In vitro and in vivo Repair Activities of Undifferentiated and Classically and Alternatively Activated Macrophages

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Introduction

Wound healing is a vital process aimed at the restoration of tissue integrity and homeostasis following injury. Immune and nonimmune cell types are implicated in this process. Macrophages promote several essential aspects of wound repair [1–3]. They clear invading microorganisms, scavenge debris and support tissue repair by releasing a variety of growth factors: transforming growth factor β (TGF-β), insulin-like growth factor I, hepatocyte growth factor and vascular endothelial growth factor [4]. As a consequence, depletion of macrophages during experimental dermal injury results in delayed re-epithelialization, reduced collagen deposition, impaired angiogenesis and decreased cell proliferation in the healing wounds [2, 3]. Likewise, the repair of colon ulcers induced by the oral administration of dextran sodium sulfate (DSS) [5] is severely delayed following macrophage depletion [6]. Our laboratory recently reported that the healing of DSS-induced colon ulcers is significantly enhanced by the administration of granulocyte-macrophage colony-stimulating factor, a factor that promotes rapid accumulation of macrophages within the ulcerated colon mucosa [7]. Taken together, these results show that wound repair is a process that critically depends on macrophages.

Key Words
Colitis · Dextran sodium sulfate · Macrophages · Wound healing

Abstract
Objective: Macrophages play a critical role in wound repair. However, the specific role of the different macrophage subtypes in wound repair remains incompletely understood. The aim of this study was to compare the wound repair activities of undifferentiated macrophages (M0), classically activated macrophages (M1) and alternatively activated (M2) macrophages.

Methods: The macrophage repair activities of intestinal wounds were evaluated using in vitro and in vivo models.

Results: All three macrophage subtypes enhanced wound closure in vitro, with the M2 macrophages demonstrating greater repair activities than the M0 and M1 macrophages. Injection of M0 and M2 macrophages into mice with experimental dextran sodium sulfate-induced colitis significantly enhanced ulcer repair when compared to control mice. In contrast, injection of M1 macrophages did not affect ulcer repair.

Conclusions: These results underscore the wound repair capacity of different macrophage subsets. Notably, wound repair activity is not restricted to M2 macrophages, as the current literature suggests.

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Mosser and Edwards [8] described four major subtypes of macrophages: nondifferentiated macrophages (M0), regulatory macrophages, classically activated macrophages (M1) and alternatively activated macrophages (M2). Macrophage differentiation depends on the cytokine environment. Differentiation of regulatory macrophages requires the presence of glucocorticoids and TGF-β [9]. Regulatory macrophages modulate the immune response by producing high levels of interleukin-10 (IL-10) [9]. M1 macrophages are generated mainly during Th1 cell-mediated responses by interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). They are considered ‘pro-inflammatory’ macrophages, as they release high levels of inflammatory mediators and cytokines like nitric oxide (NO) and IL-1β, among others [10, 11]. Conversely, M2 macrophages are considered ‘anti-inflammatory’ due to a markedly lower production of inflammatory cytokines, and they are generated during Th2 cell responses by IL-4 [12, 13]. Of note, M2 macrophages have long been considered to be ‘wound repair macrophages’ due to their high expression of arginase 1 (Arg1) [8]. Arg1 is an enzyme that converts L-arginine into urea and L-ornithine, which are then converted by ornithine aminotransferase into L-proline, an essential component of collagen and a central substrate in wound repair [14]. M0 and M1 macrophages, however, do not express notable levels of Arg1 and are currently not considered to be relevant players in wound repair.

In order to clarify the respective roles of different macrophage subtypes in wound repair, we systematically compared the repair activities of M0, M1 and M2 macrophages using established in vitro and in vivo models of intestinal wounds. M1, M2 and M0 macrophages were differentiated from bone-marrow-derived progenitor cells in vitro in the presence of IFN-γ, IL-4 or with no addition of an exogenous cytokine, respectively. Repair activities were studied by quantifying (1) wound re-epithelialization in vitro and (2) ulcer repair in a DSS-induced colitis model in vivo.

**Materials and Methods**

**Mice**

Specific-pathogen free female BALB/c mice (aged 9–12 weeks) were obtained from Harlan (Ad Horst, The Netherlands). The animal experiments were approved by the State Veterinary Office (authorization No. 1748.1).

