Original Article

Vessel-associated myogenic precursors control macrophage activation and clearance of apoptotic cells

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Summary

Swift and regulated clearance of apoptotic cells prevents the accumulation of cell remnants in injured tissues and contributes to the shift of macrophages towards alternatively activated reparatory cells that sustain wound healing. Environmental signals, most of which are unknown, in turn control the efficiency of the clearance of apoptotic cells and as such determine whether tissues eventually heal. In this study we show that vessel-associated stem cells (mesoangioblasts) specifically modulate the expression of genes involved in the clearance of apoptotic cells and in macrophage alternative activation, including those of scavenger receptors and of molecules that bridge dying cells and phagocytes. Mesoangioblasts, but not immortalized myoblasts or neural precursor cells, enhance CD163 membrane expression *in vitro* as assessed by flow cytometry, indicating that the effect is specific. Mesoangioblasts transplanted in acutely or chronically injured skeletal muscles determine the expansion of the population of CD163+ infiltrating macrophages and increase the extent of CD163 expression. Conversely, macrophages challenged with mesoangioblasts engulf significantly better apoptotic cells *in vitro*. Collectively, the data reveal a feed-forward loop between macrophages and vessel-associated stem cells, which has implications for the skeletal muscle homeostatic response to sterile injury and for diseases in which homeostasis is jeopardized, including muscle dystrophies and inflammatory myopathies.

Keywords: apoptosis, inflammation, macrophage, phagocytosis

Introduction

Tissue regeneration after acute muscle damage is controlled by a fine-tuned interaction between muscle stem cells and macrophages (MFs), which dispose of myofibre debris and apoptotic cells [1–3]. The regenerating skeletal muscle represents an optimal scenario to study the functional plasticity of tissue-recruited MFs. Macrophages that infiltrate the

tissue early after injury produce inflammatory cytokines, such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β, and effectively clear myofibre remnants and apoptotic cells while sustaining the activation and the proliferation of muscle stem cells [1]. As such, their features reflect those of the classically activated inflammatory macrophages, also referred to as 'M1 cells'. At later times, in contrast, 'alternatively activated' or 'M2' macrophages predominate, i.e. cells that play a key role in the tissue homeostasis by regulating development, healing and termination of the inflammatory responses [4]. They produce IL-10 and transforming growth factor (TGF)-β and sustain the fusion of myoblasts and the formation and assembly of novel myofibres [1,5] and are required for eventual effective myofibre regeneration [6]. The ability to clear cell remnants and macrophage alternative activation both abate when adenosine monophosphate-activated protein kinase (AMPK)-α1, a trimeric AMP kinase, is inhibited by genetic or pharmacological tools, indicating that the two events are causally linked [7], further supporting the contention that the recognition and clearance of apoptotic cells represent critical events in the M1/M2 shift.

Of importance, the pattern of MF activation controls the expression of several iron-related genes and MF ability to manage iron [8–10], and alternative activation is typically associated with an increased expression of scavenger receptors, in particular the haemoglobin/haptoglobin receptor CD163 [11,12], a pattern recognition receptor that, on one hand, physiologically scavenges haptoglobin by prompting the endocytosis of haptoglobin/haemoglobin complexes, with processing and eventual rescue of haem–iron components, and on the other hand contributes indirectly to the anti-inflammatory response associated with alternative activation [13,14].

Macrophages in injured skeletal muscles interact productively with vessel-associated stem cells (mesoangioblasts) [5,15], myogenic precursors defined by the expression of pericyte markers and by their ability to cross vessel walls [16]. In this study we show that mesoangioblasts instruct MFs, sustaining their conversion into cells that acquire CD163 receptor expression, clear apoptotic cells and promote tissue remodelling.

Materials and methods

Macrophage–mesoangioblast culture

Bone marrow-derived macrophages and mesoangioblasts were isolated and differentiated as described previously [5]. Briefly, bone marrow precursors from C57BL/6 female mice were isolated and propagated for 7 days in α-modified Eagle's medium (MEM) (Gibco/Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) in the presence of recombinant mouse macrophage colony-stimulating factor (rm-M-CSF) (100 ng/ml). Adult

mesoangioblasts were isolated from tibialis anterior muscles of C57BL/6 female mice and cloned by limiting dilution at 0·3 cell/well. Mesoangioblasts were added to macrophages at a 1:10 ratio either in physical contact (coculture) or in the upper chamber of a 0·4 μm membrane-separated Transwell system for 48 h (Transwell; Nunc, Waltham, MA, USA). Macrophages were also challenged at the indicated ratio with immortalized C_2C_{12} mouse myoblasts, mouse embryonic fibroblasts ($10T_{1/2}$), and mouse neuronal precursor stem cells (NPC), propagated as described [17].

