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Local Arginase 1 Activity Is Required for Cutaneous Wound Healing

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Chronic nonhealing wounds in the elderly population are associated with a prolonged and excessive inflammatory response, which is widely hypothesized to impede healing. Previous studies have linked alterations in local L-arginine metabolism, principally mediated by the enzymes arginase (Arg) and inducible nitric oxide synthase (iNOS), to pathological wound healing. Over subsequent years, interest in Arg/iNOS has focused on the classical versus alternatively activated (M1/M2) macrophage paradigm. Although the role of iNOS during healing has been studied, Arg contribution to healing remains unclear. Here, we report that Arg is dynamically regulated during acute wound healing. Pharmacological inhibition of local Arg activity directly perturbed healing, as did Tie2-cre-mediated deletion of *Arg1*, revealing the importance of Arg1 during healing. Inhibition or depletion of Arg did not alter alternatively activated macrophage numbers but instead was associated with increased inflammation, including increased influx of $iNOS^+$ cells and defects in matrix deposition. Finally, we reveal that in preclinical murine models reduced Arg expression directly correlates with delayed healing, and as such may represent an important future therapeutic target.

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INTRODUCTION

Expanding global elderly and diabetic populations combined with a continued lack of effective treatment modalities means the incidence of chronic wounds is increasing. Chronic wounds are associated with an excessive inflammatory response, which is widely accepted to be a major causative factor in the multifactorial healing pathology (Loots *et al.*, 1998; Diegelmann, 2003). Macrophages, the key mediators of the inflammatory response to infection and repair, display clear plasticity that permits development into a spectrum of phenotypes depending on environmental and cytokine signals. Seminal studies have classified the major macrophage subtypes that lie at the polar ends of the spectrum, these include (a) Th1-induced classically activated macrophages (CAMs)—IFN-γ and tumor necrosis factor-α induced with enhanced antimicrobial capacity and proinflammatory cytokine production (Mosser and Zhang, 2008) and (b) Th2-induced alternatively activated macrophages (AAMs)—IL-4 and/or IL-13 induced with anti-inflammatory ''tissue'' repair functions (Gordon and Martinez, 2010). Although the disease relevance of macrophage polarization (or lack of) has been demonstrated in numerous tissue pathologies and clearly linked to disease progression (Hesse *et al.*, 2001; Pesce *et al.*, 2009; Sindrilaru *et al.*, 2011), the contribution of these macrophage subtypes to chronic wound pathology remains unclear.

CAMs and AAMs are phenotypically different with AAMs identified through the expression of cell surface receptors IL4Rα chain and mannose receptor and intracellular enzymes *Retnla* (encoding Fizz1/RELMα), *Chi3l3* (Ym1), and *Arg1* (Gordon and Martinez, 2010). CAMs and AAMs are thought to have different functions during the host response, mediated partly by the upregulation of intracellular enzymes, inducible nitric oxide synthase (iNOS) in CAMs, and arginase (Arg) in AAMs. Although Arg1 is predominantly associated with AAMs, its expression has also been observed in CAMs in chronic parasitic and bacterial infection (El Kasmi *et al.*, 2008; Gordon and Martinez, 2010). Interestingly, iNOS and Arg can compete for their common substrate, the amino acid L-arginine, which is a key component of the urea cycle. L-arginine metabolism by iNOS, through substrate competition with Arg, produces L-citrulline and nitric oxide, a critical mediator of immunological and physiological aspects of tissue repair. It is noteworthy that iNOS-deficient mice display altered epithelial and endothelial cell proliferation and migration (Ziche *et al.*, 1994; Yamasaki *et al.*, 1998). Arg exists as two isoforms with Arg1 previously linked to tissue regeneration (Peranzoni *et al.*, 2007). Both Arg1 and Arg2 metabolize L-arginine into L-ornithine and urea. L-ornithine is a

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Abbreviation: AAM, alternatively activated macrophage; Arg, arginase; CAM, classically activated macrophage; iNOS, inducible nitric oxide synthase; nor-NOHA, N(omega)-hydroxy-nor-L-arginine

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precursor of proline and polyamines, which promote collagen synthesis and cell proliferation, respectively, key aspects of tissue regeneration (Jenkinson *et al.*, 1996; Witte *et al.*, 2002). The expression and activity of Arg and iNOS must therefore be tightly regulated to provide tissues with the appropriate biological mediators. Indeed, a dysregulated balance between the local iNOS and Arg activity has been suggested to promote chronic disease (Unal *et al.*, 2005; Maarsingh *et al.*, 2006; Naura et al., 2010; Redente et al., 2010) and potentially impair wound healing in elderly subjects (Childress *et al.*, 2008; Debats *et al.*, 2009).