**Administration of DSS**

Groups of 5 mice were given 4% (wt/v) DSS (mol wt 47,000; Tdb Consultancy, Uppsala, Sweden) in drinking water ad libitum for 7 days. On day 7, mice were either sacrificed or returned to tap water and then sacrificed on day 10.

**Evaluation of Ulcer Repair**

Ulcer repair was evaluated on hematoxylin and eosin (HE)-stained step sections of 5–7 complete colon rings per animal. Histological analysis was performed by a pathologist (H.-A.L.) in a strictly blinded fashion. Repair scores were defined as follows: score 1 = incomplete ulcer re-epithelialization without crypt restoration, score 2 = complete ulcer re-epithelialization without underlying crypt restitution, score 3 = complete ulcer re-epithelialization with beginning crypt regeneration, score 4 = complete ulcer re-epithelialization with advanced crypt regeneration re-establishing most of the former ulcer base, score 5 = restitution ad integrum and score 6 = no ulcers. In this way, 29–31 colon sections were scored for each group of mice (5 mice per group).

**Macrophage Differentiation**

Macrophages were generated by seeding 3 × 10^6 mouse bone-marrow cells in bacteriological Petri dishes with 10 ml of Iscove’s modified Dulbecco’s medium GlutaMAX™ medium (GIBCO, Basel, Switzerland) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), β2-mercaptoethanol (50 μM), 10% heat-inactivated fetal calf serum and 10 ng/ml macrophage colony-stimulating factor (R&D Systems, Minneapolis, Minn., USA). On day 3, 10 ml of fresh medium was added to the culture. On day 7, macrophages were harvested by scraping, and were then seeded in new bacterial Petri dishes (up to 10 × 10^6 cells/dish) for 48 h with 20 ml of fresh medium supplemented (or not) with cytokines. Macrophages were then cultured with IFN-γ (20 ng/ml, R&D Systems) to generate M1 macrophages, or with IL-4 (20 ng/ml, R&D Systems) to generate M2 macrophages. The macrophages cultured without any cytokines were considered as M0 macrophages.

**Macrophage Transfer**

Macrophages subtypes were harvested by scraping and after two PBS washes, 1 × 10^6 cells were administered intravenously to mice on days 3 and 4 of DSS exposure, as previously described [7]. Noninjected DSS-administered mice and nonmanipulated mice were used as controls. For the in vivo tracking experiments, the mice were injected on day 3 of DSS exposure with 1 × 10^6 macrophage subtypes labeled using the PKH26 red fluorescence cell-linker kit (Sigma). Recipient mice were sacrificed 24 h later and their colons were frozen in Tissue-Tek OCT compound (Sakura-Finetek, Zoeterwoude, The Netherlands). Sections of 5-μm thickness were cut at –20°C and fixed with 2% paraformaldehyde solution, counterstained with DAPI (Roche Diagnostics) for 5 min to identify cell nuclei and finally analyzed with a fluorescence microscope (Olympus IX81).
**In vitro Wound Closure Assays**

In vitro wound closure assays were performed as previously described [15]. Briefly, CMT-93 mouse rectal epithelial cell monolayers were wounded using a razor blade and then cocultured with 2.5 × 10^5 M0, M1 or M2 macrophages. The plate was fixed with paraformaldehyde 24 h after wounding (t = +24). Pictures of the wounds were taken at t = 0 and t = +24 h and wound surfaces were measured using Photoshop software. The percentage of wound repair was calculated using the following formula: 

\[
\text{Repair} \% = \left( \frac{\text{wounded area } t = 0 - \text{wounded area } t = +24}{\text{wounded area } t = 0} \right) \times 100
\]

Cells cultured without macrophages had a percentage of wound closure between 30–40%.