Gene expression profile and data analysis

Total cellular RNA was extracted from M0 and M0 Transwell mesoangioblasts (MAB) using the RNeasy midi kit, following the manufacturer's recommendations. Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit, Santa Clara, CA, USA) were used to determine the concentration and purity/integrity of RNA samples using the Agilent 2100 bioanalyser. cDNA synthesis, biotin-labelled target synthesis, HG-U133 plus 2·0 GeneChip (Affymetrix, Santa Clara, CA, USA) array hybridization, staining and scanning were performed according to the standard protocol supplied by Affymetrix. The GeneChip mouse expression, set 430 2·0, which provides comprehensive coverage of the mouse transcriptome, was used. Raw data were acquired using the Affymetrix® GeneChip® Command Console® (AGCC) software. Data processing and appropriate statistical analysis and all data quality controls were performed using the R (Bioconductor) and Partek® Genomic Suite. The functional analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, http://www.ingenuity.com).

Intramuscular mesoangioblast transplantation

Tibialis anterior and quadriceps muscles of 6-week-old C57Bl/6 mice were injured by injection of cardiotoxin, as described [5]. Mesoangioblasts (5×10^5 /mouse) were transplanted 1 day after treatment with cardiotoxin or 15 days before killing in αSG$^{-/-}$ dystrophic mice (8–10 weeks old). Immunohistochemistry on muscle sections, isolation of leucocytes from the skeletal muscle and flow cytometry were performed as described previously [5]. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the San Raffaele Scientific Institute.

Phagocytosis

RMA cells were plated (5×10^5 cells/ml) in RPMI + 10% FCS. Cells were then irradiated using an ultraviolet (UV) source at 0·1 J/cm^3. After 16 h, apoptosis was verified as described [18] and cells stained with CMTMR (Invitrogen,

Table 1. Values refer to the Affymetrix® GeneChip® Command Console® (AGCC). Macrophages alone (Mφ) or cultured in Transwell (Tw) for 48 h with mesoangioblast stem cells (Mφ Tw MAB) were screened for gene array analysis. Signals for transcripts with a present call are shown in gradient-coloured cells from blue (low abundance) to dark red (very highly abundant transcript). Expression of macrophages genes that were modulated in a statistically significant manner after culture with mesoangioblasts are reported. Data are from three independent experiments.

Gene title	Symbol	MΦ	MΦ Tw MAB	Significance log ratio
Haptoglobin–haemoglobin receptor	Cd163	109,6	269,5	
Macrophage scavenger receptor 1	Msr1	700,6	1374,1	
Mannose receptor, C type 1	Mrc1	4329,7	10822,5	
CD36 antigen	Cd36	383,1	428,5	
Matrix metallopeptidase 9	Mmp9	317,7	435,8	
Myeloperoxidase	Mpo	126,1	244,8	
CD74 antigen	Cd74	6145,6	4051,5	
CD83 antigen	Cd83	1081,7	755,6	
Fc receptor, lgG, high affinity I	Fcgr1	2869,2	2118,1	
Interleukin 6 receptor, alpha	Il6ra	942,2	695,4	
Interleukin 7 receptor	Il7r	1309,9	668	
Interleukin 4 receptor, alpha	Il4ra	535,2	946,3	
Interleukin 13 receptor, alpha 1	Il13ra1	96,6	274,9	
Interleukin 17 receptor A	Il17ra	223,2	323	
Interleukin 21 receptor	Il21r	205,4	262,4	
Interferon (alpha/beta) receptor 1	Ifnar1	647,4	486,3	
Interferon gamma receptor 1	Ifngr1	1535,9	1923,5	
Interferon gamma receptor 2	Ifngr2	706,7	890,9	
Transforming growth factor, beta r	Tgfbr2	306,7	492,2	
Insulin-like growth factor I receptor	Igf1r	194,3	342,5	
Thrombospondin 1	Thbs1	1833,9	4112,1	
Annexin A3	Anxa3	3708,3	4287,4	
Annexin A6	Anxa6	528,3	645,2	
Growth arrest specific 6	Gas6	3616,4	5684,2	
Protein S (alpha)	Pros1	393	448,3	
AXL receptor tyrosine kinase	Axl	337,5	256,3	
c-mer tyrosine kinase	Mertk	3012,4	2309,3	