Recent studies have begun to focus on the role of macrophage activation/polarization during healing, with Miao *et al.*, (2012), reporting altered macrophage activation in diabetic mouse wounds. Data in this area remain somewhat contentious with iNOS-deficient mice displaying delayed healing or no effect on healing depending on the wound model investigated (Yamasaki *et al.*, 1998; Most *et al.*, 2002). Surprisingly, although Arg has been found to be functionally important in multiple disease pathologies (Abeyakirthi *et al.*, 2010; Maarsingh *et al.*, 2006; Pesce *et al.*, 2009), little is known about the role of Arg1 in normal skin repair. Here, we report the effects of both functional Arg inhibition (via local nor-NOHA treatment) and genetic ablation of Arg1 (cell-specific deletion *T2C;Arg1*$^{fl/fl}$) during skin repair. In both models, Arg deficiency delays healing associated with an altered inflammatory response and abnormal matrix deposition.

RESULTS

Arg1 is dynamically regulated during acute healing

Previous studies have suggested that macrophage phenotype is temporally regulated during wound healing, with CAMs present at early stages and AAMs more dominant during later stages (Albina *et al.*, 1990; Daley *et al.*, 2010). We confirmed this temporal profile in our C57/Bl6 excisional wound model using immunohistochemistry for Arg1 and iNOS, widely accepted markers of CAM and AAM activation, respectively (Gordon and Martinez, 2010). In the acute healing model, iNOS levels peaked at 3 days post wounding, whereas Arg1 remained high until 7 days (Figure 1). These time points correlate with the transition from a proinflammatory extracellular milieu to a phase of matrix deposition (Shaw and Martin, 2009). To corroborate these findings further, we analyzed Arg enzymatic activity, which provides a

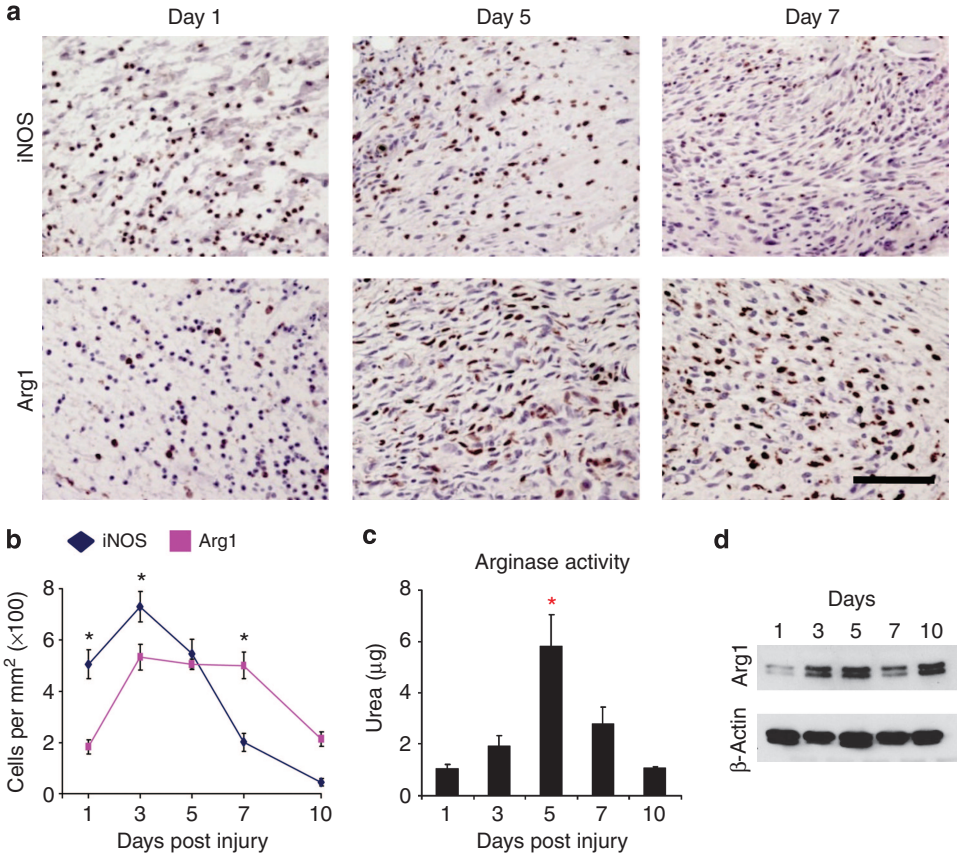

Figure 1. Arginase1 (Arg1) is dynamically regulated during healing. (**a**) Images representing the experimental group mean for inducible nitric oxide synthase-positive (iNOS$^+$) and Arg1$^+$ cells in 1-, 5-, and 7-day excisional wound granulation tissue. (**b**) Quantification of iNOS$^+$ and Arg1$^+$ dermal inflammatory cells reveals differing temporal profiles. Immunohistochemical quantification data are derived from the mean of five randomly selected high-powered fields per wound and two wounds per mouse. (**c**) Arginase activity from isolated excisional wound tissue (measured through urea production) peaks at 5 days post wounding. (**d**) Western blot analysis of total Arg1 protein in excisional wounds reveals increased expression at 3, 5, and 10 days post wounding. (**b**) Data presented indicate mean + SEM of $n = 5$–6 mice per group or (**c**, **d**) three replicates per group across two individual experiments. Bar = 100 μm. (**b**) *$P < 0.05$ comparing iNOS with Arg1, (**c**) *$P < 0.05$ compared with days 1, 3, and 10.