**Quantitative Reverse-Transcription Polymerase Chain Reaction**

Total RNA was isolated using RNasy Plus Mini kit (Qiagen, Valencia, Calif., USA). Total RNA samples (1 μg) were submitted for reverse transcription using the ThermoScript RT-PCR system (Invitrogen Life Technologies, Carlsbad, Calif., USA) according to the manufacturer’s protocol, and oligo-dT as primers. PCR amplification was performed on a MyiQ iCycler (Bio-Rad, Hercules, Calif., USA) using the iQ SYBR Green Supermix (Bio-Rad) and primers for GAPDH, TNF-α, HPRT, resistine-like/FIZZ1, IL-6, inducible nitric oxide synthase (iNOS), IL-1β, Arg1 and interferon regulatory factor 4 (IRF-4;  sequences available upon request). For each individual sample, mRNA quantification was performed by normalizing the number of mRNA copies obtained for the gene of interest per million of mRNA copies obtained for the housekeeping genes GAPDH or HPRT for in vivo and in vitro samples, respectively. Relative mRNA expression levels were calculated for each experimental condition by normalizing individual data to the mean value obtained with the control group of untreated macrophages (in vitro experiments) or control mice (in vivo experiments).

**Myeloperoxidase Quantification**

Total proteins were extracted from colon samples using 0.5% Cetavlon, morpholine-3-propanesulfonic acid (10 mM/ml, pH = 7) extraction buffer and then quantified using a bicinchoninic acid (Biorad). Myeloperoxidase (MPO) activity was determined on 10 μg of colon proteins by spectrophotometry as previously described [16].

**Statistical Analysis**

Analyses of in vitro and in vivo data, as well as qPCR results, were performed using a Kruskal-Wallis test to assess the difference between experimental conditions. If there was a statistical difference, experimental conditions were compared by pairs using the Mann-Whitney U test with the Bonferroni correction.

**Results**

**Differentiation of Macrophage Subtypes**

M0, M1 and M2 macrophages were generated in vitro from mouse bone-marrow cells with no exogenous cytokine or in the presence of interferon-γ or IL-4, respectively [8]. In order to confirm macrophage differentiation, mRNA expression levels of M1 markers were analyzed by qPCR. M1 macrophages expressed high levels of mRNA encoding iNOS (fig. 1a), TNF-α (fig. 1b) and IL-1β (fig. 1c) [8], while M2 macrophages expressed high levels of found in inflammatory zone 1 (FIZZ1; fig. 1d), interferon regulatory factor 4 (IRF-4; fig. 1e) [17] and Arg1 (fig. 1f). The M0 macrophages had lower mRNA expression levels of M1 and M2 marker genes (fig. 1), reflecting the absence of differentiation.

**In vitro Repair Activities of Macrophage Subtypes**

The efficacy of different macrophage subtypes in promoting wound re-epithelialization was evaluated in vitro. CMT-93 rectal epithelial cell monolayers were wounded using a razor blade (fig. 2a) and then cocultured with M0, M1 or M2 macrophages. CMT-93 monolayers cultured in the absence of macrophages as with CD4 T cells isolated from the spleen [7] exhibited minimal wound closure 24 h after wounding (fig. 2a). The addition of M0, M1 or M2 macrophages to wounded epithelial monolayers accelerated wound closure (fig. 2b). Remarkably, M2 macrophages promoted wound closure at a higher level when compared to M0 and M1 macrophages (fig. 2b). Taken together, these results indicate that M0, M1 and M2 macrophages are able to promote intestinal wound re-epithelialization in vitro. However, M2 macrophages show a stronger repair activity than M0 and M1 macrophages.

**In vivo Repair Activities of Macrophage Subtypes**

We previously reported that intravenous injection of splenic CD11b+ cells, a heterogeneous cell population containing 30% of cells expressing the macrophage marker F4/80, promoted repair of colonic ulcers induced by the oral administration of DSS [7]. Remarkably, injected splenic CD11b+ cells [7] and in vitro bone marrow-derived macrophages [18] colonized the inflamed colon mucosa, engaging in immediate contact with colonic epithelial cells. This observation suggested a direct cross-talk between macrophages and intestinal epithelial cells. We used this same experimental setting in this study to evaluate the in vivo repair activities of M0, M1 and M2 macrophages.

DSS-treated mice were injected with fluorescently labeled macrophages in order to evaluate the ability of M0, M1 and M2 macrophages to home into the inflamed colon mucosa (fig. 3). Comparable numbers of fluorescent M0, M1 and M2 macrophages were detected in the murine colons 24 h after injection (fig. 3). Similar results were obtained at 6 and 12 h after injection (data not shown). This result suggests that M0, M1 and M2 macrophages home into the inflamed colon equally.
**Fig. 1.** In vitro differentiation of macrophage subtypes. mRNA expression levels of the M1-associated marker genes iNOS (a), TNF-α (b) and IL-1β (c) and of the M2-associated marker genes FIZZ1 (d), IRF-4 (e) and Arg1 (f) were quantified by qPCR. Data are expressed as means ± SEM, with the number of independent macrophage subtypes cultures analyzed for each gene shown in parentheses. p values were calculated using the Mann-Whitney U test.

