isonitrosopropiophenone (Sigma Aldrich) was added and incubated for 45 minutes at 100 °C in the dark. Absorbance was measured at 570 nm using a MRXII (Dynex Technologies, Worthing, UK). Arginase activity was determined using a standard curve generated from known urea concentrations.

Wounding experiments

Inflammatory cell-specific *ERα* and *ERβ-null* mice (*LysM-ERα*$^{-/-}$ and *LysM ERβ*$^{-/-}$) were generated by crossing *ERα*$^{fl/fl}$ and *ERβ*$^{fl/fl}$ mice (Campbell *et al.*, 2010) with the well-characterized *LysM-Cre* mice (Clausen *et al.*, 1999). Female heterozygote littermates were used as controls. Twelve-week-old female mice that had undergone ovariectomy 1 month previously were wounded (two equidistant 1-cm full-thickness skin incisional wounds made through skin and panniculus carnosus muscle using a scalpel blade (Swann-Morton, Sheffield, UK) and left to heal by secondary intention) according to our established protocol (Emmerson *et al.*, 2009). Exogenous estrogen was administered at the time of wounding by subcutaneously implantation of a 0.05-mg, 21-day, slow-release 17β-estradiol pellet (Innovative Research of America, Sarasota, FL) with successful estrogen replacement confirmed by enzyme immunoassay on serum samples.

Animal euthanasia and tissue collection

Animals were killed by rising carbon dioxide overdose and confirmed by cervical dislocation. Wounds were excised and bisected (laterally at the midpoint) at days 3 and 7 post wounding, with one half fixed in formalin-fixative solution for histology and the remaining half snap-frozen in liquid nitrogen and stored at −80 °C before RNA isolation.

Histology and immunohistochemistry

Five-micrometre sections were cut from bisected wound tissue embedded in paraffin wax and stained with hematoxylin and eosin or subjected to immunohistochemistry with rat anti-neutrophil (ThermoScientific), rat anti-Mac-3 (Becton Dickinson, Oxford, UK), rabbit anti-iNOS, goat anti-arginase 1 (Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-nitrotyrosine (Millipore, Watford, UK), or goat anti-Ym1 (R&D Systems, Abingdon, UK) and the appropriate biotinylated secondary antibody followed by ABC-peroxidase reagent (Vector Laboratories, Peterborough, UK) with Novared substrate and counterstaining with hematoxylin. For immunofluorescence, labeled secondary antibodies were used (Invitrogen). Wound area and cell numbers were quantified with the Image Pro Plus software (Media-Cybernetics, Silver Spring, Maryland), as previously described in detail (Emmerson *et al.*, 2012). Briefly, wound area was measured from the panniculus carnosus muscle, to the margins of normal skin on either side of the wound, and under the epidermis or eschar. Re-epithelialization was calculated as a percentage by using the measurement of newly formed epidermis divided by the distance that the epidermis would migrate to fully close the wound.

Statistical analysis

Statistical differences were determined using analysis of variance (one-way and two-way) (SIMFIT, The University of Manchester). A *P*-value of <0.05 was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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