−1·5   −1·0   −0·5   0·0   0·5   1·0   1·5   2·0

MΦ:MΦ tw MAB down ←   → MΦ:MΦ tw MAB up

Carlsbad, CA, USA), according to the manufacturer's instructions. Bone-marrow derived macrophages were seeded in the presence or absence of MAB in a Transwell system as described above. After 48 h macrophages were challenged with apoptotic cells for 2 h at a 1:1 ratio at 37°C or 4°C. Cells were washed and phagocytosis verified by flow cytometry after staining with allophycocyanin(APC)-conjugated anti-CD11b (clone M1/70; BD Biosciences, San Jose, CA, USA). The phagocytosis was calculated as %CD11b⁺CMTMR⁺ cells at 37°C – %CD11b⁺CMTMR⁺ cells at 4°C. Data were acquired on a fluorescence activated cell sorter (FACS)Calibur (BD Biosciences).

Statistics

All data are presented as mean ± standard error of the mean (s.e.m.). The two-tailed Student's *t*-test was used for comparisons between experimental groups. Significant differences were defined at $P < 0·05$.

Results

Mesoangioblasts promote the expression of genes associated with macrophage alternative activation

We assessed the gene expression of macrophages propagated from the mouse bone marrow that had been challenged or not with mesoangioblasts in a Transwell system, which prevents direct cell-to-cell contact but allows the diffusion of soluble moieties. The expression of scavenger receptors involved in: (i) the alternative activation of macrophages (CD163, CD206) [11,19,20] and (ii) the phagocytic clearance of soluble and particulate substrates, including cell remnants (MSR1, CD36) [21,22] is significantly up-regulated (Table 1). Moreover, macrophages exposed to mesoangioblasts express significantly higher amounts of genes coding for thrombospondin 1 (TSP-1) and Gas6, which bridge macrophages to the apoptotic substrate (Table 1).