**Fig. 2.** M0, M1 and M2 macrophages promote in vitro intestinal epithelial repair. a Confluent epithelial cell monolayers were wounded with a razor blade and cultured for 24 h in the absence (left) or presence (right) of $2.5 \times 10^5$ M0 macrophages. Arrows indicate the limits of the wounded area. b Graphic rendition of wound closure data of 3 independent experiments. Data are expressed as means ± SEM, with the number of wounds used to determine the repair activity of macrophage subtypes in parentheses. p values were calculated using the Mann-Whitney U test.
In the next series of experiments, unlabeled M0, M1 and M2 macrophages were injected intravenously into DSS-treated mice. The mice were sacrificed on days 7 and 10 and their colons were recovered and processed for subsequent histomorphological analysis. Intravenous injection of M0 macrophages into DSS-treated mice significantly accelerated ulcer repair on both day 7 and day 10 when compared to control mice (fig. 4). Injection of M2 macrophages promoted ulcer repair on day 10 but not on day 7 (fig. 4). In contrast, injection of M1 macrophages into colitic mice did not affect ulcer repair, neither on day 7 nor on day 10. Taken together, these results show that M0 and M2, but not M1 macrophages, promote ulcer repair activities in vivo.

Effect of Macrophage Injection on Colon Inflammation

The development of ulcers within the colon mucosa is associated with the translocation of bacteria across the damaged mucosa into the colon lamina propria. This bacterial invasion triggers the development of colon inflammation, the recruitment of inflammatory cells (i.e. neutrophils) and the production and release of inflammatory mediators. As it is well known that M1 and M2 macrophages sustain and decrease inflammation, respectively, we explored the effects of macrophage injection on colon inflammation. To this end, mRNA expression levels of inflammatory markers and MPO activity were quantified in colon extracts. MPO is the major com-
ponent of neutrophil granules and greater MPO activity in the colon indicates a massive infiltration of neutrophils within the colon mucosa. Injection of M0, M1 and M2 macrophages into DSS-treated mice did not reduce MPO activity either at day 7 or day 10, suggesting that macrophage injection did not affect colonic neutrophil recruitment (fig. 5a).

Next, mRNA expression levels of inflammatory markers were quantified by qPCR. On day 7 of DSS exposure, colitic mice demonstrated markedly high expression levels of mRNA encoding IL-1β, IL-6, iNOS, TNF-α and IFN-γ-inducible 10-kD protein (IP-10) when compared to control mice (fig. 5). As expected, the mice had reduced expression levels of inflammatory cytokines on day 10, i.e. 3 days after discontinuing the administration of DSS. Remarkably, macrophage injections into DSS-treated mice had levels of inflammatory markers very similar to the control mice, both on day 7 and day 10 (fig. 5). Indeed, mRNA expression levels encoding IL-6, IP-10, TNF-α and iNOS were virtually identical in the four groups of mice (fig. 5). However, on day 7, there was a significant reduction of IL-1β mRNA expression levels in the colons of the M0-macrophage-injected mice as well as a significant decrease of TNF-α expression in the colons of M2-
macrophage-injected mice compared to the control mice. Therefore, on day 7, M0 and M2 injections very modestly decreased the extent of colon inflammation. At the same time point, M1 macrophage injection did not modify colon inflammation. On day 10, injections of M0, M1 and M2 macrophages did not modulate colon inflammation. Taken together, these data show that colon inflammation in mice with experimental DSS colitis at an early stage improved after M0 and M2 macrophage injections, albeit to a moderate degree, and that M1 macrophage injection did not affect colon inflammation.

Discussion

This study aimed at evaluating the repair activities of M0, M1 and M2 macrophages. We provide evidence herein that all macrophage subtypes promote epithelial repair in vitro, but that M2 macrophages exhibit the strongest repair activity. In vivo, the injection of M0 or M2 macrophages into DSS-treated mice accelerated colon ulcer repair, while M1 macrophages showed no beneficial effect.