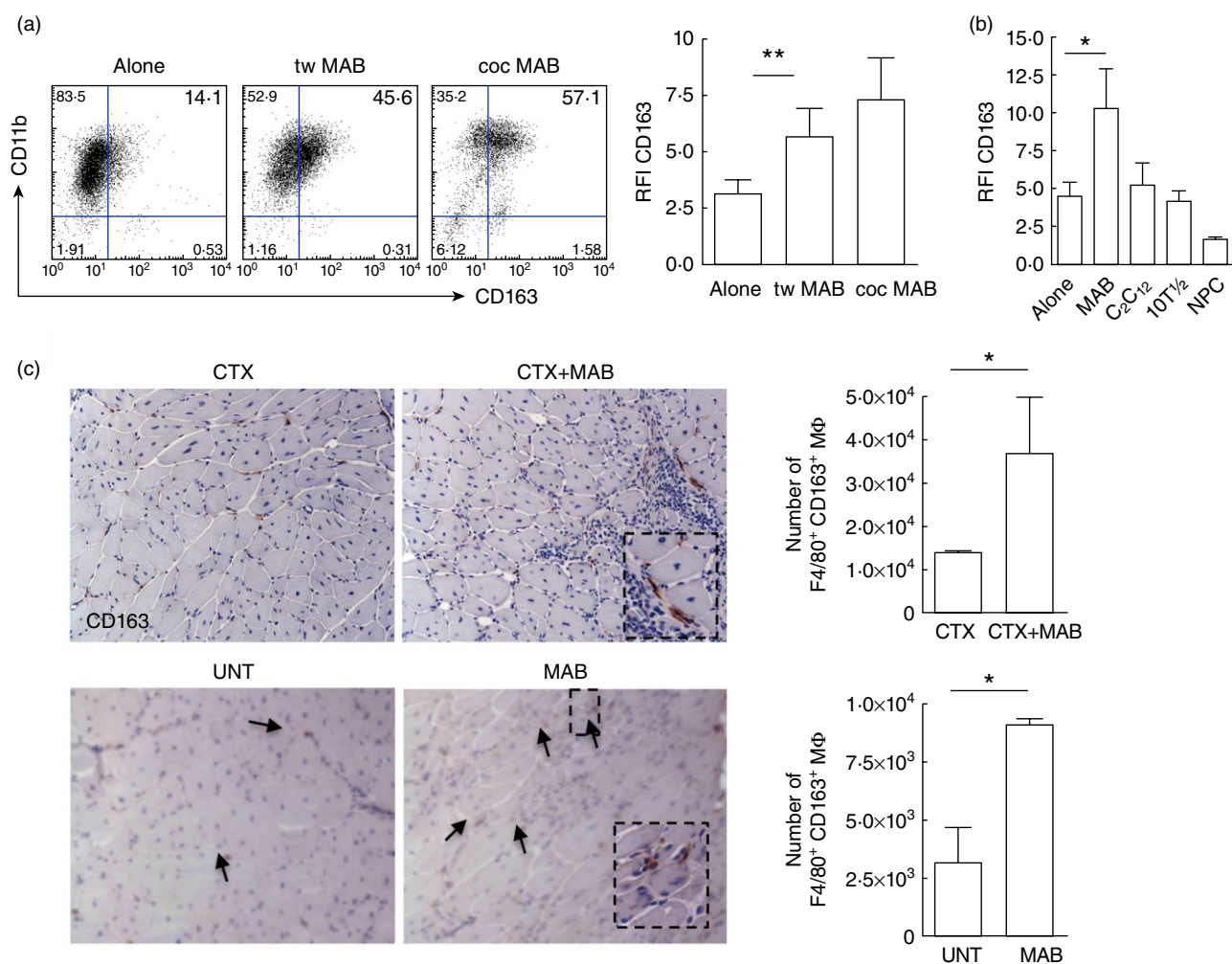
Fig. 1. Mesoangioblasts regulate macrophage CD163 expression. Macrophages cultured for 48 h alone (alone), or challenged with mesoangioblasts (MAB) either in culture conditions allowing physical cell-to-cell contact (co-culture, coc) or separated by a semi-permeable membrane (Transwell, Tw) were analysed by flow cytometry for CD163 expression. (a) Representative dot-plots indicating frequency of CD11b$^+$CD163$^+$ cells and CD163 relative fluorescence intensity (RFI). Error bars indicate the mean ± standard error of the mean (s.e.m.) of 11 independent samples. **$P < 0.01$. (b) CD163-associated fluorescence (RFI) was assessed in macrophages cultured alone or challenged with MAB, C$_2$C$_{12}$ immortalized myoblasts, 10T$_{1/2}$ embryonic fibroblasts or neural precursor cells (NPC). Error bars indicate the mean ± s.e.m. of three independent experiments. *$P < 0.05$. (c) CD163 expression was assessed by immunohistochemistry: in the skeletal muscle of C57Bl6 mice in which mesoangioblasts had been transplanted (CTX+MAB) or not (CTX) 24 h after injury (top panels); in the skeletal muscle of C57Bl6 αSG$^{-/-}$ dystrophic mice (bottom panel) transplanted (MAB) or not (untreated, UNT) with mesoangioblasts. (d) Mononuclear cells were retrieved after enzymatic digestion of the skeletal muscle. F4/80$^+$CD163$^+$ macrophages were identified within CD45$^+$CD11b$^+$ leucocytes by flow cytometry. Error bars indicate the mean ± s.e.m. of three independent experiments with three mice per group. *$P < 0.05$.

Mesoangioblasts modulate macrophages phenotype and function *in vitro* and *in vivo*

The fraction of macrophages expressing the haptoglobin–haemoglobin receptor CD163 and the overall CD163-associated fluorescence are both significantly higher in macrophages challenged with mesoangioblasts than in those cultured alone (Fig. 1a). CD163 up-regulation is similar when macrophages are in direct contact with mesoangioblasts or physically separated via a Transwell

system (Fig. 1a). In contrast, immortalized myoblasts (C$_2$C$_{12}$), embryonic primary fibroblasts (10T$_{1/2}$) and NPC fail to modulate macrophage CD163 expression (Fig. 1b). Mesoangioblasts transplanted in muscles damaged by the injection of cardiotoxin or dystrophic because of the genetic deficiency of alpha-sarcoglycan not only favour tissue regeneration (not shown and [16]), but also expand the fraction of inflammatory phagocytes expressing the CD163, as detected by immunohistochemistry and by flow cytometry (Fig. 1c). Macrophages that had been challenged
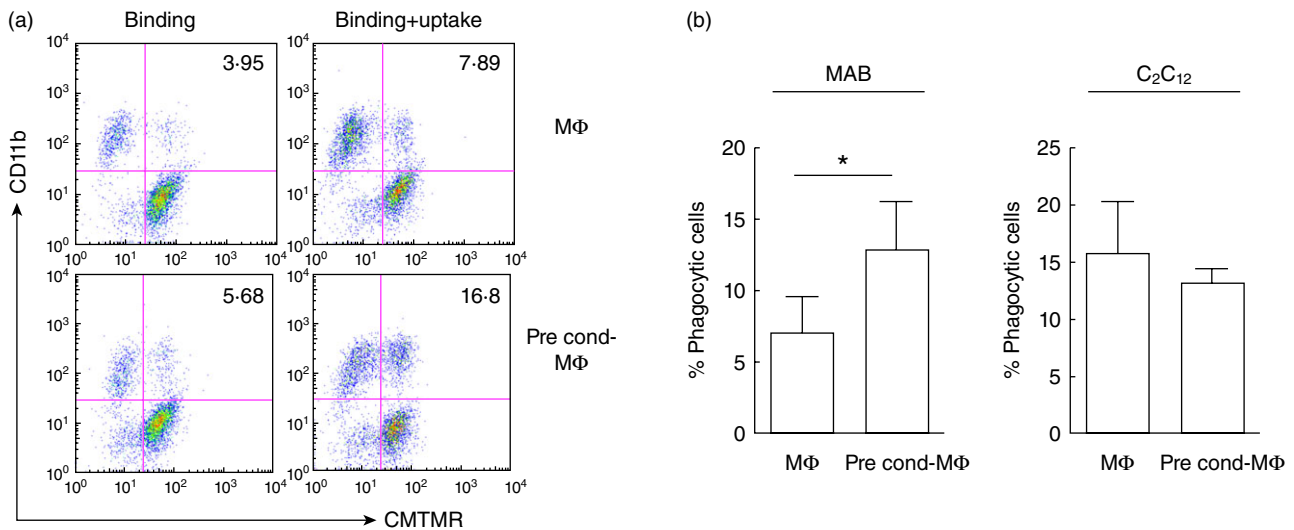
Fig. 2. Mesoangioblasts increase macrophage capacity to phagocyte apoptotic cells. Macrophages were cultured alone (Mφ) or challenged with mesoangioblasts (pre-cond-Mφ) for 48 h. Macrophages were then incubated with CellTracker CMTMR-labelled apoptotic RMA cells for 2 h. (a) Physical interaction and phagocytosis were verified by flow cytometry at 4°C (binding) and 37°C (binding+uptake). Percentage of CD11b$^+$CMTMR$^+$ phagocytic cells is normalized with the control (binding at 4°C). (b) Macrophages cultured alone or in the Transwell system for 48 h with immortalized myoblasts (C$_2$C$_{12}$) were subjected to phagocytosis assay, as reported above.

previously with mesoangioblasts phagocytose apoptotic CMTMR-labelled RMA cells significantly more effectively than macrophages challenged with satellite cells or cultured alone (Fig. 2). The mechanism by which mesoangioblasts modulate macrophage function remains to be elucidated. However, they express an array of inflammatory molecules, which are important for their ability to migrate to sites of injury and to interact with inflammatory and tissue cells. Inflammatory molecules expressed by mesoangioblasts comprise signals that are known to modulate the alternative activation of macrophages, in particular TGF-β and M-CSF (Supporting information, Table S1).

Discussion

Macrophages reprogramme their function in response to signals derived from microbes [23,24], damaged tissues [25] and resting or activated lymphocytes [26–28], an event which is critical for tissue plasticity [29]. The interaction between mesoangioblasts and macrophages has been investigated in previous studies [5,15], and macrophages have been shown to orchestrate the survival and the differentiation of mesoangioblasts transplanted into injured skeletal muscles. The latter event is strictly linked to the alternative activation of macrophages within the tissue [5,7]. Here we reveal the existence of a feed-forward loop, by which mesoangioblasts sustain the activation program of macrophages which, in turn, enable them to provide stem cells with survival and differentiation signals. Of interest, the action of mesoangioblasts on macrophages appears somewhat selective, as other sources of stem cells, including NPCs, which have well-characterized immunoregulatory properties [17], fail to modulate macrophage characteristics.

Mesoangioblasts are derived from a subset of pericytes found in the skeletal muscle, and pericytes have been shown to regulate the traffic of leucocytes through the subendothelial matrix in inflamed tissues [30–32]. It is tempting to speculate that mesoangioblasts' ability to modulate the activation of macrophages reflect a more complex action of pericytes associated physiologically with muscular vessels. Further studies are necessary to verify whether this is the case and whether the up-regulation of bridging proteins that have been implicated in the phagocytic synapse elicited by mesoangioblasts controls macrophage behaviour *in vivo*.

Disclosure

The authors have no financial conflicts of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Values refer to the Affymetrix® GeneChip® Command Console® (AGCC). Mesoangioblasts were screened for gene array analysis. Signals for transcripts with a present call are shown in gradient-coloured cells from blue (low abundance) to dark red (very highly abundant transcript). Data are from two independent experiments.