In our in vitro model of wound repair, epithelial wound closure relies predominantly on epithelial restitution [19]. Restitution is the first stage of wound repair and a key step allowing re-epithelialization of the intestinal mucosa following epithelial cell loss [20]. We observed that M2 macrophages have very strong repair activities in vitro (fig. 2), suggesting a higher efficacy in promoting restitution when compared to M0 and M1 macrophages. The greater repair activity of M2 macrophages might be associated with the increased secretion of repair-promoting factors like TGF-β, insulin-like growth factor I or hepatocyte growth factor and/or by the high expression of Arg1 (fig. 1) [8]. Daley et al. [21] showed that the macrophages purified from wounds do not exclusively express M2 markers but present a mixed phenotype characteristic of both M1 and M2 markers. Interestingly, our results showed that M0 and M1 macrophages also promote epithelial restitution, suggesting that the promotion of epithelial repair is an intrinsic feature of macrophages and does not depend on the macrophage subtype or phenotype.

Since M0, M1 and M2 macrophages promoted epithelial repair in vitro, their capacity to promote ulcer repair was then evaluated in vivo. Remarkably, intravenous injection of M0 macrophages into DSS-treated mice promoted ulcer repair on day 7 when compared to control mice or to mice injected with M1 or M2 macrophages (fig. 3). On day 10, the promotion of wound repair was only observed in mice injected with M0 or M2 macrophages (fig. 3). These results are in line with previous studies that show that macrophage injections promote wound repair in mice suffering from skin or kidney injuries [22, 23]. Despite exhibiting repair activity in vitro, injection of M1 macrophages into colitic mice failed to promote wound repair in vivo, on day 7 and on day 10. These results confirm the work of Wang et al. [24] who showed that injection of M1 macrophages did not promote repair in mice suffering from renal injuries. It is known that activated M1 macrophages promote inflammation, so it is tempting to suggest that the discrepancy between in vitro and in vivo finding might be explained by the absence of macrophage activation in vitro.

Injection of M2 macrophages into colitic mice significantly promoted epithelial repair on day 10 but not on day 7 when compared to control mice (fig. 3). The absence of increased ulcer repair on day 7 following M2 macrophage injection is potentially linked to the anti-inflammatory profile of M2. Although not enhancing inflammatory processes, injected M2 macrophages may be less efficient in clearing bacteria, an essential prerequisite for ulcer healing. On day 10, the anti-inflammatory properties of M2 macrophages may be beneficial to complete ulcer healing. This scenario is not purely speculative, but is based on the work of Hunter et al. [18] who showed that, in a murine model of dinitrobenzene sulfonic acid-induced colitis, the transfer of M2 macrophages markedly reduced the clinical signs of colitis, an effect that was abolished by the injection of functionally blocking anti-IL-10 antibodies [18].

Remarkably, the promotion of intestinal repair by M0 macrophage injection into colitic mice was detected on days 7 and 10 (fig. 4). M0 macrophages are undifferentiated macrophages that can still be ‘educated’ to differentiate into specific macrophage subtypes. Therefore, one has to assume that during the first days of DSS exposure, M0 macrophages home into the site of colonic inflammation and undergo M1 and M2 differentiation for the effective control of bacterial invasion, tissue destruction and to release anti-inflammatory molecules and factors promoting angiogenesis and epithelial cell proliferation and differentiation. The acquisition of M1 and M2 features could be a pivotal phenomenon to efficiently promote the different stages of wound repair [8]. Although all macrophages maintain phenotypic plasticity, M0 might have a higher and/or faster differentiation capacity than M1 or M2 macrophages, and this could then explain the greater
repair activities of M0 macrophages when compared to M1 and M2 macrophages.

The observation that M0 and M2 macrophage injections promote ulcer repair warrants translation into clinical practice. In the last few years, clinical data have shown that promoting mucosal healing plays a key role in inducing and maintaining remission in Crohn’s disease or ulcerative colitis patients [25]. Therefore, from a clinical perspective, we agree with the proposal by Hunter et al. [18] that injections of M0 or M2 macrophages into these patients may indeed promote mucosal healing in the short or long term, alone or in combination with conventional therapies.

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References


